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Peptic Ulcer Disease

Edited by Jianyuan Chai

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Contributors

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Notice

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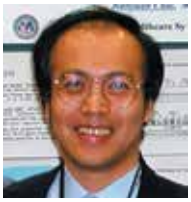
Meet the editor



Dr. Chai received his Ph.D from the City University of New York in 1998 and completed his post-doctoral training at Harvard University in 2001. Currently, he is a principal investigator of the U.S Department of Veterans Affairs and a faculty member of the Department of Medicine at University of California in Irvine. Dr. Chai has published over 30 peer-reviewed articles and book chapters in diverse areas including protozoology, parasitology, arachnology, marine biology, cardiology and gastroenterology. His current research focuses on acid reflux induced esophageal cancer. He holds professional membership with AGA, AHA, and ASBMB, has been a solicited reviewer for multiple journals and grants, and also serves as Associate Editor-in-Chief for World Journal of Gastrointestinal Oncology, editorial board member of World Journal of Gastroenterology, and Chairman of the VA Long Beach IACUC.

Contents

Preface

Our knowledge about peptic ulcer can be traced back at least to the medieval era (~ 11th century) when the great Iranian physician, Avicenna, described the association between stomachache and mealtimes in his book, *The Cannon of Medicine*, and suggested that the pain could be all due to the presence of a gastric ulcer. Later it was the invention of microscope (~ 16th century) that gave the early investigators the edge to draw a connection between gastric ulcers and the bacteria living in the stomach. Nowadays, when you search the literature for the cause of peptic ulcers, almost every article tells you that it is the bacterium *Helicobacter pylori* that is responsible for the vast majority of ulcers. The main argument for this notion is the high frequency of *H. pylori* found in ulcer patients. However, we should not ignore the fact that although more than half of the world population is infected by *H. pylori*, only less than 10% of them develop peptic ulcer disease. We should also remember that peptic ulcers sometimes occur in the absence of the bacteria. Use of NSAIDs is an example. Some estimate that more than 16,000 people die of NSAIDs-induced ulcers each year. The common effect of *H. pylori* and NSAIDs on the stomach is that both can disarm the mucosal protection, exposing the epithelium directly to the gastric fluid that is highly acidic (~ pH 1-2). As early as in 1940s, scientists already learned through animal experiments that exposure of any living tissues, including the stomach wall, to this fluid is detrimental. The reason that our stomach can normally tolerate such hostile environment is the mucus overlaid on the surface of the gastric epithelium, which shields gastric tissue from gastric acid. In addition, both the stomach and the duodenum contain cells that can produce bicarbonate to neutralize the acid intimate to the mucosal surface. When *H. pylori* dwells in the stomach or duodenum, it produces toxins that trigger the inflammatory response from the host, which can lead to mucosal atrophy, making the epithelial cells prone to the acidic damage. Similarly, NSAIDs inhibit production of mucus and bicarbonate, which can result in epithelial denudation. In both cases, the ultimate cause of peptic ulcers is the acid. Zollinger-Ellison Syndrome is an extreme case in which peptic ulcers are simply induced by over secretion of gastric acid.

This book is a snapshot of the current view of peptic ulcer disease. Although the book is divided into five sections, it is only for the convenience of the readers and the content of each section is tightly connected to the rest of the book. *Peptic Ulcer Disease* opens with a discussion on the causes of the disease. The first chapter by Silva & de

Souza (Federal University of Ceará, Brazil) introduces the offense-defense mechanisms that protect mucosal integrity and discusses each factor that is known to jeopardize this balance. Out of all the offensive factors, *H. pylori* infection and NSAID use are two most common ones. The chapter by Wang & Wang (Zhejiang University, China) analyze these two major etiologies, debate on their relationship whether synergistic, antagonistic or independent, and point out several elements that may influence the outcome of this interaction, such as age of the patient, type of NSAIDs, strain of the bacteria, etc. The following two chapters examine each one of these two causes in more detail respectively. Tsang (Northwestern University, USA) focuses his attention on the No. 1 factor – *H. pylori*, thoroughly describes what is known about this bacterium, including its microbiology, pathophysiology, epidemiology and eventually the methods of clinical testing. The large body of references provides further readings for those who are eager to know more about *H. pylori*. Whereas, Ferraz-Amaro & Diaz-Gonzalez (Hospital Universitario de Canarias, Spain) give a systematic review on the second cause of peptic ulcer disease – NSAIDs. They unfold a complete picture of NSAIDs including their history, pharmacological features and their effects on gastrointestinal system. In Chapter 5, a group of clinical researchers (Istanbul University, Turkey) present an actual set of data that shows a consistent association of peptic ulcers with *H. pylori* infection and NSAID use. The last chapter of this section by Lukes et al (Charles University in Prague, Czech) reports an unusual location of *H. pylori* infection, the oral cavity, which is outside its common gastro-duodenal territory and a little beyond our central topic of this book – peptic ulcer disease, however, the paper raises an interesting question: is *H. pylori* really interested only in acidic environments? After all, the oral cavity is just the upper end of the gastro-duodenal tract.

The second part of the book collects seven chapters devoting to the molecular mechanisms of peptic ulcer development and healing. Chapter 7 by Fornai et al (University of Pisa, Italy) explains how mucosal defense system at the molecular level protects gastric tissue from gastric acid damage in normal people, then discusses how NSAIDs break down this barrier and induce ulceration, and finally describes how ulcer healing proceeds. The next chapter (Chapter 8) by Chai (Department of Veterans Affairs, USA) gives a systematic review on the molecular and cellular mechanisms of ulcer healing and introduces a powerful regulator – SRF, a transcription factor that has influence on hundreds and possibly thousands of genes, many of which are important to ulcer healing. Chapter 9 by Gisbert group (Hospital Universitario de la Princesa, Spain) takes a look at peptic ulcer from a very different point of view – oxidative stress and apoptosis, and presents strong evidence that ulceration, regardless of the cause, is mediated through excessive production of reactive oxygen species, and therefore, therapeutic strategies to eliminate the oxidative stress should benefit the ulcer patients. The following two chapters (Chapter 10 & 11) provide strong data that elucidate hormonal involvement in ulcer healing. Brzozowski group (Jagiellonian University Medical College, Poland) studied ghrelin, orexin-A and obestatin and found that these newly discovered gastrointestinal hormones all have positive effects on gastric ulcer

healing. Filaretova (Pavlov Institute of Physiology, Russia), who has spent many years to study the effect of glucocorticoids on ulcer development, raises a strong argument that these hormones are gastro-protective rather than ulcerogenic, which is contradictory to many other studies. Chapter 12 by Matsukawa & Kato (Nihon University School of Medicine, Japan) presents a set of clinical data that show interesting correlations of serum immunoglobulin levels vs. *H. pylori* infection, smoking and peptic ulcer respectively. This section ends with the work of D'Elios (University of Florence, Italy) and Bernard (University of Padua, Italy) about *H. pylori* infection induced host immune response, an important aspect for the development of efficient vaccines against the bacteria.

The third section of the book contains four articles that focus on the clinical management of peptic ulcer patients. In the first article (Chapter 14), Salles (Hospital Xavier Arnozan, France) discusses peptic ulcer in elderly patients, which is the population suffered mostly from the disease because both *H. pylori* infection and NSAID use increase with age. This chapter also puts together a list of common methods for diagnosis and treatment of *H. pylori* infection. Although the prevalence of peptic ulcer overall is declining, the incidence of ulcer complications, such as perforation, bleeding and obstruction, remains unchanged. The next two chapters (Chapter 15 & 16), written by Saber (Port-Fouad General Hospital, Egypt) and Rensburg & Marais (Stellenbosch University, South Africa) respectively, concentrate on these issues individually and also provide management protocols associated. This section closes with a case study (Chapter 17) reviewed by Peace (Georgia Southern University, USA), which shows negative associations of duodenal ulcer healing vs. smoking status, as well as duodenal ulcer healing vs. ulcer size.

Peptic ulcer treatment usually involves a combination of antibiotics, acid suppressors, and mucosa protectors. The next section includes eight chapters dealing with peptic ulcer treatment and prevention strategies. The first chapter (Chapter 18), prepared by Ozguney (University of Ege, Turkey) respectively, provide extensive discussions on how drugs should be designed for ulcer treatment, which is very useful information for pharmaceutical industries when they develop new drugs for gastrointestinal use. Because of the cost and side-effects of current treatment regimens, more and more researchers have started to search for alternative therapy. The next five chapters (Chapter 19-23) introduce a few natural products that have been found useful for ulcer treatment, including spices that we use to prepare our meals (Mofleh, King Saud University, Soudi Arabia), plants (Novaes & Leite, Federal University of Vicosa, Brazil), fruits (Hamauzu, Shinshu University, Japan), and herbs (Abdel-Sater, Al-Azhar University, Egypt). Use of natural products to treat ulcers has a long history; however, their chemical and biological mechanisms are still not quite understood. There is much room to allow further investigation. These five chapters give the readers an update in this area, including possible toxicity that requires precautions. The last chapter (Chapter 24) of this section by Morsy & El-Sheikh (Minia University, Egypt) provides a take-home message on peptic ulcer prevention including our daily habits, internal protection and therapeutic interventions.

The final section of the book includes a unique paper by Souza & Pinto (Federal University of Vicosa, Brazil) on the ulcer development in horses and ponies due to the use of NSAIDs, giving additional values to this book so that not only physicians and researchers but veterinarians can also be benefited from it.

The contributors to this book come from 15 countries spread out in Africa, Asia, Europe, North America and South America, which gives the book an excellent geographic representation. Some of the authors are senior investigators, while others are junior or mid-career researchers. Every one of them provides views from different disciplines, including gastroenterology, pharmacology, microbiology, epidemiology, pathology, and molecular/cellular biology, giving each chapter a unique value. Readers may not agree with everything in this book, but if college students find it educational, medical professionals find it helpful in their practices, and researchers find more interesting questions to investigate, our effort will be worthwhile.

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Part 1

Pathogenesis of Peptic Ulcer

1

Gastric Ulcer Etiology

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1. Introduction

A gastric ulcer, also called stomach ulcer, is a break in the normal gastric mucosa integrity that extends through the muscularis mucosa into the submucosa or deeper. The incidence varies with the age, gender, geographical location and is associated with severe complications including hemorrhages, perforations, gastrointestinal obstruction, and malignancy. Thus, this clinical condition represents a worldwide health problem because of its high morbidity, mortality and economic loss (Brown & Wilson, 1999; Dimaline & Varro, 2007).

The normal stomach mucosa maintains a balance between protective and aggressive factors. Some of the main aggressive factors are gastric acid, abnormal motility, pepsin, bile salts, use of alcohol and nonsteroidal anti-inflammatory drugs (NSAID), as well as infection with microorganisms (*Helicobacter pylori* and others). On the other hand, mucus secretion, bicarbonate production, gastroprotective prostaglandin synthesis and normal tissue microcirculation protect against ulcer formation. Although in most cases the etiology of ulcer is unknown yet, it is generally accepted that gastric ulcers are multifactorial and develop when aggressive factors (endogenous, exogenous and/or infectious agents) overcome mucosal defense mechanisms (Allen & Garner, 1980; Wallace, 1992; Peskar & Marici, 1998; Tulassay & Herszényi, 2010).

In this context, the present chapter aims to address etiologies multiples of gastric ulcer development, clarifying how the imbalance between aggressive and defensive factors leads to this clinical condition. For this purpose, we review basic components of gastric mucosal defense and discuss conditions in which mucosal injury is directly related to impairment in mucosal defense.

2. Gastric protective factors

The stomach is lined by a complex epithelium that forms a selective barrier between the external environment (lumen) and the body, which is folded into several branching, tubular gastric glands that reach deep into the muscularis mucosa. The diverse range of functions performed by gastric epithelial cells is maintained in the face of a hostile luminal environment that can contain up to 150 mm HCl and aggressive proteases, which are capable of digesting tissue, as well as a variety of noxious pathogens (Dimaline &Varro, 2007). Despite continuous exposure to these injurious factors, under normal conditions a

large number of defense mechanisms prevent local damage and maintain structural and functional mucosal integrity (Tulassay & Herszényi, 2010).
In general, gastric defense mechanisms consist of a gastric mucosal "barrier". It is a multilayer system, which include a preepithelial mucus-bicarbonate "barrier", an epithelial "barrier" (surface epithelial cells connected by tight junctions), and a subepithelial component including blood flow and nerves. (Henriksnäs et al., 2006; Dimaline & Varro, 2007; Nayeb-Hashemi & Kaunitz, 2009).

2.1 Gastric mucosal "barrier"

2.1.1 Preepithelial mucus-bicarbonate "barrier"

The regular exposure of the stomach to endogenously produced acid and degrading enzymes requires the presence of an efficient gastric mucosal "barrier". Since the first experimental evidence for the mucus bicarbonate barrier was reported about three decades ago (Allen, 1978; Allen & Garner, 1980), it has become firmly established as a key component of the gastroduodenal mucosal protective mechanisms against noxious agents (Allen & Flemström, 2005). This barrier constitutes the first line of mucosal defense and is formed by mucus gel, bicarbonate ($HCO3^-$), and surfactant phospholipids, which cover the mucosal surface (Lichtenberger, 1999; Allen & Flemström, 2005).
The gastric mucus consists of a viscous, elastic, adherent and transparent gel secreted by apical expulsion from surface epithelial cells. It is formed by ~ 95% water and ~ 5% mucin glycoproteins that covers the entire gastrointestinal mucosa, and its luminal surface is coated with a film of surfactant phospholipids with strong hydrophobic properties. The $HCO3^-$ is secreted by surface epithelial cells and its role is to neutralize acid diffusing into a stable, adherent mucus gel layer and to be quantitatively sufficient to maintain a near-neutral pH (~ 7.0) at the mucus-mucosal surface interface (Figure 1) (Hills et al., 1983; Lichtenberger 1999; Repetto & Llesuy, 2002; Tulassay & Herszényi, 2010).
In contrast to stomach acid, pepsin has received relatively little attention as the other endogenous aggressor in gastric juice. Pepsin damage is characterized by focal areas of discontinuity in the adherent mucus layer, localized hemorrhagic punctuate ulcers with bleeding into the lumen, and no evidence of reepithelialization or mucoid cap formation (Allen & Flemström, 2005). Thus, the unstirred mucus gel layer is also a physical barrier to luminal pepsin accessing the underlying mucosa. It retains $HCO3^-$ secreted by surface epithelial cells, preventing penetration of pepsin and therefore proteolytic digestion of the surface epithelium (Tulassay & Herszényi, 2010). Therefore, a dissipation of the mucus gel and phospholipid layer by ulcerogenic substances (such as aspirin and bile salts) leads to both acid back-diffusion and mucosal injury. (Darling et al., 2004; Allen & Flemström , 2005).
Moreover, if some oxygen radicals are generated in surface epithelium containing mucus, intracellular mucus could scavenge them, acting as an antioxidant and thus reducing mucosal damage mediated by oxygen free radicals. (Penissi & Piezzi, 1999; Repetto & Llesuy, 2002). Even when cells containing mucus are damaged by extracellular oxygen radicals, intracellular mucus may be released into the gastric tissue and prevent additional damage by scavenging them (Seno et al., 1995).
The efficacy of protective properties of the mucus barrier depends not only on the gel structure but also on the amount or thickness of the layer covering the mucosal surface (Penissi & Piezzi, 1999; Repetto & Llesuy, 2002). The thickness of this layer is the result of a dynamic balance between its secretion and its erosion mechanically by shear forces of the

digestive process and by proteolytic degradation, particularly from luminal pepsin in stomach. Compared with other gastrointestinal secretions, the adherent mucus gel form is physically unique. Studies have shown that adherent mucus gels from stomach, duodenum, and colon are all well-defined viscoelastic gels that do not dissolve on dilution (Allen et al., 1976; Allen, 1989; Allen & Flemström, 2005). They flow over a relatively long time (30–120 min), reannealing when sectioned. Thus, mucus gels are known stable substances, and exposure of isolated gastric mucus gel to pH 1–8, hypertonic salt, or bile does not disperse or affect its rheological properties. In functional terms, these recognized properties contribute to the adherent mucus gel layer forming a continuous and effective protection over the mucosa (Allen & Flemström, 2005)

The mucus bicarbonate barrier is the only preepithelial barrier between epithelium and lumen. When it is overwhelmed or breaks down in different disease conditions, the next series of protective mechanisms come into play, including epithelial repair, and maintenance and distribution of mucosal blood flow (Tulassay & Herszényi, 2010).

2.1.2 Epithelial "barrier"

Subsequent to mucus-bicarbonate "barrier", the next line of mucosal protection is formed by a continuous layer of surface epithelial cells, which secrete mucus and bicarbonate and generate prostaglandins (PGs), heat shock proteins, trefoil factor family peptides (TFFs), and cathelicidins. This epithelial barrier serves to separate the digestive lumen from the internal compartments of the organism. Its main role is to maintain a selective exchange of different substances (secretions, nutrients, etc.) between these two compartments, and to assure the protection of the organism against the penetration of micro-organisms and other exogenous antigens, essentially contained in food. In this context, two crucial elements of the digestive epithelial barrier assure these functions: the epithelial cells and the intercellular junctions (tight junctions). Both structures provide two pathways for transepithelial transport: transcellular and paracellular routes, respectively (Figure 1). (Matysiak-Budnik, et al., 2003; Laine et al., 2008; Tulassay & Herszényi, 2010). Because of the presence of phospholipids on epithelial cells surfaces, these cells are hydrophobic and therefore repel acid- and water-soluble damaging agents (Lichtenberger et al., 1983).

The paracellular pathway seems to be the major route of transepithelial macromolecular permeation. This route is a complex array of structures that are mainly controlled by tight junctions between epithelial cells, which appear to be key regulators of gastrointestinal permeability to macromolecules such as endotoxin and other bacterial products. Also, interconnected by tight junctions, the surface epithelial cells form a "barrier" preventing back diffusion of acid and pepsin (Farhadi et al., 2003; Werther, 2000; Laine et al., 2008). The physiology of this tightly regulated conduit is not fully known. However, this dynamic gateway is able to change its size under various physiological and pathological conditions. For instance, an earlier study (Madara, 1983) showed that increases in guinea pig intestinal transepithelial resistance induced by osmotic loads were accompanied by alterations in absorptive-cell tight junction structure. This alteration in intestinal permeability after meal ingestion enhances the ability of the small intestine to harvest the maximal amount of nutrients, as well as also increase the risk of exposure to luminal proinflammatory compounds. Tight junctions are also composed of other structural proteins including actin anchoring protein (ZO-1) and occludin, which could be the target of oxidative or other toxin injury and result in disruption of gastrointestinal barrier integrity (Farhadi et al., 2003; Nusrat et al., 2001).

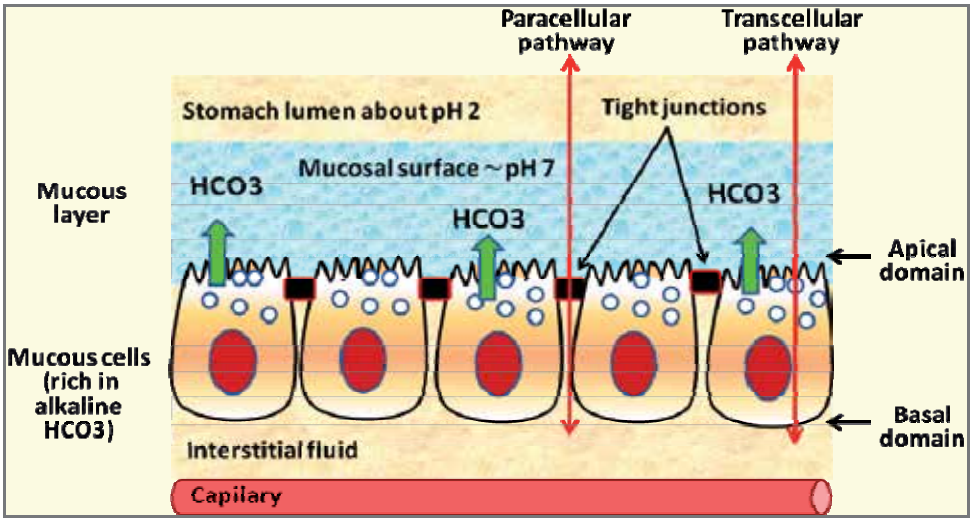

Fig. 1. Bicarbonate rich mucous secreted by surface epithelial cells.

Heat shock proteins generated by gastric epithelial cells are essential for the maintenance of cellular homeostasis during normal cell growth and for survival during various cellular stresses, such as increased temperature, oxidative stress, and cytotoxic agents, preventing protein denaturation and protecting cells against injury. Activation of heat shock protein response is one of the mucosal protective mechanisms of the antacid hydrotalcite. Also, cathelicidin and ß defensins are cationic peptides that play roles in the innate defensive system at mucosal surfaces preventing bacterial colonization. These elements have been demonstrated in gastric epithelial cells, and they accelerate ulcer healing (Tarnawski et al., 1999; Oyaka et al., 2006; Tanaka et al., 2007; Tulassay & Herszényi, 2010).

The trefoil factor family (TFFs) comprises a group of small peptides (6.5–12 kDa) secreted abundantly by surface epithelium, which has been demonstrated play an important role in mucosal integrity (Taupin & Podolsky, 2003). They regulate reepithelialization by stimulating cell migration and exert mucosal protective action from a broad range of toxic chemicals and drugs (Laine et al., 2008), as well as inhibiting apoptosis and inflammation, and augmenting the barrier function of mucus (Taupin & Podolsky, 2003; Hernández et al., 2009; Tulassay & Herszényi, 2010).

Maintenance of epithelial integrity requires a precise balance between cell proliferation and cell death. Thus, the epithelium is continually renewed by a well-coordinated and controlled proliferation of progenitor cells that enables replacement of damaged or aged surface epithelial cells. In this context, the gastric epithelium is populated by a variety of functionally-mature cells derived from proliferation of stem cells, such as mucous cells in the stomach, which show rapid turnover rates, and die within only a few days after their formation. Cell proliferation of progenitor cells is controlled by two peptides that have received attention for their potential role in barrier maintenance: epidermal growth factor (EGF), derived from salivary, esophageal and duodenal glands, and transforming growth factor alpha (TGF-alpha). Both peptides stimulate epithelial cell proliferation in case of injury as well as also enhance mucus secretion and inhibit acid production in the stomach (Murphy, 1998; Laine et al., 2008). However, after superficial injury, restitution of the surface epithelium occurs within minutes by migration of preserved epithelial cells located in the

neck area of gastric glands. Such migration precedes and is independent of proliferation of progenitor cells, which occurs hours after injury (Lacy & Ito, 1984; Blikslager & Roberts, 1997; Laine et al., 2008).

Prostaglandins (PGs) are also synthesized by gastric mucosal epithelial cells from arachidonate metabolism through the action of cyclooxygenases (COX). The ability of exogenous PGs to attenuate or even completely prevent mucosal damage caused by corrosive substances such as absolute ethanol, concentrated bile or hiperosmolar solutions has been termed "cytoprotection" (Farhadi et al., 2003). Particularly prostaglandin E2 and prostacyclin have long been known to have "cytoprotective" effects on the gastrointestinal epithelium and therefore they can be crucial for the maintenance of the gastric integrity. In fact, it is well established that inhibition of their synthesis results in the reduction of gastric mucosal blood flow and gastric mucosal damage (Abdel Salam et al., 1997).

Thus, although the precise mechanism of cytoprotective action of prostaglandins remained unknown, it appears to result from a complex ability to stimulate mucosal mucus and bicarbonate secretion, to increase mucosal blood flow and sulfhydryl compounds and, particularly in the stomach, to limit back diffusion of acid into the epithelium (Tarnawski et al., 1985; Farhadi et al., 2003; Kato et al., 2005). Earlier studies confirm not only these finding but also document that certain growth factors, especially EGF, could be considered as gastroprotective because they were capable of reducing nonsteroidal anti-inflammatory drugs (NSAID)-induced gastric ulcerations in animals when endogenous PGs were completely inhibited by administration of these drugs (Konturek et a., 1981). Also, growth factors stimulated prostaglandin production in rat endometrial cells through a mechanism that involves an increase in cyclooxygenase activity (Bany & Kennedy, 1995). Moreover, previous studies (Matsuda et al. 2002; Sánchez et al. 2006), including that conducted by our group (Silva et al., 2009), reported that endogenous prostaglandins are involved in the protective effect of different natural or semi-synthetic terpenes.

In addition, other mediators such as nitric oxide (NO), calcitonin gene related peptide (CGRP) as well as some hormones including gastrin and cholecystokinin (CCK), ghrelin, leptin and gastrin-releasing peptide (GRP) have been also found to protect gastric mucosa against the damage induced by corrosive substances. This protective action has also been attributed in part to the release of PGs because it could be abolished by the pretreatment with indomethacin (a nonselective inhibitor of COX 1 and 2) and restored by the addition of exogenous PGE2 (Farhadi et al., 2003).

2.1.3 Subepithelial components (microcirculation and sensory innervations)

The modulation of the gastric mucosal microcirculation plays an essential role in the maintenance of gastric integrity, especially for delivering oxygen and nutrients and removing toxic substances. At the level of the muscularis mucosae, most gastric arteries branch into capillaries, which enter the lamina propria and travel upward in proximity to gastric glandular epithelial cells. At the base of surface epithelial cells, capillaries converge into collecting venules (Laine et al., 2008; Tulassay & Herszényi, 2010). Thus, blood flow is essential for many protective mechanisms. For instance, restitution, a process whereby denuded areas of the mucosa are covered by rapidly migrating cells from adjacent mucosa, depends to a large extent on adequate blood flow (Lacy & Ito, 1984; Guttu et al., 1994; Abdel-Salam et al., 2001). Also, exposure of the gastric mucosa to an irritant or acid back-diffusion occurrence leads to a marked increase in mucosal blood flow. This increase allows

removal and/or dilution of the back-diffusing acid and/or noxious agents and seems to be essential for mucosal defense because its abolition through mechanical restriction of blood flow leads to hemorrhagic necrosis (Holzer, 2006, Laine et al., 2008).

The endothelial cells are also able to generate potent vasodilators agents such as nitric oxide (NO) and prostacyclin (PGI2). NO is produced from L-arginine in a reaction catalyzed by the enzyme nitric oxide synthase (NOS) (Bredt & Snyder, 1990). It is an important biological signaling molecule that influences circulation by regulating vascular smooth muscle tone and modulating systemic blood pressure. Therefore, it has been shown to exert positive effects on mucosal defense in the gastrointestinal system (Berg et al., 2004). Both NO and PGI2 oppose the mucosal damaging action of vasoconstrictors such as thromboxane A2, leukotriene C4, and endothelin. Consequently, these agents maintain viability of endothelial cells and prevent platelet and leukocyte adherence to the microvascular endothelial cells, preventing compromise of the microcirculation and thus protecting the gastric mucosa against injury (Laine et al., 2008). In addition to maintaining gastric blood flow, NO protects the gastrointestinal tract by inhibiting gastric acid secretion from parietal cells, stimulating mucus and bicarbonate secretion and by promotion angiogenesis in *vivo* and in *vitro* (Brown et al., 1993; Ma and Wallace, 2000).

Thus, for all its functions on gastric mucosal, NO has been shown to be beneficial in gastric ulcer healing. In fact, previous studies have demonstrated that NOS inhibition by *N*(G)-nitro- L-arginine (L-NNA) or *N*(G)-monomethyl-L-arginine (L-NMMA) significantly delayed ulcer healing, impaired angiogenesis in the granulation tissue and reduced gastric blood flow around the ulcer (Konturek et al., 1993). Also, NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) has been showed to increase ethanol-induced gastric lesions in mice (Bulut et al., 1999; Silva et al., 2009). On the other hand, administration of an NO donor (glyceryl trinitrate) or L-arginine (the substrate of NOS) significantly reverses NOS inhibitor induced delayed healing and enhances healing (Elliott et al., 1995; Brzozowski et al., 1995; Moura Rocha et al.; 2010).

In addition to local mucosal protection factors, gastric mucosal defense is also regulated, at least in part, by the central nervous system and hormonal factors (Stroff et al., 1995; Peskar, 2001; Mózsik et al., 2001). Gastric mucosa and submucosal vessels are innervated by primary afferent sensory neurons and nerves forming a dense plexus at the mucosal base. Afferent neurons constitute an emergency system that is requested when the gastric mucosa is endangered by noxious agents. Thus, activation of these nerves in presence of gastric acid promotes releasing of neurotransmitters such as substance P and calcitonin gene-related peptide (CGRP), which relax the smooth muscle surrounding the arterioles, resulting in an elevation of mucosal blood flow, increase in mucus gel and surface cell intracellular pH in stomach. This mucosal protective action occurs most likely through vasodilatation of submucosal vessels mediated by NO generation. In this sense, interference with any aspect of the sensory innervations impairs the hyperemic response and therefore diminishes resistance of the gastric mucosa to injury (Tanaka et al., 1997; Holzer, 2007; Laine et al., 2008; Tulassay & Herszényi, 2010).

3. Etiologies multiples of gastric ulcer development

Despite its robust and multi-faceted nature, many factors directly related to impairment in mucosal defense can alter the epithelial barrier and encourage the formation of mucosal injury, the most important of which are acid secretion, bacteria and their products, non-

steroidal anti-inflammatory drugs, alcohol, reactive oxygen species, as well as different chemical compounds. Their effects on the gastric barrier represent important mechanisms of the pathogenesis of gastric ulcers, chronic gastritis and other gastric diseases, which are frequently generated through an imbalance between mucosal aggressive and defensive factors (Figure 2) (Wallace, 1992; Peskar & Marici, 1998; Tulassay & Herszényi, 2010).

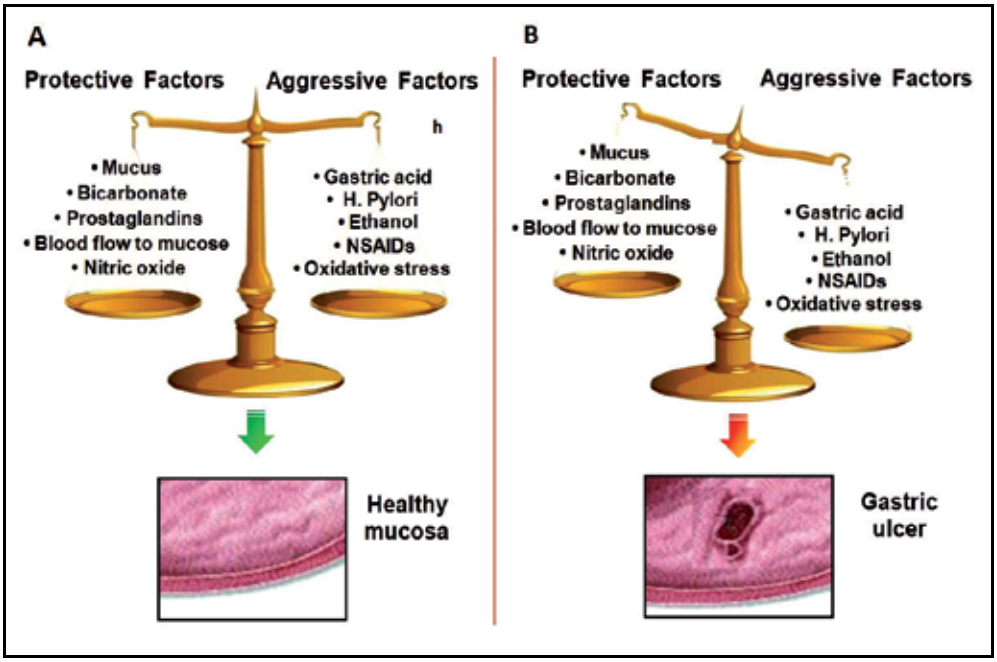

Fig. 2. (A) Healthy gastric mucosa: balance between mucosal aggressive and protective factors. (B) Gastric ulcer formation: imbalance between mucosal aggressive and protective factors.

3.1 Helicobacter Pylori

Helicobacter pylori is a common human pathogen and public health problem associated with the pathogenesis of gastritis and peptic ulcers. With a prevalence of up to 90% in developing populations, this microorganism is the second most common pathogen for human beings. It is a nonsporulating, gram-negative microaerophilic bacilli, spiral-shaped, having one to six polar-sheathed flagellae emerging from one of its rounded ends and a smooth surface (Dye et al.; 1989). This pathogen multiplies with great efficiency in the hostile environment within the stomach but survives poorly in the gastric lumen. It is mainly found under the mucous layer and in close proximity, or even attached, to gastric superficial epithelial cells, without substantial invasion of host tissue (Dubois, 1995).

H. pylori induces chronic gastritis of varying severity in infected subjects, which in around 10-15% progresses to peptic ulcer, while in 1-2% of subjects ultimately results in MALT lymphoma or gastric adenocarcinoma. The initial response to infection is an interaction of the host epithelial cells with the bacteria, however, the pathogenetic mechanisms of chronic infection with *H. pylori* and gastric ulcer are yet to be full determined (Parsonnet et al., 1991; Ernst & Gold, 2000; Calvino-Fernández & Parra-Cid, 2010).

A characteristics feature of this pathogen is the synthesis of urease, which was its first virulence factor studied. This enzyme may explain the extraordinary ability of bacteria to colonize the gastric mucosa and survive in an acid environment (Smoot, 1991). Because the ecologic niches of these bacteria are rich in urea, it catalyzes urea hydrolysis with the formation of ammonium (NH3), carbon dioxide and hydroxyl ions. By this mechanism, *H. pylori* neutralizes the surrounding gastric acid and protects itself from the strong acidicity of the stomach (Smoot, 1991). On the other hand, although the neutralization of gastric acid benefits the bacteria, metabolites from urease activity are toxic to gastric epithelial cells (Figure 3). The formed ammonium reacts with OCl^- produced by activated neutrophils to form highly toxic monochloramine (NH2Cl) in the stomach, a hallmark of *H. pylori* infection. In fact, inhibition of *H. pylori* urease has been showed significantly decrease this toxicity, suggesting that ammonia is at least partially responsible for the cytotoxicity found in association with this bacterium. Moreover, hydroxide ions are also considered toxic to gastric epithelial cells (Smoot, 1991; Handa et al., 2010).

Besides urease activity, further important virulence factors from *H. pylori* are their spiral shape and the motility of their flagellae, which render them resistant to peristaltic flushing of the gastric contents and enable them to persist in the mucous layer. Additionally, this pathogen produces other enzymes including catalase, oxidase, protease, and phospholipase, as well as it synthesizes specific adhesion proteins that enable them to adhere to mucous and epithelial cells (Boren et al., 1993; Dubois, 1995). In this context, although *H. pylori* typically colonizes the human stomach for many decades without adverse consequences, as referred above, the presence of this pathogen is associated with an increased risk of several diseases, including peptic ulcers, noncardia gastric adenocarcinoma, and gastric mucosa associated lymphoid tissue (MALT) lymphoma (Cover & Blaser, 2009).

The risks of developing gastric diseases are determined in part by the presence or absence of specific genotypes of the *H. pylori* strains with which an individual is colonized. For that reason, *H. pylori* pathogenicity may differ with respect to each of its virulence factors and this diversity is likely to contribute to variation in colonization or disease. (Dubois, 1995; Marshall & Windsor, 2005; Cover & Blaser, 2009). Thus, a number of *H pylori* strains can express multiple factors that interact with host tissue and therefore are associated with increased gastric mucosal inflammatory cell infiltration and increased gastric epithelial injury, whereas strains that lack these factors would be relatively noninteractive with the host. When virulent *H. pylori* strains are present, organisms adhere to the gastric epithelium, which disrupts membrane integrity and induces host cells to release toxic proteins, cytotoxins, platelet activating factor, , and lipopolysaccharides that all further damage the gastric mucosa. These changes would accelerate apoptosis and proliferation in the mucosal layer (Figure 3) (Crabtree, 1996; Kohda et al., 1999; Makola et al., 2007). However, it has been shown that this inflammation resolves after eradication of the infection, and presumably the concentrations of the pro-inflammatory and antisecretory cytokines also decrease. Thus, once the eradication of the bacterium is always followed by resolution of gastritis, the aim of treatment is eradication of the pathogen, defined as negative tests for the organism for one month after completion of the course of the antimicrobial (Pakodi et al., 2000; Kuipers et al., 2003; Brzozowski, et al., 2006).

Although much research and understanding have been gained in the last decades since the discovery of *H. pylori*, more questions have been raised than answered. The long road towards deciphering the fundamental mechanisms underlying the development of gastritis,

intestinal metaplasia and gastric cancer has only just begun. Thus, currently it is apparent that infection with *H. pylori* negatively influences several of the important defense mechanisms in the gastric barrier, however, the exact mechanisms leading to the development of pathological changes by *H. pylori* remain to be further investigated.

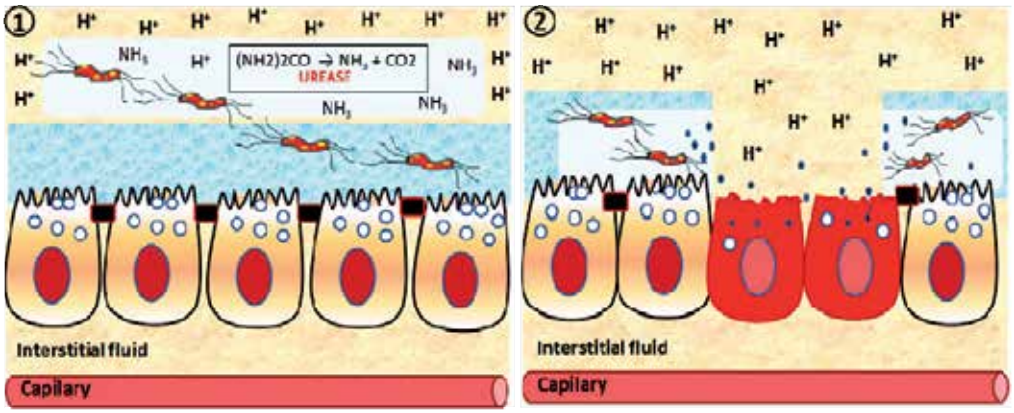

Fig. 3. Gastric ulcer formatted by *Helicobacter pylori*. (1) *H. pylori* catalyzes urea hydrolysis with the formation of ammonium (NH3) that neutralizes the surrounding gastric acid and protects itself from the strong acidicity of the stomach. (2) *H. pylori* penetrates the mucus layer of stomach, adhere the surface of gastric mucosal epithelial cells, prolifirate and finally form the infectious focus. The gastric lesion is developed by destruction of mucosa, inflammation and mucosal cell death.

3.2 Non-steroidal anti-inflammatory drugs (NSAIDs)

Another important factor directly related to gastric injury initiated by impairment in mucosal defense is the prominent non-steroidal anti-inflammatory drugs (NSAIDs) use. As the prevalence of *H. pylori* infection has declined, because of continued efforts to eradicate the organism, the prevalence of NSAID-induced ulcers has risen and is taking on greater clinical importance. Studies show that NSAIDs are among the most commonly used drugs in the world. In United States, approximately 70 million prescriptions are written each year, while in Europe these medications represent more than 7.7% of all prescriptions (Graumlich, 2001; Jones, 2001). NSAIDs use is more frequent among women and increases with age, as does the incidence of rheumatic diseases. In fact, more than 90% of prescriptions for NSAIDs are made to patients aged >65 years. The major problem with the use of these drugs is that they induce predictable gastric mucosal injury, including complications in both upper and lower gastrointestinal tract (Laine et al., 2008; Sostres et al., 2010).

The major mechanism via which NSAIDs cause ulcers and gastrointestinal complications is thought to be by inhibition of cyclooxygenase (COX), a key enzyme in the biosynthesis of prostaglandins (PGs). There are two well identified isoforms of COX, COX 1 and COX 2 (Laine et al., 2008; Sostres et al., 2010). COX 1 isoform is expressed in most tissues, producing prostaglandins that play an essential protective role in the stomach by stimulating the synthesis and secretion of mucus and bicarbonate, increasing mucosal blood flow and promoting epithelial proliferation. So, the COX-1-mediated PG synthesis is mainly responsible for maintaining gastric mucosal integrity at baseline. On the other hand, COX2

has little or no expression in most tissues but is rapidly induced in response to inflammatory stimuli. Therefore, this isoform is the primary target for anti-inflammatory drugs.

In this context, the traditional NSAIDs nonselective inhibitors of both COX-1 and COX-2, such as indomethacin or ibuprofen, cause damage in the stomach with a marked decrease in the gastric mucosal PGE2 content. This effect occurs via COX 1 isoform inhibition, creating a gastric environment that is more susceptible to topical attack by endogenous and exogenous factors (Vane & Botting, 1995). Moreover, the inhibition of the COX 1 blocks platelet production of thromboxane, which increases bleeding when an active gastrointestinal bleeding site is present (Lanas & Scheiman, 2007; Sostres et al., 2010). This contention was further supported by the fact that COX-2 selective inhibitors, which do not inhibit COX-1 at therapeutic doses, do not affect the mucosal PGs production and do not produce gross gastric damage in experimental models (Laine et al., 2008).

Therefore, the development of NSAIDs which selectively inhibit COX-2 (Coxibs), while having little to no effect on COX-1, should result in effective pain relief with reduced adverse gastrointestinal effects. In fact, data from large gastrointestinal outcomes studies reveal that Coxibs significantly decrease gastroduodenal ulcers as compared with nonselective NSAIDs. Ulcer rates were significantly decreased with 5 tested Coxibs (celecoxib, valdecoxib, rofecoxib, lumiracoxib, etoricoxib) and when these drugs were tested against any of the most commonly used NSAIDs (diclofenac, naproxen or ibuprofen) (Rostom et al., 2007). Large randomized controlled outcome trials demonstrate a considerable reduction in upper gastrointestinal complications and overall upper gastrointestinal clinical events with Coxibs compared to traditional NSAIDs (Silverstein et al., 2000; Laine et al., 2007). Moreover, the results of another large outcomes study, celecoxib vs naproxen and diclofenac in osteoarthritis patients, confirmed the significantly better safety profile of celecoxib compared with traditional NSAIDs (Singh et al., 2006).

However, has been recognized that prostaglandins derived from COX-2 can be generated at the ulcer margin and appear to play an important role in ulcer healing through triggering the cell proliferation, promotion of angiogenesis and restoration of mucosal integrity (Konturek et al., 2005, Sostres et al., 2010). These observations indicate that, in contrast to the initial concept, COX-2 plays an important role in gastric mucosal defense. Accordingly to this, experimental studies have reported that inhibition of both COX-1 and COX-2 is required for NSAID-induced gastric injury (Wallace et al., 2000; Tanaka et al., 2001; Peskar et al., 2001) and therefore Coxibs markedly decrease but do not eliminate NSAIDs associated gastric and duodenal ulceration (Silverstein et al, 2000; Sostres et al., 2010). In fact, indomethacin and similar NSAIDs, which inhibit both isoforms of the COX enzyme, produce more severe damage in gastric tissue, even gastrointestinal bleeding, than more selective drugs (Delaney et al., 2007). Therefore, indomethacin became one of the first choice drugs to produce an experimental ulcer model (Sigthorsson et al., 2000; Suleyman et al., 2004). In this sense, the fact that nimesulide, which is considered to be less selective for COX-2 compared to Coxibs, is able to inhibit NSAID-induced gastric damage, while Coxibs agents (more selective for COX-2) are unable to inhibit these ulcers, indicates that it is impossible to attribute the gastrointestinal side effects of indomethacin and other NSAIDs to the inhibition of only the COX-1 enzyme (Suleyman et al., 2002).

In addition, studies have evidenced that NSAIDs may induce tissue and cell injury by mechanisms independent of prostaglandin inhibition, which include the inhibition of phosphorylating enzymes (kinases), inhibition of oxidative phosphorylation in

mitochondria, and/or activation of apoptosis (Husain et al., 2001). These mechanisms, in combination with those related to prostaglandin suppression, lead to microvessel occlusion and subsequent hyperproduction of reactive oxygen metabolites. Such agents are then able to induce oxidative tissue injury that seems to play a prominent role in the development of mucosal ulceration caused by NSAIDs (Blandizzi et al., 2005). Furthermore, with the decrease in arachidonic acid metabolism via the COX pathway in NSAID users, arachidonic acid metabolism may be shifted to the alternative 5-lipoxygenase pathway, with a resultant increase in leukotriene production. In this way, a potential role for leukotrienes in NSAID induced gastric injury also has been postulated, since licofelone, an inhibitor of COX-1, COX-2, and 5-lipoxygenase, did not increase gastric mucosal injury (Bias et al., 2004).
Thus, because the prevalence and severity of NSAID-related gastrointestinal complications, recent efforts have been directed at the prevention of mucosal injury induced by these agents. In this sense, since NSAID and even Coxibs therapy delay the healing of active peptic ulcers, the best way to prevent mucosal injury is to avoid the use of NSAIDs or replace it with an agent less toxic to the gastroduodenal mucosa (Sostres et al., 2010).

3.3 Gastric acid secretion

For decades, surgeons were taught and believed that peptic ulcer disease was caused by acid, since the cure rate of peptic ulcer disease by acid-reduction operations (such as partial gastrectomies, gastroenterostomies or vagotomies) was substantial, impressive, and reported in the literature repeatedly, as described by Latarjet (1922) and Herrington et al. (1984). Thus, acid was meticulously measured in an attempt to better understand and treat peptic ulcer disease, the major clinical challenge at that time (Dotevall & Walan, 1971). In this sense, when pharmacologic means were developed such as histamine-2 blockers drugs (like cimetidine), which effectively eliminated acid and thus many patients found that their ulcer disease was healed, these observations validated the dictum "no acid, no ulcer"(Gustafson and Welling, 2010).
Over time, the prevalence as well as the management of these disorders has changed. With the advent of newer pharmacological therapy (potent antisecretory medications such as proton pump inhibitors) and the understanding of the role of *Helicobacter pylori* in the pathogenesis of peptic ulcer disease, more ulcers were successfully treated medically and the number of surgical cases drastically decreased (Lorentzon et al., 1987; Lindberg et al., 1990; Meyer-Rosberg et al., 1996; Fock et al., 2008; Schubert & Peura, 2008). As a result, the quantitative measurement of gastric acid secretion, for the most part, has become obsolete. Nevertheless, although there are multiple processes involved in the development of gastric lesions, the presence of acid hypersecretion continues to be a necessary condition for ulcer production and for a variety of common gastrointestinal disorders, since medical therapy for these illnesses involves both removing the injurious agent (eg, NSAIDs or *H. pilory*) and inhibiting acid secretion (Richardson et al., 1998; Schubert & Peura, 2008).
Parietal cells secrete hydrochloric acid at a concentration of approximately 160 mmol/L or pH 0.8. Acid facilitates the digestion of proteins and absorption of calcium, iron, and vitamin B-12, as well as it is the first line of mucosal defense to avoid microorganisms colonization thus preventing the bacterial overgrowth and consequent enteric infection (such as by *Helicobacter pylori*). However, when levels of acid (and pepsin) overwhelm mucosal defense mechanisms, common and potentially serious acid-related clinical conditions occur, including gastroesophageal reflux disease, Barrett's esophagus, where the

usual squamous mucosal lining becomes replaced by columnar epithelial cells of putative specific aspect, peptic ulcer disease, and stress-related erosion/ulcer disease (Schubert & Peura, 2008; Schubert, 2008).

Acid is thought to gain access to the lumen by means of channels in the mucus layer created by the relatively high intraglandular hydrostatic pressures generated during secretion (approximately 17 mm Hg) (Johansson et al., 2001). Thus, luminal acid interferes with the process of restitution, resulting in the conversion of superficial injury to deeper mucosal lesion and inactivates the acid-labile growth factors important for maintenance of mucosal integrity and repair of superficial injury. A large amount of studies show that the rate of acid secretion by the human stomach changes little with aging unless there is coexisting disease of the oxyntic mucosa such as atrophic gastritis, infection with *H. pylori* or both (Trey et al., 1997; Schubert & Peura, 2008).

To prevent acid-induced mucosal damage, gastric acid must be precisely regulated through a highly coordinated interaction of neural, hormonal, and paracrine pathways (Schubert & Peura, 2008). In this sense, the principal stimulants of acid secretion include gastrin, histamine, gastrin-releasing peptide (GRP), orexin, ghrelin, and glucocorticoids, while the main inhibitor is somatostatin, released from oxyntic and pyloric D cells (paracrine). Gastrin, released from antral G cells into the blood stream during meals, stimulates acid secretion primarily by releasing histamine from histaminesecreting enterochromaffin-like (ECL) cells. GRP, released from antral nerve fibers in response to proteins, stimulates gastrin secretion. Ghrelin and orexin appear to stimulate acid secretion, although their physiologic roles in the stomach are not known. Glucocorticoids stimulate acid secretion acting via phosphoinositide 3 kinase, serum-inducible kinase and glucocorticoid-inducible kinase (Schubert, 2008). In addition, acetylcholine (Ach), released from postganglionic enteric neurons (neuronal stimulation), acts directly stimulating parietal cell acid secretion, as well as indirectly, by eliminating the inhibitory paracrine influence of somatostatin on parietal and ECL cells (Chuang et al., 1993; Schubert, 2008; Schubert & Peura, 2008).

Acid secretion by the parietal cell involves intracellular elevation of calcium, cyclic AMP, or both followed by a cascade that triggers the translocation of the proton pump, H^+K^+-ATPase, from cytoplasmic tubulovesicles to the apical plasma membrane. This pump is an integral membrane protein that transports hydronium ions from the cytoplasm into the canaliculus of the parietal cell in exchange for potassium. Most of the adult population chronically infected with *H. pylori* produce less than normal amounts of acid probably due to increased apoptosis via secreted mediators (such as VacA cytotoxin and lipopolysaccharide), induction of proinflammatory mediators (such as IL-1b), and inhibition of the H^+K^+-ATPase activity (Schubert, 2008). This condition may cause further reduction of acid production and, eventually, atrophy of the stomach lining, which may lead not only gastric ulcer but also increased risk for stomach cancer (Suerbaum & Michetti, 2002; Peek & Crabtree, 2006). Conversely, approximately 10% to 15% of patients chronically infected with this pathogen have antral predominant inflammation and are predisposed to duodenal ulcer. They produce increased amounts of acid as a result of reduced antral somatostatin content and elevated basal and stimulated gastrin secretion. Gastrin stimulates the parietal cells in the corpus to secrete even more acid into the stomach lumen and chronically may cause the number of parietal cells to also increase. The increased acid load ulcerations the duodenum (El-Omar, 2006; Schubert & Peura, 2008).

Thus, dosed before mealtime, proton pump inhibitors drugs are the most effective acid inhibitors currently available and are the most widely prescribed class of gastrointestinal

medications. Not only can peptic ulcers be healed more rapidly with these agents, but refractory ulcers have all but disappeared. However, many clinical studies do support an accelerating effect of proton pump inhibitors on the development of atrophic gastritis in *H. pylori*-positive patients, while other evidences suggest that long-term acid suppression would result in relatively greater bacterial colonization in the corpus leading to diffuse or corpus-predominant gastritis or to acute gastroenteritis (Moayyedi et al., 2000; Rosh & Hassall, 2006; Schubert, 2008). Such observations have important implications given the extensive use of these drugs worldwide. Thus, continued progress in understanding of gastric acid secretion in health and disease is needed and this knowledge will be used to develop new more effective strategies to prevent and manage gastric disorders.

3.4 Alcohol

Throughout the world, alcohol has been used for centuries in social, medical, cultural, and religious settings. Currently, it is considered to be one of the most commonly abused drugs, related to a wide range of physical, mental, and social harms, and responsible for 3.8% of deaths and 4.6% of disability-adjusted life years lost worldwide. The World Health Organization (WHO) has estimated that there are about 2 billion people worldwide who consume alcoholic beverages and 76.3 million with diagnosable alcohol use disorders (Stermer, 2002; WHO, 2004, 2008; Rehm et al., 2009).

Among the various organ systems that mediate alcohol's effects on the human body and its health, the gastrointestinal tract plays a particularly important role. The alcohol absorption into the bloodstream occurs throughout the gastrointestinal tract and its direct contact with the mucosa can induce numerous metabolic and functional changes. These alterations may lead to marked mucosal damage, which can result in a broad spectrum of acute and chronic diseases, such as gastrointestinal bleeding and ulcers (Bode & Bode, 1997). In this context, pathogenesis of ethanol-induced gastric lesions is complex. Alcohol may interact directly with the gastric mucosa or it may act through a more general mechanism affecting the release of hormones and the regulation of nerve functions involved in acid secretion (Bode & Bode, 1997; Chari et al. 1993).

Intragastric application of absolute ethanol has long been used as a reproducible method to induce gastric mucosa lesions in experimental animals (Szabo et al., 1981; Arafa & Sayed-Ahmed, 2003). The effects of acute administration of absolute ethanol to rats and mice on the gastric mucosa are dose-dependent and the damage appears as early as 30 minutes after ingestion and reaches a peak at about 60 minutes. The ethanol-induced gastric musosal lesions and erosions are similar to those occurring in gastric ulcer (Stermer, 2002; Repetto & Llesuy, 2002). Thus, alcoholic gastritis leads to the impairment of the integrity of gastric mucosal barrier, contributing to acid reflux into the subluminal layers of the mucosa and submucosa (Oh et al., 2005).

Chari et al. (1993) relate that intravenous, oral, and intragastric alcohol at a concentration of up to 5% increases acid secretion principally by stimulating the secretion of gastrin and to a lesser extent by a direct effect on the parietal cells. On the other hand, an alcohol concentration of higher than 5% has no effect on gastric acid secretion (Stermer, 2002). Also, oxidative stress and depletion of non-protein sulfhydryls concentration, modulation of nitric oxide system and reduction of gastric mucosal blood flow frequently underlie the development of gastric lesions (Arafa & Sayed-Ahmed, 2003). According to Bode et al. (1996), the decreased formation of prostaglandins might also play a role in alcohol-induced

mucosal injury, while other studies have indicated that an alcohol-dependent increase in the production of leukotrienes also might contribute to the development of alcohol-induced damage. It is important emphasize that changes induced by short-term exposure to alcoholic beverages are rapidly reversible while prolonged alcohol exposure leads to progressive structural mucosal damage (Bode & Bode, 1997).

Oxidative stress and depletion of anti-oxidants have been considered a crucial step in alcohol-induced mucosal damage and so they have been widely investigated in a number of studies (Hirokawa 1998; La Casa et al., 2000; Arafa & Sayed-Ahmed, 2003). Ethanol treatment induces intracellular oxidative stress and produces mitochondrial permeability transition and mitochondrial depolarization, which precede cell death in gastric mucosal cells. Thus, considering that ethanol is involved in the formation of oxidative stress generated extracellularly and/or intracellylary, the cytoprotective role of anti-oxidants in the prevention and healing of gastric lesions has also been widely investigated (Santos & Rao, 2001; Silva et al., 2009). In this sense, various studies point to intracellular antioxidants, such as glutathione (an endogenous sulfhydryl compound, as described below), as significant protective agents against ethanol in gastric mucosal cells (Repetto & Llesuy, 2002). Intragastric administration of superoxide dismutase was also able to protect the gastric mucosa against the damaging effect of ethanol (Terano et al., 1989). Also, ethanol-induced oxidative stress may account for the decreased NO release, because NO may be shunted toward scavenging free radicals. In this context, response to ethanol was prevented by increased production of nitric oxide and inducible NOS (Kato et al., 2000). In addition to ethanol-induced gastrointestinal tract alterations, alcohol consumption has been linked to increased risk of tumors in the pharynx, esophagus, stomach and colon (Stermer, 2002).

3.5 Oxidative stress

It is well documented in the literature that reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, are involved in the etiology and physiopathology of several human diseases including neurodegenerative disorders, viral infections, inflammation, autoimmune pathologies, as well as in digestive disturbances such as gastrointestinal inflammation and gastric ulcer (Repetto & Llesuy, 2002).

During gastric oxidative stress, the imbalance of aggressive and defensive factors in the stomach plays a pivotal role in gastric hemorrhage and ulcer formation (Hung, 2005). Overproduction of ROS has been concerned as one of the major pathogenic factors that directly results in oxidative damage, including lipid peroxidation, protein oxidation, and DNA damage, which can lead to cell death. Additionally, these agents are known to act as second messengers to activate diverse redox-sensitive signaling transduction cascades, including mitogen-activated protein kinases (MAPKs) and downstream transcription factors such as NF-kB and AP-1, which regulate the expression of several pro-inflammatory genes and, thereby, lead to the elaboration of chemical and humoral mediators of tissue inflammation and injury (Sun & Oberley, 1996; Ali & Harty, 2009). This is frequently evidenced by pro-ulcerative factors in the stomach and gut such as *H pylori*, use of NSAIDs, ethanol, smoking, psychological stress, corticosteroid use, and loss of sleep, while defensive factors involve glutathione (GSH), an important endogenous sulfhydryl compound, and mucus biosynthesis (Hung, 2005; Olaleye et al., 2007).

In the illness state, oxidative stress of the stomach may occur and result in an elevation of mucosal lipid peroxide that are generated from the reaction of oxyradicals and cellular

polyunsaturated fatty acid, while GSH may act to prevent this aggressive action that can damage gastric mucosal cells. Malondialdehyde (MDA) is an end product resulting from peroxidation of polyunsaturated fatty acids and related esters within cell membranes, and the measurement of this substance represents a suitable index of oxidative tissue damage. On the other hand, sulfhydryl compounds such as GSH are involved in the maintenance of gastric integrity, particularly when reactive oxygen species are implicated in the pathophysiology of tissue injury (Blandizzi et al., 2005). Thus, the appearance of lipid free radicals and MDA in the blood and gastric juice could result from ROS-initiated chain reactions or initiated by indirect mechanisms that suppress the antioxidant capacity in both blood and gastric wall to scavenge ROS (Dotan et al., 2004; Tuorkey & Abdul-Aziz, 2011). In fact, numerous studies have demonstrated a decrease in GSH level in inflammatory and ulcerated gastric mucosa, as well as the protective effect of GSH on gastric damage induced by ethanol, nonsteroidal anti-inflammatory drugs, or lipopolysaccharide has been well documented (Hung, 2000; Hung, 2005; Silva et al., 2009; Al-Hashem, 2010).

On the other hand, a large body of research in both animal and human studies has examined the effect of psychological stress on the gastrointestinal tract. For instance, in accordance to Levenstein et al., (1999), susceptibility to gastric lesions is increased in rats by social stressors as premature separation of the rat pup from its mother (Ackerman et al., 1975). Also, subjects with psychological distress, self-described "stress or strain," or concrete life stressors at baseline have increased incidence of ulcer over 9 to 15 years (Levenstein et al., 1995; Levenstein et al., 1999). In this sense, according to Chang (2008), stressors can be acute or chronic and range from daily hassles to life-threatening situations like natural disasters and violence that trigger the "fight or flight" response. Over time, recurrent stress results in an increase demand on physiologic systems. Thus, several terms have been used to describe stress-related mucosal damage in critically ill patients, including stress ulcers, stress gastritis, stress erosions, hemorrhagic gastritis, erosive gastritis, and stress-related mucosal disease (Ali & Harty, 2009).

Stimulation of gastric acid secretion has historically been considered a mechanism by which physiological stress increases susceptibility to gastroduodenal ulceration. It is also known to modify gastric blood flow, which plays an important role in the gastric mucosal barrier, and to affect possible mediators such as cytokines, corticotropin-releasing hormone and thyrotropin- releasing hormone. Furthermore, stress seems to have different effects on gastric motility including delayed gastric emptying, which could increase the risk of gastric ulcer, while accelerated emptying could increase the net acid load delivered to the duodenum, enhancing the risk of duodenal ulcer. Psychological stress may also promote the growth of *H. pylori* in the duodenum if it increases duodenal acid load, since the *H. pylori*–inhibitory effects of bile seem to be reversed by acid (Levenstein et al., 1999).

4. Conclusion

Despite continuous exposure to several noxious factors, under normal conditions the gastric mucosa is able to maintain structural integrity and function. However, gastric mucosal injuries may occur when harmful factors overcome an intact mucosal defense or when the mucosal defensive mechanisms are impaired. Thus, much importance is attached to interactions and relationships among various ulcer-related factors, as well as to the individuality of the patients, including infections by *H. pylori*, alcohol and NSAIDs consume, and even smoking use, or stress-related disease.

Significant knowledge over the past three decades regarding gastric mucosal attack and defense mechanisms has led to the development of current and potential future therapies to reduce gastrointestinal injury and improve the quality of ulcer healing. Therefore, the incidence of gastric ulcers has declined, possibly as a result of the increasing use of proton pump inhibitors and decreasing rates of *Helicobacter pylori* infection. However, although there are many studies on gastroprotective therapies, their clinical effectiveness remains unclear. Thus, because gastric ulcer is a multifactorial disease, its medical management should not be based on a simple cause-effect relationship, instead a bio-psychosocial approach adjusted for the individual patient should be applied, with careful consideration of the association of this disease with many personal factors.

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2

Effects of *Helicobacter pylori* and Non-Steroidal Anti-Inflammatory Drugs on Peptic Ulcer

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1. Introduction

Peptic ulcer (PU) was a local gastrointestinal lesion due to gastric fluid, gastric acid and pepsin insult. The lesion may involve in mucosal layer, submucosal or even muscle and plasma layer in duodenum and stomach. It was characterized as not only easy to relapse but also hard to prevent (Wang *et al*., 1998). Its etiology and mechanism was very sophisticated due to the imbalance between offensive factors (gastric acid, pepsin, *H. pylori* and NSAIDs) and defensive factors (gastric mucus, bicarbonates and blood flow of gastric mucosa) (Hoogerwerf & Pasricha, 2006). There were at least 3 defensive barriers in the gastric wall to resist gastric acid and pepsin: the mucus-bicarbonates barrier that includes mucus and the bicarbonates grade in the mucus, the mucosa barrier that is the tight conjunction structure among gastric epithelial cells, and the blood flow in mucosa that provides oxygen and nutrition to mucosa and support the turnover of gastric epithelium and mucus. *H. pylori* and NSAIDs were gastric mucosa's offensive factors (Tytgat, 2000). Although they cause peptic ulcer by destroying the gastric barrier function, the mechanism was not clear. There were arguments for their simultaneous effects on peptic ulcer (Fendrick *et al*., 2001). Therefore, it is important to clarify the relationship between *H. pylori* and NSAIDs, especially when both cause simultaneously the damage to gastric mucosa.

2. *H. pylori* and peptic ulcer

H. pylori results in peptic ulcer through damaging gastric mucus and mucosa barriers, and enhancing gastric acid secretion.

2.1 Damages of gastric mucus and mucosa barriers

When *H. pylori* infects the stomach, it can produce cytotoxin-associated gene A protein (CagA), vacuolating cytotoxin A (VacA), urease, mucus enzyme, lipase and phospholipase to injure the gastric mucus and mucosa barriers, and finally results in the peptic ulcer with the combination of gastric acid and pepsin.

Vacuolating cytotoxin and cytotoxin-associated protein: VacA is expressed in *H. pylori* and results in vacuolar degeneration in gastric epithelium through interfering ion transport protein, eg. vacuolar ATPase (Leunk *et al*., 1988). CagA is up-regulated in $VacA^+$ strain and related to VacA activity. 60~70% of *H. pylori* strains express CagA and thereby induce host

cells to produce cytokines, enhance inflammatory reaction and damage the gastric mucosa (Ghira *et al.*, 1995). CagA is immunogenic and induces gastric epithelial cell to produce interleukine-8 (IL-8) that causes strong immunoreactions and results in gastric mucosa injury (Ernst *et al.*, 1994).

Urease: *H. pylori* can produce urease. This enzyme locates on the *H. pylori*'s surface and introcytoplasma (Phadnis *et al.*, 1996; Bode *et al.*, 1989) and hydrolyses urea into ammonia. Ammonia can decrease the content of mucin in mucus and destroy the integrity of the ion in mucus, and finally decline the function of mucus barrier that results in the diffusion of hydrogen ion back to the stomach wall and the erosion of mucosa layer (Hazell *et al.*, 1986). Ammonia can also deprive alpha-ketoglutaric acid that is a middle metabolic substance in Kreb's cycle, and thus, this cycle is blocked and the metabolism of cells is interfered, and finally the ATP production decreases and the Na^+-K^+ pump on the cellular membrane is out of order. It may result in cellular edema, degeneration and necrosis, and the barriers of mucus and mucosa are finally destroyed and the ulcer is formed (Marshall, 1994). The hydroxy created from ammonia and water has cytotoxic effect on gastric mucosa. A high concentration of ammonia can cause cellular vacuolar degeneration (Xu *et al.*, 1990). Urease can also cause directly the tissue damage of host (Windsor *et al.*, 2000). *H. pylori* can stimulate neutrophils and cause oxidative burst, and thereby result in the production of H_2O_2 and oxygenate oxy-chloride ion. This ion can further combine ammonia and form more toxic monochloramine that participates in the process of mucosa injury (Sarosiek *et al.*, 1989). The urea may serve as leukocyte chemotactic factor to attract inflammatory cells, cause local inflammation in the stomach, and damage indirectly the gastric epithelium.

Mucus enzyme and pepsin: *H. pylori* can produce mucolytic enzyme that causes the gastric mucous degradation. The degraded mucus losses its viscosity and elasticity and thus, allows the diffusion of hydrogen ion back to the stomach wall and the erosion of mucosa layer. The decreased viscosity of mucus benefits the movement of *H. pylori* and makes *H. pylori* easier to plant on the stomach wall. The decomposed mucus can also provide nutrition necessary to *H. pylori* (Beales *et al.*, 1996). *H. pylori* can also generate an extracellular protease that was able to split the polymer of glycoprotein in gastric mucus and wipe out the gastric mucous barrier so as to allow the gastric epithelium to contact directly with attack factors such as gastric acid, pepsin, cholic acid and drugs. Finally, the gastric epithelial erosion appears (Kawano *et al.*, 1990).

Lipase and phospholipase: the normal cell membrane is composed of double phospholipid layers. *H. pylori* can produce phospholipase A that hydrolyzes palmityl lecithin into free palmitic acid and lysolecithin and thereby destroy the integrity of the cellular membrane (Goggin *et al.*, 1991). The lipid and phospholipid in the gastric mucus play an important role in the maintenance of mucous viscosity and hydrophobic characters and in the prevention of hydrogen ion from diffusion back to the stomach wall (Goggin *et al.*, 1991). The lipase and phospholipase A can hydrolyze lipid and phospholipid in the mucus and thus obliterate the function of mucous barrier. Phospholipase A can also enhance the release of arachidonic acid and generate inflammatory media such as prostaglandin and thromboxane that induce inflammatory reaction. The metabolism of phospholipid, such as lysolecithin, also has cytotoxic effect (Lewis *et al.*, 1990).

Alcoholic dehydrogenase: Alcoholic dehydrogenase generated by *H. pylori* can oxygenate alcohol into acetaldehyde that is a strong oxidizer and causes the injury of mucosa.

Lipopolysaccharide (LPS): LPS produced by *H. pylori* can stimulate gastric epithelial cell to secrets IL-8 that can induce local inflammatory reaction in infected stomach. LPS also stimulates the pepsinogen secretion in gastric epithelium. Pepsin hydrolyses the protein in gastric epithelium, originates epithelial injury and causes ulceration (Young *et al.*, 1992). LPS from *H. pylori* has similar antigenic determinants to human being, such as Lewis type 2, e.g. Lewis X and Lewis Y. These similar antigenic determinants also distribute in the surface of parietal cell and gastric gland. The patient with *H. pylori* infection may generate antibody for Lewis antigenic determinants. Therefore, the mucosa barrier will be injured by autoimmune reaction (Appelmelk *et al.*, 1996).

Free radical: When *H. pylori* infects the stomach, it can adhere to gastric epithelium via its surface structure such as N-acetylneuraminic lactose fibril haemogglutinin, extra cellular S adhesin and Lewis B blood-group antigen adhesin, etc (Lundstrom *et al.*, 2001; Domingo *et al.*, 1999; Dundon *et al.*, 2001; McGee *et al.*, 1999) . Neutrophil chemotactic factors such as VacA, CagA and neutrophil activating protein (NAP) are released (Atherton *et al.*, 1997; Naito *et al.*, 2002; Satin *et al.*, 2000; Yoshikawa *et al.*, 2000). Furthermore, *H. pylori* stimulates gastric epithelium to secret interleukin-8 (Shiotani *et al.*, 2002; Bhattacharyya *et al.*, 2002) that is a strong neutrophil chemotactic factor. These white blood chemotactic factors result in the occurrence of inflammation. Neutrophils, monocytes, lymphocytes and macrophage may infiltrate into mucosa and release a big amount of free radicals. Lipids and proteins in epithelium are peroxidized, and the cellular structure and function were damaged, and finally the epithelial barrier was destroyed.

Hemolysin: *H. pylori* can secret hemolysin that has cytotoxic effects, induces inflammatory reaction, and results in the injury of epithelial barrier (Wetherall *et al.*, 1989).

In addition, *H.pylori* inhibits the expression of constitutive nitric oxide (cNOS) and enhances the expression of inducible NOS (iNOS) that may lead to the overproduction of NO and the excessive generation of toxic radical peroxynitrate the is involved in the gastric cell inflammatory response and cellular damage (Brzozowski *et al.*, 2006).

2.2 Increases of gastric acid

H. pylori causes the release of urease and the formation of ammonia. Ammonia increases the pH on the surface of epithelial. Consequently, the gastrin secretion increases. Gastrinemia stimulates parietal cell to secret gastric acid. Persistent gastrinemia causes the proliferation of parietal cell and the further production of gastric acid. Gastric acid is a strong attack factor and causes ulceration formation (Levi *et al.*, 1989). Other study demonstrated that *H. pylori* inhibit the secretion of somatostatin (SS) in sinus ventriculi D cells. SS inhibits the secretion of gastrin in sinus ventriculi G cells. The reduction of somatostatin weakens the control of gastrin secretion and thus prolongs the postprandial gastric acid secretion and causes ulceration (Kaneko *et al.*, 1992) . *H. pylori* has a growth inhibitory factor that inhibits the turnover of mucous epithelial cells.

3. NSAIDs and ulceration

NSAIDs, such as aspirin and indometacin, are effective drugs for anti-inflammation, anti-rheumatics, antipyretics and analgesics. Furthermore, NSAIDs, due to their effect on anti platelet aggregation, are regular prophylaxis drugs for cardiac and brain vascular diseases (Tarnawski *et al.*, 2003). NSAIDs can also decrease the rate of colonial and rectal cancer (Husain *et al.*, 2002) and Alzheimer disease (Tarnawski *et al.*, 2003b). Therefore, NSAIDs

are widely used. NSAIDs, however, have a serious side-effect causing gastric mucosa damage.

3.1 NSAIDs direct mucosa damage

Most of NSAIDs are weak organic acids as non-ion status under acidic environment in the stomach. NSAIDs can freely pass cellular membrane to intracellular where the environment is neutral. Intracellular NSAIDs can be dissociated into water soluble ion status. The intracellular concentration of NSAIDs is much higher than the extracellular one. Therefore, NSAIDs have a direct cytotoxic effect on gastric mucosa cells (Scheiman, 1996). Furthermore, NSAIDs inhibit mitochondrial oxidative phosphorylation so as to interfere energy metabolism, inhibit the expression of heat shock proteins (HSP) related to cellular membrane integrity (Wallace, 1997), originate the injury of epithelium and the cellular exfoliation, induce the release of various inflammatory factors, such as leukotriene B4 and histamine, and finally damage capillary vascular, increase vascular permeability and reduce blood flow into the mucosa (Wallace *et al.*, 1990; Wallace *et al.*, 1995). Another effect is that NSAIDs trigger gastric epithelium to release tumor necrosis factor alpha (TNF-α). TNF-α increases adhesive molecules and activates neutrophils (Wallace *et al.*, 1995), which result in the gastric mucosa neutrophil infiltration, the submucosa capillary vascular constriction, the mucosa ischemia and hypoxia, the abnormal metabolism in epithelial cells, and finally the functional damage of mucus and mucosa barrier.

3.2 NSAIDs inhibit the syntheses of prostaglandin

Arachidonic acid may be produced from phospholipids in the membrane under catalysis of phospholipase A_2. Arachidonic acid generates leukotrienes through lipoxidase and generates PGI_2 and PGE_2 through cyclo-oxygenase. Leukotrienes are involved in allergic reaction, leukocyte chemotaxis and inflammation. PGI_2 has the effects on vasodilatation and platelet aggregation. PGE_2 is capable of inducing inflammation, fever, pain, vasodilatation and gastric mucosa protection (Scheiman, 1996).

NSAIDs may inhibit COX activity, interfere the metabolism of arachidonic acid, and decrease PG syntheses (Figure 1). Therefore, NSAIDs have the effects of anti-inflammation, antipyretics and analgesics. COX has two isoforms, one is constitutive or COX-1 and another is inducible or COX-2. COX-1 constantly expresses in gastrointestinal tract and platelets, controls the syntheses of PGI_2, PGE_2 and TXA_2, regulates angiotasis, protests the mucosa of digestive tract from assault factors, and maintains the mucosa's integrity. There is little or almost no COX-2 in the mucosa of stomach and intestine and the platelets in healthy people. LPS, interleukin-1 (IL-1) and many other inflammatory factors, however, can induce its production. COX-2 can increase dramatically in local inflammatory lesion. It may result in the increase of PGI_2 and PGE_2 that also participate in inflammatory reaction. The classic NSAIDs had no selective inhibition effect on COX-1 and COX-2. The inhibition of COX-2 results in anti-inflammation, while inhibition of COX-1 causes side effects, i.e. to decrease PGI_2 and PGE_2 that have mucosa protective effects, decline the blood flow in gastric mucosa, decrease the provision of oxygen and nutrition, slow the turnover of mucosa cells, lessen the syntheses and secretion of mucus, damage the mucus and mucosa barriers, prolong the mucosa reparation, and finally cause mucosa erosion, ulceration and hemorrhage (Pawlik *et al.*, 2002). Besides above effects, aspirin prolongs the recovery of ulceration. Its mechanism underlies on the inhibition of PG syntheses (Wang *et al.*, 1989), the reduction of cellular

proliferation (Penney *et al.*, 1994) and the decrease of blood flow at the ulcer margin (Hirose *et al.*, 1991).

4. The simultaneous effects of *H. pyori* and NSAIDs on gastric mucosa

From above discussion, we know that *H. pylori* and NSAIDs are two important factors assaulting gastric mucosa and have the pivotal role in the peptic ulcer. Each has a different way to injure the gastric mucosa. It has been confirmed that *H. pylori* and NSAIDs are two independent offending factors (Grymer *et al.*, 1984). However, the exact relationship remains to be clarified (Laine, 2002). *H. pylori* and NSAIDs may be irrelevant, additive or synergistic, or possibly antagonistic (Ji *et al.*, 2003).

4.1 *H. pylori* and NSAIDs are irrelevant

Some studies demonstrated that NSAIDs should not impact *H. pylori*'s plantation (Maxton *et al.*, 1990). The infection of *H. pylori* dose not increase the ulcerative risk in long term NSAIDs user (Kim *et al.*, 1994). The epidemic investigation showed that NSAIDs did not affect the patient's susceptibility to *H. pylori* (Graham *et al.*, 1991; Barkin, 1998; Wilcox, 1997). NSAIDs dose not enhance the gastrointestinal toxicity to *H. pylori* carrier (Rybar *et al.*, 2001). Clinical data demonstrated that *H. pylori* infection did not impinge on the degree and type of gastric mucosa injury by NSAIDs (Barkin, 1998; LANZA *et al.*, 1991).

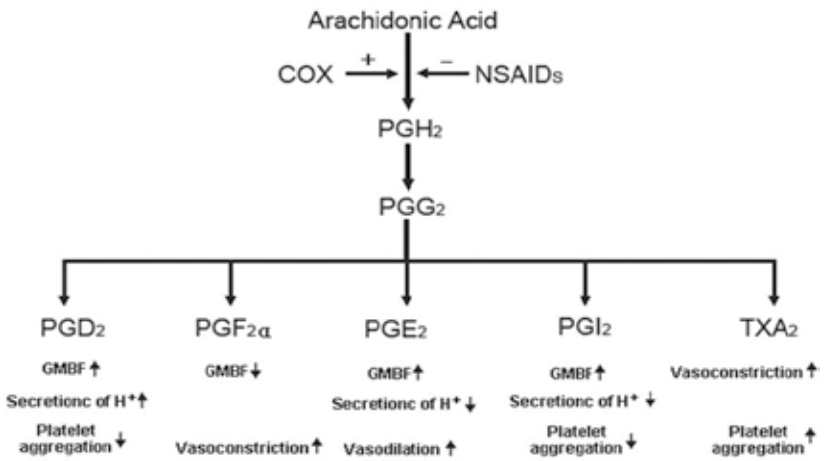

Fig. 1. NSAIDs inhibit COX activity

4.2 There is additive or synergistic relationship between *H. pylori* and NSAIDs

H. pylori and NSAIDs are strong offensive factors (Chan *et al.*, 1998). Both of them can destroy gastric barrier function. Eradication of *H. pylori* before using NSAIDs reduces the ulcerative rate (Bazzoli *et al.*, 2001). The ulceration is easier to relapse in NSAIDs takers with *H. pylori* than those without *H. pylori* infection (Chan *et al.*, 1998b). Furthermore, both *H. pylori* and NSAIDs increase permeability in the gastric epithelial cellular junction, and thus allow the gastric acid, pepsin and other endogenous offending factors to injure the gastric mucosa (Barr *et al.*, 2000). *H. pylori* and NSAIDs act synergistically through pathways of inflammation in the development of ulcers and in ulcer bleeding (Figure 2).

4.3 Antagonistic action between *H. pylori* and NSAIDs

NSAIDs have bacteriostatic and bactericidal activity against *H. pylori* (Shirin *et al.*, 2006). NSAIDs inhibit COX-1 and COX-2. This inhibition declines PG synthesis, while *H. pylori* infection stimulates gastric mucosa to express COX-2 (Takahashi *et al.*, 2000) so as to enhance PG syntheses. *H. pylori* accelerates healing of gastric ulcer induced by NSAIDs in rats due to that *H. pylori* stimulates the overexpression of COX-2 and the increase of PG synthesis, and consequently increases the production of vascular endothelial growth factor (VEGF) and the vascular proliferation. Meanwhile, PG also increases transforming growth factor alpha that causes the increase of cellular proliferation and the decrease of gastric acid (Konturek *et al.*, 2002), and finally enhances the recovery of injured gastric mucosa. The clinical trial also demonstrated that the *H. pylori* infection rate was lower in NSAIDs user than those people without NSAIDs administration (Bianchi *et al.*, 1996). It also verifies that there is antagonistic action between *H. pylori* and NSAIDs.

In conclusion, *H. pylori* and NSAIDs are individual strong factors causing peptic ulcer, and their final mechanism is to wipe out barrier function. However, the investigations are conflicts when *H. pylori* and NSAIDs coexist. The reasons leading to this conflict may be the patient's age, the different type of NSAIDs, the administration length of NSAIDs and the different strain of *H. pylori* and so on.

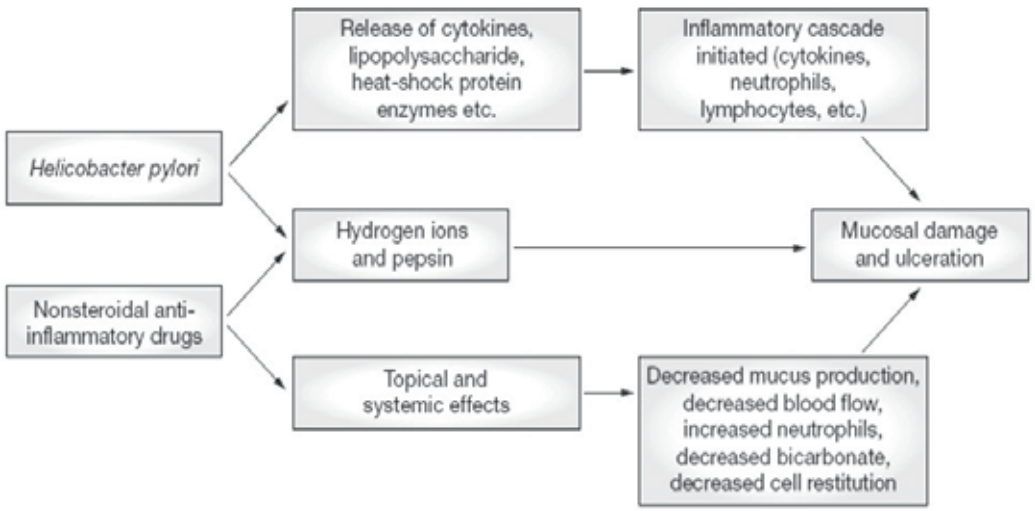

Fig. 2. *Helicobacter pylori* and nonsteroidal anti-inflammatory drugs have synergistic effects on gastric mucosal damage. Both *H. pylori* infection and NSAID use have been found to independently and significantly increase the risk of gastric and duodenal mucosal damage and ulceration. *H. pylori* and NSAIDs act synergistically through pathways of inflammation in the development of ulcers and in ulcer bleeding (Yuan *et al.*, 2006).

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3

Helicobacter Pylori Infection in Peptic Ulcer Disease

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1. Introduction

1.1 Background

Helicobacter pylori infection is one of the most common bacterial infections worldwide.[1,2] Nearly 50% of the world's population is affected.[3] Though the prevalence of this infection appears to be decreasing in many parts of the world, H. pylori remains an important factor linked to the development of peptic ulcer disease, gastric malignancy and dyspeptic symptoms.[4] Majority of H. pylori infected persons remain asymptomatic. Approximately 10-15% of the infected persons develop associated illnesses, 1 to 10% developing peptic ulcer disease, 0.1 to 3% developing gastric cancer and less than 0.01% developing gastric mucosa-associated lymphoid tissue (MALT) lymphoma.

There are several lines of evidence implicating H. Pylori in the development of gastric and duodenal ulcers.

1. H. Pylori is found in most patients who have peptic ulcers in absence of NSAID use.
2. Presence of H. Pylori is a risk factor for the development of ulcer.
3. Eradication of H. Pylori significantly reduces the recurrence of gastric and duodenal ulcers.
4. Treatment of H. Pylori infection leads to more rapid and reliable ulcer healing than does treatment with anti-secretory therapy alone.[15,21]

Early studies have estimated the rate of H. Pylori infection in patients with duodenal ulcer to be as high as 90% and in gastric ulcer to be as high as 70 to 90%.[5,6,7,29] Despite the decreasing prevalence of H. Pylori infection in developed countries, it is still an important factor in the aetiology of non-iatrogenic peptic ulcer disease. Up to 80% of duodenal ulcers and 70% of gastric ulcers are associated with H. Pylori infection. Several studies have shown that a pre- existing H. Pylori infection increases the risk for developing peptic ulcer disease.[8,9,10,11] In one study, 11% of patients with H. Pylori gastritis developed peptic ulcer disease compared to 1% of persons without gastritis.[10] Eradication of H. Pylori infection significantly reduces the recurrence of gastric and duodenal ulcers.[12,13,14,21] One study reviewed the relationship between H. Pylori eradication and reduced recurrence of duodenal and gastric ulcers. Ulcer recurrence was significantly less common among H. Pylori cured patients versus non-cured patients (6% versus 67% for patients with duodenal ulcers; 4% versus 59% for patients with gastric ulcers).[12]

H. Pylori has also been linked to the development of idiopathic thrombocytopenic purpura, ischemic heart disease and cerebrovascular accident. However, if confounding factors are taken into consideration, the strength of these associations is reduced.[16, 17, 221]

2. Bacteriology

Helicobacter pylori is a unipolar, multiflagellate, spiral shaped, microaerophilic, gram negative bacterium.[18] The bacterium was first isolated by Marshall and Warren in 1983 from gastroscopy biopsy specimens, which they described as a new species related to the genus Campylobacter.[18] The new genus Helicobacter was first published in October 1989. At least 22 species are now included in this genus, the majority of which colonise mammalian stomachs or intestines.

Helicobacter pylori is a slow growing bacterium. It can be cultured on non-selective agar media, such as blood agar, chocolate agar or on selective agar media, such as Skirrows media incubated in a humidified, micro-aerobic (5% oxygen) atmosphere at 35 to 37 degree centigrade for three to seven days.[19] Small, translucent circular colonies form and organisms are identified as Helicobacter pylori based on typical cellular morphology and positive results for oxidase, catalase and urease tests.

Under stress and nutritional deprivation, H. Pylori undergoes a morphological transformation from spiral bacilli to inactive coccoids.[19] H. Pylori cell wall enzyme Ami A, a peptidoglycan hydrolase, is involved in this morphologic transition.[20] Coccoid forms may be indicative of a dormant state. Coccoid forms may enable the organism to survive outside the human host in faeces or in water.

3. Epidemiology

Helicobacter pylori is one of the most common bacterial infections worldwide. At least 50% of the world's population is infected. The prevalence of H. Pylori infection in a community is related to three factors: 1. Rate of acquisition of infection, i.e. the incidence 2. the rate of loss of the infection 3. the prolonged prevalence of the bacterium in the gastro-duodenal mucosa between infection and eradication. [Prevalence is directly related to incidence and duration of illness].[2] Acute H. Pylori infection invariably passes undetected. Thus, the incidence of infection is determined indirectly from epidemiological studies. The incidence of H. Pylori infection is estimated to be approximately 0.5% per year in adults of developed countries. This incidence has been decreasing over time. However, the incidence of H. Pylori infection continues to be high in developing countries (3% to 10% per year).[25]

The infection is usually acquired in the first few years of life. Once acquired, infection persists indefinitely unless treated. In developing countries, the majority of children become infected during childhood and chronic infection continues during adulthood.[2,26] By age 1 year, approximately 20% are infected and by age 10 years, 50% are infected.[26] The prevalence of H. Pylori infection may be as high as 80% in adults.[30,31] However, in developed countries, such as, the United States, evidence of infection is rare before age 10, but increases to 10% between 18 and 30 years of age and to 50% in those older than age 60.[2] The higher prevalence in older age groups is thought to reflect a cohort effect related to poorer living conditions of children in previous decades. Within any age group, H. Pylori infection is more common in non-Hispanic blacks and Hispanics compared to the white population, which may be related to socioeconomic factors.[27, 28]

Important risk factors for H. Pylori infection are socioeconomic status and living conditions during childhood. Lower socioeconomic status and poor living conditions during childhood have been associated with higher risk of acquiring H. Pylori infection.[38,39,40,41] There may also be genetic susceptibility to H. Pylori infection.[42,43] Twin studies support hereditary susceptibility to infection, but this has not been proven. Individuals of certain ethnic groups including Hispanics and blacks have a higher rate of infection than Caucasians, which are not entirely explained by differences in socioeconomic status.[44]

4. Helicobacter pylori transmission

The mode of transmission of H. Pylori infection is poorly known.[1,45] Various modes of transmission have been suggested, such as person-to-person, water-borne, food-borne and zoonotic transmission.[45,46,47,48,49,50,51,52,53,54] The transmission of H. Pylori seems to be direct from person-to-person via faecal-oral or oral-oral routes.[45,46] Certain epidemiological studies have suggested water-borne and food-borne transmissions.[51,52,53,54] Zoonotic transmission has also been suggested based on isolation of H. Pylori from primates, domestic cats and sheep. [47,48,49]

Person-to person transmission is supported by the increased prevalence of infection among family members of patients with H. Pylori and among institutionalized patients. Isolation of genetically identical strains of H. Pylori from infected members of the same family and in patients in a chronic care facility further support this hypothesis.[32,33,34,35,36,37] Faecal-oral transmission is a possibility as H. Pylori has been cultured from faeces[54,55] and the organism seems to survive in water in non-culturable forms[50,51,52] (detected by PCR techniques). There is some indirect, but scarce evidence for oral-oral transmission.[45] H. Pylori has been identified in dental plaques[56], but it is unknown if this location can serve as a reservoir. Gastro-oral route of transmission through vomitus has also been suggested based on presence of bacteria in gastric secretions.[57,58]

Studies employing microbiological techniques have demonstrated that Helicobacter pylori is present in water and other environmental samples all over the world. Epidemiological studies have shown that water source and exposures related to water supply, including factors related to sewage disposal and exposure to animals, are risk factors for infection.[51] Children who swim in rivers, streams, pools, drink stream water or consume raw vegetables are more likely to be infected.[53] H. Pylori has also been detected in various food samples. So it has been hypothesised that food or water may be a reservoir in H. Pylori transmission.

Iatrogenic transmission has also been documented after the use of inadequately disinfected endoscopes and endoscopic accessories.[58]

5. Patho-physiology

5.1 Patho-physiology of gastric ulcers

Up to 70% of gastric ulcers are associated with H. Pylori infection. Three types of gastric ulcers have been described. Type I ulcers occur in the body of the stomach and are not related to other gastro-duodenal disease. Type II ulcers also occur in the body of the stomach and are associated with a duodenal ulcer scar or active ulcer. Type III ulcers occur in the immediate pre-pyloric area. Type II and III ulcers are associated with higher levels of gastric acid secretion as seen in patients with duodenal ulcers, but type I ulcers tend to be associated with normal or low levels of gastric acid secretion. Role of H. Pylori in these

different types of gastric ulcer is not known. Gastric acid secretion may not be the most important factor in the development of gastric ulcers as gastric ulcers have been seen in the presence of achlorhydria.[59] It has also been observed that basal and stimulated gastric acid secretion is within normal limits in groups of patients with gastric ulcers

5.2 Patho-physiology of duodenal ulcers

The mechanism by which Helicobacter pylori predisposes to duodenal ulcer is unclear. The pathogenesis of duodenal ulcer appears to be multi-factorial, involving an imbalance between "damaging" (e.g. acid, pepsin) and "protecting" (e.g. mucus, mucosal barrier, bicarbonate production, blood flow, cellular regeneration) factors.[60]The bacterium seems to affect different aspects of gastric and intestinal mucosal physiology that may contribute to development of ulcer disease. Disturbances in gastric acid secretion, gastric metaplasia, host inflammatory and immune response and down-regulation of various mucosal defence factors may contribute to ulcer formation. Various bacterial, host and environmental factors may also have a role in the pathogenesis of duodenal ulcer.

5.2.1 Disturbances in gastric acid secretion

Gastric acid secretion is elevated in patients with duodenal ulcers.[61,70] Helicobacter pylori infection can alter acid secretion in both directions. Acid secretion decreases temporarily during acute infection and may dwindle later if H. Pylori causes gastric atrophy.[63] In patients with duodenal ulcers, H. Pylori produces inflammation of non-acid secreting antral region of the stomach, whereas the more proximal acid-secreting fundic mucosa is relatively spared.[70,71] This may explain the increased gastric acid secretion in patients with duodenal ulcers. When compared to H. Pylori negative subjects, patients with duodenal ulcers have elevated basal acid output, peak acid output, fasting and meal-stimulated gastrin concentrations.[61,62,70]

H. Pylori infection is thought to change the physiological control of acid secretion. H. Pylori infection has been found to decrease the local expression of the inhibitory peptide somatostatin[63] and to increase the release of the acid-stimulating hormone, gastrin.[62,70] Hypergastrinemia, in addition to decreased inhibitory somatostatin, may be responsible for the increased gastric acid secretion. Hypergastrinemia may result from a decrease in the inhibitory peptide somatostatin.[64] Bacterial factors that inhibit somatostatin release have not been recognised, although TNF-alpha induced by H. Pylori infection may play a role in inhibiting somatostatin release.[65] In patients with H. Pylori infected duodenal ulcers, there is an exaggerate response to stimulation by gastrin.[61,70,71] This may be due to increased parietal cell mass in patients with duodenal ulcers[60,66,71] (Duodenal ulcer patients have approximately twice the normal parietal cell mass). But it is unclear whether or not this is due to H. Pylori infection.[67] Increased parietal cell mass may be due to trophic effects of hypergastrinemia over time or it may be related to host factors.

5.2.2 Gastric metaplasia

Elevated gastric acid secretion increases the duodenal acid load, which damages the duodenal mucosa, causing ulceration and gastric metaplasia. Gastric metaplasia occurs in the duodenum in response to acidic PH (when PH is less than 2.5).[68] Metaplastic gastric epithelium allows H. Pylori to colonise the duodenal mucosa, where it produces an acute inflammatory response. Colonization of these areas of gastric metaplasia by H. Pylori may significantly increase the risk of ulceration.[69]

However, gastric metaplasia is found in most, but not all patients with duodenal ulcers.[72,73,74] Gastric metaplasia can also be commonly found in the duodenum of healthy persons.[73,74,75] Studies have found a similar prevalence of gastric metaplasia among patients with duodenal ulcers and non ulcer dyspepsia.[76] Therefore, the role of gastric metaplasia in the pathogenesis of duodenal ulcer disease is unclear.

5.2.3 Host immune and inflammatory response

Host immune system responds to H. Pylori infection by production of inflammatory cytokines, such as interleukin(IL)-1, IL-6, tumor necrosis factor alpha, IL-8. These inflammatory cytokines may have a role in the development of duodenal ulcer.

5.2.4 Down-regulation of mucosal defence factors

i. Mucus- Mucus is a protective coat overlying the intestinal mucosa. Helicobacter pylori produces proteolytic enzymes that degrade this mucus layer, thus exposing the underlying mucosa to damaging effects of acid.[77]
ii. Bicarbonate- Most patients with duodenal ulcers have impaired proximal duodenal mucosal bicarbonate secretion. Impaired bicarbonate secretion in patients with duodenal ulcers could be caused by a cellular and/or physiological regulatory transport defect possibly related to H. Pylori infection as eradication of the infection normalises proximal mucosal bicarbonate secretion.[78]
iii. Cellular regeneration- Epidermal growth factor (EGF) and transforming growth factor-alpha(TGF-alpha) are potent gastric acid inhibitors and stimuli of mucosal growth and protection. H. Pylori may contribute to ulcerogenesis by affecting these factors for cellular regeneration as eradication of H. Pylori infection has shown to increase mucosal content and expression of TGF-alpha, EGF and EGF receptor (EGFr).[79]
iv. Blood flow- Thrombotic occlusion of surface capillaries is promoted by a bacterial platelet activating factor. Circulating platelet aggregates and activated platelets were detected in patients with H. Pylori infection. Platelet activation and aggregation may contribute to microvascular dysfunction.[84] This may play a role in producing mucosal damage and ulcer.

5.2.5 Other contributing factors

i. Bacterial factors- Various bacterial factors, such as the bacterial strain may play role in the pathogenesis of duodenal ulcer. For example, Strains with the cytotoxin-associated gene A(cag A) are associated with duodenal ulcer. Approximately 95% of patients with duodenal ulcers have cag A+ strains compared to 65% of infected patients without ulcers.[80]
ii. A specific Helicobacter pylori gene, duodenal ulcer promoting gene (dupA) is associated with an increased risk of duodenal ulcer. One study found that dup A was present in 42% of patients with duodenal ulcer versus 21% of patients with gastritis (adjusted odds ratio[OR]=3.1, 95% confidence interval; CI-1.7-5.7).[81] Its presence was also associated with more intense antral neutrophil infiltration and interleukin-8 levels and was a marker for protection against gastric atrophy, intestinal metaplasia, and gastric cancer.[81,82]
iii. Host factors- Host factors may be important in the development of duodenal ulcer. For example, patients with Helicobacter pylori who develop duodenal ulcer have higher

parietal cell mass or sensitivity to gastrin than Helicobacter pylori infected healthy persons.[60,66,71]

iv. Environmental factors, such as NSAID use and smoking may also increase the risk of duodenal ulcer in patients with Helicobacter pylori infection.[83]

5.3 Pathogenesis of H. Pylori-induced peptic ulcer disease

H. pylori causes three major gastric morphologic changes.[87] The extent and distribution of H. Pylori-induced gastritis ultimately determine the clinical outcome. The commonest morphologic change is the "simple or benign gastritis", characterized by mild pangastritis with little disruption of gastric acid secretion. This form of gastritis is commonly seen in asymptomatic people with no serious gastrointestinal disease. Up to 15 % of infected subjects develop an antral-predominant gastritis with relative sparing of the acid producing corpus mucosa. Subjects with antral-predominant gastritis have high antral inflammatory scores, high gastrin levels, relatively healthy corpus mucosa and very high acid output.[70] These abnormalities lead to the development of peptic ulcers, particularly duodenal and a large proportion of pre-pyloric ulcers. Up to 1% of infected subjects develop a corpus predominant pattern of gastritis, gastric atrophy and hypo- or achlorhydria.[85] These abnormalities develop as a direct result of the chronic inflammation induced by the infection and increase the risk of gastric cancer.

It is believed that the complex interplay between the host and the bacterium determines the disease outcome. Various bacterial factors have been described which aid in the colonisation of the gastric mucosa and subsequent modulation of the host's immune response. Studies have investigated the impact of these bacterial factors on inflammation and disease outcome. Role of bacterial factors for disease outcome remains limited, with most "virulent" strains being found in asymptomatic subjects.[86] Therefore, the variation in the host's inflammatory and immune response to infection may play a key role in determining the disease outcome. Nonetheless, the bacterium is required to initiate the host's response.

5.3.1 Bacterial factors

i. Colonisation/ Bacterial attachment

H. pylori is very sensitive to acid and it dies rapidly in the acidic PH found in the gastric lumen. Bacterial motility, urease and its ability to adhere to gastric epithelium are the factors that allow it to survive in the acidic environment.[87]

Various changes are observed in H. Pylori expression of genes following exposure to low PH. There is an increase in the expression of genes encoding proteins involved in the motility apparatus as well as genes encoding urease and proteins associated with the optimal function of the urease.[88] These observations suggest that the bacterial genes are turned on in the gastric mucosa.

H. pylori is capable of swimming freely within the mucus gel by utilising its polar flagella. It seems that the bacterium is able to sense and respond to PH gradients by swimming away from the acidic PH.[89] This allows the bacterium to swim away from the acidic PH in the gastric lumen to the close proximity of gastric epithelium, where the PH is near normal. In this environment, it enjoys the same cytoprotective mechanism as the gastric epithelium.

Other remarkable feature of H. Pylori is its ability to produce large amounts of cytosolic and cell surface associated **urease**. The urease produced by H. Pylori functions optimally at 2 different PH values, 7.2 and 3.[91]Cell-surface associated urease hydrolyses gastric luminal

urea to ammonia that helps neutralise gastric acid and form a protective cloud around the bacterium.[92] Within H. Pylori's urease gene cluster, there is a specific gene, Ure I, which encodes for a PH dependent urea channel.[22] The urea channel allows movement of urea from gastric lumen into the cytoplasm. The metabolism of urea by the cytosolic urease generates ammonia ions, which buffer hydrogen ions as they reach the cytoplasm of the organism.[93,94]

H. pylori infects gastric type epithelium to which it adheres closely. **Adherence** of the bacterium to the gastric epithelium is an important virulence factor and is necessary for the induction of pro-inflammatory responses. Adhesion to gastric epithelium may be beneficial to the bacterium in many ways. It may protect the bacterium against the mechanical clearance. Adhesion may promote invasion and persistence. The bacterium may use the cell surface as a site of replication. Increased inflammation and cellular damage caused by adhesion may release nutrients for H. Pylori. Adhesion also plays a major role in the delivery of toxins such as, Cag A and Vac A to host epithelial cell.[87]

Approximately 20% of H. Pylori in the stomach are found attached to the surfaces of mucus epithelial cells.[90] Adhesion is mediated by specific interactions between bacterial adhesin(s) and host receptor(s).[23,24] Over 30 genes in H. Pylori genomes are dedicated to the expression of outer membrane proteins (OMPs). Several of these OMPs have been classified as adhesins. Best described adhesins are BabA, Oip A and SabA. The leb-binding adhesin, BabA mediates binding to fucosylated Lewis b(Leb b) histo-blood group antigen on gastric epithelial cell.[95] Epidemiological studies also provide evidence in support of interaction between Leb and Bab A. For example, Strains of H. Pylori with BabA2 genotype are associated with inflammation, duodenal ulcer and gastric cancer.[96,97] Outer membrane protein (Oip A) coded by HPO638 gene may act as adhesins as well as promote inflammation by inducing IL-8 production.[98] However, their receptors have not yet been characterized. Sialic acid-binding adhesin (SabA) mediates binding to sialyl-dimeric-Lewix X glycosphingolipid in gastric epithelial cell.[99] Many strains of H. Pylori express a vacuolating cytotoxin Vac A, which may serve as a ligand for bacterial attachment. Although the majority of the Vac A is secreted, some may remain on the surface of the bacteria and serve as a ligand for bacterial attachment to epithelial cells, via an interaction with protein tyrosine phosphatases.[100] The Alp A and Alp B proteins have also been described as as adhesins in vitro.[101] However, there is a marked heterogeneity in H. Pylori adhesion system.[102] No individual adhesin is necessary for attachment to the gastric mucosa. Expression of adhesins is diverse between strains and variable within a single strain over time and these mechanisms of variability and adaptation are controlled at the genetic level by on/off switching of adhesin gene expression, gene inactivation or recombination.[87,102,103,104,105]

Le antigens expressed by host cells may serve as the major receptor for bacterial binding.[106,107] Bab A mediates binding to Leb receptor on host cell. However, there may be other host molecules besides Le antigens that can bind H. Pylori as it has been seen that the binding of H. Pylori to epithelial cells freshly isolated from human gastric biopsy specimens is unaffected by the expression of Le antigen[108] and individuals who do not express Leb can clearly be infected with H. Pylori.[109] One such host molecule may be class II major histocompatibility (MHC) molecule expressed on the surface of gastric epithelial cell.[110] H. Pylori can bind to class II MHC molecules on the surface of gastric epithelial cells and induce apoptosis.[111] A family of pathogen-associated molecular pattern receptors, the Toll-like receptors(TLRs) have also been examined for their role in binding of H. Pylori to the

host epithelial cells. 11 TLRs have been described.[86] Each one appears to have a different specificity for various bacterial molecules.[112] These receptors may bind bacterial products and thereby, enhance both bacterial binding and signalling. For example, TLR5 binds bacterial flagellins[113], TLR4 binds bacterial lipopolysaccharide(LPS).[114,115] The gastric trefoil protein TFF1, predominantly expressed in the gastric mucosa and the gastric mucus may serve as another receptor for H. Pylori.[116] A host cell glycosylphosphatidylinositol(GPI)-anchored glycoprotein, DAF has also been described as a potential receptor for binding H. Pylori.[117]

ii. Virulence factors

H. pylori induced gastritis and damage to the gastric mucosa is probably secondary to immune recognition of the bacteria and damage from various bacterial products.[87] Various bacterial products have been described as "toxins" based on biological activity.

Vac A

Many strains of H. Pylori express a pore forming cytotoxin, Vac A.[118] Vac A has been shown to cause cell injury in vitro and gastric tissue damage in vivo.[23,120,121] However for Vac A to cause cell damage, it must be secreted from the bacteria and delivered in an active form to host cell membranes where it assembles into pores that allow the leakage of chloride ions.[122] The Vac A gene shows a considerable genetic diversity. The activities of different alleles of the toxin vary in their toxicity. For example, strains harbouring s1 types of Vac A are highly associated with ulcers and gastric cancer.[123] M1 types of Vac A are also associated with ulcers.[123] Although the majority of Vac A is secreted, some remain associated with the bacterial cell surface. The Vac A molecules that remain on the surface of the bacteria are functional and delivered to host cells by direct contact between adhered bacteria and the host cell membrane.[124] As described earlier, Vac A on the surface of the bacteria may also serve as a ligand for bacterial attachment via an interaction with protein tyrosine phosphatases.

Several toxigenic properties of Vac A have been described that may contribute to the development of the disease.[125,126] Vac A may lead to vacuolation of epithelial cells, probably through its effect on endosomal maturation.[87] Vac A also induces apoptosis of host cells, probably through the activation of pro-apoptotic signalling molecules[127] and pore formation in mitochondrial membranes.[128] Vac A may disrupt the barrier function of tight junctions, leading to the leakage of ions and small molecules, such as iron, sugars and amino acids.[129] Vac A was also found to be a powerful inhibitor of T-cell activation in vitro.[130]

Cag A and the cag Pathogenicity island (Cag PAI)

Cag A is an important virulence factor associated with H. Pylori. It was initially thought to be the most important virulence factor as patients with antibodies against this protein showed higher rates of peptic ulcer disease[119] and gastric cancer. [131,132,133] Cag A positive strains have also been associated with increased inflammation[134], cell proliferation[135] and gastric metaplasia.[136] However, 30 to 60% of patients infected with CagA + strains do not develop any significant disease.[80] Therefore, Cag A may not be the most important virulence factor.

Cag A is a 128 to 140 kd protein, that can activate a number of signalling mechanisms and thus, affect the structure, differentiation and behaviour of epithelial cells.[87] Cag A is translocated into the host cell by the type 4 secretion system(TFSS). Genes within the cag pathogenicity island(PAI) encode proteins for the type 4 secretion apparatus(TFSS), also

referred to as Cag E.[125,137] These genes are co-transcribed and are genetically linked to Cag A.[120] TFSS allows bacterial macromolecules, such as Cag A, peptidoglycan to be translocated into the host cell.[125,137] The intact cag PAI of H. Pylori plays an important role in the pathogenesis of gastritis.[125,137,138] For example, Mutations of H. Pylori cag region were associated with decreased gastric mucosal inflammation in vivo and reduced activation of IL-8 or apoptosis in vitro.[139] It is believed that cag PAI results in the activation of nuclear factor(NF-kb) and AP-1, which in turn, regulate the expression of a wide variety of pro-inflammatory cytokines.[140,141] Cag PAI may collaborate with other bacterial factors, such as Oip A to enhance IL-8 production.[142] Bacterial peptidoglycan may also leak into the cell through the TFSS, resulting in the activation of Nod-1 mediated inflammatory response.[143] Once inside the host cell, Cag A is tyrosine phosphorylated by host Src kinases.[144] Src kinases are normally involved in controlling basic cytoskeletal process, cell proliferation and differentiation. After its tyrosine phosphorylation, it interacts with a number of host proteins, triggering growth receptor-like signalling. Through these signal transduction events, Cag A affects the proliferative activities, adhesion and cytoskeletal organisation of epithelial cells.[87,145,146,147] Cag A also perturbs cell cycle control.[148] Cag A may also have a phosphorylation independent effect on gene transcription.[149] Independently of tyrosine phosphorylation, Cag A can form complexes with several junction proteins such as Zo-1, JAM and E-cadherin and can perturb the assembly and function of both the tight junction and the adherens junctions.[150,151] Phenotypically, this leads to the deregulation of epithelial cell-cell adhesion and loss of epithelial polarity.[152] Cag A, independent of cag TFSS, can activate the nuclear factor, NF-kb leading to activation of pro-inflammatory signal and IL-8 secretion.[140,141] Cag A may also induce DNA damage and apoptosis of gastric epithelial cells via oxidative stress.[171]

Other virulence factors

Most persons infected with H. Pylori strains that produce Vac A and possess Cag A genotype nonetheless remain asymptomatic, suggesting that additional virulence factors are important in virulence. Several other H. Pylori virulence factors, such as ice A, Bab A2, Oip A have been described.[153,154,155,156,157] For example, "induced by contact with epithelium" ice A has been linked to peptic ulcers and increased mucosal concentrations of IL-8.[153,154,155] H. Pylori strains with "blood group antigen binding adhesin" Bab A2 genotype are associated with inflammation, duodenal ulcer and gastric cancer[156]. Oip A has been associated with duodenal ulcers.[157] However, the importance of these virulence factors in the life of H. Pylori is poorly understood.

iii. Mechanism of persistence

In order to colonise the human stomach, H. Pylori must overcome the physical and chemical barriers as well as innate and adaptive immune responses that are triggered in the stomach by its presence.[87] H. Pylori urease functions mainly as a protective buffering enzyme against gastric acidity. Several bacterial factors including catalase and urease antagonise innate host immune responses.[158] H. Pylori may decrease the expression of the antibacterial molecule secretory leukocyte protease inhibitor.[159] H. Pylori produces an enzyme, arginase that inhibits nitric oxide production and may favour bacterial survival.[160] Virulent strains of H. Pylori may alter mucus production[161] and phagocytosis.[162] A number of H. Pylori factors may actually contribute to reduce inflammation or recognition by the immune system. Molecular mimicry may be an important mechanism employed by the bacterium to evade recognition by the host immune system. For example, H. Pylori flagellar proteins have

evolved to avoid being recognised by toll-like receptors.[163] H. Pylori lipopolysaccharides mimic host molecules such as Lewis antigens.[164] H. Pylori virulence factors elicit both pro-inflammatory cytokines such as INF-gamma, TNF-alpha and anti-inflammatory cytokines, such as IL-4, !L-10 and transforming growth factor-beta. These anti-inflammatory cytokines may impair immune responses and may favour persistence.[86] However, these anti-inflammatory cytokines, IL-4, IL-10 and TGF-b are not expressed to the same levels as pro-inflammatory cytokines.[165,166,167,182] Hence, it has been hypothesized that H. Pylori induces a robust, but specific form of chronic inflammation that is ineffective in clearing the infection while avoiding forms of inflammation that would eliminate it.[87] This may be due to inappropriate T-cell responses or a lack of coordination in T-cell responses required for immunity.[86] A number of host polymorphisms may also lead to variations in the immune response.[87]

5.3.2 Role of host response in H. Pylori induced disease

As described earlier, the host response to H. Pylori infection is an important component in the pathogenesis of gastro-duodenal disease. H. Pylori induce chronic inflammation in the gastric mucosa, mediated by an array of pro- and anti-inflammatory cytokines. Heterogeneity in the regions of genome that control the magnitude of inflammation is thought to determine an individual's ultimate clinical outcome. For example, genetic polymorphisms in the regions controlling IL-1 beta were associated with an increased incidence of hypochlorhydria, gastric cancer and decreased occurrence of duodenal ulcer.[168,169] Il-1 beta has a profound pro-inflammatory effect and it is also a powerful acid inhibitor.[170] The pro-inflammatory genotypes of TNF-alpha, IL-8 and IL-10 were associated with the development of gastric cancer.[168]

i. Epithelial cell response to H. Pylori infection

The epithelial cell response to H. Pylori infection is determined by several variables: bacterial virulence factors, the signalling linked to specific receptors that recognise the bacterial components and the local effects of hormones, neurotransmitters, immune/inflammatory cytokines and stromal factors.[86] These responses include changes in epithelial cell morphology[175], increased epithelial cell proliferation[176], increased rates of epithelial cell death via apoptosis[177], disruption of the tight junctional complexes150, the production of inflammatory cytokines[137] and induction of numerous genes, most importantly genes involved in the regulation of the immune/inflammatory responses, epithelial cell turn over including apoptosis and proliferation and those affecting physiological properties in the stomach.[178,179,180,181] The expression of these genes in epithelial cells is modulated by transcription factors that are controlled by a series of signalling mechanisms. For example, nuclear factor kb(NF-kb) and AP-1 regulate the expression of pro-inflammatory cytokines and cellular adhesion molecules in response to infection.[182,183] These transcription factors are controlled by several signalling mechanisms including mitogen-activated protein kinases(MAPKs).[138,184] The MAPK cascades regulate several cell functions including proliferation, inflammatory responses and cell survival. ERK and P38 MAPK pathways regulate IL-8 production in gastric epithelial cells.[185,186] ERK and P38 also regulate the expression of other inflammatory response genes. Specific bacterial products as described earlier activate different transcription factors, which collaborate to enhance IL-8 production.[86] Interleukin-8 and related peptides in chemokine family secreted by gastric epithelial cells recruit and activate neutrophils and macrophages.

ii. Host responses in the lamina propria

Although H. Pylori resides predominantly in the gastric lumen, it induces a robust inflammatory and immune response. The magnitude of the host inflammatory response cannot be explained solely based upon the host epithelial cell responses to the bacterium. Significant amounts of bacterial product may leak around epithelial cells and reach the lamina propria, where it can activate phagocytes, including macrophages and neutrophils.[86] Disruption of epithelial tight junctions may enhance bacterial antigen delivery to the lamina propria. Several studies have demonstrated the ability of H. Pylori to invade gastric epithelial cells in vitro and in vivo.[172,173] Transmission electron microscopy and immunogold detection have shown H. Pylori to be in direct contact with immune cells of the lamina propria in the majority of cases of gastritis.[174] Engulfment of H. Pylori infected epithelial cells by phagocytes may be one of the mechanisms by which H. Pylori can activate the host immune response.[187]

Several bacterial products have been shown to trigger immune response within the lamina propria. A broad array of cytokines is released in the lamina propria in response to intact bacteria or bacterial factors. One such bacterial factor is H. Pylori neutrophil-activating protein, a 150 kilodalton protein, which promotes neutrophil adhesion to endothelial cells and stimulates chemotaxis of monocytes and neutrophils.[188] Bacterial urease can induce he production of IL-6 and TNF-alpha by macrophages.[189] Heat shock protein 60 induces the production of IL-6.[190] Intact bacteria can induce the production of chemokines that recruit T-cells[191] as well as IL-12[192] and IL-18[193], that favour the selection of Th1 cell. Increased IL-1, IL-6, IL-8 and TNF-alpha in response to H. Pylori infection recruit and activate monocytes and neutrophils. Release of neutrophil mediators may in turn, disrupt epithelial cells and contribute to ulcer formation.

iii. Gastric T-cell responses

Bacterial activation of epithelial cells, monocytes, macrophages and neutrophils leads to a T-helper cell type of adaptive response.[194,195] Different T-helper cell subsets emerge in response to infection with characteristic cytokine production. In H. Pylori infection, T-cell response is predominantly of T-helper cell 1(Th1) type.[138,196] Th1 cells promote cell-mediated immune responses, mainly through the production of INF-gamma and TNF-alpha while Th2 cells promote humoral immunity through the production of cytokines, such as IL-4, IL-5, IL-10 and IL-13. Previously, it was thought that the gastric mucosa is pre-conditioned to favour Th1 cell development.[165,192,197] One possible hypothesis is that H. Pylori selectively blocks Th2 development by interfering with STAT6 activation by IL-4.[198] IL-12 and IL-18 induced in response to infection may positively select for the Th1 response.[86] Activated Th1 cells produce INF-gamma and TNF-alpha which increase the expression of many pro-inflammatory genes in the epithelium including IL-8.[182] These cytokines also enhance bacterial binding[110] and may contribute to increased bacterial load.[199] Th1 cells may induce epithelial cell death through Fas-Fas L interactions.[200] In summary, Th1 activation may contribute to more severe inflammation and mucosal damage. However, Th1 type of T-cell response is a type of cell-mediated immunity against the control of intracellular pathogens.[196] It is unlikely to be effective against H. Pylori which is largely an extracellular pathogen. Hence, Th1 cell activation may produce inflammation, but not effective one which would clear the infection. In addition to Th1 cells, a subset of anti-inflammatory T-cells may be activated by H. Pylori infection. These cells may impair excessive inflammation which would otherwise lead to the clearance of the organism.[86]

iv. Gastric B-cell responses

Gastric T-cells can modulate B-cell responses, leading to the production of specific antibodies to a variety of H. Pylori antigens. During infection with H. Pylori, Ig G, Ig A and Ig M types of antibodies can be detected.[201,202] The role of these antibodies in the disease is poorly understood. Ig G class of antibody can activate complement and may contribute to immune-complex mediated inflammation.[203] In addition to producing antigen-specific antibodies, B-cells have also been shown to produce auto-reactive antibodies, that may be pathogenic.[204,205]

6. Indications for H. pylori testing

H. pylori is a common worldwide infection. The vast majority of patients with H. Pylori infection do not develop clinically significant gastroduodenal disease. Therefore, routine testing for H. Pylori is not recommended. When to test a patient for H. Pylori infection is an important question for a clinician. Guidelines from the American college of Gastroenterology [ACG] and the European Helicobacter study group [EHSG] have been published to assist clinicians in making this decision.

ACG recommendations[206]

Testing for H. Pylori should only be performed if the clinician plans to offer treatment for positive results.
Testing is indicated in patients with

1. Active peptic ulcer disease(gastric or duodenal ulcer)
2. Confirmed history of peptic ulcer disease(not previously treated for H. Pylori)
3. Gastric MALT lymphoma(low grade)
4. After endoscopic resection of early gastric cancer
5. Uninvestigated dyspepsia(depending upon H. Pylori prevalence)

The test-and-treat strategy for H. Pylori infection is a proven management strategy for patients with uninvestigated dyspepsia who are under the age of 55 yr and have no "alarm features" (bleeding, anaemia, early satiety, unexplained weight loss, progressive dysphagia, odynophagia, recurrent vomiting, family history of GI cancer, previous esophagogastric malignancy)
Deciding which test to use in which situation relies heavily upon whether a patient requires evaluation with upper endoscopy and an understanding of the strengths, weaknesses, and costs of the individual test.

EHSG recommendations[207]

Testing is indicated in patients with

1. Gastroduodenal diseases such as peptic ulcer disease and low grade gastric MALT lymphoma
2. Atrophic gastritis
3. First degree relatives of patients with gastric cancer
4. Unexplained iron deficiency anaemia
5. Chronic Idiopathic thrombocytopenic purpura (ITP)

The test-and-treat strategy using a non-invasive test is recommended in adult patients with persistent dyspepsia under the age of 45 and no "alarm symptoms".
Testing is not recommended in GORD. However, testing should be considered in patients on long-term maintenance therapy with PPIs.

Testing should be considered in patients who are naive NSIADs users.
Testing should be considered in patients who are long-term aspirin users who bleed.
Children with recurrent abdominal pain, who have a positive family history of peptic ulcer and gastric cancer should be tested for H. Pylori after exclusion of other causes.

Duodenal and gastric ulcer

Testing for H. Pylori is indicated in patients with confirmed gastric or duodenal ulcers. As described earlier, H. Pylori has been established as a major risk factor for both duodenal and gastric ulcers. H. Pylori eradication has also shown to reduce the recurrence of peptic ulcer disease. Therefore, both ACG and EHSG recommend testing patients with peptic ulcer disease for H. Pylori.

Gastroduodenal bleeding

A meta-analysis performed by Sharma et al showed that H. Pylori treatment decreased recurrent ulcer bleeding by 17% and 4% compared with ulcer healing treatment alone (bismuth, ranitidine or omeprazole) or ulcer healing treatment followed by maintenance therapy respectively.[208] Another study performed in Taiwanese patients with a history of ulcer bleeding showed that maintenance acid suppression was not routinely necessary to prevent ulcer recurrence after successful H. Pylori cure and ulcer healing.[209] Therefore, patients with a bleeding duodenal or gastric ulcer should be treated for H. Pylori.

Uninvestigated dyspepsia

The Cochrane Systematic review confirmed that there is a small benefit of eradicating H. Pylori in patients with non-ulcer dyspepsia.[210] Eradication of H. Pylori may also reduce the incidence of peptic ulcer in patients with ulcer-like functional dyspepsia.[211] Therefore, the test-and-treat strategy is recommended in patients with uninvestigated dyspepsia who are under the age of 55 yrs or 45 yrs (depending upon the specific set of guidelines) and have no "alarm features". However, this strategy has been criticised. In a placebo-controlled trial of empirical treatment involving 294 patients with uninvestigated dyspepsia and a positive H. Pylori breath test, the 1-year rate of symptom resolution was 50% in those receiving H. Pylori eradication therapy, as compared with 36% of those receiving placebo (p=0.02)[212]; 7 patients would need to receive eradication therapy for 1 patient to have a benefit. This suggests that most patients treated with the test-and-treat strategy would incur the inconvenience, costs and potential side-effects of therapy without a benefit.

Long-term maintenance therapy with PPIs

EHSG suggests H. Pylori testing in patients on long-term maintenance therapy with PPIs. Patients who are infected with H. Pylori and maintained on a PPI may be at risk for the development of atrophic gastritis.[213]However, the findings have not been confirmed in other studies.[214]

Persons using NSAIDS or Aspirin

EHSG suggests H. Pylori testing in patients who are naive NSAIDs users. A meta-analysis of five studies including 939 patients showed that H. Pylori eradication was associated with a reduced incidence of peptic ulcer in patients taking NSAIDs(OR 0.43, 95% CI 0.20-0.93). Sub-analyses demonstrated that risk reduction was evident in NSAID-naive individuals, but not for those previously taking NSAIDs.[215,219]

Iron-deficiency anaemia

EHSG recommends H. Pylori testing and eradication in patients with unexplained iron deficiency anaemia. There is emerging evidence to suggest that eradication of H. Pylori can improve iron deficiency anaemia[216,217], but the available data do not prove cause and effect.

Chronic ITP

EHSG recommends H. Pylori testing and eradication in patients with chronic ITP. The available data support an association between H. Pylori infection and ITP.[218] Studies have also shown that there is a significant increase in platelet count in patients with ITP after H. Pylori eradication.[220,222,223,224]

Prevention of gastric cancer

ACG recommends H. Pylori testing after endoscopic resection of early gastric cancer. EHSG recommends H. Pylori testing and eradication in first-degree relatives of patients with gastric cancer. Whether H. Pylori eradication reduces the risk of developing gastric cancer is unknown. H. Pylori eradication may protect against the progression of premalignant gastric lesions.[225,226] H. Pylori eradication may decrease the risk of developing cancer in individuals without precancerous lesions from high risk populations.[227] However, this may not apply to low-risk populations.

7. Diagnostic tests for H. pylori infection

Diagnostic tests for H. Pylori can be divided into endoscopic and non-endoscopic tests. Various diagnostic tests for H. Pylori infection are shown in Table 1-2. All the methods currently available for the detection of H. Pylori have their advantages and disadvantages regarding sensitivity, specificity, convenience, cost and immediacy. Choosing among these tests depends upon the clinical circumstance, the pre-test probability of infection, the accuracy of the tests, the availability and the relative costs.

General recommendations from ACG

When endoscopy is indicated, the test of first choice is the rapid urease test (RUT) in patients who have not been on a PPI within 1-2 week or an antibiotic or bismuth within 4 week of endoscopy.

For patients who have been taking a PPI, antibiotics or bismuth, it is appropriate to obtain biopsies from the gastric body and antrum for histology with or without RUT or plan testing with Urea breath test(UBT) or faecal antigen test(FAT) at a later date after withholding the offending agents for an appropriate period of time.

Culture or PCR is not routinely recommended.

UBTs and faecal antigen tests provide reliable means of identifying active H. Pylori infection before antibiotic therapy.

In the setting of acute upper GI bleeding, a positive RUT indicates the presence of active H. Pylori infection, whereas a negative RUT and/or histology should be confirmed with another test. An antibody test provides a reasonably sensitive testing option. Alternatively, patient can undergo a UBT or FAT at a later date after withholding medications that can negatively affect the sensitivity of these tests for an appropriate period of time.

Antibody testing for H. Pylori is appropriate in patients with uninvestigated dyspepsia in regions where the prevalence of H. Pylori infection is high. In low prevalence populations

(prevalence less than 20%), antibody tests should be avoided altogether or positive results should be confirmed with a test that identifies active infection, such as UBT or FAT prior to initiating eradication therapy.

Tests	Advantages	Disadvantages
Non- Endoscopic		
1.Urea Breath Test (13_C & 14_C)	• Rapid, inexpensive and identifies active infection. • Excellent PPV regardless of H. Pylori prevalence. • Useful after H. Pylori therapy.	• False negative results may be observed in patients who are taking PPIs, bismuth or antibiotics. • May not be available consistently.
2.Serological Test or Antibody Test	• Widely available. • Least expensive test. • Excellent NPV.	• The PPV is greatly influenced by the prevalence of H. pylori infection. Not recommended for confirming eradication as positive results may reflect past rather than current infection.
3. Fecal Antigen Test	• Identifies active H. pylori infection. • High positive and negative predictive values. • Useful before and after H. Pylori treatment.	• Collecting stool may be unpleasant to patients. • False negative results may be observed in patients who are taking PPIs, bismuth or antibiotics. • Polyclonal test less well validated.
Endoscopic		
1.Rapid Urease Testing	• Rapid, inexpensive and accurate in properly selected patients.	• False negative results may be observed in patients who are taking PPIs, bismuth or antibiotics.

2.Histological assessment	• High sensitivity and specificity.	• Expensive. • Requires trained personnel.
3. Culture	• Excellent specificity. • Allows determination of antibiotic sensitivities.	• Sensitivity variable. • Requires infrastructure and trained personnel. • Expensive, time consuming, difficult to perform and not widely available.
4. Polymerase Chain Reaction	• High sensitivity and specificity. • Provides opportunity to test for antibiotic sensitivity.	• False positive results may be due to contamination, homologous DNA sequences among various species, non-specific amplifications. • False negative results may be due to reaction failure. • Methodology not standardized across laboratories. • Not widely available.

Table 1.

Test	**Sensitivity**	**Specificity**
Non-Endoscopic Tests		
1.Urea Breath Test	90%-96%	88%-98%
2.Antibody Test	88%-94%	74%-88%
3.Fecal Antigen Test	86%-96%	92%-97%
Endoscopic Tests		
1.Rapid Urease Test	88%-95%	95%-100%
2.Histology	93%-96%	98%-99%
3.Culture	80%-98%	100%
4.Polymerase Chain Reaction	>95%	>95%

Table 2.

Confirmation of eradication is indicated in any patients with an H. Pylori-associated ulcer, persistent dyspeptic symptoms despite the test-and-treat strategy, H. Pylori-associated MALT lymphoma and in individuals who have undergone resection of early gastric cancer. If testing to prove eradication were performed in the setting of endoscopy, histology or the combination of histology and RUT would be appropriate.

UBT is the most reliable non-endoscopic test to document eradication of H. Pylori infection. The monoclonal FAT provides another non-endoscopic means of establishing H. Pylori cure. Testing to prove H. Pylori cure appears to be most accurate if performed at least 4 wk after the completion of antibiotic therapy.

7.1 Endoscopic diagnostic tests

Currently available biopsy-based diagnostic methods for H. Pylori infection are the rapid urease test, histology, culture and polymerase chain reaction (PCR).

7.1.1 Rapid Urease test (RUT)

Rapid Urease tests depend on the activity of bacterial urease. Endoscopic biopsy specimens are placed into an agar gel or on a reaction strip containing urea, a buffering agent and a PH sensitive dye. If H. Pylori is present, its urease cleaves urea to liberate ammonia and bicarbonate, leading to an increase in the PH and change in the colour of the dye. CLO test, Hp Fast, HUT-test, Pyloritek and Pronto Dry are some of the commercially available RUT kits. The overall performance of these tests is comparable.[228,229]

Although RUTs are rapid, inexpensive and easy to perform, their sensitivity is reduced under certain circumstances. The tests may produce a false negative result in patients with active or recent bleeding from the upper gastrointestinal tract when gastric contents are contaminated with blood.[230,231,232] Furthermore, these tests may give a false negative result in patients who have recently been taking proton pump inhibitors (PPIs), H2-receptor antagonists (H2RAs), antibiotics, or bismuth containing compounds.[228]In these patients, the RUT is usually combined with other endoscopic or non-endoscopic tests to determine the presence or absence of the infection. It is also recommended to obtain biopsies from two sites, the body of the gastric angularis and greater curvature of the antrum.[233] This may increase the sensitivity of the test. An alternative is to withhold the offending agents, such as PPIs or antibiotics for an appropriate period of time prior to endoscopy. The duration of the deleterious effects of medications on the sensitivity of the RUT is unknown. However, based on data from UBT, it is probably reasonable to withhold a PPI for 1-2 weeks and bismuth and antibiotics for four weeks prior to the RUT.[234,235]

7.1.2 Histology

Histological testing of gastric biopsy specimens is another method of diagnosing H. Pylori infection. A significant advantage of histology over other diagnostic tests is the ability to evaluate for pathological changes associated with H. Pylori infection, such as gastritis, atrophy, intestinal metaplasia and malignancy.[236]The presence of type B chronic gastritis (non-atrophic diffuse antral gastritis or atrophic pangastritis) may be used as a surrogate marker for the infection when organisms are not detected whereas the absence of chronic gastritis may be used as a marker for the absence of infection. However, the sensitivity of histology is affected by several factors, such as the site, number and size of gastric biopsies, method of staining, level of training of the examining pathologist and use of medications, such as bismuth, antibiotics and PPIs. It is therefore recommended to obtain a minimum of three biopsies, one from the greater curvature of the corpus, one from the greater curvature of the antrum and one from the angularis to maximize the diagnostic yield of histology.[237]

7.1.3 Brush cytology

Brush cytology may be used as an alternative to histology for the diagnosis of H. Pylori infection, especially in patients who have an increased risk of bleeding following forces biopsy. Data with endoscopic brush cytology are encouraging, with reported sensitivity and specificity of more than 95%.[238]

7.1.4 Culture

Bacterial culture is highly specific method for detecting active H. Pylori infection. In addition to identifying infection, it permits testing for sensitivity to anti-microbial agents.[239] However, bacterial culture is relatively insensitive[240,241] and seldom performed in routine clinical practise. Not all hospital laboratories have necessary expertise or resources available to offer routine culturing. Furthermore, culturing H. Pylori is difficult, time consuming and expensive.

Culture and sensitivity testing may be useful in patients with refractory disease since the incidence of resistance is very high in this subgroup.

7.1.5 Polymerase chain reaction (PCR)

Detection of H. Pylori by PCR is based on the amplification of a target DNA sequence in the bacterial genome. The use of PCR for the detection of H. Pylori from environmental samples is well documented.[269,270] PCR can also be used to detect H. Pylori in biopsy specimens.[261,262,265] In fact, PCR may be more sensitive than other biopsy-based diagnostic techniques in diagnosing H. Pylori infection.[264,265] PCR testing may be more sensitive than other biopsy based tests in detecting H. Pylori infection in patients who are taking PPIs, H2 RAs, antibiotics or bismuth containing compounds.[263] The testing is also highly specific and allows testing for antibiotic sensitivities.[266,267,268]

Although PCR has many advantages, its clinical use is limited due to its tendency towards false positive and false negative results. False positives can result from clinical or laboratory contamination, carry over contaminations and most importantly, similarities between the primer binding regions of H. Pylori and other organisms especially at the 3′ ends. False negatives can result from low number of target organisms, the presence of a specific PCR inhibitor, degenerated target DNA, and polymorphisms in the primer binding regions, especially at the 3′ ends, that prevent the amplification of the target DNA. Furthermore, the test is not widely available and the methodology is not standardized across laboratories.

Newer PCR techniques, such as multiplex PCR assays may reduce false positive and false negative results and thereby improve the accuracy of the test. For example, TZAM HP multiplex PCR assay amplifies 10 DNA fragments from 5 DNA regions in the genome of H. Pylori at the same time. Amplifying more than one DNA region increases the sensitivity because the probability of amplifying several selected DNA regions is much higher than the chance of amplifying only one region. It also increases the specificity because probing different loci at the same time more accurately distinguishes one pathogen from another.[265]

7.2 Non-endoscopic diagnostic tests

Currently available non-endoscopic diagnostic tests for H. Pylori infection are urea breath test (UBT), antibody test and fecal antigen test (FAT).

7.2.1 Urea breat test (UBT)

The urea breath test, like the RUT, depends on the activity of bacterial urease. The test involves the ingestion of urea, labelled with either the non-radioactive isotope ^{13}C or the radioactive isotope ^{14}C, which is converted to labelled carbon dioxide by the bacterial urease. The labelled carbon dioxide can then be measured in expired air.[242,243,244] Although the dose of radiation exposure in ^{14}C UBT is small, the ^{13}C UBT is preferred in children and women of child bearing potential.[242,243]

The UBT has excellent sensitivity and specificity[242,243] therefore, it is considered to be the most reliable test to document H. Pylori infection. It can be used to screen for infection as well as to confirm eradication after H. Pylori treatment.[244,245,246,247] However, UBTs may produce a false negative result in patients who are taking PPIs, bismuth or antibiotics. It is currently recommended to withhold bismuth and antibiotics for at least 28 days and a PPI for 7-14 days prior to UBT to reduce false negative results.[234,235,248] It is unknown whether H2RAs affect the sensitivity of the UBT[249], although these drugs are generally stopped for 24-48 hours before the UBT.

A urease blood test, using a 13 C- bicarbonate assay also reliably detects active H. Pylori infection before and after treatment. In the presence of H. Pylori, the ingestion of a 13 C-urea rich meal results in the production of labelled bicarbonate, which can be measured in serum.[250,251]

7.2.2 Serological test or antibody test

Antibody testing is based upon the detection of H. Pylori specific Ig G antibodies in serum, whole blood or urine. Antibodies to H. Pylori can be quantitatively assessed using laboratory-based ELISA and latex agglutination techniques or qualitatively assessed using office-based serological kits.

Antibody testing is cheap, widely available and easy to perform. However, there are several factors limiting its usefulness in clinical practice. The test is less accurate when compared with other diagnostic tests.[252] The test has high sensitivity (88-94%), but variable specificity (74-88%) with accuracy ranging from 83 to 98%. In general, office based serological kits are less accurate than laboratory-based quantitative tests. The PPV of the test is greatly influenced by the prevalence of H. Pylori infection.[253] In a population with low prevalence of H. Pylori infection, a positive antibody test is more likely to be a false positive test. Finally, serological tests are unreliable indicators of H. pylori status in patients who have received treatment for the infection.[254]Although antibody titres fall in most patients after successful eradication, the rate and extent of the decline are highly variable and unpredictable.

7.2.3 Fecal antigen test (FAT)

H. pylori infection can be diagnosed by identifying H. Pylori specific antigens in the stool by enzyme immunoassay with the use of polyclonal or monoclonal anti-H. Pylori antibodies.[255,256] The FAT is a reliable test to diagnose H. Pylori infection as well as to confirm eradication after treatment and can be used interchangeably with the UBT. Both polyclonal and monoclonal tests have excellent sensitivity, specificity, positive and negative predictive values for diagnosing infection before treatment.[255] However, in the post-treatment setting, only the monoclonal test appears to have sensitivity, specificity and predictive values of greater than 90%. The polyclonal test appears to have less satisfactory sensitivity and positive predictive value.[255] Therefore, in the post-treatment setting, the monoclonal FAT is more reliable than the polyclonal test. The FAT may be effective in confirming eradication as early as 14 days after treatment[257] but, the general recommendation is to perform the test more than 4 weeks after treatment.[255]

The FAT has its own disadvantages. Like the UBT, the FAT may produce a false negative result in patients who are taking PPIs, antibiotics or bismuth.[258,259] To reduce false negative results, it is generally recommended to withhold bismuth and antibiotics for at least 4 weeks

and a PPI for 2 weeks prior to the FAT. The FAT may produce a false positive result in patients with acute upper gastrointestinal bleeding.[231,260] This may be due to cross-reactivity with blood products. Furthermore, the process of stool collection may be unpleasant to patients.

8. References

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4

NSAIDs and Peptic Ulcer Disease

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Spain

1. Introduction

In 1897 Felix Hoffman, a 29-year-old scientist working for the Bayer Company patented a chemical procedure that enabled the acetylation of salicylic acid with the enough purity to be used commercially (Wallace, 1997). The new product was evaluated by Dreser Heinrich, head of marketing at Bayer, who - despite some initial misgivings - gave his approval for marketing acetylsalicylic acid under the name "Aspirin" in 1899. The new compound was commercialized by Bayer as an effective therapy for fever and aches, one that, unlike its source molecule (salicylic acid), had no gastric side effects. For over six decades aspirin remained the mainstay of non-narcotic analgesic treatment and nonsteroidal anti-inflammatory drug (*NSAIDs*) therapy. Beginning with the sale of indomethacin in 1963, used for the treatment of rheumatoid arthritis, at least twenty other *NSAIDs* with aspirin-like actions have been developed over the past half-century. Although aspirin's preeminence as an over-the-counter analgesic has been progressively displaced by new *NSAIDs,* new studies exploring its antiplatelet effect led to aspirin's development as a major antithrombotic agent (Patrono et al., 2005). Today, *NSAIDs* are popular because of their versatile effectiveness as analgesics, antipyretics, and as anti-inflammatory agents, and they remain among the most frequently prescribed medications worldwide.

The long-standing confidence in the gastric safety of aspirin went unchallenged for 40 years until 1938 when two researchers at Guy's Hospital in London, Douthwaite and Lintott, showed unquestionably that it had a major gastro-erosive activity (Douthwaite & Lintott 1938). Unfortunately, most of the *NSAIDs* currently available on the market can injure the gastric and duodenal mucosa (Cryer & Feldman, 1992; Soll et al., 1991), much like aspirin, with considerable rates of morbidity and mortality. The standard evolution of peptic ulcers resulting from *NSAIDs* ranges from resolution without intervention to the development of complications, such as bleeding and perforation. To variable degrees aspirin and *NSAIDs* inhibit the cyclooxygenase (COX) enzymes COX-1 and COX-2, which synthesize the inflammatory mediators known as prostaglandins and thromboxane. Prostaglandin inhibition plays a critical role in the pathogenesis of *NSAIDs*-induced gastric injury. Beginning ten years ago new specific inhibitors of COX-2 became available, compounds that have significantly reduced gastrointestinal (GI) side effects compared with COX-1 inhibitors (Bombardier et al., 2000; Laine et al., 2007; Silverstein et al., 2000).

Peptic ulcers are defects in the GI mucosa that extend throughout the muscularis mucosae, and which are often associated with *Helicobacter pylori* (*H. pylori*) infection or with

consumption of *NSAIDs*. As the prevalence of *H. pylori* infection has declined in Western countries, gastric ulcers have increasingly been linked to *NSAID* use, with acetylsalicylic acid (Yuan et al., 2006) being an important cause of morbidity and rising health care costs related to work loss, hospitalization, and outpatient care (Ramakrishnan & Salinas, 2007). Management of peptic ulcer disease has improved radically during the past few decades, culminating in the widespread use of proton pump inhibitor (PPI)-based triple therapy for *H. pylori* eradication. However, prescriptions for drugs such as aspirin and *NSAIDs* have also increased over this same time period and adherence to gastroprotection protocols to prevent *NSAID*-induced peptic ulcer disease still seem to be far from optimal (Jones, 2001).

2. Epidemiology

2.1 Ulcers and NSAIDs. Incidence and prevalence

In the United States alone sales of over-the-counter analgesics approach 3 billion dollars annually, with *NSAIDs* accounting for about 60% of sales (the other 40% is attributed to acetaminophen) (Laine, 2001). An estimated 14 million patients use *NSAIDs* for the relief of symptoms associated with arthritis alone (Wolfe, 1996), and among patients older that 65, as many as 70% take NSAIDs at least once a week, with at least 34% of this population taking a *NSAID* tablet daily (Talley et al., 1995).

A systematic review of the worldwide literature estimated that the annual incidence of peptic ulcer disease ranged from 0.1 to 0.19 percent for physician-diagnosed and 0.01 to 0.17 percent when based upon hospitalized patients (Sung et al., 2009). Upper GI symptoms, such as dyspepsia, occur in 15% to 60% of NSAID users, twice as often as in individuals not taking NSAIDs. The prevalence of gastric or duodenal ulcers in patients taking NSAIDs regularly is approximately 15% to 30%. The annual incidence of NSAID-related clinical upper GI events (complicated and symptomatic ulcers) is approximately 2.5% to 4.5%, with the annual incidence of serious complications (severe bleeding, perforation, and obstruction) running about 1% to 1.5% (Laine, 2002).

2.2 Trends

The epidemiology of peptic ulcer disease largely reflects environmental factors, primarily *H. pylori* infection, but also *NSAID* use and smoking. The incidence of *H. pylori* below age 50 is falling dramatically in developed countries due, in part, to improved hygiene and socioeconomic conditions; however, the prevalence of *H. pylori* infection remains high for older individuals and in certain predisposed subpopulations. The epidemiology of peptic ulcer disease has changed in the past decade due to the enormous efforts made to eradicate *H. pylori* infection, which is the single largest cause of peptic ulcers. As the prevalence of *H. pylori* infection has declined, the proportion (and actual numbers) of patients with *H. pylori*-negative idiopathic ulcers, and with ulcers attributed to the use of NSAIDs, has risen (Chow & Sung, 2007). A prospective cohort study in Hong Kong (Chan et al., 2001; Hung et al., 2005) demonstrated that the number and proportion of *H. pylori*-associated ulcers decreased from 50.3% in 1997–1998 and to 33.4% in 2000–2001. This study also showed that there was a 4.5-fold increase in the absolute number of idiopathic ulcers, from 4.1% to 18.8% during that same time period. By contrast, the relative proportion of *NSAID*-associated ulcers remained constant (45.5% in 1997–1998 versus 47.8% in 2000–2001). On the basis of these data, the incidence of non-*H. pylori*, non-*NSAID* peptic ulcer bleeding is thought to be on the rise in Asia. Similar prospective studies are awaited in the West; however, it is clear that the global

prevalence of idiopathic ulcers is on increasing. Although *H. pylori* is the predominant cause of peptic ulcer disease worldwide, it appears that there are regional differences in prevalence that cannot be explained by this infection per se. Patterns of *NSAID* use and smoking are likely to be important, too.
At a minimum, smoking clearly exacerbates *H. pylori*-associated ulcer disease. The decline in smoking in younger individuals, particularly in males, and the concomitant increase in women, may be a factor in the declining male/female ratio of ulcer disease. Smoking does not appear to be a factor in the ulcerative complications found in older women or in *NSAID*-related ulcers. It should also be borne in mind that *NSAID* use increases as a function of age and is an independent risk factor for ulcers. In addition, older subjects are more likely not only to develop complications from *NSAID*-related ulcers, but also to suffer increased morbidity and mortality from these complications because of co-morbidities.

3. Mechanisms of therapeutic action of NSAIDs

Based on their chemical structure, there are now at least 20 different *NSAIDs* from six major groups available for use in humans (Table 1). All of them are absorbed completely, have negligible first-pass hepatic metabolism, are tightly bound to albumin, and have small volumes of distribution. The half-lives of *NSAIDs* vary, though in general can be divided into "short" (less than six hours) and "long" (more than six hours) lasting drugs. These compounds differ in their doses, interaction with other drugs, and some specific side effects. The inhibition of prostaglandin synthesis via the blockade of cyclooxygenase (COX) has been widely accepted as the main mechanism of action and toxicity utilized by these compounds (Moncada et al., 1973). However, during the last decade, many groups have described a number of non-prostaglandin-mediated anti-inflammatory effects from NSAIDs, suggesting that COX inhibition is not the only explanation for either the anti-inflammatory action or the gastric toxicity observed with this group of therapeutic agents (Abramson & Weissmann, 1989; Amin et al., 1995; Kopp & Ghosh, 1994; Mahmud et al., 1996).

Non-selective nonsteroidal anti-inflammatory (NSAID) agents
Salicylate (acetylated): Aspirin
Salicylates (non-acetylated): Diflunisal, Choline magnesium trisalicylate, Salsalate
Propionic acids: Ibuprofen, Naproxen, Ketoprofen, Flurbiprofen, Oxaprozin
Acetic acids: Diclofenac, Etodolac, Tolmetin, Sulindac, Ketorolac, Indomethacin
Oxicams (enolic acids): Meloxicam, Piroxicam
Fenamates (anthranilic acids): Meclofenamic acid, Mefenamic acid
Non-acidic (naphthylalkanone): Nabumetone

Selective COX-2 inhibitors:
Celecoxib, Eterocoxib, Parecoxib

Table 1. NSAID families

3.1 COX inhibition

As stated above, NSAIDs target COX, and hence the synthesis of prostaglandins, particularly prostaglandin E2 (PGE2). This inflammatory molecule lowers pain thresholds,

and the primary goal of NSAIDs is to reduce pain. Tissue prostaglandins are produced from membrane arachidonic acid via two pathways: cyclooxygenase-1 (COX-1) and COX-2. The COX-1 pathway is the predominant constitutive pathway; prostaglandins derived from this enzyme mediate many effects, most notably they facilitate gastroduodenal cytoprotection, renal perfusion, and platelet activity. In contrast, the COX-2 pathway is inducible by inflammatory stimuli and mediates effects through prostaglandins, which results in inflammation, pain, and fever. COX-2 induces at least two orders of magnitude more PGE2 than COX-1. Specific inhibitors of COX-2 represent a major advance in the treatment of pain, particularly in patients with osteoarthritis or rheumatoid arthritis. For the most part, COX-2 inhibitors have significantly reduced gastrointestinal side effects compared with COX-1 inhibitors (Rostom et al., 2007).

All of the non-salicylate, non-COX-2 selective *NSAIDs* including aspirin can interfere with platelet aggregation and secretion (McQueen et al., 1986; O'Brien, 1968) through the inactivation of platelet COX-1. In platelets, this enzyme is a rate-limiting step in the transformation of arachidonic acid into thromboxane A2, a potent platelet-aggregating agent. However, aspirin inhibits platelet COX-1 in an irreversible manner and thus has proven beneficial in reducing the risk of secondary thrombotic cardiovascular events (Berger et al., 2008). These properties may be important enough to warrant its continued use in those patients also needing common *NSAIDs*. Thus, if needed, such patients can be continued on low dose aspirin. Administration of some *NSAIDs* may interfere with the desirable antiplatelet effects of aspirin (Catella-Lawson et al., 2001).

3.2 Nonprostaglandin-mediated effects

Although *NSAIDs*' degree of potency as inhibitors of prostaglandin synthesis *in vitro* tends to reflect their anti-inflammatory potency *in vivo*, several experimental and clinical studies suggest that prostaglandin inhibition is only part of the story. The necessary dose of any given *NSAID*, notably aspirin, which is essential for suppressing inflammation, may well exceed that required to substantially inhibit prostaglandin synthesis, at least in plasma. In this regard, salicylate, which is a weak inhibitor of COX activity, appears to be as effective as aspirin, a potent inhibitor of COX activity, in controlling inflammation in patients with rheumatoid arthritis (Brooks & Day, 1991). These and other data have led to the suggestion that these drugs are driven by prostaglandin-independent mechanistic actions, particularly in terms of their anti-inflammatory properties.

Therefore, several non-prostaglandin-mediated *NSAID*-induced mechanisms of action may also be important. These mechanisms are generally related to the ability of *NSAIDs* to insert themselves into biological membranes and disrupt a wide range of cell functions and cell-cell interactions. For example, *NSAIDs* might interfere with neutrophil-endothelial cell adherence by decreasing the availability of L-selectins in the membranes of neutrophils, thereby removing a critical step in the migration of granulocytes to sites of inflammation (Diaz-Gonzalez et al., 1995). *NSAIDs* also inhibit nuclear factor *kappa B (NF-kB)*-dependent transcription *in vitro*, resulting in a decrease in available nitric oxide synthetase (Amin et al., 1995). This enzyme produces nitric oxide in large amounts, thereby causing vasodilation, cytotoxicity, and increased vascular permeability (Hawkey, 1995). The anti-nociceptive actions of some *NSAIDs*, though not all, appear to involve L-arginine-NO-cyclic GMP-potassium channel pathways (Ortiz et al., 2003).

The role and importance of these non-prostaglandin-mediated processes in clinical inflammation remains unclear. Also unknown is whether any of these potential

mechanisms of action might explain the great variability in individual patient response to *NSAIDs*.

4. Mechanisms underlying NSAID-induced gastrointestinal toxicity

NSAIDs damage gastrointestinal mucosa by causing both local injuries and by systemically inhibiting prostaglandin production. However, current consensus on the pathogenesis of symptomatic peptic ulcer disease resulting from exposure to *NSAIDs* holds that it is mainly a consequence of systemic (post-absorptive) inhibition of GI mucosal COX activity rather than a local effect (van Oijen et al., 2008).

4.1 COX inhibition

NSAIDs induce gastrointestinal cytotoxicity through the inhibition of COX enzyme activity, a correlation which has been well established (Warner et al., 1999). In addition, while it is now known that certain prostaglandins such as *PGE2* reduce gastric acid secretion, the onset of hypochlorhydria does not entirely explain the mucosal protection observed with PGE2. This has led to the notion that prostaglandins have antisecretory-independent effects that have been collectively referred to as "cytoprotection". Some of the cytoprotective mechanisms employed by prostaglandins include stimulation of glycoprotein (mucin), bicarbonate and phospholipid production by epithelial cells, enhancement of mucosal blood flow and oxygen delivery to epithelial cells via local vasodilation, increased epithelial cell migration towards the luminal surface (restitution), and enhanced epithelial cell proliferation (Robert et al., 1979). Inhibition of the COX-1 pathway blocks production of prostaglandins in epithelial cells and, consequently, these compounds impair any protective capabilities, resulting in a gastric environment that is more susceptible to topical attack by endogenous factors, such as acid, pepsin, and bile salts. Many *NSAIDs* block COX-1 and COX-2 more or less equally (i.e., are non-selective) and thus they may impair gastric prostaglandin production even at low concentrations. Since prostaglandins are essential to both the maintenance of intact GI defenses and normal platelet function, *NSAIDs* promote ulcer formation as well as bleeding. Drugs that more selectively inhibit COX-2 than COX-1 have less suppressive effects on gastric prostaglandin synthesis. As a result, selective inhibitors of COX-2 preserve prostaglandin-mediated GI mucosal protection. However, COX-2 selective inhibitors may still block COX-1 at clinically recommended doses, and thus have the potential to also block COX-1 in the stomach and duodenum and thereby cause damage.

4.2 Nonprostaglandin-mediated effects

Most *NSAIDs* are carboxylic acid derivatives. As a result, they are not ionized at the acidic pH levels found in the gastric lumen and can thus be absorbed across the gastric mucosa. Once the drug moves from the acidic environment of the gastric lumen into the pH–neutral mucosa, the drug ionizes and is trapped temporarily in epithelial cells where it may inflict damage.

The ability to uncouple mitochondrial oxidative phosphorylation is a common characteristic of NSAIDs containing an ionizable group. NSAIDs are able to interfere with mitochondrial oxidative phosphorylation (Krause et al., 2003), reducing intracellular ATP synthesis *in vivo* (Mingatto et al., 1996), an effect that has been postulated as representing an early pathogenic event in NSAID-mediated enteropathy (Mahmud et al., 1996; Rainsford, 1980). Recently,

novel pathways, independent of COX inhibition, have been identified for some NSAIDs, as has their ability to bind and disrupt cell membranes. *In vitro* exposure of gastric epithelial cells to different concentrations of NSAIDs can result in altered cell-membrane permeability. This can lead to profound and rapid changes in cell morphology, suggesting that the cytotoxicity and biological actions of NSAIDs are mediated by the cell membrane and are not dependent upon COX (Zhou et al.).

4.3 Role of Helicobacter pylori infection

Until recently, data analysis on the role of *H. pylori* infection as a risk factor for GI bleeding in *NSAID* users was complicated by a failure, in many studies, to account for the variable influence of multiple, co-existing risk factors. Not surprisingly, therefore, these studies have yielded conflicting results (Graham et al., 1992).

A comprehensive meta-analysis of 16 case–controlled studies demonstrated that the risk of peptic ulcer bleeding was increased by a factor of 1.79 with *H. pylori* infection, by 4.85 with *NSAID* usage, and by 6.13 in the presence of both *NSAID* use and *H. pylori* infection, strongly suggesting the possibility of an additive effect (Huang et al., 2002). Further evidence of *H. pylori* infection's additive role in the context of *NSAID* use comes from trials measuring the impact of *H. pylori* eradication. Indeed, the eradication of *H. pylori* in high-risk patients prior to the initiation of *NSAID* therapy has been shown to significantly reduce the risk of subsequent ulceration (Bazzoli et al., 2001; Malfertheiner et al., 2002). Two systematic reviews have clearly shown that eradication of *H. pylori* is superior to placebo therapy in the primary prevention of peptic ulcers among *NSAID* users (risk ratio (95 % CI) 0.35 (0.20 – 0.61)) (Leontiadis et al., 2007). Using a Markov model, Leontiadis *et al.* (Leontiadis et al., 2007) showed that the most cost-effective strategy for primary prevention of *NSAID*-associated ulcers was *H. pylori* eradication in patients over age 50. Interestingly, sensitivity analysis showed that eradication therapy remained cost-effective even when the *H. pylori* prevalence was as low as 5%. However, eradication seems less effective than treatment with a maintenance PPI for preventing non-steroidal anti-inflammatory drug-associated ulcers (Vergara et al., 2005).

It must be added that many patients take *NSAIDs* intermittently and often for only short periods at a time. Whether a "test-and-treat" strategy would be cost effective for such a large population group is unknown. Furthermore, it has also been noted that eradication of *H. pylori* infection alone is not sufficient for the secondary prevention of peptic ulcer bleeding in chronic *NSAID* users (Vergara et al., 2005).

5. Risk of GI complications in patients taking different NSAIDs

Recent studies suggest that the risk of GI complications may be lower with the use of some *NSAIDs*, including ibuprofen, nabumetone, meloxicam, and etodolac, but higher with others such as sulindac, piroxicam, and ketorolac (de Abajo & Garcia Rodriguez, 2001; Garcia Rodriguez & Hernandez-Diaz, 2001; Hernandez-Diaz & Rodriguez, 2000). In the case of ibuprofen, this may be due to the use, in general, of lower analgesic doses, especially in relation to ibuprofen preparations that are available over-the-counter. Nabumetone, meloxicam, and etodolac may possess some degree of COX-2 selectivity, whereas sulindac, piroxicam, and ketorolac may owe their increased toxicity to the presence of relatively long plasma half-lives, which would thereby result in more prolonged mucosal exposure (Simon & Mills, 1980). With respect to aspirin, its use at low doses alone, in the absence of other risk

factors, is also associated with an increased risk for both GI bleeding and death from GI complications. Numerous studies in patients taking low-dose aspirin alone have shown a relative risk of 2–4 for GI bleeding (Lanas et al., 2005). Furthermore, a large percentage of patients on low-dose aspirin are elderly, have multiple co-morbidities, and cardiovascular disease, in particular, and are likely to be concurrently taking anticoagulants, *NSAIDs* and corticosteroids, any one of which will elevate their relative risk for GI events to several times that of low-dose aspirin alone. It is important to emphasize that physicians are often unaware that patients are self-medicating with low-dose aspirin when they prescribe *NSAIDs* for pain relief or anti-inflammatory effects.

In regards to COX-2 inhibitors, there have been several large randomized, controlled, outcome trials comparing COX-2 inhibitors to traditional NSAIDs. The CLASS study (Silverstein et al., 2000) compared celecoxib 400 mg b.i.d. with ibuprofen 800 mg t.i.d., or diclofenac 75 mg b.i.d., in osteoarthritis or rheumatoid arthritis patients. A non-significant 50% reduction in ulcer complications was observed in the celecoxib group compared with those who received the conventional *NSAID* after 6 months of therapy. After 1 year, however, there was little or no difference between the three groups. Another large trial, the VIGOR study (Bombardier et al., 2000), compared outcomes in rheumatoid arthritis patients taking either 500 mg of naproxen b.i.d. or 50 mg of rofecoxib daily. At 6 months, rofecoxib was associated with a significantly lower incidence of GI events (2.1 *vs.* 4.5%, $P < 0.001$), and GI complications (0.6 *vs.* 1.42 %, $P = 0.005$). The TARGET study (Schnitzer et al., 2004) compared lumiracoxib with traditional *NSAIDs* in patients with osteoarthritis. After 1 year, a significant reduction in ulcer complication rates was noted for lumiracoxib among the entire study population (0.3 *vs.* 0.9%), as well as among those who were not taking aspirin (0.2 *vs.* 0.9%), but not in those taking aspirin. In a report summarizing the results of eterocoxib, based on three prospective randomized, double-blind trials (Laine et al., 2007), 34,701 arthritic patients were treated with 60 or 90 mg of etoricoxib or 150 mg of diclofenac daily. This study included patients on low-dose aspirin and/or PPI therapy. It was found that the overall incidence of uncomplicated GI events was significantly less with etoricoxib than with diclofenac (Hazard ratio 0.69, 95 % CI; 0.57 – 0.83) ($P < 0.001$). There were no differences between the groups for complicated events (bleeding, perforation, and/or obstruction).

A Cochrane systematic review of GI safety revealed that COX-2 inhibitors produced significantly fewer gastroduodenal ulcers (relative risk, 0.26; 95 % confidence interval, 0.23 – 0.30) and ulcer complications (relative risk, 0.39; 95 % confidence interval, 0.31–0.50), as well as fewer withdrawals caused by GI symptoms when compared with nonselective *NSAIDs* (Rostom et al., 2007).

6. Clinical spectrum of gastroduodenal mucosal injury by NSAIDs

NSAIDs are valuable agents in the treatment of arthritis and many other musculoskeletal disorders, and as analgesics in a wide variety of clinical scenarios. However, as stated above, their use has been limited mainly by their association with mucosal injury to the upper gastrointestinal tract, including the development of peptic ulcer disease and its complications, most notably upper gastrointestinal hemorrhage and perforation (Cryer & Feldman, 1992; Soll et al., 1991).

6.1 Gastric damage

Upper GI symptoms, such as dyspepsia, occur in 15% to 60% of *NSAID* users, twice as often as in individuals not taking *NSAIDs*. Dyspepsia occurs in three common patterns: ulcer-like

or acid dyspepsia (e.g., burning, epigastric hunger pain with food, antacid, and antisecretory agent relief); indigestion (also called functional dyspepsia or dysmotility-like dyspepsia, with postprandial belching, bloating, epigastric fullness, anorexia, early satiety, nausea, and occasional vomiting); and reflux-like dyspepsia. These patterns overlap considerably. Although poorly correlated with endoscopic lesions and clinical events, dyspepsia and other GI symptoms limit the use of *NSAIDs*, affect quality of life, and frequently require medical co-therapy with H2-receptor antagonists or PPIs. For example, it has been estimated that 5% to 15% of patients with rheumatoid arthritis can expect to discontinue *NSAID* therapy because of dyspepsia (Singh & Triadafilopoulos, 1999).

Endoscopically visible lesions associated with *NSAID* use include subepithelial hemorrhages, erosions, and ulcers. Subepithelial hemorrhages appear as bright red areas without any clear break in the mucosa. Microscopically, they appear as large numbers of red blood cells in the superficial portion of the mucosa, beneath the epithelium. Erosions are breaks in the mucosa that remain confined therein. Subepithelial hemorrhages occur in virtually 100% of people within 15 to 30 minutes after ingestion of a single 650-mg dose of aspirin. Repeated dosing of aspirin (650 mg 4 times daily) leads to development of gastric erosions within 24 hours in virtually all patients (Laine, 1996). With longer-term therapy, adaptation may occur, although gastric erosions are still found in approximately 40% to 60% of patients taking regular doses of *NSAIDs*.

An ulcer is defined histologically as a break that extends into the submucosa or deeper, and endoscopically as a break in the mucosa of >3 mm in diameter with unequivocal depth. For this reason, only ulcers can cause serious GI complications, such as bleeding, perforation, and obstruction. Subepithelial hemorrhages and erosions do not cause major GI bleeding because they are confined to the mucosal layer, where there are no blood vessels of significant size. Furthermore, they cannot cause the most dreaded of GI complications, perforation, since perforation requires an extension of the break in the mucosa through all 4 layers of the GI tract (mucosa, submucosa, musclaris propria, and serosa) (Laine, 2002). Ulcers may be formed in healthy subjects within 1 week of regular *NSAID* use. The prevalence of ulcers in patients taking *NSAIDs* regularly is approximately 15% to 30% (Laine, 1996; Larkai et al., 1987). The cumulative incidence of *NSAID*-associated ulcers in recent double-blind trials has been as high as 45% after 6 months. Most ulcers do not cause clinically important GI events. In fact, large outcome studies of arthritis patients indicate that the annual incidence of *NSAID*-related clinical upper GI events (complicated and symptomatic ulcers) is approximately 2.5% to 4.5%, while the annual incidence of serious complications (severe bleeding, perforation, and obstruction) is about 1% to 1.5%(Bombardier et al., 2000; Silverstein et al., 2000). Although these percentages are relatively small, the consumption of *NSAIDs* is so extensive that a large number of GI events are caused by *NSAIDs*, making GI complications the most important concern limiting use of *NSAIDs*.

6.2 Duodenal damage

In contrast to the stomach, damage to the duodenal mucosa by aspirin and *NSAIDs* seems to largely depend upon gastric acid. The "classic" symptoms of a duodenal ulcer occur when acid is secreted in the absence of a food buffer. Food is usually well emptied by two to three hours after meals, although food-stimulated acid secretion can persist for three to five hours; thus, classic duodenal ulcer symptoms occur two to five hours after meals or on an empty stomach. Symptoms also occur at night when the circadian stimulation of acid secretion is maximal. The ability of alkali, food, and antisecretory agents to produce relief suggests that

acid plays a role in this process. Thus, "acid dyspepsia" is a fitting term. Symptomatic periods lasting a few weeks, followed by symptom-free periods of weeks or months, is a pattern characteristic of classic duodenal ulcers.
Numerous findings suggest that *NSAIDs* represent the most relevant factor in duodenal ulcers not associated with *H. pylori* infection. The history of *NSAID* use is more common in duodenal ulcer patients who have a normal, non-infected stomach than in those in whom the ulcer is associated with *H. pylori* gastritis. Several studies have found either that *NSAID* intake is present in 25–75% of the *H. pylori*-negative duodenal ulcer patients or that *NSAIDs* are the most frequently identifiable cause in non-infected duodenal ulcers (Gisbert & Calvet, 2009).

6.3 Death rates associated with NSAIDs

A very large study carried out by the Spanish National Health System reported a death rate of 15.3 persons per 100,000 *NSAID* users, including aspirin. Approximately 50% of the patients who died in the Spanish study had a prior history of one, or more, of the following risk factors: peptic ulceration, GI bleeding, dyspepsia, cardiac disease, or hypertension. The average age of patients dying from *NSAID* complications was 70±13.5 years and 89.7% of those who died were above age 60 (Lanas et al., 2005). In the United States the reported death rate associated with *NSAIDs* is three times higher than in Spain (Singh & Triadafilopoulos, 1999), probably because this figure was extrapolated from a small sample of rheumatoid arthritis patients and it is well-known that rheumatoid arthritis alone has been associated with increased mortality, independent of *NSAID* use.

7. Risk factors for NSAID-related GI complications

Risk factors for GI complications associated with *NSAIDs* have been identified through a series of case–control and cohort studies that compared outcomes for patients taking these agents with those of control groups. A series of nested case–control studies based on incidence rates for hospitalization for GI bleeding in patients above age 65 showed an increased risk not only for this population group (odds ratio 4.7), but also for those on higher doses of *NSAIDs* (odds ratio 8.0), those who had a relatively short-term history of *NSAID* use (less than 1 month; odds ratio 7.2), and those concurrently taking corticosteroids (odds ratio 4.4) or anticoagulants (odds ratio 12.7) (Griffin et al., 1991; Griffin et al., 1988; Piper et al., 1991; Shorr et al., 1993) . In a large study series based on autopsy findings on patients with a history of *NSAID* use, gastric and duodenal ulcers were found to be more common among those who had consumed *NSAIDs* for less than 3 months (Allison et al., 1992). Although the risk of ulcer complications decreases after the first few months of *NSAID* use, it does not disappear with long-term therapy. One approach to risk stratification has been proposed (Table 2) (Lanza et al., 2009). Gastrointestinal risk is arbitrarily stratified into low (i.e., no risk factors), moderate (presence of one or two risk factors), and high-risk groups (multiple risk factors, a history of ulcer complications, or concomitant use of corticosteroids or anticoagulants).

8. Treatment and prevention of NSAID-related GI complications

If a patient develops an ulcer while on *NSAIDs*, the relevant *NSAID(s)* should be stopped if at all possible and traditional ulcer therapy with a PPI or an H2 antagonist started. PPIs are

generally preferred as they are associated with more rapid healing. As in all patients with peptic ulcers, the individual's *H. pylori* status should also be assessed; if positive, appropriate therapy should be instituted. For patients who must remain on *NSAID* therapy or on low-dose aspirin, randomized trials have shown that ulcer healing occurs more rapidly with a PPI rather than with an H2 antagonist (Agrawal et al., 2000; Yeomans et al., 1998), misoprostol (Hawkey et al., 1998), or sucralfate (Bianchi Porro et al., 1998).

8.1 Prevention

Most experts in the field agree that patients with a recent complicated peptic ulcer are at very high risk and it is best in such cases to avoid *NSAID* treatment entirely; however, if anti-inflammatory treatment must be undertaken, a COX-2 inhibitor plus misoprostol or a PPI therapy should be employed. Patients with a history of peptic ulcer disease, with or without complications, at any time in the past, and concurrent use of aspirin (including low

High risk

1. History of a previously complicated ulcer, especially if recent
2. Multiple (>2) risk factors

Moderate risk (1-2)

1. Age > 65
2. High-dose NSAID therapy
3. A previous history of an uncomplicated ulcer
4. Concurrent use of aspirin (including low dose), corticosteroids, or anticoagulants

Low risk

1. No risk factors

H. pylori is an independent and additive risk factor and needs to be addressed separately

Table 2. Patients at increased risk for NSAID GI toxicity (Guidelines for the Prevention of NSAID-related Ulcer Complications) (Lanza et al., 2009)

dose), antiplatelet drugs (e.g., clopidrogel), anticoagulants (e.g., warfarin), or corticosteroids, or two or more risk factors also fall in the high-risk categories; here, treatment should consist of a COX-2 inhibitor and either misoprostol or PPI therapy (Lanza et al., 2009). As shown in Table 2, patients considered to be at moderate risk must be treated with a COX-2 inhibitor alone or with an *NSAID* plus misoprostol or a PPI. Patients without risk factors are at low risk for *NSAID*-related peptic ulcer complications and no protective measures are required (Lanza et al., 2009) (Table 3).

As mentioned above, multiple studies have evaluated the relationship between *H. pylori* and the risk of gastric ulcers in *NSAID* users. Based upon the available evidence, patients with a history of uncomplicated or complicated peptic ulcers (gastric, duodenal) should be tested for *H. pylori* prior to beginning a course of *NSAIDs* or low-dose aspirin therapy. If present,

Gastrointestinal risk level		
Low	Moderate	High
NSAID alone (the least ulcerogenic NSAID at the lowest effective dose)	NSAID + PPI/misoprostol	Alternative therapy if possible or COX-2 inhibitor+PPI/misoprostol

Table 3. Treatment recommendations (Guidelines for the Prevention of NSAID-related Ulcer Complications) (Lanza et al., 2009)

H. pylori should be treated with the appropriate therapy, even if it is believed that the prior ulcer was due to *NSAIDs*. In asymptomatic patients who have no history of ulcers and who are not currently taking a *NSAID*, physicians can consider *H. pylori* testing prior to beginning long-term therapy with a *NSAID*. One review of this topic found that eradication of *H. pylori* was beneficial in patients who were naïve to *NSAIDs*, while little benefit was observed in patients already taking and tolerating *NSAIDs* (Kiltz et al., 2008). This "test-and-treat" approach may be more useful in populations with a relatively high prevalence of *H. pylori* infection.

8.2 Misoprostol

Misoprostol is a synthetic prostaglandin E_1 analog that replaces the protective prostaglandins ingested during prostaglandin-inhibiting therapies. It was the first agent approved for the prevention of *NSAID*-related ulceration. Early studies in normal volunteers showed a marked reduction in the primary prevention of gastroduodenal lesion in patients receiving *NSAIDs* in combination with misoprostol, compared with those who received *NSAIDs* and a placebo (Lanza et al., 1989). A more recent meta-analysis revealed that co-therapy with misoprostol reduced the incidence of duodenal ulcers by 53% and gastric ulcers by 74%, when compared with placebo therapy (Rostom et al., 2002). Another study that compared a standard dose of misoprostol (200 mcg q.i.d.) with the PPI lansoprazole (in doses of 15 and 30 mg daily) in long-term *NSAID* users showed that 93% of patients taking misoprostol were protected from developing a gastric ulcer compared with 80% and 82% in the two lansoprazole groups, respectively, over 12 weeks, and without any statistical significance among treated groups. Patients who were ulcer free after 12 weeks of therapy were kept on the same regimen for another 12 weeks, and at the end of that time 43% of those on placebo, 83% on misoprostol, 83% on lansoprazole 30 mg, and 89% on lansoprazole 15 mg were still ulcer free (Graham et al., 2002) .

Although misoprostol has proven to be effective in the prevention of GI complications induced by *NSAIDs*, it should be noted that its usefulness is limited in clinical settings by the occurrence of GI side effects, primarily cramping and diarrhea, and by compliance problems related to multiple dosage.

8.3 Proton pump inhibitors (PPIs)

PPIs have been utilized extensively as a co-therapy for the prevention of *NSAID*-induced peptic ulcers. Two large randomized controlled trials (Hawkey et al., 1998; Yeomans et al., 1998) have been performed in osteoarthritis and rheumatoid arthritis patients comparing omeprazole with placebo therapy, misoprostol, and ranitidine in the secondary prevention

and healing of gastric and duodenal ulcers. In the first study, omeprazole (20 mg or 40 mg daily) co-therapy resulted in a significant reduction in the total number of *NSAID*-related ulcers when compared to ranitidine (150 mg orally twice a day). In the second study, omeprazol (20 mg daily) was more effective than misoprostol (800 mg daily) in preventing duodenal ulcers and in reducing gastric ulcers.

The results of two similar multicenter randomized controlled trials have recently been published jointly (Scheiman et al., 2006). They compared esomeprazole 20 or 40 mg/daily with placebo therapy in preventing ulcers in patients taking *NSAIDs* or COX-2 inhibitors over a 6-month period. In the first study, which involved 844 patients, ulcer rates were 20.4%, 5.3%, and 4.7% for placebo, esomeprazole 20 mg, and esomeprazole 40 mg, respectively. In the second study, which involved 585 patients, the respective ulcer rates were 12.3%, 5.2%, and 4.4 %. Patients in both studies were *H. pylori*-negative and were considered at-risk on the basis of age (above 60), or due to a history of documented gastric or duodenal ulcerations within 5 years of entry into the study. Both studies concluded that in at-risk patients, esomeprazole was effective at preventing ulcers in long-term users of *NSAIDs*, including COX-2 inhibitors.

Thus, although full-dose misoprostol (200 mcg q.i.d.) is very effective in preventing *NSAID*-related ulcers and their complications, as stated above, GI side effects limit its clinical use. Lower doses of misoprostol are not associated with cramps or diarrhea, but appear to be no more effective than standard PPI therapy. For all of these reasons, PPIs have assumed a dominant role in *NSAID*-related upper GI injury prophylaxis and therapy. A randomized trial of *NSAID* users who had *H. pylori* infection and prior ulcer bleeding (Chan et al., 2001) demonstrated that co-therapy with omeprazole was effective at preventing recurrent ulcer bleeding. Data from observational studies and secondary analyses of a large-scale randomized trial also indicate that PPIs reduce the risk of *NSAID*-associated ulcer bleeding. Maintenance therapy is indicated in patients who remain on or who resume *NSAID* treatment.

There is no data suggesting that any of the available PPIs is more effective than another in treating NSAID-related GI damage.

8.4 H2 receptor antagonists

In most reports, standard doses of H2 receptor antagonists have shown no effectiveness in preventing NSAID-induced gastric ulcers. Systematic reviews have shown that double-dose (e.g., famotidine 40 mg two times daily) but not single-dose H2 receptor antagonists are effective at reducing the risk of NSAID-induced endoscopic gastric ulcers (Leontiadis et al., 2007; Rostom et al., 2002). In patients taking low-dose aspirin, famotidine 20 mg twice daily can reduce the development of oesophagitis, gastric and duodenal ulcers by 80% in an average-risk population, when compared with placebo therapy (Taha et al., 2009). In contrast, a separate study from Hong Kong showed that high-dose famotidine (40 mg twice daily) was inferior to pantoprazole (20mg daily) in the prevention of gastroduodenal ulcers in patients at high risk of aspirin-related ulcers. Recurrent symptomatic or bleeding ulcers (20% versus 0%) and gastrointestinal bleeding (7.7% versus 0%) were more common in patients on famotidine than in those on pantoprazole (Ng et al., 2010). However, economic modeling suggests that co-therapy with H2 receptor antagonists may be a cost-effective strategy for the prevention of ulcer bleeding in *NSAID* users (Brown et al., 2006). Like PPIs, there have not been any randomized, clinical outcome trials that evaluate the efficacy of H2 receptor antagonists in chronic *NSAID* users.

9. Conclusions

In high-risk patients, gastrointestinal complications associated with the use of NSAIDs are often caused by the concomitant use of aspirin or multiple NSAIDs, a failure to properly identify a patient's risk factors, and the underutilization of gastroprotective agents. The latter includes the use of PPIs in patients at high risk of gastrointestinal bleeding and the eradication of *H. pylori* in patients with a prior history of ulcers. PPIs have been shown to significantly reduce both gastric and duodenal ulcers and their attendant complications in patients taking not only NSAIDs, but also COX-2 inhibitors. Misoprostol, when given at full doses (800 mg/day) is very effective in preventing ulcers, and ulcer complications, in patients taking NSAIDs. However, its usefulness is limited in clinical practice due to its GI side effects. COX-2 inhibitors are associated with a significantly lower incidence of gastric and duodenal ulcers when compared to traditional NSAIDs. However, these beneficial effects are abrogated when the patient is concomitantly taking low-dose aspirin.

10. References

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5

The Etiological Factors of Duodenal and Gastric Ulcers

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1. Introduction

1.1 Background

Peptic ulcer disease (PUD) had a tremendous effect on morbidity and mortality until the last decades of the 20th century. Development of new effective and potent acid suppressants and the discovery of Helicobacter pylori (H. pylori) are two important steps that caused a reduction in the prevalence of peptic ulcer. With the discovery of H. pylori, causes, pathogenesis and treatment of PUD have been defined again in the last 25 years. However, this condition continues to be an important clinical issue because of common use of nonsteroidal anti-inflammatory drugs (NSAID) and acetylsalicylic acid at low doses. The rare but increasingly problematic issue is H pylori-negative and NSAID-negative ulcers (1). Despite progress in diagnosis and treatment, peptic ulcer disease (PUD) remains a common reason for hospitalization and operation (2).

Peptic ulcer disease (PUD) affects 10% of the world population. Helicobacter pylori infection and the use of a nonsteroidal anti-inflammatory drug (NSAID) are the principal factors associated with PUD (3). The declining global prevalence of peptic ulcer disease (PUD) might be because of the decreasing prevalence of Helicobacter pylori (Hp) infection (4). The decreasing prevalence of H. pylori could lead to a relative increase in the number of patients with this NSAID associated and idiopathic peptic ulcer disease (IPUD (5). Another view is that incidence of peptic ulcers decreased and an increasing proportion was related to NSAID and the Mortality was high (6). It is also known that the incidence of idiopathic peptic ulcer disease has increased (7,8).

H pylori infection causes both gastric and duodenal ulcers (35-27). Current data shows that H pylori infection plays a major role in peptic ulcer disease and non-ulcer dyspepsia (9,10). Apart from these diseases, H pylori are thought to play a role in the etiology of atrophic gastritis, gastric adenocarcinoma and lymphoma. In our country, H. pylori prevalence remains an important health problem (11).

The aim of this study is to determine the etiology of patients with duodenal and gastric ulcers.

2. Materials-methods

Between April 2002 and April 2009, 140 patients who referred to our endoscopy laboratory with dyspeptic complaints for gastroscopy and diagnosed with peptic ulcer (duodenal and/or gastric) were enrolled to this prospective study. Before the procedure, medical history, cigarette smoking and alcohol consumption and nonsteroidal anti-inflammatory drugs (NSAIDs) and acetylsalicylic acid use within the last month were queried. Patients with a history of gastric operation, with malign ulcer or another malign disease and who were not willing to participate the study were excluded.

Two biopsy specimens were collected from antrum and corpus for histology and one for rapid urease testing and stool samples were analyzed for Helicobacter pylori (H pylori) antigen using Laboquick H pylori antigen test kit. A patient was classified as being H. pylori positive if any of the three test methods were positive. NSAID and/or acetylsalicylic acid use within the last month was associated with ulcer, if any. Inflammatory activity, intestinal metaplasia, atrophy and H. pylori were evaluated in histopathological examination. Serum calcium and gastrin levels were also analyzed.

SPSS 13.0 statistical program was used for statistical assessments. Mean ± standard deviation (SD) or median were used for quantitative variables. For independent group comparisons, intergroup variations were analyzed with non parametrical Mann –Whitney U test. Inter group variations were evaluated using Wilcoxon test (for dependent group comparison). Correlation analyses were performed using Pearson and Spearman correlation tests. Results with "p value" less than 0.05 were accepted as statistically significant.

3. Results

82 of the patients (58%) were male and 58% were female (42%). Mean age was 47.70 ± 15.03 (range 16-92). 62 of the patients (44%) were smoking and 18 (13%) were drinking alcohol. 132 of the patients (94%) were from urban whereas 8 (6%) were from rural areas. 14 patients (10%) had a family history of PUD, whereas 4 (0.3%) had a family history of stomach cancer. Ulcer was located in duodenum in 96 patients (69%), in stomach in 40 patients (28%), and both in duodenum and stomach in 4 patients (3%).

Rate of patients tested positive for H pylori antigen in stool, positive in urease testing and tested positive for H. pylory presence in antral and corpus samples were 48%, 52%, 67% and 60% respectively (see Table 1). 107 patients (76%) were positive for H. pylori in one of the test methods.

Method	**Incidence**
Stool sample for H pylori positive	48%
Rapid urease testing positive	52%
H pylori positive antral histology	67%
H pylori positive corpus histology	60%
H pylori positive with any method	76%

Table 1. Incidence of H. pylori in peptic ulcer using various methods.

Among 64 patients (46%) with a story of nonsteroidal antienflammatory drug (NSAID) use within the last 1 month, 48 (75%) were Hp positive and 16 were (25%) negative (see Table 2).

Mean age of patients on NSAID therapy higher 51.26 ± 15.60 (range 21-92) compared to the non-users 45.32 ±14.25 (range 16-80) ($p<0.05$).

H pylori positive PUD	107	76%
NSAID use	64	(46%)
NSAID+H pylori positive	48	(75%)
NSAİİ+ H pylori negatifliği	16	12% (25%)
Idiopathic PUD	17	12%

Table 2. Peptic ulcer (PUD) etiology

Incidence of inflammatory activity, atrophy and intestinal metaplasia were 65%, 17.5% and 11% in antral biopsies and 66%, 6.5% and 1.5% in corpus samples, respectively (see Table 2).

	Antrum	**Corpus**
Inlammatory activity	65%	66%
Atrophy	17.5%	6.5%
Intestinal metaplasia	11%	1.5%
H. pylori incidence	67%	60%

Table 3. Histology findings of the biopsy samples

Histopathologically inflammatory activity was correlated with H. pylori ($p<0.05$). Mean levels of calcium and gastrin were 9.29 ± 0.40 (7.90-10.20) and 73.96 ± 89.88 (12.86-562.50) respectively. In patients with elevated gastrin levels, no hypersecretory condition was detected. Elevated levels of gastrin were correlated with inflammatory activity and presence of H. pylori ($p<0.05$).

19 of patients (13.6%) were negative for H pylori, NSAID use and hypersecretory illness and classified as idiopathic. Mean age of these pateints were 51.52 ± 13.88 (range 16-78). Ulcer was located in the duodenum of 13 patients (68%) and in stomach of 6 patients (32%). 11 of the patients (58%) were male and 8% were female (42%). 9 of the patients (47%) were smoking and 3 (16%) were drinking alcohol. 1 patient had a family history of PUD and 1 had a family history of stomach cancer. Mean age of these patients was higher compared to patients with a known etiology ($p<0.05$), however there were no statistical differences in terms of ulcer location, gender, smoking, alcohol consumption, and family history ($p>0.5$). Mean gastrin level of 60.07 ± 64.13 (12.86-183.61) was lower compared to the patients with a known etiology ($p<0.05$) whereas calcium levels of 9.33 ± 0.6 (7.9-10.2) were similar ($p>0.5$).

4. Discussion

54 (19%) of 277 consecutive patients had evidence of peptic ulcer disease (34 gastric ulcer, 14 duodenal ulcer and 6 both gastric and duodenal ulcer) in a similar study where demographic and endoscopic characteristics of patients with Helicobacter pylori positive and negative chronic peptic ulcer disease were evaluated (12). The most common finding in this study was gastric ulcer whereas in our study, among 140 patients with PUD 96 (69%) had duodenal ulcer, 40 had (28%) gastric ulcer and 4 had (3%) both duodenal and gastric

ulcer in our study. These variations may be associated with the regional characteristics, lower number of patients evaluated in the other study and etiological differences.

Helicobacter pylori infection and the use of a nonsteroidal anti-inflammatory drug (NSAID) are the principal factors associated with PUD (3). Similarly, etiologic factor in 88% of patients in our study was H pylori and/or NSAID use.

While incidence of PUD associated with H pylori infection is decreasing especially in western countries (5,7,8), in our country most common cause is H pylori (76%). It may be associated with the fact that H. pylori prevalence remains an important health problem in our country and prevalence in the community is very high. In a study performed in our country with 9239 patients who underwent gastrointestinal endoscopy, H. pylori incidence was 41.44% using the CLO test (11).

In a study where demographic and endoscopic characteristics of peptic ulcer were evaluated, urease, culture, histology and serum anti-H pylori IgG antibody were evaluated in patients and demographic data as well as NSAID use within the last 3 months were assessed. 56% of patients were H pylori positive and 22% were using NSAIDs (70% were H pylori positive) (12). In our study H. pylori was evaluated using urease testing, H pylori antigen in stool and histology and rate of patients who are H pylori positive and using NSAIDs within the last month were higher (76% and 46% respectively). Similarly, some patients using NSAID (75%) were also H pylori positive.

H. pylori induces chronic inflammation of the gastric mucosa, but only a proportion of infected individuals develop peptic ulcer disease or gastric carcinoma Reasons underlying these observations include differences in bacterial pathogenicity as well as in host susceptibility (13). Meta-analyses showed that Helicobacter pylori eradication therapy was effective for healing and prevention of recurrence of peptic ulcers in H. pylori-positive patients and that treatment of H. pylori infection was more effective than antisecretory non-eradicating therapy (with or without long-term maintenance therapy) in preventing recurrent bleeding (14). H pylori eradication has been associated with decreased risk of gastric cancer in patients with peptic ulcer diseases (15). In our study, H pylori were also correlated with inflammatory activity. When it is considered that the H pylori associated PUD is a common disease in our country, we should give more importance to eradication therapy both for effective treatment of PUD and for cancer prophylaxis.

NSAIDs have known detrimental side effects on the gastrointestinal system. The risk is increased with older age and history of PUD. Helicobacter pylori infection and cardioprotective acetylsalicylic acid have additive risks in the presence of NSAID use (16). The development of PUD was observed earlier in the combined H pylori and NSAID group than in patients with only NSAID use and this suggests a synergic effect between the two risks factors in the development of PUD (3). In our study, 75% of patients with history of NSAID therapy were H pylori positive. This finding suggest that H pylori and NSAID usage, when together, increases the risk of PUD.

Apart from H pylori and NSAID, risk factors such as smoking, alcohol intake, age, and male gender were reported as contributing to the gastric and duodenal ulcer development (17-20). In our study, patients who are supporting these findings were at middle or older age and 58% of them were male. Almost half of the patients (44%) were smokers and 13% were drinking alcohol.

Rate of patients classified as idiopathic (H pylori negative, NSAID negative) were reported between 4 and 20% (5, 8, 17). Among 140 patients 19 were (13%) H pylori and NSAID

negative. Idiopathic ulcer was reported among younger patients in a study (12) where as 19 patients classified as idiopathic in our study were older compared to patients with a known etiology.

Apart from H pylori, poor socio-economic status has been reported as an important risk factor for PUD infection while genetic factors do not influence the risk of PUD (21). Although the socio-economic status of the patients enrolled in our study has not been investigated in detail, 132 of the patients (94%) were from the urban regions while 8 (6%) were from the rural area and 14 (10%) had a history of ulcer.

As a consequence, most common cause of duodenal and gastric ulcer is H. pylori and is responsible for three-fourths of the cases. About half of the patients had a history of NSAID use and NSAID and H. pylori are both responsible for the ulcer in three-forths of these patients. In one tenth of the patients, NSAİD use was the cause of ulcer alone and about one-tenth of the ulcers were classified as idiopathic.

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6

Helicobacter pylori – Not Only a Gastric Pathogene?

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1. Introduction

Helicobacter pylori is a spiral, microaerophillic, Gram-negative bacterium. Infection by *H. pylori* has been established as the major cause of chronic gastritis and plays important role in the pathogenesis of other gastroduodenal diseases such as peptic ulceration, gastric lymphoma, and gastric cancer (Israel and Peek 2001). *H. pylori* is considered to be the most common chronic bacterial infection in humans (Cave 1996). The prevalence has been estimated to range from 40 to 80% and it varies widely by geographic area, age, race, ethnicity, and socioeconomic status (Bures et al. 2006). In most cases the infection is silent, clinical manifestation appears in only 10-15% of infected individuals. This is due to different strength of virulence of *H. pylori* strains and different host immune system response (Stromberg et al. 2003).
The stomach was supposed to be the only reservoir of infection in humans. Nevertheless *H. pylori* infection was detected in other sites recently. It was found in dental plaque and saliva (Kim et al. 2000) and also in oropharyngeal lymphatic tissue (Pavlik et al. 2007). This finding is of great importance because of known carcinogenic potential of *H. pylori*. It was declared type I carcinogen by IARC (1994). The question of direct contribution of *H. pylori* to oral and oropharyngeal diseases was not resolved yet.

2. *H. pylori* pathogenesis

Immunological changes caused by *H. pylori* in the stomach mucosa were explained recently (Tummala et al. 2004). There are no more detailed data about effect of *H. pylori* in the oral or oropharyngeal mucosa. *H. pylori* has several mechanisms to elude host defences (Portal-Celhay and Perez-Perez 2006). It is able to survive the acidic gastric environment by producing the enzyme urease, which metabolizes urea to carbon dioxide and ammonia to

buffer the gastric acid. *H. pylori* moves across gastric mucus and can adhere to epithelial cells using a variety of adhesin-like proteins (Sachs et al. 2003). Once adhered to epithelial cells, *H. pylori* induces a strong immune system response (Crabtree 1996). This response does not lead to elimination of the bacterium, but causes development of chronic inflammation. *H. pylori* is not eradicated unless an infected individual is treated with a combination of antibiotics (Portal-Celhay and Perez-Perez 2006). Chemical products of *H. pylori* attract cells of the immune system into lamina propria (Blanchard et al. 2004). It was shown that *H. pylori* can induce the maturation and activation of monocyte-derived dendritic cells. This activity is mediated by TLRs (Toll-like receptors) expressed on antigen presenting cells and leads to promotion of NK and Th1 effector responses (Portal-Celhay and Perez-Perez 2006). IFN – gamma producing Th1 polarized T cells and activated NK cells have been suggested to play an important role for development of severe pathologies (Hafsi et al. 2004).

H. pylori infection in gastric mucosa is associated with the production of both proinflammatory and immunomodulatory cytokines. Changes in secretion of IL-8, IL-1beta, IL-6, TNF-alpha, TGF-beta were described (Stromberg et al. 2003). These cytokines are produced by both the immune system and epithelial cells. The response of host cells is dependent on production of *H. pylori* virulence factors (Blanchard et al. 2004). The most important virulence factors, which are associated with gastric diseases, are CagA (cytotoxin associated gene A) and VacA (vacuolizating cytotoxin A).

3. *H. pylori* virulence factors

Genome sequence analysis led to identification of genes encoding these virulence factors grouped in the so-called pathogenicity island (cagPAI). It is a genomic region containing about 30 genes including genes for type IV secretion system (Mobley 1996). *H. pylori* strains producing CagA are associated with increased risk of severe gastric pathologies compared with CagA negative strains (Portal-Celhay and Perez-Perez 2006). Injection of bacterial proteins into the gastric cells by a type IV bacterial secretion system (a multi-molecular complex that mediates the translocation of bacterial factors into the host cell) has been described (Segal et al. 1999; Oliveira et al. 2006). In this way, CagA protein can get inside the host cells and stimulate cell signalling through interaction with several host proteins. This interaction leads to increased cytokine and regulatory molecule production (Guillemin et al. 2002) and could be related to initiation of tumour transformation (Segal 1997; Tummala et al. 2004; Hatakeyama 2006).

VacA is another important *H. pylori* virulence factor. This bacterial toxin with multiple activities is inserted into the host cell membrane, inducing cytoplasmic vacuolation (Cover and Blaser 1992). This toxin is coded by *vacA* gene, which is present in all *H. pylori* strains. Only about 50% of strains produce VacA protein. This is due to variability of *vacA* sequence. (Portal-Celhay and Perez-Perez 2006). There are several types of signal region (s1a, s1b, s1c, s2) and two types of midregion (m1 or m2). *H. pylori* strains with different forms of *vacA* differ in association with diseases. Strains with s1 signal sequence allele produce intact VacA toxin, s2 strains have low cytotoxic activity. Strains with s1/m1 allele combination have highest cytotoxic activity and they are associated with gastric ulceration and gastric carcinoma (Miehlke et al. 2000). s1/m2 strains are characterized by medium or low VacA production and s2/m2 strains do not produce VacA at all (Van Doorn et al. 1999). s2/m1 strains was found only sporadically (Letley et al. 1999; Martinez-Gomis et al. 2006).

Other virulence factors are e.g. adhesins, which help *H. pylori* to adhere to mucosal epithelial cells (Gisbert and Pajares 2004). Important is BabA protein which binds Lewis^b antigen, which is present in individuals with 0 blood group. Presence of *BabA* gene is connected to increased prevalence of gastric ulcers and gastric carcinoma in Lewis^b positive individuals (Blanchard et al. 2004). *BabA* often coexists with *vacA* s1 and *cagA* alleles (Kusters et al. 2006).

4. *H. pylori* induced carcinogenesis

H. pylori is a declared type I carcinogen (IARC, 1994). However, the exact way of carcinogenesis is not yet fully understood. There are three supposed ways of *H. pylori* carcinogenic action:

1. *H. pylori* could act as direct mutagen. Interaction of intracellular signalling molecules and *H. pylori* CagA may predispose cells to accumulate multiple genetic and epigenetic changes that promote multistep carcinogenesis (Hatakeyama 2006).
2. *H. pylori* produced VacA can cause immunosuppression by blocking proliferation of T cells (Boncristiano et al. 2003),
3. *H. pylori* can induce cell proliferation by increasing levels of several cytokines and regulatory molecules, which are involved in tumour formation and cell transformation (Konturek et al. 1997; Sakaguchi et al. 1999; Keates et al. 2001; Gobert et al. 2002; Schiemann et al. 2002; Wang et al. 2002). Current information about regulation mechanism of epithelial tissue by cytokines and regulatory molecules focus an interest mainly on Epithelial Growth Factor (EGF), Transforming Growth Factor (TGF) and NO synthases (NOS) (Gallo et al. 1998; Rubin Grandis et al. 1998; Sakaguchi et al. 1999; Gobert et al. 2002; Schiemann et al. 2002).

5. Methods of *H. pylori* detection in the oral cavity and pharynx

Diagnostics of *H. pylori* is significantly developed in gastroenterology. Attempts of *H. pylori* detection in other sites encountered diverse success rates (Dowsett and Kowolik 2003). Routinely used tests can be divided into non-invasive and invasive group. When detecting extragastric presence of *H. pylori*, invasive tests must be used based on the detection of bacteria in biopsy specimen. These invasive tests are often used to detect extragastric *H. pylori* presence:

Histology – Several staining methods are in use. These include e.g. haematoxylin and eosin, modified Giemsa, Warthin Starry, Gimenez, and Genta (Rotimi et al., 2000). These staining methods achieve high sensitivity and specificity rates (up to 96%) (Hep 2003) in case of gastric mucosa specimens, where no other bacterial strains are supposed to be present, but provide low specificity in the case of oral specimens, where other bacterial strains are often found (Dowsett and Kowolik, 2003). Differentiation of *H. pylori* from other bacteria can be very difficult.

Rapid Urease Test (RUT) or Campylobacter-like Organism (CLO) test is based on detection of urease production by *H. pylori*. When viable *H. pylori* bacteria are present, urea is being cleaved and the change of pH is visualized by colour indicator (Qureshi et al. 1992). This is very useful method when dealing with gastric mucosa specimens, in case of other specimens results may show high false-positive rate because of some other urease-producing species presence, e.g. *Streptococcus spp., Haemophilus spp.* a *Actinomyces spp.* (Dowsett and Kowolik 2003).

Culture is currently accepted gold standard for the diagnosis of gastric *H. pylori* (Makristathis et al., 2004). This method achieve 80-90% sensitivity and 90-100% specificity rates (Hep 2003). Culture of *H. pylori* from the oral cavity or oropharynx showed to by highly difficult to perform and has met with limited success (Dowsett and Kowolik, 2003). Use of special transport medium, microaerophilic environment, supplemented media for culture and three to seven days incubation is mandatory. Overgrowth by other bacterial species often appears. Direct inhibition of *H. pylori* by oral species in vitro has also been reported (Ishihara et al., 1997). Transformation of *H. pylori* into unculturable, coccoid form in the unfavourable environment was described (Shahamat et al., 2004).
Immunohistochemistry – to detect extragastric *H. pylori* is being used only experimentally. Tissue sections are incubated with rabbit polyclonal anti-*H. pylori* antibodies followed with the use of streptavidin-biotin-peroxidase kit and haematoxylin and eosin counterstaining (Akbayir et al. 2005).
Molecular methods – are currently generating most possibilities of detection and also typing of *H. pylori* strains. Various modifications of the polymerase chain reaction (PCR) are in use. These methods are used only for experimental purposes in the detection of extragastric *H. pylori*. In experiments on the detection of oral and pharyngeal *H. pylori* many variations of PCR diagnosis has been used with a detection rate ranging between 0-90% (Dowsett and Kowolik 2003). The lack of uniformity of laboratory procedures can play a role in the reported inconsistencies. The described modification exerted different primers and probes for the detection of different DNA segments of *H. pylori* DNA. Various primers were used (for example, urease gene, 16S ribosomal RNA genes and others). Specificity and sensitivity of different primers, however, can vary significantly (Song et al., 1999). PCR genotyping makes it possible to distinguish different *H. pylori* strains and their carriage of genes encoding virulence factors (Pavlik et al. 2007). The discrepancy of published PCR results shows the importance of finding suitable PCR assay. Tissue specimens collection and especially immediate immersion into proper transport medium is essential for successful test results (Pavlik et al. 2007). It has to be considered that PCR allows the detection of a low number of bacteria or nonviable bacteria, which cannot influence progress of diseases.

6. *H. pylori* in oropharyngeal lymphatic tissue

Several studies have explored the presence of *H. pylori* in tonsillar and adenoid tissue. The results of these studies were inconsistent with different detection rates. The discrepancies are due to different detection methods used. Some of the methods are believed to be unsuitable for detection of extragastric *H. pylori* (e.g. RUT or CLO test). PCR assay is now considered the most appropriate method for detection of pharyngeal *H. pylori*. However, differences in the primers and probes used in published studies do not allow drawing specific conclusions. Table 1. shows an overview of published papers focused on the detection of *H. pylori* in tonsillar and adenoid tissue. According to above mentioned data PCR assay is considered most valuable detection method for extragastric *H. pylori* detection. In their study Di Bonaventura et al. (2001) used PCR for investigation of tonsillar swabs and biopsy specimens with no evidence of *H. pylori* presence. Cirak et al. (2003), Bulut et al. (2006) found *H. pylori* in tonsillar and adenoid tissue by PCR (*16S rRNA* gene and *glmM* gene respectively). They found *H. pylori* strains positive for *cagA* gene. Bitar et al. (2005) investigated adenoid tissue specimens by RUT, histology and nested PCR (*ureA* gene). They found positivity by RUT and histology, but no positivity by nested PCR. In their next study

these authors investigated middle ear fluids and adenoid tissue specimens using culture, RUT and PCR (*ureC* and adhesion subunit genes). All middle ear fluids were negative. In adenoids they found positivity by RUT, but none by PCR (Bitar et al. 2006). Yilmaz et al. (2004) found *H. pylori* in middle ear effusions and in one adenoid tissue specimen using PCR (*23S rRNA* gene). Yilmaz et al. (2006) found *H. pylori* in 64% of adenoid and tonsillar specimens by PCR (*16S rRNA* gene). Kusano et al. (2007) showed *H. pylori* positivity in 126 (72.9%) tonsillar specimens using PCR (*16S rRNA* gene). They also demonstrated the presence of coccoid forms of *H. pylori* in tonsillar crypts using immunoelectron microscopy. Eyigor et al. (2009) found 5,5% of adenoid and tonsillar specimens positive for *H. pylori* by RUT, but none of them positive by PCR (*glmM* gene). Vilarinho et al. (2010) found 3 adenoid and tonsillar specimens positive by RUT, 2 positive by immunohistochemistry, but none positive by fluorescence in situ hybridization or PCR (*vacA* gene). Abdel-Monem (2011) found 16 (53.3%) adenoid and tonsillar specimens positive by RUT and 5 (16.6%) specimens positive by PCR (*ureC* gene). Other studies mentioned in Table 1. used different diagnostic methods with different detection rates.

The relationship between *H. pylori* infection and gastric tumour pathogenesis has been well described. It was supposed that *H. pylori* could act the same way in progression of oropharyngeal tumourigenesis. Some authors tried to identify a correlation between *H. pylori* and cancers of head and neck (Table 2.). Tests which determined serum levels of anti-*H. pylori* antibodies in patients with head and neck spinocellular carcinoma (HNSCC) brought inconsistent results (Grandis et al. 1997; Aygenc et al. 2001; Rubin et al. 2003; Nurgalieva et al. 2005). Okuda et al. (2000) proved the presence of *H. pylori* in oral swab specimens and oral cancer specimens using RT PCR (reverse transcriptase polymerase chain reaction) and culture. On the other hand Kanda (2005) found no HNSCC specimen positive using PCR, culture and immunohistochemical analysis. Kizilay et al. (2006) did not find *H. pylori* in laryngeal SCC and non-neoplastic specimens using haematoxylin and eosin stain or modified Giemsa stain. Akbayir et al. (2005) found *H. pylori* in specimens collected from laryngeal cancers and benign laryngeal disorders by histopathological methods, but not by immunohistochemical methods. Only one study performed PCR genotyping of *H. pylori* strains in specimens collected from the oropharynx. Tonsillar tissue specimens were collected from patients with chronic tonsillitis, obstructive sleep apnea syndrome (OSAS) and tonsillar cancer. The detected *H. pylori* strains differ from strains found in the stomachs of Czech patients with gastric diseases (Pavlik et al. 2007).

7. Comparison of oral and oropharyngeal genotypes

It is supposed that *H. pylori* is spread from person to person by oral-oral or faecal-oral route (Brown 2000), this hypothesis has not yet been convincingly demonstrated. Assuming the oral cavity and oropharynx as a gateway of infection, we can assume that in the oral cavity and oropharynx of the same individual we can find *H. pylori* strains of the same genotype. Initial works focused on comparison of oral and gastric *H. pylori* strains used endonuklease restriction analysis, single strand conformation polymorphism analysis (SSCP) or PCR (Shames et al. 1989; Khandaker et al. 1993; Zhang and Lu 1997; Kim et al. 2003). Identical strains have been found in gastric mucosa and oral cavity. The first comparison of gastric and oral *H. pylori* strains using PCR genotyping performed Wang et al. (2002) and, consequently, Burgers et al. (2008). Different genotypes in the stomach and oral cavity were found in both studies. PCR assays used by these authors could be considered more accurate

Author	Year	Subjects	Specimens	Diagnostic Method	Number of Subjects positive for *H.pylori*
Di Bonaventura	2000	36	tonsillar swabs	culture, imunohistochemistry	0 (0%)
Di Bonaventura	2001	75	tonsillar swabs and biopsy	PCR	0 (0%)
Unver et al.	2001	19	adenoid tissue	CLO test	11 (58%)
Skinner et al.	2001	50	tonsillar tissue	CLO test, imunocytochemistry	0 (0%) CLO test and imunocytochem.
Uygur-Bayramicli	2002	27	tonsillar tissue	histology, imunohistochemistry	0 (0%) histology and imunohistochemistry
Cirak	2003	23	tonsillar and adenoid tissue	PCR (16S ribosomal RNA, CagA)	7 (30%) positive for *H. pylori*
					5 of them (71%) positive for Cag A gene
Yilmaz et al.	2004	50	tonsillar and adenoid tissue	CLO test	0 (0%)
Yilmaz et al.	2005	38	adenoid tissue,	PCR (23S ribosomal RNA)	12 (67%) in middle ear effusion.
			middle ear effusions		1 (5%) in adenoid tissue
Pitkaranta	2005	20	adenoid tissue and middle ear fluid	culture	0 (0%)
Khademi et al.	2005	56	tonsillar and adenoid tissue	CLO test	27 (48%)
Bitar	2005	25	adenoid tissue	RUT, histology and nested PCR	21 (84%) positive by RUT,
				(UreA)	4 (16%) positive by histology
					0 (0%) positive by nested PCR
Bulut	2006	71	tonsillar and adenoid tissue	PCR (CagA - glmM gene)	29 (24,6%) postitive for *H. pylori*
					17 of them (58,6%) CagA positive
Bitar	2006	28	adenoid tissue and middle ear	culture, RUT, PCR (urease-C,	0 (0%) middle ear fluids
			fluid	adhesion subunit genes)	10 (77%) adenoid tissue by RUT
					0 (0%) by PCR
Yilmaz et al.	2006	22	middle ear fluid, promontorium	culture, PCR (16S RNA)	middle ear fluids: 2 positive by culture,
			mucosa,adenoid and tonsillar		7 by PCR
			tissue		mucosa: 1 by culture, 7 by PCR
					adenoids 11 (50%) by culture,
					14 (64%) by PCR
					tonsillar tissue:12 (55%) by culture,
					14 (64% by PCR)
Kusano et al.	2007	173	palatal tonsils	immunohistochemistry,	126 (72,9%) positive
				immunoelectron microscopy,	
				in-situ hybridization,	
				PCR (16S RNA gene)	
Vayisoglu et al.	2008	91	tonsillar and adenoid tissue	RUT, immunohistochemistry	2 (2,2%) adenoid tissue,
					0 (0%) tonsillar tissue using RUT,
					0(0%) immunohostochemistry
Eyigor et al.	2009	55	35 adenoids, 20 tonsils	RUT, PCR (glmM gene)	RUT 5,5% positive,
					0% PCR positive
Ozcan	2009	25	adenoid tissue, middle ear fluid	CLO, immunohistochemistry	0 (0%) CLO positive,
					0 (0%) immunohistochemistry positive
Jabbari Moghaddam	2009	285	tonsillar tissue	RUT, histopathology	113 (39.6%) positive by histopatology
					40 (14%) positive by RUT
Vilarinho et al.	2010	62	adenoid and tonsillar tissue	RUT, immunohistochemistry,	3 positive by RUT,
				fluorescence in situ hybridization	2 positive by immunohistochemistry,
				(FISH), PCR-DNA hybridization	0 positive by FISH,
				assay (vacA gene)	0 positive by PCR
Abdel-Monem	2011	20	adenoid and tonsillar tissue	RUT, PCR (ureC gene)	16 (53.3%) positive by RUT,
					5 (16.6%) positive by PCR

Table 1. Studies focused on detection of pharyngeal presence of *H. pylori*

Author	Year	Subjects	Specimens	Diagnostic Method	Number of Subjects Positive for *H. pylori*
Grandis et al.	1997	42	21 SCC	serology - IgG antibodies	57% with SCC
			21 controls without SCC		62% controls
Okuda et al.	2000	116	116 gastric and oral samples	RT-PCR, culture	46,6% gastric samples
			including 58 oral cancers		12,1% oral swab samples
					100% oral cancer swabs
Aygenc et al.	2001	58	26 laryngeal SCC	serology - IgG antibodies	73% with SCC
			32 controls without SCC		41% controls
Rubin et al.	2003	61	6 severe laryngeal dysplasia,	serology	38 seropositive (including all tonsillar SCC)
			5 tonsillar SCC, 50 other SCC		
Akbayir et al	2005	100	50 laryngeal SCC	histopathological and	0 (0%) imunihist.
			50 benign laryngeal disorders	imunohistochemical methods	28 SCC, 1 benign by histol.
Kanda et al.	2005	31	31 SCC	PCR, culture, immunohistochemical	21 seropositive
				analysis, serology - from urine	0 PCR, culture , immunohist.
Nurgalieva et al.	2005	230	119 laryngeal or pharyngeal SCC	serology - IgG antibodies	32,8% with SCC
			111 controls without SCC		27,0% controls
Kizilay et al.	2006	99	69 laryngeal SCC	histology - HE, modified Giemsa stain	0%
			30 nonneoplastic controls		
Pavlik et al.	2006	7	3 chronic tonsillitis	serology IgA, IgG, IgM	2 of 3 chronic tonsillitis serolgicaly
			3 tonsillar SCC	PCR genotyping	2 of 3 chronic tonsillitis, 3 of 3 SCC and 1 of 1
			1 OSAS		OSAS by PCR

Table 2. Studies focused on possible role of *H. pylori* in head and neck carcinogenesis

(Schabereiter-Gurtner et al. 2004). Findings of Lukes et al. (2009) are in concordance with these results. In four of six individuals different genotypes of *H. pylori* strains were found in the stomach and oropharynx. The results also show that from 20 individuals with proven

oropharyngeal *H. pylori* infection, only 8 had concurrent gastric infection. This confirms the findings of Burgers et al. (2008), who report that only 38% of persons with demonstrated presence of *H. pylori* in the oral cavity also had the infection in the stomach. These authors also reported the finding of 10 cases with positive *H. pylori* in saliva, with no detectable specific anti-*H. pylori* antibodies in serum. This is consistent with the results obtained by Lukes et. al. (2009). *H. pylori* was found in the oropharynx in 12 patients with no demonstrable antibody response.

8. Conclusions

Oral cavity (saliva and dental plaque) is now considered a possible extragastric reservoir of *H. pylori*. The published works dealing with oropharyngeal and nasopharyngeal detection of *H. pylori* infection have yielded contradictory results. Pharyngeal detection of *H. pylori* was reported in the range of 0-90%. Regarding that the various authors used different methods of detection, it is not possible to reach valuable conclusions. Frequently used tests like CLO test and RUT appears to be inappropriate methods for diagnosis of pharyngeal *H. pylori*. The presence of other urease-producing bacterial strains in the pharynx can lead to false positive results. Culture has proved to be very difficult and not very resistant to external influences, which may even prevent a successful detection. Molecular diagnostics (PCR) can be regarded as a method with sufficient sensitivity and specificity. Results achieved by these methods demonstrated the presence of *H. pylori* in the lymphoid tissue of oropharynx and nasopharynx. PCR method allows not only detect the presence of *H. pylori* infection, but also genotyping of strains within the tissue. The fact remains that the PCR methods allow determine the presence of bacterial DNA but can not determine whether the DNA comes from live or dead bacteria. Results of culture despite the very low numbers of positive results indicate the possible presence of viable bacteria capable of reproduction. High susceptibility of *H. pylori* in adverse effects during transport of specimens or during handling in the laboratory can explain low numbers of positive results of culture. Also, a frequent colonisation of oropharyngeal tissue by other bacterial species can have a significant influence on the failure of the culture of *H. pylori.*

The assumption that the oropharyngeal *H. pylori* infection may contribute to oropharyngeal carcinogenesis as a direct mutagen was not confirmed yet. An analogous situation, however, occurs in the stomach, where prevalence of *H. pylori* infection among the population is reported between 40-80%, serious stomach problems such as gastroduodenal ulcer disease or gastric cancer has only 10 -15% of infected.

Virulence of *H. pylori* strains varies according to the production of toxins. This production is due to the presence of virulence factor genes. Most important are the *cagA* gene and *vacA* gene. The main carcinogenic effect of *H. pylori* is declared to be associated with the presence of *cagA* gene and s1/m1 combination of alleles of *vacA* gene. Recent studies indicate that *H. pylori* may exist in the oropharynx independently to the gastric infection. Comparison of genotypes of *H. pylori* in the oral cavity, oropharynx, and stomach showed that an individual can host more than one strain of *H. pylori* in various locations. Differences were found in the presence of *cagA* gene and in the structure of *vacA* gene.

The findings of *H. pylori* in the oral cavity and oropharynx without demonstrable specific anti-*H. pylori* antibodies in serum are remarkable. This could be explained by an early detection of *H. pylori* presence after primary infection, when the antibody response has not started yet. Next, the possibility that *H. pylori* could colonize the oral cavity and the

oropharynx without inducing the host immune response must be considered. Another possible explanation is the presence of *H. pylori* coccoid forms. These are viable form of bacteria that can not be cultivated by conventional microbiological techniques and are characterized by a reduced virulence.

The question of transmission of *H. pylori* has not been satisfactorily resolved yet. If we consider the oral-oral or faecal-oral route as a way of transmission, we can assume finding of the same *H. pylori* strains in the oropharynx and stomach in the same individual. The findings of different genotypes in both locations still lack an accurate explanation. Inoculation of mixtures of *H. pylori* strains and consequently their different settlements in the different areas according to sensitivity of the strains could be one of the possible explanations. It can be assumed that the area of the oropharynx is less favourable for *H. pylori*, and can only be colonized by more resistant strains. One of the negative factors for growth and reproduction of *H. pylori* is the presence of other bacterial strains that were able to stop the growth of *H. pylori* during in-vitro experiments. A variety of bacterial colonization in the oral cavity and oropharynx can be assumed.

Epidemiological data on the prevalence of *H. pylori* infection published in the literature are often based on serological detection of specific anti-*H. pylori* antibodies. The prevalence of infection is reported 40-80%. The presence of anti-*H. pylori* antibodies was given in relation only to gastric infection. The newly obtained data prove the possibility of the presence of *H. pylori* infection in other locations independently to the gastric infection. This should be considered in future epidemiological studies. Not only antibodies should be evaluated but also identification of the exact location of the infection must be done.

In the future it would be appropriate to focus attention on local effects of *H. pylori* in oropharyngeal lymphoid tissue. Changes in the expression of some cytokines caused by *H. pylori*, which were described in the gastric mucosa, can be expected in the oropharyngeal tissue. Another study focused on oropharyngeal *H. pylori* genotyping should be done. In case that high virulent *H. pylori* strains can survive in oropharyngeal tissue, translocation of toxins into the oropharyngeal mucosa cells with subsequent cytokine response can be expected. Nevertheless this assumption has not been confirmed nor refuted yet.

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Part 2

Molecular Mechanisms of Peptic Ulcer Development and Healing

7

Pathophysiology of Gastric Ulcer Development and Healing: Molecular Mechanisms and Novel Therapeutic Options

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1. Introduction

The stomach plays a pivotal role in the digestion of foods that we eat. With the exception of rare cases, this organ can resist to a large variety of noxious factors, including hydrochloric acid, refluxed bile salts and alcohol, with a wide range of temperatures and osmolality. This high resistance to injuries depends on a number of physiological responses elicited by the mucosal lining against potentially harmful luminal agents, as well as to the ability of rapidly repairing the mucosal damage when it does occur (Laine et al., 2008). Nevertheless, when these protective mechanisms are overwhelmed by injurious factors, a gastric mucosal lesion may develop. Major detrimental effects on gastric mucosa are exerted by non-steroidal anti-inflammatory drugs (NSAIDs). These drugs are able not only to exert gastric injuring effects, but also to delay the healing of ulcer lesions through a variety of local and systemic mechanisms (Musumba et al., 2009).

Since the discovery that prostaglandin biosynthesis could be inhibited by NSAIDs through the blockade of cyclooxygenase enzymes, there has been a great interest in the contribution of prostaglandins to the mechanisms of gastric mucosal defense. Thus, it has been appreciated that these lipidic mediators are able to modulate virtually every factor involved in mucosal protection, and the importance of this contribution is made evident by the increased susceptibility of the stomach to injury following the intake of NSAIDs. Indeed, chronic treatments with these drugs can be associated with the development of ulcers in the stomach, and research over the past two decades has helped to identify some of the key events, triggered by cyclooxygenase blockade, which take part to ulcer formation and/or impairment of ulcer healing. Since many years, it has been recognized that NSAIDs can interfere with gastric mucosal physiology also through injuring mechanisms unrelated to the inhibition of prostaglandin biosynthesis, such as oxidative stress and changes in epithelial cell proliferation/apoptosis balance.

Following the discovery of two isoforms of cyclooxygenase (COX-1 and COX-2), and based on the assumption that COX-2 was an inducible enzyme responsible for inflammation, but devoid of gastroprotective functions (Vane et al., 1998), selective COX-2 inhibitors (coxibs, including celecoxib, rofecoxib, valdecoxib, parecoxib, etoricoxib and lumiracoxib) were

clinically developed as novel anti-inflammatory/analgesic drugs characterized by reduced gastric toxicity (Dubois et al., 2004). These advances have then fostered intensive preclinical and clinical research supporting the view that coxibs may confer advantages over conventional non-selective NSAIDs in terms of gastrointestinal risk reduction. Nevertheless, there are still a number of unresolved issues in this field, and the criteria for an appropriate use of coxibs in patients with various degrees of gastrointestinal risk, including ongoing gastric ulcerations, remain matter of discussion.

Another relevant topic, regarding the integrity of gastric mucosa, is represented by the use of proton pump inhibitors (PPIs). These drugs have been proven not only to prevent NSAID-induced upper gastrointestinal injury, but also to promote the healing process once the damage has occurred, even in the presence of a continued NSAID administration. The beneficial effects of PPIs can be largely ascribed to their ability to maintain a sustained inhibition of gastric acid secretion. However, there is also evidence to suggest that pharmacodynamic properties unrelated to acid inhibition may contribute to the therapeutic actions of these drugs (Blandizzi et al., 2008).

Recent research has highlighted the fact that, beside prostaglandins, gastric mucosal protective functions can be accomplished by other mediators, with particular regard for the gaseous mediators nitric oxide (NO) and hydrogen sulfide (H_2S). Moreover, anti-inflammatory drugs endowed with dual cyclooxygenase/5-lypooxygenase inhibitory effects, such as licofelone, could represent novel therapeutic strategies helping to drive the development of safer anti-inflammatory drugs and effective therapies to accelerate and improve the quality of ulcer healing (Blandizzi et al., 2009).

This chapter is focused on the available evidence on the molecular mechanisms underlying the pathophysiology of gastric injury development and healing, as well as on novel therapeutic options for prevention and treatment of gastric ulcers.

2. Mechanisms of gastric mucosal defense

The mechanisms of gastric mucosal defense include several local and neurohormonal protective factors, which allow the mucosa to resist against frequent exposures to damaging factors (Laine et al., 2008). In the following sections, a detailed description of the mucosal defense mechanisms is provided.

2.1 Local mechanisms of gastric mucosal defense

2.1.1 Mucus-bicarbonate-phospholipid barrier

The first line of gastric mucosal defense is represented by the mucus-bicarbonate-phospholipid barrier (Lichtenberger, 1999). The surface of gastric mucosa is covered by a layer formed by mucus gel, bicarbonate anions and surfactant phospholipids. This unstirred layer is capable of retaining the bicarbonate ions secreted by surface epithelial cells and maintaining a microenvironment with a pH near to 7 at the mucus-mucosa interface. The mucus layer is also able to prevent the penetration of pepsin, thus avoiding the proteolytic digestion of epithelium (Allen and Flemstrom, 2005). In addition, the luminal surface of mucus gel is covered by a film of surfactant phospholipids which confers hydrophobic properties to the mucus layer (Lichtenberger, 1999).

The mucus gel is secreted by surface epithelial cells and is formed by a large amount of water (about 95%) and various kinds of mucin glycoproteins (i.e., MUC2, MUC5AC, MUC5B and MUC6), the production of which may vary in different regions of the gastric

mucosa (Allen and Flemstrom, 2005; Ho et al., 2004). Gel-forming mucin units polymerize into large mucin multimers, which are essential for gel formation. The mucus gel is secreted along with low-molecular weight trefoil factor (CRF) family (TFF) peptides, which play a relevant role in the formation of the mucus layer (Newton et al., 2000). For example, TFF2 is known to increase the viscosity of gastric mucin and stabilize the gel network (Thim et al., 2002). The secretion of gastric mucus is regulated also by various gastrointestinal hormones, including gastrin and secretin, as well as prostaglandins and acetylcholine (Allen and Flemstrom, 2005).

The secretion of bicarbonate into the mucus gel layer is essential to maintain a pH gradient at the epithelial surface, which represents a first line of defense against gastric acid (Allen and Flemstrom, 2005). Bicarbonate secretion from the apical membrane of surface epithelial cells is mediated by a Cl^-/HCO_3^- anion exchanger, and it is stimulated by various factors, including prostaglandins (via EP_1 receptors), luminal acid, corticotrophin-releasing factor, melatonin, uroguanylin and orexin A (Allen and Flemstrom, 2005; Montrose et al., 2006).

The mucus-bicarbonate barrier is the only system which segregates the epithelium from the gastric lumen. Therefore, when this protective barrier breaks down during pathological events or upon detrimental actions by injuring agents, a second line of protective mechanisms comes into play. They include intracellular acid neutralization, rapid epithelial repair, and maintenance of mucosal blood flow.

2.1.2 Epithelial cells

The continuous layer of surface epithelial cells represents the next line of mucosal defense. This epithelial tissue is responsible for the production of mucus, bicarbonate and other components of the gastric mucosal barrier. These cells are hydrophobic in nature, being able to repel acid- and water-soluble injuring agents, owing to the presence of phospholipids on their surface (Lichtenberger, 1999). Surface epithelial cells are also closely interconnected by tight junctions, forming a continuous barrier, which prevents back diffusion of acid and pepsin (Allen and Flemstrom, 2005). Another relevant protective factor, available in the epithelial cells, is represented by heat shock proteins, which are activated in response to stress, including temperature increments, oxidative stress and cytotoxic agents (Tanaka et al., 2007). These proteins can prevent protein denaturation and protect cells against injury. Cathelicidin and beta-defensin are cationic peptides which play a relevant role in the innate defensive system at the mucosal surface, preventing bacterial colonization (Yang et al., 2006). In addition, TFFs secreted by epithelial cells regulate the re-epithelization process and exert mucosal protective actions (Taupin and Podolsky, 2003).

2.1.3 Mucosal cell renewal

The integrity of gastric epithelium is maintained by a continuous process of cell renewal ensured by mucosal progenitor cells. These cells are subjected to a continuous, well coordinated and controlled proliferation, which ensures the replacement of damaged or aged cells on the epithelial surface. The process of complete epithelial renewal takes about 3-7 days, while the overall glandular cell replacement requires months. However, the restitution of surface epithelium after damage occurs very quickly (i.e., few minutes) and results by migration of preserved cells located in the neck area of gastric glands (Laine et al., 2008).

The process of cell turnover is regulated by growth factors. In particular, a marked expression of epidermal growth factor receptor (EGF-R) has been detected in gastric progenitor cells. Such a receptor can be activated by mitogenic growth factors, such as

transforming growth factor-α (TGF-α) and insulin-like growth factor-1 (IGF-1) (Nguyen et al., 2007). In addition, PGE_2 and gastrin are able to transactivate the EGF-R and promote the activation of mitogen-activated protein kinase (MAPK) pathway, with consequent stimulation of cell proliferation (Pai et al., 2002). Notably, the presence of EGF has not been detected in the normal mucosa, although it is contained in the gastric juice, as a product of salivary and esophageal glands, and can stimulate mucosal cell proliferation in case of injury (Milani and Calabrò, 2001). In addition, mucosal progenitor cells do express survivin, an antiapoptotic factor, which inhibits apoptotic cell death (Chiou et al., 2005).

2.1.4 Mucosal blood flow

Mucosal blood flow is essential to deliver oxygen and nutrients and to remove toxic metabolites from gastric mucosa. Arteries embedded into the muscularis mucosae branch into capillaries, which then enter the lamina propria and travel toward the proximity of glandular epithelial cells. Endothelial cells, lining these microvessels, produce NO and prostacyclin (PGI_2), which act as potent vasodilators, thus protecting the gastric mucosa against damage and counteracting the detrimental effects of various vasoconstrictors, including leukotriene C_4, thromboxane A_2, and endothelin. In addition, NO and PGI_2 maintain the viability of endothelial cells and inhibit platelet and leukocyte adhesion to the microvasculature, thus preventing the occurrence of microischaemic phenomena (Laine et al., 2008).

When the gastric mucosa is exposed to irritants or acid back-diffusion, a massive and rapid increase in mucosal blood flow occurs. This process allows removal and dilution of back-diffusing acid or noxious agents. The increase in blood flow is regarded as a pivotal mechanism for preventing gastric mucosal cell injury, and its decrease results in the development of tissue necrosis. The increase in mucosal blood flow is mediated by NO release, and there is experimental evidence demonstrating that NO protects the gastric mucosa against injury induced by ethanol or endothelin 1, while the inhibition of NO synthase enhances mucosal injury (Holzer, 2006). It has been also observed that another endogenous compound, H_2S, can exert protective actions against gastric mucosal injury. In particular, this compound has been shown to reduce the expression of tumor necrosis factor α (TNF-α), to decrease leukocyte adhesion to vascular endothelium, and to prevent NSAID-induced gastric mucosal damage (Fiorucci et al., 2006).

2.1.5 Sensory innervation

The vasculature of gastric mucosa and submucosa is innervated by extrinsic primary afferent sensory neurons, which are arranged in a plexus at the base of the mucosal layer (Holzer, 2007). The nerve fibers stemming from this plexus run along with capillary vessels and reach the basal membrane of surface epithelial cells. These nerves can detect luminal acidity or back-diffusing acid through acid-sensing channels. The activation of such sensory nerves modulates the contractile tone of submucosal arterioles, thus regulating the mucosal blood flow. In particular, the stimulation of sensory nerves leads to the release of calcitonin gene-related peptide (CGRP) and substance P from nerve terminals surrounding large submucosal vessels (Holzer, 2007). CGRP then contributes to the maintenance of mucosal integrity through the vasodilation of submucosal vessels mediated by NO release. Sensory innervation plays a prominent role in the protection of gastric mucosa from injury, as demonstrated by studies where the ablation of sensory transmission (i.e., with capsaicin)

impaired the vasodilatatory response and increased the sensitivity of gastric mucosa to injuring agents (Holzer, 2007).

2.1.6 Prostaglandins

The gastric mucosa represents a source of continuous prostaglandin production, such as PGE_2 and PGI_2, which are regarded as crucial factors for the maintenance of mucosal integrity and protection against injuring factors (Halter et al., 2001; Brzozowski et al., 2005a). It has been demonstrated that prostaglandins have the potential to stimulate almost all the mucosal defense mechanisms. In particular, they reduce acid output, stimulate mucus, bicarbonate and phospholipid production, increase mucosal blood flow, and accelerate epithelial restitution and mucosal healing (Brzozowski et al., 2005a). Prostaglandins are also known to inhibit mast cell activation as well as leukocyte and platelet adhesion to the vascular endothelium (Halter et al., 2001; Brzozowski et al., 2005a). The beneficial actions exerted by PGE_2 have been shown to be mediated by activation of specific EP receptor subtypes. In particular, the activation of EP_1 receptors mediates the most important protective effects of prostaglandins, through an increase in bicarbonate secretion and mucosal blood flow in the damaged mucosa and a decrease in gastric motility (Takeuchi et al., 2002). Other EP receptor subtypes are also involved in the protective actions of PGE_2. For example, EP_3 receptors inhibit the gastric acid secretion, while EP_4 receptors stimulate the secretion of mucus (Kato et al., 2005).

2.2 Neurohormonal mechanisms

Gastric mucosal defense is supported by mechanisms activated, at least in part, by the central nervous system and hormonal factors (Laine et al., 2008). Experimental studies have demonstrated that central vagal activation stimulates mucus secretion and increases intracellular pH in the surface epithelial cells of in the stomach. In addition, while the CRF pathway is involved in endocrine responses to stress (Chatzaki et al., 2006). In addition, peripheral CRF contributes significantly to the regulation of gastric defense mechanisms, in particular, the CRF2 receptor is known to mediate antiapoptotic effects in gastric epithelial cells as well as to inhibit gastric emptying and motility (Chatzaki et al., 2006).

Other hormone mediators, including gastrin-17, cholecystokinin, thyrotropin-releasing hormone, bombesin, EGF, peptide YY and neurokinin A, play significant roles in the regulation of gastric protective mechanisms, which can be blunted by afferent nerve ablation, CGRP receptor blockade, and inhibition of NO synthase (Peskar, 2001; Moszik et al., 2001). Ghrelin, a hormone peptide produced by gastric A-like cells in rodents and P/D1 cells in humans, is involved in the regulation of growth hormone secretion and appetite stimulation (Brzozowski et al., 2005b). Moreover, it is also able to exert significant protective effects at gastric level, including the enhancement of mucosal blood flow via stimulation of NO and CGRP release from sensory afferent nerves (Brzozowski et al., 2005b).

Glucocorticoids have been shown to support the mechanisms of protection at gastric level. These hormones are involved in the response to stress, and represent potent gastroprotective factors against injury (Filaretova et al., 1998). Consistently with this contention, glucocorticoid antagonists enhanced the severity of stress-induced erosions, further supporting a protective role of these hormones during stress (Filaretova et al., 2001). The mechanisms through which glucocorticoids exert their protective effects include the maintenance of glucose homeostasis, the increase in mucosal blood flow and mucus

secretion, and the attenuation of both enhanced gastric motility and microvascular permeability (Filaretova et al., 2007).

3. Mechanisms of gastric mucosal damage

Gastric mucosal injury may occur as a consequence of various conditions, including alcohol intake, refluxed bile salts, stress, aging and *Helicobacter pylori* infection, although the most important agents known to impair the mechanisms of gastric mucosal defense are represented by NSAIDs. For this reason, in the following sections a detailed description of NSAID-related mechanisms of gastric injury is provided.

3.1 Effects of NSAIDs on gastric mucosa

The pathophysiology of gastric injury associated with NSAID administration depends partly on cyclooxygenase inhibition and partly on cyclooxygenase-independent mechanisms, which result mainly from local direct actions (Scarpignato and Hunt, 2010). Cyclooxygenase blockade has been shown to increase the susceptibility of gastric mucosa to NSAID-induced injury by suppression of a number of prostaglandin-mediated protective functions. For instance, prostaglandins reduce the activation of neutrophils and the local release of reactive oxygen species (ROS). The production of prostacyclin by the endothelium of mucosal microcirculation is also highly relevant in ensuring a tonic inhibition of neutrophil adhesion. Therefore, NSAIDs can shift the mucosal balance toward the recruitment and endothelial adhesion of circulating neutrophils through the inhibition of prostaglandin biosynthesis (Whittle, 2002). Once adhered, neutrophils clog the microvasculature causing a local decrease in mucosal blood flow and a marked release of tissue damaging factors, including proteolytic enzymes and leukotrienes, which enhance the vascular tone, exacerbate tissue ischaemia, stimulate the production of ROS, and promote the destruction of intestinal matrix, leading to a severe degree of focal tissue necrosis, particularly in the presence of a low luminal pH (Whittle, 2002; Jimenez et al., 2004).

As anticipated above, cyclooxygenase-dependent inhibition of bicarbonate secretion contributes also to the gastric mucosal injury elicited by NSAIDs. Indeed, the secretion of bicarbonate ions in the mucus gel layer generates a pH gradient on the mucosal surface, thus providing a first line defense against luminal acid (Allen and Flemstrom, 2005). A number of studies have demonstrated the expression of bicarbonate/chloride ion exchangers in the apical membranes of gastric surface epithelial cells, and shown that cyclooxygenase-derived prostaglandins stimulate bicarbonate secretion via activation of EP_1 receptors (Takeuchi et al., 1997; Rossmann et al., 1999).

Most NSAIDs are weakly acidic in nature and this property accounts for their local cyclooxygenase-independent injuring actions on the gastric mucosa. In the presence of gastric acidity, the undissociated lipophilic form of acidic NSAIDs can impair the hydrophobic surface barrier of the stomach. This transformation of the gastric mucosal surface from a non-wettable to a wettable state appears to be linked with the ability of acidic NSAIDs to destabilize the extracellular lining of zwitterionic phospholipids, particularly phosphatidylcholine, which are present within and on surface of the mucus gel layer (Lichtenberger et al., 2007). Previous studies have demonstrated that such an effect contributes significantly to NSAID-induced gastric injury in experimental models, and that it can persist for prolonged periods after discontinuation of NSAID administration (Lichtenberger, 2001). There is also consistent evidence that the protonophore actions of

aspirin and other acidic NSAIDs take a significant part in the topical damage to gastric mucosa. In particular, upon exposure to the acidic environment of gastric lumen, the undissociated lipid-soluble form of aspirin is able to penetrate cell membranes and accumulate into epithelial cells, where the inner pH is at a physiological level of 7.4. At this pH value, aspirin dissociates and remains segregated within cells. This accumulation enhances the inhibition of prostaglandin biosynthesis, and it brings also into play other properties of aspirin, such as the uncoupling of mitochondrial oxidative phosphorylation. The consequences of such mitochondrial dysfunction are a decrease in ATP production and an increase in AMP and ADP levels, which are then responsible for increments of intracellular calcium concentration. These changes are followed by mitochondrial injury, increased generation of ROS and alterations in the Na^+/K^+ balance, which lead to weakening of the mucosal barrier and cellular necrosis (Wallace, 2001; Bjarnson et al., 2007).

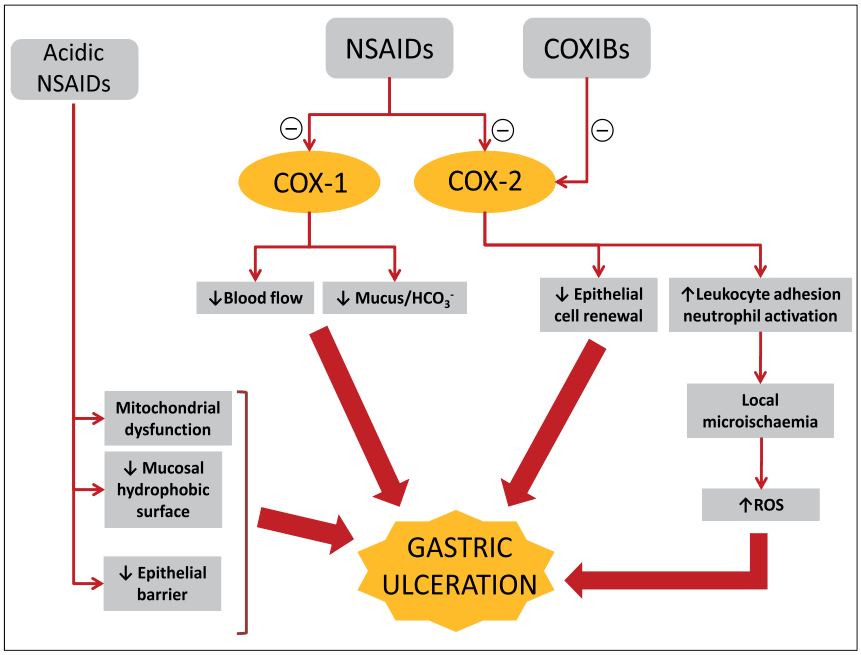

Fig. 1. Pathophysiology of gastric injury induced by non-selective NSAIDs. These anti-inflammatory drugs exert their detrimental effects on the gastric mucosa through two key mechanisms: simultaneous inhibition of COX-1 and COX-2, and direct topic cytotoxic effects. The topic injuring actions depend on the acidic chemical structure of NSAIDs. Coxibs do not harm the gastric mucosa owing to their ability to selectively inhibit COX-2, while not affecting the protective functions of COX-1. ROS: reactive oxygen species.

An additional mechanism, involved in the injurious effects of NSAIDs on gastrointestinal mucosa, is related to the detrimental actions of these drugs on the integrity of epithelial tight junctions, which are known to segregate the apical from basolateral cell surface domains, in order to establish cell polarity and provide a barrier function against the back diffusion of acid and other solutes through the paracellular space (Schneeberger and Lynch, 2004). It has been suggested that cyclooxygenase inhibition may be implicated in NSAID-induced

alterations of intercellular epithelial permeability (Joh et al., 2003). However, recent evidence indicates that aspirin can elicit gastric epithelial barrier dysfunction through down-regulation of claudin-7, a member of the claudin protein family, which play important roles in the formation of tight junctions (Oshima et al., 2008).
Coxibs do not alter the integrity of normal gastric mucosa in preclinical models, and their clinical development was based on the assumption that COX-2 is not expressed in the gastric mucosa (Laine et al., 2008). However, this initial hypothesis has not been supported by subsequent observations, demonstrating the constitutive presence of both COX-1 and COX-2 in human and rodent gastric mucosa (Zimmermann et al., 1998). In addition, studies on COX-1-knockout mice have provided no evidence of spontaneous gastric injury and demonstrated the ability of NSAIDs to damage the gastric mucosa via COX-2-dependent mechanisms (Langenbach et al., 1995). Wallace et al. (2000) investigated further the functional roles of COX isoforms in the gastric mucosa, showing that COX-1-dependent prostaglandins are involved in the maintenance of mucus/bicarbonate secretion and blood flow, while COX-2 protects the mucosa from leucocyte endothelial adhesion and supports epithelial renewal. In addition, these Authors observed that selective COX-1 or COX-2 inhibitors did not damage the stomach when tested alone, while NSAIDs or the combined administration of COX-1 plus COX-2 selective inhibitors resulted in gastric erosions (Wallace et al., 2000). A schematic diagram illustrating the mechanisms of gastric mucosal injury exerted by cyclooxygenase inhibitors is provided in Figure 1. Overall, it is currently acknowledged that NSAIDs can impair gastric protection via a concomitant blockade of COX-1 and COX-2, while coxibs lack damaging actions on gastric mucosa by preserving COX-1-dependent prostaglandin production (Wallace, 2006).

4. Mechanisms of gastric ulcer healing

Gastric ulcer results from mucosal tissue necrosis triggered primarily by ischemia, with cessation of nutrient delivery and ROS formation. Tissue necrosis and subsequent release of arachidonic acid metabolites from injured cells, including leukotrienes B, attract leukocytes and macrophages, which then phagocitize the necrotic tissue and release pro-inflammatory cytokines, which in turn activate local fibroblasts, endothelial cells and epithelial cells to attempt a tissue restoration (Cotran et al., 1999; Tarnawski, 2005). Morphologically, gastric ulcer consists of two components: the margin, sorrounded by adjacent non-necrotic mucosa, and the base, consisting of granulation tissue, which is a connective tissue rich in macrophages, fibroblasts and proliferating microvessels (Cotran et al., 1999). Ulcer healing is a complex process, in which the tissue repairs itself after injury, attempting a restitution towards integrity. It has been proposed that such a process can be distinguished in sequential, partly overlapping, phases: haemostasis, inflammation, proliferation and remodeling (Stadelmann et al., 1998). According to Schmassmann (1998), the phases and time course of ulcer healing can be described as follows: ulcer development phase (within 3 days from injury), characterized by tissue necrosis, inflammatory infiltration, formation of ulcer margin (de-differentiation) and development of granulation tissue; healing phase (after 3-10 days from injury), which includes an early healing (rapid migration of epithelial cells and contraction of ulcer base) followed by a late healing (angiogenesis in ulcer bed, remodeling of granulation tissue and complete re-epithelialization of ulcer crater); reconstruction phase (day 20-40 after ulceration) consisting of the reconstruction of glands, muscularis mucosae and muscularis propria; maturation phase (40-150 days after

ulceration), characterized by maturation and differentiation of specialized cells (Schmassmann, 1998).

In general, following the ulcerative injury, a set of complex biochemical events takes place to provide support for cellular migration from ulcer margin and attachment to the ulcer base, with subsequent cellular proliferation and restoration of the epithelial layer. Ulcer healing is initiated by formation of the 'healing zone', consisting of dilated glands, whose cells undergo de-differentiation, express epidermal growth factor receptor (EGF-R) and starts to actively proliferate. At this stage, inflammatory infiltration occurs closely to the necrotic tissue and ulcer crater. In response to growth factors, the ulcer margin is formed, cells adjacent to the margin de-differentiate, and granulation tissue develops at the ulcer base. During healing, the granulation tissue undergoes continuous remodeling, contraction and changes in cellular composition, whereby the inflammatory cells, appeared in the early phase of healing, are replaced by fibroblasts and microvessels in the late healing phase (Cotran et al., 1999). Wong et al. (2000) analyzed the sequential expression of various genes during ulcer healing and were able to distinguish the following arrays: *genes involved in early response* (EGF-R, c-fos, c-jun, egr-1, sp-1, trefoil factor-2/spasmolytic peptide [TFF-2/SP]), which are all activated shortly after ulcer formation (i.e., within 30 minutes-2 hours); *intermediate response genes* (EGF, basic fibroblast growth factor [bFGF], platelet derived growth factor [PDGF] and vascular endothelial growth factor [VEGF]), which become activated within 6 hours-2 days; *late response genes* (hepatocyte growth factor [HGF], intestinal trefoil factor [ITF], c-met/hepatocyte growth factor receptor [HGF-R]), which are activated within 14 days (Wong et al., 2000). The subsequent proliferation step is initiated within 3 days from ulceration, and it is essential for the healing process, since it supplies the epithelial cells needed for re-epithelialization mucosal surface and gland reconstruction of (Cotran et al., 1999). There is evidence that mucosal ulceration leads to the development of a novel cell lineage designated as *ulcer associated-cell lineage,* which stems from the base of surviving crypts (Cotran et al., 1999). These cells, which express EGF-R and initiate the synthesis of EGF, HGF, trefoil peptides and other growth factors, promote epithelial tube formation, migration and invasion of granulation tissue, and ultimately drive gland reconstruction within the ulcer scar (Tarnawski, 2005). Time-sequence analysis has shown that trefoil peptides are expressed much earlier than EGF following the induction of tissue ulceration. Furthermore, receptor analysis, using radioligand binding assays and immunohistochemistry, has shown a rapid increase in EGF-R expression and a rapid decrease in somatostatin receptor density in the ulcer margin (Reubi et al., 1994).

The major stimuli for cell migration and ulcer re-epithelialization are mediated by growth factors which are produced by platelets, injured tissue and macrophages. Current evidence suggests also that the epithelium of ulcerated mucosa can be regenerated by bone marrow-derived adult stem cells, since biopsy specimens of gastric mucosa, obtained from female patients receiving bone marrow transplants from male donors, were found to contain cells equipped with chromosome Y (Okamoto et al., 2002). The migration of epithelial cells from the ulcer margin, to restore the continuity of epithelial lining, is essential for ulcer healing, and it is subjected to a fine regulation, since it generates a barrier protecting the granulation tissue from any mechanical and chemical damage. Notably, cell migration requires complex cytoskeletal rearrangements. In particular, it has been appreciated that cytoplasmic microfilaments, consisting of G-actin, polymerize into F-actin and the latter, together with myosin II, provides contractile bundles through which cell motility can take place (Chai et al., 2004). A schematic diagram showing the main factors involved in gastric ulcer healing is provided in Figure 2.

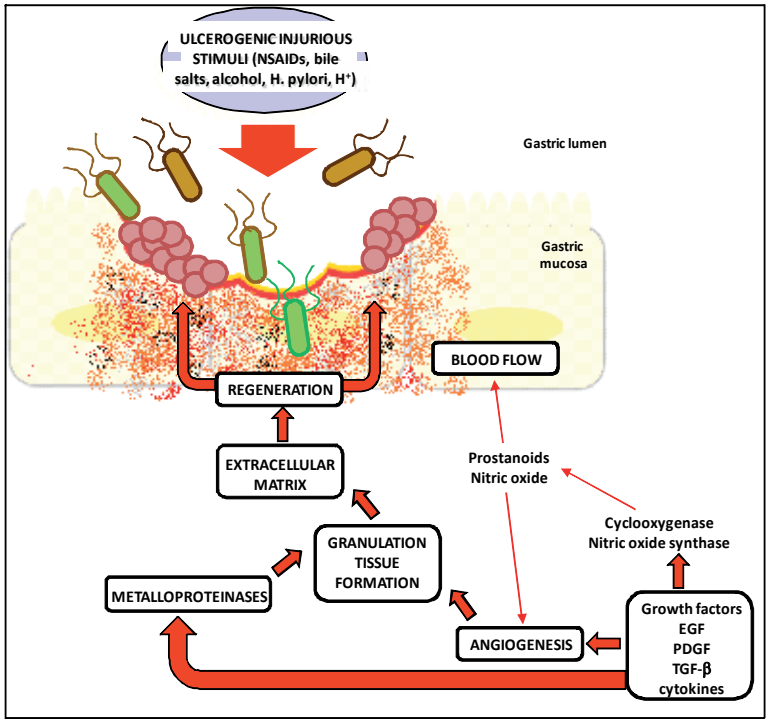

Fig. 2. Main mechanisms involved in gastric ulcer healing. EGF: epidermal growth factor; PDGF: platelet-derived growth factor; TGF-β: transforming growth factor- β.

4.1 Early primary response genes: protooncogenes

Ulcer healing depends on a long-term array of responses, which requires de novo mRNA and protein synthesis as well as cell replication. Changes in gene regulation, in response to wounding or ulceration, result in an increase in cell proliferation to replace lost cells. To accomplish this task, the damaged tissue induces early primary response genes, belonging to the family of protooncogenes, which code for sequence-specific DNA-binding nuclear proteins, having the potential of directly influencing the expression of specific genes at the transcriptional level. Although a low basal expression of the nuclear protooncogenes *c-fos*, *c-jun* and *c-myc* is usually observed in most cells, their expression can be rapidly and transiently up-regulated following tissue wounding (Wang and Johnson, 1994). In the rat stress ulcer model, it has been demonstrated that exposure to stress resulted in a rapid increase in *c-fos* and *c-myc* mRNA levels, up to 3-4-fold the basal value. The change in the expression of these protooncogenes was found to precede an increased rate of DNA synthesis (Wang e Johnson, 1994). In another study, based on in situ ibridization, Ito et al. (1990) examined the changes in protooncogenes expression during gastric regeneration after stress injury. In this setting, cells expressing *c-myc* mRNA were identified as mucous neck, parietal, chief and enterochromaffin-like cells, and the distribution of cells in S-phase coincided with that of protooncogene expressing cells (Ito et al., 1990). The exact signal transduction pathways, leading to protooncogene up-regulation following tissue injury, are still unclear, but they are thought to result from modulation of gene transcription by the polyamines, spermine, spermidine and putrescine. These low-molecular-weight organic

cations are ubiquitous in eukariotic cells and able to bind negatively charged macromolecules, such as DNA, RNA and proteins, thus influencing the chromatin structure and sequence-specific DNA-protein interactions, with consequent changes in the regulation of initiation, elongation and termination of gene transcription (Li et al., 2001).

4.2 Angiogenesis and angiogenic growth factors

Following gastric ulcerative insults, all mucosal components, including microvessels, undergo destruction within the necrotic area. The healing of such deep mucosal lesions requires the reconstruction of surface epithelium and glandular epithelial structures, the restoration of lamina propria and the reconstruction of mucosal microvascular network, which is essential for delivery of oxygen and nutrients to the healing site (Tarnawski, 2005). The latter goal is achieved through angiogenesis, a finely regulated process, in which microvascular endothelial cells migrate from preserved microvessels at the wound edge, proliferate and attempt to re-establish a microvascular network through de novo vessel formation (Folkman and D'Amore, 1996). Angiogenesis occurs via a series of sequential steps, which include: degradadation of capillary basement membranes by activation of matrix metalloproteinases (MMPs); endothelial cell migration into the perivascular space and proliferation; formation of microvascular tubes followed by anastomoses; establishment of lamina propria and basement membranes and, ultimately, formation of a novel capillary network (Folkman and D'Amore, 1996). The growth of granulation tissue and generation of new microvessels through angiogenesis is stimulated by bFGF, VEGF, PDGF, angiopoietins, other growth factors and cytokines, including IL-1 and TNF-α (Risau, 1997). Gastric mucosal angiogenesis is strongly stimulated by prostacyclin and human recombinant bFGF. Furthermore, the induction of mucosal injury triggers the activation of bFGF and its receptors, and enhances bFGF protein expression in the mucosa-bordering necrosis (Tarnawski, 2005).

VEGF is a pivotal regulator of angiogenesis. It binds at least two specific receptors, VEGF-R1 or flt-1 and VEGF-R2 or flk/KDR, which are expressed mainly on endothelial cells and initiate the phosphorylation of cytosolic proteins involved in signal transduction promoting endothelial cell proliferation, migration and microvascular formation (Ferrara, 2004). VEGF production is stimulated by PDGF, TGF-α, cytokines, NO and prostaglandin E_2. Hypoxia is one of the best characterized stimuli for the induction of VEGF expression, acting via a hypoxia-inducible factor (HIF)-1 binding site located on the VEGF gene promoter (Ferrara, 2004). Jones et al. (1999) demonstrated a 4-6 fold increase in VEGF mRNA and protein in the mucosa-bordering necrosis after 24 hours from the induction of ulcer by intragastric ethanol instillation. In this study, the quantitative assessment of angiogenesis demonstrated that almost 10% of microvessels in the mucosa-bordering necrosis displayed endothelial sprouting, reflecting the ongoing angiogenesis. Moreover, treatment with anti-VEGF neutralizing antibody reduced the angiogenic response and delayed ulcer healing (Jones et al., 1999). The activation of MAPK (Erk1 and Erk2) signal transduction pathway is crucial for VEGF-induced stimulation of angiogenesis in ulcer healing, and NSAIDs have been found to interfere with the angiogenic process in part by inihibiting the MAPK/Erk pathway (Jones et al., 1999). In normal gastric microvascular cells it has been demonstrated that prostaglandins can induce VEGF mRNA through transactivation of JNK by Erk2 (Pai et al., 2001). Moreover, this stimulant effect of prostaglandins is likely to be amplified via a positive feedback mechanism, since VEGF, once induced, activates COX-2 expression via an autocrine and paracrine action (Tamura et al., 2002).

4.3 Platelets

It is becoming increasingly appreciated that the platelet has the potential of performing a large array of functions, in addition to its role in haemostasis. Tissue repair is initiated with the aggregation of platelets, formation of fibrin clot and release of growth factors from platelets, injured cells and extracellular matrix. Platelets represent one of the largest source of growth factors in the body, and it is through the release of these growth factors that, at least in part, platelets are capable of markedly influencing the processes of tissue healing. Several potent angiogenic stimulators are stored in platelets, including VEGF, platelet derived endothelial growth factor (PDEGF), EGF and PDGF (Perini et al., 2005). These factors account for the ability of platelets to stimulate endothelial cell proliferation and capillary-like formation. Factors that influence the platelet content of pro- versus antiangiogenic factors, or their release from platelets, have the potential to markedly affect angiogenesis and ulcer healing. For example, treatment of rats for 1 week with ticlopidine, an antiplatelet drug acting as adenosine diphosphate receptor antagonist, resulted in a marked increase in platelets and serum levels of endostatin, without affecting platelet VEGF levels. Moreover, this treatment resulted in a marked delay in gastric ulcer healing (Ma et al., 2001). Notably, among a number of receptors, that are important in regulating platelet adhesion, aggregation and secretion, platelet membranes have been found to express proteinase-activated receptors (PARs), which are G protein–coupled receptors, activated by proteinase cleavage at a specific site in their extracellular NH2-terminus. Four PARs have been cloned to date. The activation of PAR_1 by thrombin stimulates the release of VEGF, while inhibiting the release of endostatin. By contrast, the activation of PAR_4 mediates opposite effects on VEGF release (Ma et al., 2005). The balance in platelet and serum levels of pro- and antiangiogenic factors may influence the healing processes of gastric ulcer and raises the possibility that a selective modulation of PARs could be a viable pharmacological strategy for modulating ulcer healing.

4.4 Heat shock proteins

In response to environmental or physical stress, such as heat or ethanol, eukaryotic cells induce the synthesis of intracellular proteins designated as heat shock proteins (HSPs) or stress proteins (Tsukimi and Okabe, 2001). These proteins function as molecular chaperones, which participate in the folding and assembly of nascent proteins, the refolding of partial damaged functional proteins, and the delivery of precursor proteins to mitochondria (Hightower, 1991). HSPs are classified into four major families according to their biological activities and apparent molecular weights: HSP90, HSP70, HSP60, which are constitutively expressed, and small HSPs, including HSP27 and HSP10, which are inducible by various conditions, including oxidative stress (Hightower, 1991). Tsukimi and Okabe (2001) found that the level of HSP70 in normal mucosa was quite low, while it was significantly higher in the ulcer base at the time of ulcer development. HSP70 is expressed in proliferating cells during re-epithelialization (Soncin and Calderwood, 1996), and thus it is likely to be involved in the regeneration of ulcerated mucosa. The induction of HSP70 in the ulcer base might either contribute to the de novo synthesis of proteins or regulate the activity of key enzymes involved in ulcer healing through a molecular chaperone activity. Of note, Ethridge et al. (1998) reported that the overexpression of COX-2 by transfected cDNA inhibited the expression of HSP70 and the activation of heat shock factor-1 (HSF-1) in response to heat shock in rat intestinal epithelial cells. Such inhibition was antagonized by

the COX-2 inhibitor NS-398. Accordingly, Ethridge et al. (1998) proposed that prostaglandins derived from COX-2 might be associated with HSP70 induced by heat shock, suggesting an inverse relationship between COX-2 expression and HSP70 induction. HSP47 is a 47 kDa stress protein that specifically binds collagen (Nagata et al., 1988). Collagen biosynthesis represents an essential step for granulation tissue formation. In this regard, HSP47 was found to be expressed in the ulcer base at the time of ulcer development, and its expression decreased with the progress of ulcer healing. Based on these results, it has been suggested that HSP47 might be involved in ulcer healing by playing a role in collagen biosynthesis (Tsukimi and Okabe, 2001).

4.5 Annexin-1

Annexin-1 is a 37-kDa member of the annexin family of proteins, which bind and activate 'formyl-peptide' receptors (FPR), known to mediate immune and anti-inflammatory responses. These receptors are expressed on the surface on a variety of cells, including subepithelial myofibroblasts, smooth muscle cells, leukocytes, mast cells and T cells (Chiang et al., 2006). Annexin-1 can also exert its anti-inflammatory actions after proteolitic removal of its NH2 terminus (Martin et al., 2008). The expression of annexin-1, designated also as lipocortin, can be induced by glucocorticoids and it has been shown to contribute to their anti-inflammatory effects (Hannon et al., 2003). In mice, annexin-1 is expressed in the healthy gastric mucosa, and it is markedly up-regulated following ulcer induction by acetic-acid. In this setting, treatment of mice with an annexin-1 mimetic peptide improved gastric ulcer healing. Furthermore, although annexin-1 deficient mice did not exhibit any difference from wild-type mice in terms of susceptibility to indomethacin-induced gastric damage, the healing of such lesions was impaired in annexin-1-deficient mice (Martin et al., 2008). These data are consistent with the hypothesis that annexin-1 contributes to ulcer repair through mechanisms depending on its anti-inflammatory actions. Consistently with this view, Martin et al. (2008) observed an increased expression of the 33-kDa cleavage product of annexin-1 in concomitance with the up-regulation of annexin-1 in the gastric ulcer of mice. The expression of this cleavage product was not observed in healthy stomachs, and therefore it is likely that annexin-1 cleavage, probably due to elevated protease levels, occurred as a consequence of factors induced during the inflammatory or repair process to generate peptide retaining anti-inflammatory properties (Martin et al., 2008).

4.6 Extracellular matrix and tissue remodeling

The replacement of granulation tissue with a connective tissue scar, as well as the reconstruction of mucosal architecture, involves tissue remodeling and changes in the composition of extracellular matrix (ECM). ECM consists of fibrous structural proteins, such as collagens and elastins, adhesive glycoproteins, including fibronectin and laminin, and an amorphous gel composed by proteoglycan and hyaluronan. ECM provides the supporting structure for epithelial, endothelial and smooth muscle cells and it is an essential component of connective tissue (Cotran et al., 1999). In the acetic acid-induced gastric ulcer model, Shahin et al. (2001) demonstrated a marked increase in procollagen I 3 days after ulcer induction. Procollagen gene expression remained elevated up to day 15, while returning to the initial levels on day 30. The highest procollagen transcript levels were found in the intact submucosa surrounding the ulcer margins, followed by the muscularis propria and serosa, with the lamina propria displaying the lowest transcript levels (Shahin et al., 1997). Beside

collagens, other important components of ECM are spatially and temporally regulated during ulcer healing. MMPs include collagenases, which cleave the fibrillar collagens. These enzymes are produced by several cell types, such as fibroblast, macrophages, neutrophils, endothelial cells and some epithelial cells, and their secretion is induced by growth factors, cytokines or steroids (Cotran et al., 1999). Activated MMPs are rapidly inhibited by specific tissue inhibitors, designated as tissue inhibitors of metalloproteinase (TIMP), to prevent uncontrolled actions by proteinases (Cotran et al., 1999). It has been reported that MMP-2 RNA expression can be detected as early as 24 hours after ulcer induction, a time point that coincides with the clearance of necrotic tissue (Shahin et al., 2001). Its further enhancement at the ulcer margin, after 48 hours, parallels the increment of ulcer diameter observed after the sloughing of necrotic tissue. TIMP-1 expression has been found to be enhanced at 72 hours, suggesting that MMP-2 may promote the ulceration process through local degradation of matrix and tissue proteolysis (Shahin et al., 2001).

5. Effects of NSAIDs and coxibs on gastric ulcer healing

The pharmacological modulation of cellular and molecular targets involved in the healing process can alter ulcer repair. Cell renewal in the ulcer margin and angiogenesis in the ulcer base have been found to be significantly impaired during NSAID treatment, with significant delay in ulcer healing (Levi et al., 1990). In the acetic acid-induced ulcer rat model, Sanchez-Fidalgo et al., (2004) found that the ulcerated area was characterized by increased bFGF expression and microvessel density in the granulation tissue at the ulcer base, in concomitance with increments of both apoptotic cell death and expression of proliferation cellular nuclear antigen (PCNA), a marker of cell proliferation. In this setting, both rofecoxib (a selective COX-2 inhibitor) and ibuprofen (a non selective NSAID) delayed ulcer healing, but only rofecoxib was found to reduce all the above mentioned parameters. More recently, indomethacin was tested for its effects on ulcer healing, PCNA and activated caspase-3 expression in acetic acid-induced gastric ulcers. In this study, indomethacin was found to delay ulcer healing, and to up-regulate caspase-3 but not PCNA in ulcerated tissues, suggesting that apoptotic cell death represents a relevant mechanism whereby NSAIDs can impair ulcer repair (Colucci et al., 2009).

Prostaglandins are known to stimulate angiogenesis in vivo and in vitro (Mehrabi et al., 2001; Cheng et al., 1998). Therefore, it is likely that drugs acting as cyclooxygenase blockers, such as NSAIDs, can interfere with angiogenesis in the setting of gastric ulcer healing. Tsujii et al. (1998) showed that aspirin, a non selective NSAID, and NS398, a selective COX-2 inhibitor, blocked angiogenesis in cultured human umbilical vein endothelial cells. Some clinical and experimental data support the view that both non-selective NSAIDs and COX-2 selective inhibitors can delay gastric ulcer healing, partly by inhibiting angiogenesis in the granulation tissue at the ulcer base (Tarnawski and Jones, 2003). In particular, indomethacin significantly reduced (by >37%) the number of microvessels in the ulcer granulation tissue, and the selective COX-2 inhibitors L-745,337, celecoxib and NS398 were found to exert similar effects (Tarnawski and Jones, 2003). The mechanisms by which NSAIDs inhibit angiogenesis appear to include a local change in angiogenic growth factor expression, alterations in key regulators of VEGF, increased endothelial cell apoptosis, inhibition of endothelial cell migration and recruitment of inflammatory cells and platelets (Tarnawski and Jones, 2003). In rat primary aortic endothelial cells, indomethacin and NS398 markedly

inhibited the tube formation and Erk2 nuclear translocation. Incubation with prostaglandins partly prevented the NS398-induced effects, but not those exerted by indomethacin, suggesting that both COX-1 and COX-2 are important for the regulation of ulcer angiogenesis, and that the inhibitory action of NSAIDs on angiogenesis depends on both prostaglandin-dependent and prostaglandin-independent mechanisms (Tarnawski and Jones, 2003). Pai et al. (2001) have proposed that NSAIDs can arrest endothelial cell proliferation by suppressing cell cycle proteins, since indomethacin was found to significantly inhibit bFGF-stimulated endothelial cell proliferation by reducing cyclin D1 and increasing p21 protein expression. Furthermore, in a study carried on microvascular endothelial cells, indomethacin and NS398 were found to be able to inhibit VEGF-induced early growth response factor (Egr) 1 gene activation, which is a transcription factor activated by hypoxia in angiogenesis (Szabo et al., 2001). Ma et al. (2002) examined the effects of cyclooxygenase inhibitors on the healing of gastric ulcer in rats, angiogenesis in granulation tissue, and serum levels of VEGF and endostatin. In this study, both celecoxib, a selective COX-2 inhibitor, and flurbiprofen, a non-selective NSAID, significantly impaired angiogenesis, delayed ulcer healing and increased serum endostatin levels (Ma et al., 2002). There is also evidence that NSAIDs can interfere with ulcer healing by both acid-dependent and acid-independent mechanisms (Schmassmann, 1998). In this respect, an experimental study has shown that: the thick granulation tissue below the ulcer crater was transformed into a thinner mature scar within 2 weeks from ulceration; in the presence of NSAIDs, the thickness of granulation tissue progressively increased, indicating an inhibition of its maturation process, and the ulcer healing was delayed; such detrimental effect of NSAIDs on the remodeling of granulation tissue could be reversed by omeprazole, suggesting the involvement of acid-dependent mechanisms (Schmassmann et al., 1995).

As anticipated above, preclinical studies have shown that the impairing actions of NSAIDs on ulcer healing can be shared by COX-2 selective inhibitors, suggesting a role for COX-2 in the process of ulcer repair. However, there is also evidence supporting the view that factors other than COX-2 could be important in the detrimental effects of NSAIDs and selective COX-2 inhibitors on ulcer healing (Blandizzi et al., 2009). First of all, data regarding COX-2 expression in gastric ulcer tissue are conflicting. Furthermore, Schmassmann et al. (2006) observed that treatment with selective COX-1 inhibitors did not delay ulcer healing in COX-1 knockout mice and wild type animals. However, in the same study, the combination of selective COX-1 and COX-2 inhibitors impaired ulcer healing to a higher extent than selective COX-2 inhibitors alone, suggesting that COX-1 could also contribute to ulcer healing process under a condition of COX-2 inhibition. It has been suggested also that the detrimental action of aspirin in combination with celecoxib on ulcer healing could result from the ability of aspirin to alter surface phospholipids, without significant involvement of the cyclooxygenase pathways (Lichtenberg et al., 2007). More recently, we have obtained preliminary evidence that the ulcer healing impairing effects exerted by treatment with indomethacin (COX-1/COX-2 inhibitor) or DFU (selective COX-2 inhibitor) could depend on the ability of these drugs to induce the expression of NSAID activated gene-1 (NAG-1), which is known to promote apoptosis (Colucci et al., 2008).

6. Effects of PPIs on gastric mucosal protection and ulcer healing

Several preclinical and clinical lines of evidence have demonstrated that PPIs are highly effective in promoting the healing of gastric damage induced by NSAIDs, even in the

presence of a continued NSAID administration, through the activation of both acid-dependent and -independent mechanisms (Blandizzi et al., 2008).
PPIs are substituted benzimidazole derivatives (Figure 3) endowed with potent inhibitory effects on gastric acid secretion.

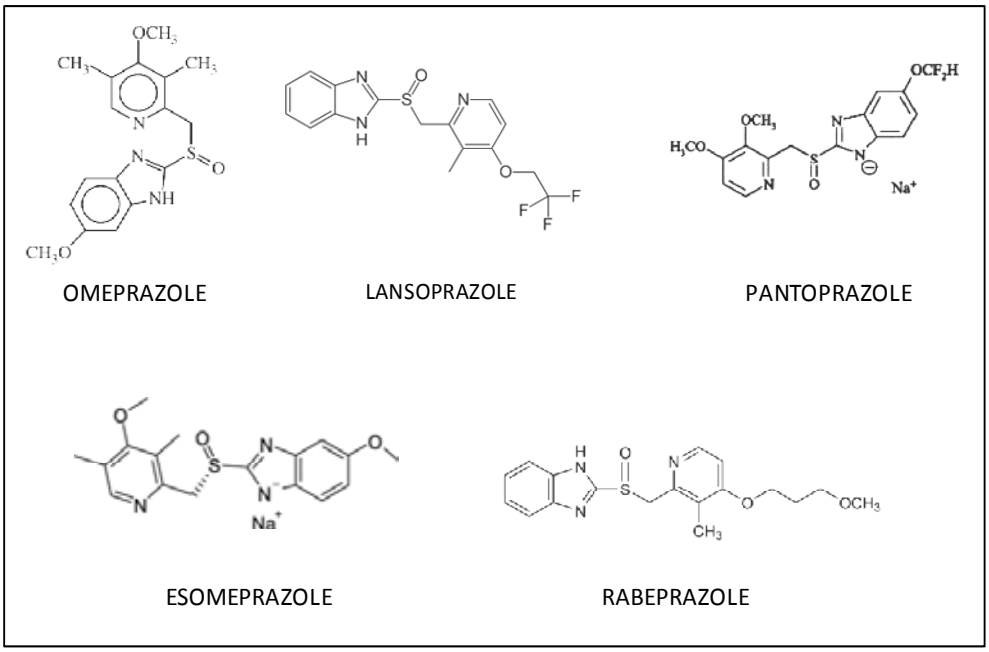

Fig. 3. Chemical structure of proton pump inhibitors (PPIs)

These drugs act primarily through the blockade of the enzyme H^+/K^+-adenosine triphosphatase (H^+/K^+-ATPase, the so-called "proton pump"), which is activated during the final step of acid secretion by the parietal cells of the stomach. PPIs are weak basic compounds, with acid dissociation constant (pKa) values ranging from 3.9 to 5.0. For this reason, they accumulate massively in the highly-acidic secretory canalicula of parietal cells, where they are rapidly converted into their active cyclic sulfenamide form. This highly reactive sulfenamide derivative binds sulfidryl groups of H^+/K^+-ATPase, leading to permanent enzyme inhibition and subsequent potent reduction of acid secretion (Boparai et al., 2008). Some studies have suggested that the beneficial effects of PPIs on ulcer healing could be ascribed to a marked inhibition of acid secretion, which can lead to a consistent increase in plasma gastrin levels, a peptide actively involved in the regulation of mucosal cell proliferation (Koh and Chen, 2000). However, the evidence supporting the involvement of gastrin in the healing action of PPIs is conflicting and there is no general consensus on the significance of this mechanism. Ito et al. (1994) initially showed that omeprazole was effective in increasing the healing rate of acetic acid-induced gastric ulcers in rats, and that this effect was related to a marked increase in serum gastrin levels. In a subsequent study, Schmassmann and Reubi (2000) observed that both omeprazole, inducing hypergastrinaemia, and exogenous gastrin-17 enhanced cell proliferation in the ulcer margin, with an acceleration of the healing process. Since these ameliorative effects were reversed by treatment with a gastrin receptor antagonist, the authors suggested that

omeprazole promoted ulcer healing through an increase in cell proliferation secondary to hypergastrinaemia. However, Okabe and Amagase (2005) provided evidence that a somatostatin analogue significantly decreased the omeprazole-induced hypergastrinaemia in rats with gastric ulcers, while not affecting the ability of this PPI to stimulate ulcer healing. In addition, the healing effect of omeprazole was not modified by gastrin receptor antagonists, thus suggesting that gastrin, released in response to omeprazole, played a marginal role in the mechanisms underlying the ulcer healing action of this PPI.

Besides the marked inhibition of gastric acid secretion, increasing evidence indicates that the beneficial effects of PPIs against NSAID-induced gastric injury could depend on acid-independent mechanisms. For instance, it has been shown that these drugs are able to counteract tissue oxidative damage in a direct or indirect manner (Lapenna et al., 1996; Natale et al., 2004). In particular, several in vitro experiments demonstrated a direct antioxidant activity of PPIs, showing that pantoprazole (Fornai et al., 2005) and lansoprazole (Blandizzi et al., 2005) concentration-dependently reduced copper-induced oxidation of human native low density lipoproteins (LDLs), while omeprazole behaved as a scavenger of hypochlorous acid (an oxidant compound generated by phagocytes) (Lapenna et al., 1996). Other studies have shown that pantoprazole is able to scavenge hydroxyl radicals, produced during a Fenton reaction, through the interaction with the hydroxyl radical generating system (Simon et al., 2006). Interestingly, in vitro experiments demonstrated that omeprazole and lansoprazole protected DNA from oxidative damage generated by hydroxyl radicals (Biswas et al., 2003). When considering the indirect antioxidant mechanisms, it has been observed that PPIs can significantly counteract the oxidative stress arising from polymorphonuclear cell activation. In this regard, omeprazole was shown to reduce neutrophil functions (Wandall, 1992), including adhesion processes to endothelial cells (Suzuki et al., 1999), phagocytosis and acidification of phagolysosomes (Agastya et al., 2000), and the production of ROS (Zedtwitz-Liebenstein et al., 2002). In addition, lansoprazole inhibited the release of free oxygen radicals from neutrophils activated by *Helicobacter pylori* (Suzuki et al., 1995). Recently, Martins de Oliveira et al. (2007) showed that omeprazole and pantoprazole inhibited H^+K^+-ATPase in neutrophils, resulting in cationic flow disturbances and subsequent suppression of migration and intracellular events, such as calcium influx and p38 MAPK activation. On the same line, Pastoris et al. (2008) demonstrated that, in addition to inhibiting acid secretion, the effects exerted by esomeprazole against indomethacin-induced gastric damage can be partly ascribed to a reduction in gastric oxidative injury.

It is also worthy to mention a novel mechanism contributing to the acid-independent beneficial effects of PPIs, which are able to induce and subsequently increase the catalytic activity of heme oxygenase-1 (HO-1) (Becker et al., 2006). The antioxidant, anti-inflammatory, anti-apoptotic, and vasodilatory properties of HO-1 pathway products, such as bilirubin and carbon monoxide, can counteract the main mechanisms of gastric damage. In particular, HO-1 plays a key role in the physiological tissue defense as well as in the modulation of ulcer healing process (Becker et al., 2006).

Mucosal depletion of sulphydryl radicals has been found to take part to the pathogenesis of gastric lesions evoked by different NSAIDs (Villegas et al., 2002), and reduced glutathione (GSH) concentrations have been detected in mucosal biopsies from patients with NSAID-induced gastric bleeding (Savoye et al., 2001). Consistently with these findings, gastric injury evoked by indomethacin in rats was shown to be associated with a significant decrease in mucosal GSH concentration, and treatment with esomeprazole protected the

gastric mucosa against indometacin-induced damage by restoring mucosal GSH levels (Pastoris et al., 2008).

The involvement of cyclooxygenase/prostaglandin pathways in the ulcer healing mechanisms activated by PPIs has been investigated with conflicting evidence. Some reports suggested that gastric mucosal levels of PGE_2 were unaffected by treatment with PPIs (Natale et al., 2004; Fornai et al., 2011). By contrast, Tsuji et al. (2002) reported that lansoprazole increased gastric COX-2 expression and PGE_2 production after repeated administrations in rats, and suggested that such increments resulted from a lansoprazole-induced increase in gastrin secretion.

Some studies have investigated the modulating effects of PPIs on several molecular markers of cell proliferation and apoptosis, in order to better characterize the mechanisms contributing to their ulcer healing actions. In this respect, Colucci et al. (2009) observed that the ability of esomeprazole to counteract the detrimental action of indomethacin on ulcer repair was ascribable to an enhancement of NF-kB activation and to a decrease in caspase-3-dependent apoptosis. Interestingly, these effects were found to likely depend on acid-independent mechanisms, since they were not reproduced by the histamine H_2 receptor antagonist famotidine, administered at an equivalent acid-inhibiting dose. More recently, in another experimental model of gastric ulceration, elicited by chronic indomethacin administration, it was confirmed that esomeprazole can exert antiapoptotic actions on gastric mucosal cells in the setting of ulcer repair (Fornai et al., 2011). In the same study, treatment with esomeprazole was also associated with a significant increase in mucosal expression of PCNA and Ki-67, both regarded as markers of cell proliferation. The beneficial influence of esomeprazole on ulcer repair has been related to mechanisms which are likely to be independent from the inhibition of acid secretion and ascribable to antioxidant properties (Fornai et al., 2011). This view is in line with previous studies reporting that both the antioxidant compound ascorbic acid and omeprazole enhanced the expression of growth factors, including TGF-α, in the gastric mucosa of rats treated with aspirin (Jainu and Mohan, 2008). In addition, these preclinical findings are consistent with the clinical evidence provided by Tsuji et al. (1995), who showed that lansoprazole, but not famotidine, induced the expression of bFGF in the gastric ulcer margin, and that PPI was more effective than famotidine in promoting ulcer healing. Other reports have suggested that several growth factors are involved in the ulcer healing effects of PPIs. In this regard, Kinoshita et al. (1998) observed that the gastric levels of HGF were enhanced by omeprazole in rats with indomethacin-induced gastric damage. Moreover, the expression of EGF was found to be increased in the gastric mucosa of mice with indomethacin-induced injury, and further enhanced by omeprazole (Banerjee et al., 2008).

6.1 Effects of PPIs on gastric ulcer healing: clinical evidence

Several clinical studies have been performed to investigate the efficacy of PPIs in promoting the healing of mucosal lesions in patients who unavoidably need to continue NSAID therapy. In a multicentre study, a subgroup of 68 gastric ulcer patients, who continued using NSAIDs, showed rapid ulcer healing when receiving omeprazole 20 and 40 mg/day, with a therapeutic advantage of 31% and 43%, respectively, after 8 weeks as compared with ranitidine 300 mg (Walan et al., 1989). Subsequently, in the ASTRONAUT trial, two doses of omeprazole (20 and 40 mg/day) were compared with ranitidine (150 mg twice daily) in patients with both gastric and duodenal ulcers. In this study, treatment with omeprazole was more effective than the H_2-receptor antagonist in terms of ulcer healing (Yeomans et al.,

1998). The clinical effectiveness of omeprazole has also been documented in comparative studies with other protective drugs. For example, a therapeutic gain of 18% in gastric ulcer patients and 22% in duodenal ulcer patients taking NSAIDs has been estimated when comparing omeprazole with sucralfate (Bianchi Porro et al., 1998). By contrast, the OMNIUM study did not display significant differences between omeprazole (20 and 40 mg/day) and misoprostol (200 μg four times daily) in terms of ulcer healing (Hawkey et al., 1998). Similar results were observed for lansoprazole (15 or 30 mg/day) in comparison with ranitidine (150 mg twice daily). Both doses of lansoprazole were significantly more effective than ranitidine for promoting the healing of gastric ulcers, in patients taking NSAIDs, after 4 and 8 weeks of treatment. In particular, after 8 weeks, the healing rate was 74% in patients treated with lansoprazole 30 mg, and 50% in patients treated with ranitidine 150 mg twice daily (Agrawal et al., 2000; Campbell et al., 2002). In a double-blind, placebo-controlled, randomized trial, patients, treated with low-dose aspirin and affected by upper digestive symptoms, were assigned to treatment with rabeprazole (20 mg once daily) or placebo for 4 weeks. At the end of this period, 47% of patients treated with rabeprazole and 43% of patients given placebo reported a complete relief of upper gastrointestinal symptoms (Laheij et al., 2003). Subsequently, two studies were performed to compare esomeprazole (20 or 40 mg once daily) with ranitidine (Goldstein et al., 2005; 2007). In the first study, gastric ulcer healing occurred in significantly higher proportions of patients treated with either 20 or 40 mg of esomeprazole, as compared with ranitidine at both 4 and 8 weeks. In particular, at the end of the 8-week treatment, the healing rate was 74% in the ranitidine group, 88% with esomeprazole 20 mg and 92% with esomeprazole 40 mg (Goldstein et al., 2005). The second study, performed by the same Authors, highlighted a significant difference in favor of both esomeprazole doses only after 4 weeks. By contrast, after 8 weeks, the healing rates were similar for esomeprazole (20 and 40 mg/day) in comparison with ranitidine (Goldstein et al., 2007).

7. Novel therapeutic options for prevention and treatment of gastric ulcer

Although the control of gastric acid secretion represents a cornerstone for the promotion of ulcer healing, an increasing interest is growing up about the characterization of the mechanisms supporting the process of ulcer repair, and the possibility that both the speed and quality of ulcer healing can be pharmacologically modulated.

At present, novel pharmacological strategies are being investigated to counteract the detrimental actions of traditional NSAIDs on the gastrointestinal tract. The main options currently under active evaluation are: (i) dual inhibitors of cyclooxygenase and 5-lipooxygenase (5-LOX), in order to prevent the mucosal injury resulting from the enhanced biosynthesis of leukotrienes, arising from the shift of arachidonic acid metabolism towards the leukotriene pathway as a consequence of cyclooxygenase inhibition; (ii) traditional NSAIDs associated with phosphatidylcholine, to minimize the destabilizing action of these drugs on the extracellular mucosal lining of zwitterionic phospholipids; (iii) NO donating NSAIDs, designated as cyclooxygenase inhibitors/NO donors (CINODs) and aimed at preventing the injurious actions of NSAIDs through the gastroprotective activity of exogenous NO; (iv) NSAIDs releasing H_2S, a gaseous mediator actively involved in the maintenance of digestive mucosal integrity and blood flow (Blandizzi et al., 2009).

Some of the above mentioned drugs are under clinical development. In particular, licofelone, a dual cyclooxygenase/5-LOX inhibitor, has been shown to spare the human gastric mucosa (endoscopic endpoint) when administered for 4–12 weeks to healthy

volunteers or patients with osteoarthritis in phase II or phase III trials controlled with placebo or naproxen (Bias et al., 2004; Becker et al., 2004). In a 4-day study, performed on healthy volunteers, the gastric injuring action of aspirin, assessed through endoscopic examination, was significantly reduced in subjects administered with soy phosphatidylcholine, although in both treatment groups prostaglandin levels in gastric biopsies were significantly reduced (Anand et al., 1999). Recently, Lanza et al. (2008) evaluated the digestive safety of ibuprofen chemically combined with phosphatidylcholine in osteoarthritic patients, observing a better tolerability of this association in comparison with ibuprofen alone.

CINODs have been developed exploiting the concept that NO, released locally in the gastric mucosa, would enhance the mucosal blood flow and reduce leukocyte adherence in the gastric microcirculation. Based on this assumption, aspirin and other traditional NSAIDs have been coupled to a nitroxybutyl or nitrosothiol group to yield novel anti-inflammatory entities which release discrete amounts of NO (Fiorucci et al., 2007). At present, the pharmacokinetic profile of these novel pharmacological entities remains unclear and deserve further investigations. However, encouraging results about the gastric safety profiles of these novel drugs arise from studies performed on healthy volunteers. In this regard, an endoscopic study demonstrated that healthy subjects treated for 7 days with NCX-4016, an NO-donating aspirin, did not display gastrointestinal toxicity (Fiorucci et al., 2003). On the same line, a trial performed on 31 healthy volunteers showed that upper gastrointestinal endoscopic events following oral administration of AZD 3582, a novel NO donating naproxen, for 12 days were significantly reduced in comparison with traditional naproxen (Hawkey et al., 2003). Moreover, Wilder-Smith et al. (2006) investigated the effects of equimolar doses of AZD3582 and traditional naproxen in healthy volunteers treated for 12 days, observing that treatment with the CINOD was endowed with a better gastroduodenal safety profile in comparison with naproxen. Clearly, further clinical studies are needed to establish whether CINODs confer actual advantages over traditional NSAIDs in terms of upper digestive safety.

Recently, an increasing attention has been paid to the beneficial effects of H_2S on the gastric mucosa. This gaseous compound, previously regarded as a toxic agent, is emerging as an endogenous modulator which seems to share almost all the beneficial actions of NO on several physiological processes. In particular, it has been demonstrated that H_2S is produced by the gastric mucosa, and that it contributes to the ability of this tissue to resist against damage induced by luminal agents (Fiorucci et al., 2007). Interestingly, several lines of evidence have shown that H_2S donors can prevent the decrease in gastric blood flow induced by NSAIDs, and reduce NSAID-induced leukocyte accumulation and adhesion in gastric microvessels, thus providing a rationale for the synthesis of H_2S-releasing NSAID derivatives as novel anti-inflammatory drugs (Fiorucci et al., 2007). As previously observed with CINODs, an H_2S-releasing derivative of diclofenac was shown to be better tolerated, in terms of gastric damage, than traditional NSAIDs. Moreover, the addition of the H_2S-releasing moiety has been found to increase the anti-inflammatory activity of diclofenac (Wallace, 2007; Li et al., 2007). Additional strategies for the prevention of NSAID-induced upper digestive damage include the ongoing clinical development of pharmaceutical products containing fixed combinations of a NSAID with a gastroprotective drug, such as naproxen/omeprazole, naproxen/lansoprazole, naproxen/esomeprazole and ibuprofen/famotidine (Blandizzi et al., 2009).

Several studies have focused their attention toward novel approaches to promote the healing of gastric ulcer. It has been widely recognized that the healing process requires

angiogenesis in the granulation tissue at the ulcer base, followed by a sustained proliferation of epithelial cells in ulcer margins and a subsequent re-arrangement of tissue architecture (Wallace, 2005). As discussed in this chapter, this complex process is finely regulated. In particular, it has been demonstrated PARs play important roles in the modulation of ulcer repair. In particular, preclinical studies have suggested PAR1 as a potential therapeutic target for promoting ulcer healing (Ma et al., 2005).

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8

Gastric Ulcer Healing – Role of Serum Response Factor

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1. Introduction

Histologically, a gastric ulcer is viewed as a necrotic lesion penetrating through the entire mucosal thickness of the stomach. Because of its great similarities with ulcers in other parts of the digestive tract, gastric ulcer is often reviewed with esophageal and duodenal ulcers together as peptic ulcer disease (PUD). Although it is not as common as duodenal ulcers, gastric ulcers are more often to develop malignancy.

PUD can be found in any part of the world and is probably the most common chronic infection in human population. It causes considerable loss of life year and creates a great economic burden (Figure 1). It had a tremendous effect on morbidity and mortality until the last few decades of the last century when epidemiological trends started to point to an impressive fall in its incidence, particularly in the Western countries. The reason why the rates of PUD decreased is thought to be the development of new effective medication, and of course, the discovery of the pathogen – *Helicobacter pylori*. It is now commonly accepted that the main cause of PUD is *H. pylori*, a helix-shaped Gram-negative bacterium, which infects more than 50% of world population and can be transmitted by contaminated food, groundwater, and even through human saliva (such as from kissing or sharing food utensils). For this reason, higher incidence of PUD is found in the third world countries and low socioeconomic groups. In the developed countries, on the other hand, although *H. pylori* infection is under controlled, thanks to the easy access to advanced treatment and better living condition, extensive use of non-steroidal anti-inflammatory drugs (NSAIDs) keeps the incidence of complicated gastric ulcer and hospitalization stable (Feinstein et al, 2010).

Treatment of PUD usually involves a combination of antibiotics (e.g. metronidazole, clarithromycin, tetracycline, amoxicillin), acid suppressors (e.g. cimetidine, ranitidine, omeprazole, lansoprazole), and mucosa protectors (e.g. bismuth subsalicylate). Unfortunately, patients have to take as many as 20 pills a day and often end up with multiple side effects including nausea, vomiting, diarrhea, dizziness, and headache. Perforated ulcers require surgical repair, while bleeding ulcers have to be taken care by endoscopic cautery, injection or clipping. In any case, healing of an ulcer normally requires multiple molecular and cellular processes to achieve. This chapter will dissect molecular and cellular mechanisms of gastric ulcer healing and focus on an important molecule – Serum Response Factor (SRF) and its role in this event.

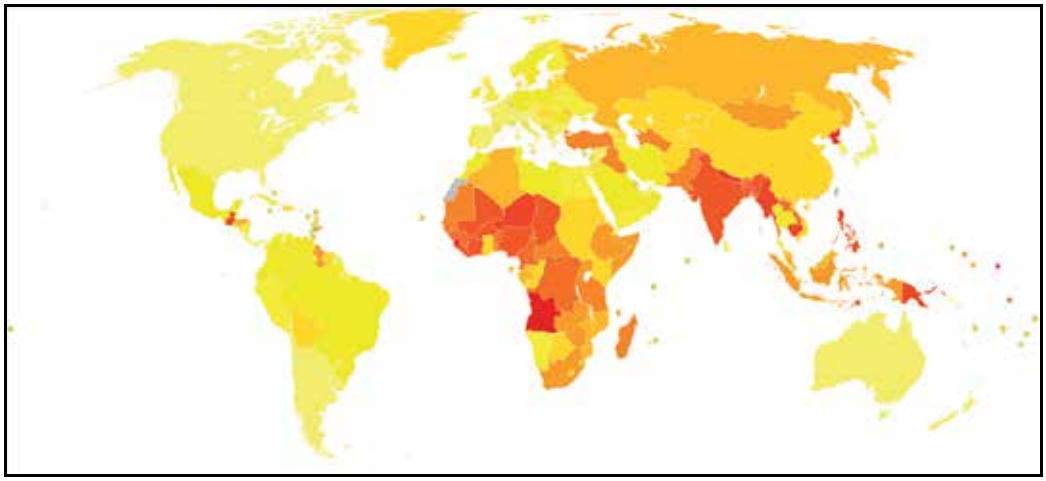

Fig. 1. World age-standardized disability-adjusted life year (DALY) for peptic ulcer disease per 100,000 inhabitants (Wikimedia Commons, based on WHO data in 2004). DALY is a term used by WHO to measure overall disease burden and is expressed as the number of years lost due to illness, disability or early death. It is calculated by summation of the years of life lost and years lived with disability. (no data less than 20 20-40 40-60 60-80 80-100 100-120 120-140 140-160 160-180 180-200 200-220 more than 220)

2. Prevalence of gastric ulcers

Our body function relies on two sources of energy, oxygen and food. Oxygen is taken into our biological system through breath and is directly utilized in the biochemical reaction, while food has to be processed in a very long and complicated structure to become useful to our body. That structure is called digestive system, which includes mouth, esophagus, stomach, small intestine, colon, liver, gall bladder, and pancreas. Any illness in these organs can cause the entire body suffering, often collapse. According to the World Health Organization (WHO), each year, more than 6 million people on earth die of various digestive disorders, making it the second most common cause of death in the world, after heart disease (~7 million). Among all the digestive fatalities, one third is caused by diarrhea, which kills 1.5 million children each year, more than AIDS, malaria and measles combined. For instance, in India, diarrhea causes 386,600 child deaths annually; and in Angola, it contributes to more than 17% of the overall death. Similar to diarrhea, PUD is also most prevalent in the third world countries, responsible for 4% of the total death toll caused by all kinds of digestive diseases combined (Figure 2). The top 15 most affected countries by PUD are listed in Table 1. Philippines is on the top of the list. In this country the PUD death rate is close to 16%, making it the third most deadly gastrointestinal disorder of the country, after diarrhea (37.4%) and liver diseases (23.6%). In the developed countries, on the other hand, the situation is totally different. Take the United States as an example, among eight major categories of digestive diseases, PUD (2%) is the least cause of death, next to diarrhea (2.4%). Instead, colorectal cancer (32.9%), liver disease (25.4%) and pancreas cancer (17.5%) become the top three deadly gastrointestinal problems (Figure 2). According to National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), PUD affects 14.5 million Americans

and about 350,000 new cases are diagnosed each year. Among them, duodenal ulcers are four times as many as gastric ulcers. The annual mortality is approximately 3,000.

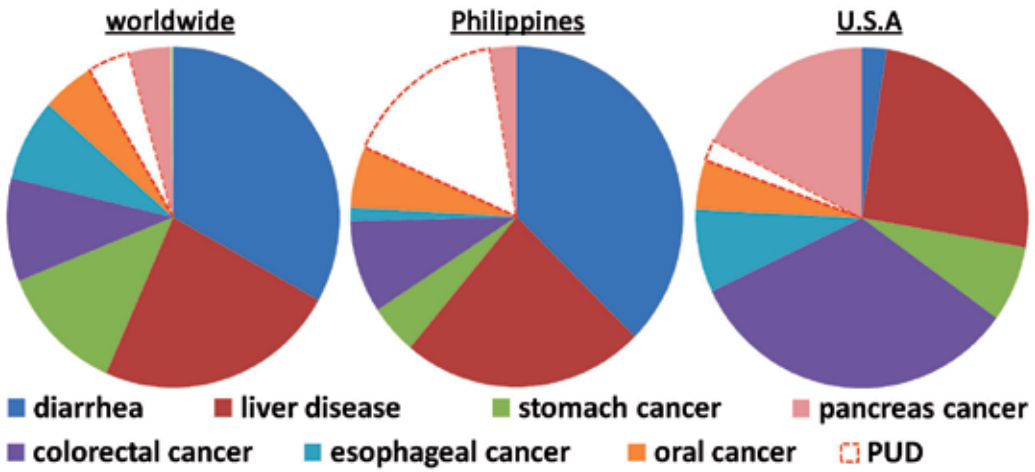

Fig. 2. Death caused by major digestive diseases. Diarrhea is the No.1 cause of death worldwide; however, in the developed countries such as the U.S.A, it becomes the least concern. So is PUD. (Data are extracted from WHO documents).

3. Causes of gastric ulcers

For decades, the causes of gastric ulcers were believed to be spicy food, stress, and excessive acid secretion. As the German Protestant theologian Karl Schwarz said "Ohne saueren Magensaft kein peptisches Geschwür", meaning no acid, no ulcer. Therefore, treatment options were confined to acid suppression medications and surgical operation. The successful rate of PUD treatment by acid suppressive operations was reported in the literature repeatedly. At the time, people did not believe that bacteria could survive in the human stomach, as the stomach produces extensive amounts of acid of strength similar to the acid found in a car battery.

By 1875, German scientists Bottcher and Letulle had examined the base of ulcers and found bacteria growing on the floors as well as on the margins of ulcers (Kidd & Modlin, 1998). They postulated, but never proved, that bacteria play a role in the development of PUD. Further effort had been made to dig the issue. In 1886, a Polish clinical researcher Jaworski found the same bacteria in the sediments of stomach washings from human and published his work in the *Handbook of Gastric diseases* in 1899, but the work had little impact because it was written in Polish (Konturek, 2003). The same bacteria were also found in the stomachs of animals including dogs (Bizzozero, 1892), cats and mice (Salomon, 1896). In 1938, Doenges discovered that 43% of 242 stomachs that he examined contained spirochete-like bacteria (Doenges, 1938); and in 1947, Freedburg and Barron confirmed this discovery in 37% of 35 specimens that they examined and they also noticed these bacteria appearing more frequently near ulcers than ulcer inside (Freedburg & Barron, 1940). Based on their observations, they concluded that the bacteria were opportunistic infections rather than the cause of PUD. However, interest in the role of bacteria in gastric diseases faded when Palmer, a pathologist at Walter Reed Army Medical Center in Washington DC, found no

bacteria at all in 1,180 biopsies taken from living individuals and he was very confident to claim that other people's discovery of bacteria in PUD was simply contamination (Palmer, 1954).

Rank	Country	Population	PUD Death Rate
1	PHILIPPINES	94,013,200	16.8
2	CAMBODIA	13,395,682	16.7
3	KIRIBATI	100,000	15.3
4	GUATEMALA	14,361,666	15.2
5	NEW GUINEA	10,324,000	14.7
6	NORTH KOREA	23,991,000	14.5
7	MYANMAR	50,496,000	14.5
8	NEPAL	28,584,975	14.3
9	TIMOR-LESTE	1,171,000	12.5
10	ANGOLA	18,993,000	10.5
11	BANGLADESH	149,804,000	10.4
12	SIERRA LEONE	5,836,000	9.9
13	NIGER	15,203,822	9.5
14	INDONESIA	237,556,363	9.0
15	LAOS	6,436,000	9.0

Table 1. Top 15 countries are mostly affected by PUD. Death rate is calculated by dividing the number of deaths caused by PUD with the total number of deaths. (Data are extracted from WHO documents).

Eager to understand the role of bacteria in gastric diseases was revived in the 1970s when Steer showed an image of the bacterium in the ultra-structure of gastric epithelia from PUD patients (Steer, 1975). Meantime, an Australian pathologist Warren and his clinical fellow Marshall were trying to isolate the bacteria and culture them *in vitro*. After numerous unsuccessful attempts, finally they succeeded in 1982 when they found colonies on their petri dishes that they accidentally left in the incubator for the Easter weekend. Marshall wanted to prove that the bacterium was a gastric pathogen, so he decided to use himself to

do an experiment. He swallowed the bacteria isolated from a 66-year-old man with known dyspepsia. Two weeks later, he found the bacteria colonized in his stomach in association with gastritis, proving his speculation (Marshall, 2002).

The bacterium was later identified as a new species named *Helicobacter pylori*, which infects upper gastrointestinal tract of more than half of the world's population, and in some regions of Africa and Asia, the prevalence can be as high as 80-90% of the local residents. In the developed countries, the rate is around 25% (Pounder & Ng, 1995). The ability of *H. pylori* surviving in the stomach comes from an enzyme – urease, which can break down urea into carbon dioxide and ammonia. The ammonia is converted into ammonium by taking a proton (H+), which leaves only hydroxyl ion. Hydroxyl ions then react with carbon dioxide, producing carbonate, which neutralizes gastric acid. Urease activity is low at neutral pH but can increase 10- to 20-fold as the external pH falls between 6.5 and 5.5, and remains high at pH 2.5 (Scott et al, 1998). *H. pylori* also expresses another protein – urel, which is a urea transporter that brings urea into the cytoplasm of the bacteria for urease to digest. About 50-70% of *H. pylori* strains in Western countries carry the *cag* pathogenicity island (*cag* PAI), a 40kb DNA segment containing more than 30 genes (Peek & Crabtree, 2006). Patients infected with this strain have a stronger inflammatory response in the stomach and are at a greater risk of developing peptic ulcers or stomach cancer than those infected with strains lacking the island (Kusters et al, 2006). The bacterium produces many different molecules that allow it to adhere to the mucosal surface. Following attachment of *H. pylori* to stomach epithelial cells, the type IV secretion system expressed by the *cag* PAI "injects" the inflammation-inducing agent, peptidoglycan, from their own cell wall into the epithelial cells. The injected peptidoglycan is recognized by the cytoplasmic pattern recognition receptor (immune sensor) Nod1, which then stimulates expression of cytokines that promote inflammatory response, such as gastritis, from the host (Viala et al, 2004). This inflammation leads to mucosal atrophy in the host, which predisposes to formation of ulcers. Therefore, eradication of the bacterium from the host has been proven to efficiently eliminate ulcer reoccurrence.

However, gastric ulcers are also found in people without *H. pylori* infection. Studies have associated this group of patients with overly use of NSAIDs. Most NSAIDs are non-selective inhibitors of cyclooxygenases (Cox-1, Cox-2), which convert arachidonic acid to prostaglandins (Pai et al, 2001). Prostaglandins are mediators of inflammation. Inhibition of prostaglandin synthesis in the stomach causes increased gastric acid secretion and decreased mucus secretion, thereby weakening gastric mucosa protection and allowing the acid to come into close contact with the mucosal epithelium.

It is currently believed that 70-90% of gastric ulcers are caused by *Helicobacter pylori* infection, and utilization of NSAIDs is responsible for the remainder. However, in both conditions, doctors have noticed that adding acid-suppressive drugs to the treatment regimen can greatly help ulcer healing and prevent ulcer reoccurrence. Some even argue that *H. pylori* itself cannot cause ulcers at all; even Dr. Robin Warren, the Noble laureate for the discovery of *H. pylori* as the pathogen of gastric ulcers, admitted that the bacteria cannot be responsible for so many ulcers without acid. Therefore, acid is still a factor. It is my belief that no matter *H. pylori* or NSAIDs, their actions lead to removal of mucosal protection, which allows the acid to come into a direct contact with the mucosal epithelium and that causes ulcer development. Zollinger-Ellison syndrome is an example,

in which gastric acid is over-secreted due to high level of hormone gastrin. Gastrin induces parietal cells to produce more acid and also stimulates parietal cell hyperplasia, which leads to severe gastric ulceration. One might conclude that the dictum "no acid, no ulcer" still holds true.

4. Molecular and cellular mechanisms of gastric ulcer healing

A gastric ulcer is a deep wound in the stomach wall that involves epithelium, endothelium, connective tissue, and smooth muscle. Therefore, healing of a gastric ulcer means a restoration of all these tissue components that have been damaged during ulceration. At the cellular level, this process requires participation of all the cell types that originally make these tissues, including epithelial cells, endothelial cells, fibroblasts, myofibroblasts, smooth muscle cells, and immune cells. All these cells are activated to move towards the ulcer to fill in the positions that had been vacant due to damage and loss. Some of these cells (e.g. epithelial cells) need to divide to make up the number, while others (e.g. immune cells) need to be differentiated from progenitor stem cells. In addition to cell proliferation and differentiation, there is a third source to get the cell supply needed to re-build the tissue, that is, cell transformation. Some of these cells, if not all, can transform from one cell type to another (Chai et al, 2010a). For example, epithelial cells can start to express mesenchymal molecules (e.g. vimentin, N-cadherin, smooth muscle α-actin) to become fibroblasts or even myofibroblasts, while fibroblasts or myofibroblasts can express epithelial markers (e.g. E-cadherin, ZO-1, γ-catenin) to connect with each other and form cellular sheets like epithelium. The former event is called epithelial-mesenchymal transition (EMT), and the later, of course, is mesenchymal-epithelial transition (MET). In a normal individual, all these events take place in a well synchronized spatial and temporal manner so that the damaged tissue is eventually replaced by new tissue precisely like the old tissue before ulceration. This job is done at the molecular level.

Like any other wounds, ulcer healing starts with a process of coagulation and hemostasis immediately after ulceration is initiated. The principal of this process is to prevent exsaguination and to provide a matrix for the cells coming into the ulcer in the later phase of healing. A dynamic balance between endothelial cells, platelets, coagulation, and fibrinolysis regulates hemostasis and determines the amount of fibrin deposited at the wound site, thereby influencing the progress of healing. Normally, endothelial cells produce heparin-like molecules and thrombomodulin to prevent blood coagulation and also nitric oxide and prostacyclin to inhibit platelet aggregation; however, when a vascular injury occurs during ulceration, these cells stop making these molecules, instead, start to secrete von Willebrand factor and thromboplastin to adhere platelets to the exposed collagen and to convert prothrombin to thrombin. Thrombin then converts fibrinogen to fibril to strengthen platelet plug. Once platelets come in contact with collagen, they become activated to release growth factors and cytokines, such as platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), Tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). These molecules act as promoters in the ulcer healing cascade by activating and attracting neutrophils and later, macrophages, endothelial cells, fibroblasts, and myofibroblasts to the ulcer area, and move

the healing process to the next phase – inflammation. The main function of neutrophils is to prevent infection. These cells can destroy and remove bacteria and damaged tissue by phagocytosis. Once this task is completed, neutrophils are eliminated by apoptosis. Then macrophages move in to clean up the cell remnants and apoptotic bodies of neutrophils. Macrophages are key regulatory cells during ulcer healing because they not only continue neutrophil's job, but also produce an abundant reservoir of potent growth factors to activate additional endothelial cells and fibroblasts.

The inflammatory phase is ended when lymphocytes attracted to the ulcer by IL-1, an important regulator of collagenase activity that is later needed for extracellular matrix (ECM) remodeling. Fibroblasts synthesize ECM to replace the provisional network of fibrin and fibronectin and form granulation tissue under the ulcer bed. Fibroblasts are attracted to the ulcer by TGF-β and PDGF that are produced by inflammatory cells and platelets. Once in the ulcer, fibroblasts proliferate rapidly and produce abundant ECM proteins, such as fibronectin, proteoglycans and procollagen, whose accumulation in the ulcer provides further support for cell migration and tissue repair. Thereafter, fibroblasts transform into myofibroblasts with thick actin bundles underneath the cell membrane which generate powerful forces to pull the wound edges together to close the ulcer. Granulation tissue is a reflection of active angiogenesis. A number of molecules released during hemostasis are angiogenic factors, such as VEGF, PDGF, bFGF, and TGF-β, which can stimulate resident endothelial cells to proliferate. The activated endothelial cells produce proteases (matrix metalloproteinases or MMPs) to digest the basal lamina in the parental vessels in order to crawl through the ECM and to re-gather to form new blood vessels in the wound center, giving bumpy appearance to the ulcer bed. Angiogenesis is essential for ulcer healing, because it provides nutrients for the healing process to move forward.

Meantime, mucosal epithelial cells at the ulcer margin are stimulated by ulceration to form a contractile actomyosin ring around the ulcer. Actomyosin ring is made of filamentous actin (F-actin) and myosin-II in association with radially organized microtubules (Mandato & Bement, 2003). F-actin cable in each epithelial cell at the ulcer margin links to neighboring cells through adherens junctions and is operated by the motor protein myosin-II, jointly like a purse string provides the force necessary to draw the wound edges together to achieve re-epithelialization (Figure 3). The whole process is regulated by the small GTPases including RhoA, Rac and Cdc42. RhoA activates the assembly of F-actin stress fibers by cortical flow, Rac is required for the rapid actin polymerization to form lamellipodia, and Cdc42 is essential for myosin-II organization and actin assembly/disassembly (Garcia-Fernandez et al, 2009; Darenfed & Mandato, 2005). The cells directly bordering the ulcer are connected by a continuous actomyosin cable, anchored at cell-cell junctions, and form lamellipodia at their leading edge (Figure 3). At the final stage of wound closure, opposing leading edge cells make contact through lamellipodia and seal the gap.

Epithelial cell migration stops once the gap is sealed. However, healing process still continues into the next phase – tissue remodeling within the ulcer. A new basement membrane starts to build underneath the epithelium. Granulation tissue is gradually replaced by regenerated tissue that more closely resembles the original tissue before ulceration. The main players in this phase are MMPs and their antagonists TIMPs. They keep in a very delicate dynamic balance and work together in a coordinated fashion to allow tissue synthesis and breakdown to take place simultaneously.

5. Serum Response Factor in gastric ulcer healing

During ulcer healing, epithelial cells proliferate and migrate from nearby to close the wound; smooth muscle cells and myofibroblasts multiply to restore the musculature; endothelial cells are motivated to generate vessels to make sure the newly generated tissue has an adequate nutrient supply; and immune cells stand by to guard the wounded area and protect from invasions of pathogens. All these cellular activities are directed and regulated by dozens of molecules including growth factors, cytokines, chemokines, and more importantly, transcription factors, because every one of these molecules has to be transcribed from its gene fundamentally and transcription factors are the ones for this job. Among many transcription factors involved in ulcer healing, Serum response factor (SRF) is the master regulator. SRF is ubiquitously expressed in every type of tissue and its targeted genes take up nearly 1% of our entire genome (Sun et al, 2006; Miano, 2010). SRF can be activated by growth factors, cytokines and chemokines, and in return, activated SRF can direct expressions of these molecules to heal ulcers in a precisely organized manner. Moreover, CagA, one of the main products of *H. pylori*, can increase SRF binding capacity by 40 fold (Hirata et al, 2002). SRF is involved in every stage of the healing process including re-epithelialization, angiogenesis and granulation tissue remodeling.

5.1 Story of SRF

SRF was first identified by a British scientist Richard Treisman in 1986 (Treisman, 1986), for which he was awarded the EMBO Medal in 1995. Treisman's discovery was built on a prior observation by Michael Greenberg, a postdoctoral research fellow at the time in Edward Ziff' lab at New York University. Greenberg's work showed that resting fibroblasts responded to serum addition with a rapid activation of *c-fos* (Greenberg & Ziff, 1984). Since its activation does not require new protein synthesis, *c-fos* was classified as an immediate early gene. Later, it was found that in addition to serum, other mitogenic agents such as growth factors have the same effect on *c-fos* activation (Rollins & Stiles, 1989). During that time, Treisman was a struggling postdoctoral research fellow at Harvard University who was interested in *c-myc* regulation (Treisman, 1995). In the summer of 1984, he met Edward Ziff and heard about Greenberg's discovery. Treisman immediately forsook *c-myc* and switched to *c-fos*. After he returned to England, Treisman rapidly proceeded with *c-fos* study by focusing on 5′ regulatory region. Several regulatory DNA elements were identified in the promoter region of *c-fos* gene, but a particular attention was given to a short sequence located about 300bp upstream of the transcription initiation site. For convenience, Treisman named this sequence Serum Response Element (SRE) and the protein that identifies this sequence Serum Response Factor (Treisman, 1986). SRE is an A/T rich core flanked by an inverted repeat, $CC(A/T)_6GG$, and for this reason, SRE is also referred to as CArG box. Treisman demonstrated that *c-fos* activation by serum requires SRF binding to SRE. By that time, several other labs also identified the existence of SRF (Gilman et al, 1986; Prywes & Roeder, 1986; Greenberg et al, 1987). Since then, SRE has been identified in many genes across our entire genome (Sun et al, 2006). The list of SRE-containing genes is still growing.

In 1986, Greenberg moved to Boston and became a faculty of Harvard Medical School with his own lab. His initial observation stimulated many researchers to look in that direction and led to a series of important discoveries in the area of gene transcriptional regulation. His colleagues wrote a song to portrait him and his work around c-fos:

"He was a bald headed man
He was brutally handsome
And they were terminally busy
They held him up
And he held them for ransom
In a lab in a cold, cold city
He had a nasty reputation
As a cru-el dude
They said he was ruthless,
Said he was crude
They had one thing in common
They were always uptight
He'd say "Faster, faster,
Let's publish by tonight"
Life in the fos lane
Surely make you lose your mind
Life in the fos lane
Eager for action
Hot for the game
The Sephadex fraction
The quest for the fame
They read all the right journals
They paid gigantic bills
They threw outrageous parties
They had infamous spills
There were bands on the Northern
But no counts could be traced
He pretended not to notice
He was caught up in the race
In every evening, until it was light
They were so tired, they faked it
He was too tired to fight about it
Life in the fos lane
Surely make you lose your mind
Life in the fos lane
Life in the fos lane
Everything, all the time
Life in the fos lane
Rapid and transient
Transcribed in a burst
In all cell responses
c-fos turns on first
He said listen Bernie
We need space to work in
We've been up and down this
hallway
And never seen Ed Lin
He said call Howard Hughes
I think I'm gonna crash
Six post-docs are coming
And I'm almost out of cash
He kept pushing them to publish
"Go for Cell" he would shout
They didn't care
They were just dying to get out
And it was
Life in the fos lane
Surely make you lose your mind
Life in the fos lane
Life in the fos lane
Everything all the time
Life in the fos lane"

5.2 Biology of SRF

The human SRF gene is 10607bp long containing 7 exons and is mapped to the chromosome 6p21.1. The full length of SRF transcript is 4201bp including exon 1 (1-871), exon 2 (872-1138), exon 3 (1139-1400), exon 4 (1401-1520), exon 5 (1521-1712), exon 6 (1713-1789), and exon 7 (1790-4201). SRF can be expressed in different isoforms due to alternative splicing and some of them appear to display tissue specificity. For instance, SRF-S, which lacks both exon 4 and 5 (Δ4, 5), has only been detected in the aorta, while SRF-I, which is the shortest isoform (missing exon 3, 4 and 5), is specific to embryonic tissues. On the other hand, SRF-M, which lacks only exon 5, has been shown as a dominant negative mutant. SRF expression is self regulated, because SRF gene promoter contains four SRE sites. Full length SRF protein (~67 kDa) contains three distinct domains: a SRE DNA binding domain, a transactivation domain and multiple phosphorylation

sites. The DNA binding domain, which also serves for dimerization and interaction with accessory factors, has been highly conserved throughout evolution, showing a 93% homology between fruit flies and humans. Phosphorylation at Serine 103, which is immediately adjacent to the DNA binding domain, was shown to greatly enhance SRF activity (Chai & Tarnawski, 2002; Modak & Chai, 2010).

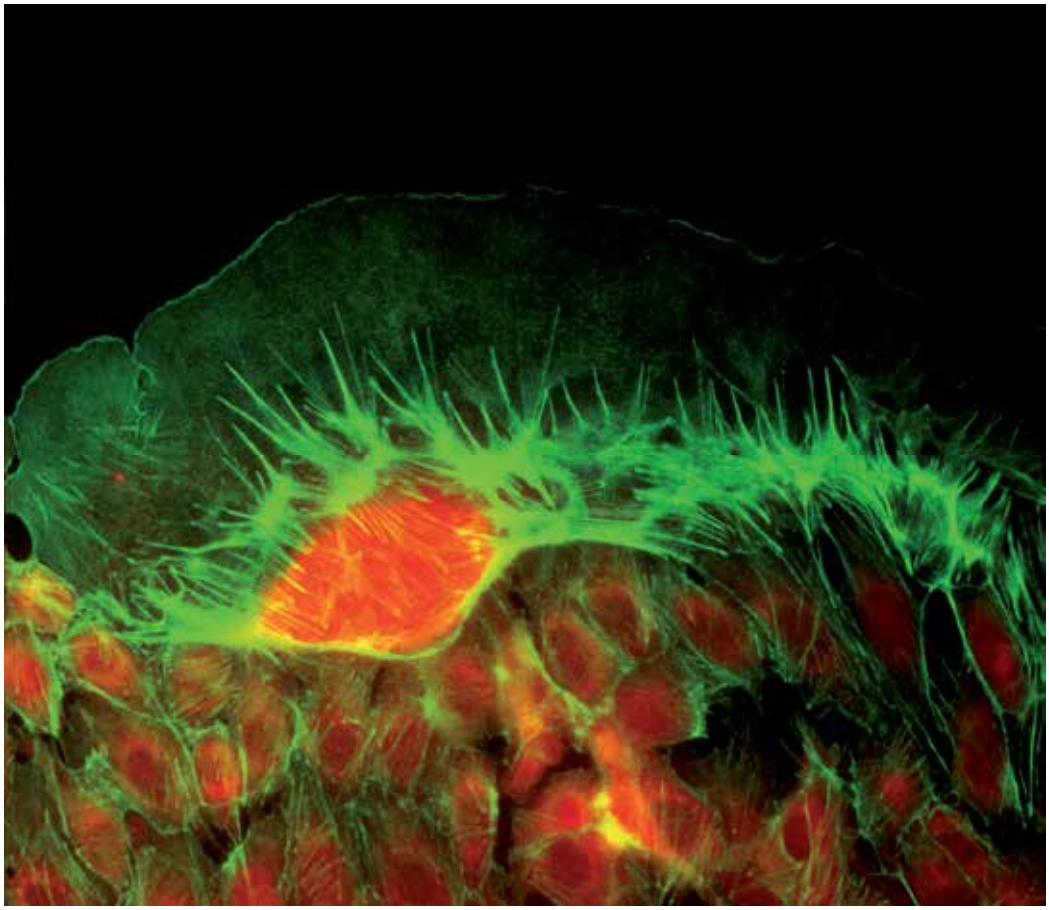

Fig. 3. Actomyosin ring formation at the edge of a wound. Monolayer of rat gastric mucosal epithelial cells (RGM1) was wounded by scratch with a pipette tip. Two hours later, cells were fixed in 3.7% formaldehyde, permeabilized with acetone, and stained for F-actin (green) with Oregon Green-conjugated phalloidin and for G-actin (red) with Texas Red-conjugated DNase I. The image shows a part of the ring and lamellipodia.

5.3 Functions of SRF

What is SRF? A few years ago, the **Medical News Today** conducted an interview with Joseph Miano from University of Rochester, one of the prominent SRF researchers, and described that "SRF is one of nature's oldest proteins and is essential for life because it supports the basic internal structure of all living cells. Its function is to carefully turn on 300 of our 30,000 genes" (Orr, 2004). In another word, about 1% of the total human genes carries

SRF target – SRE. These genes fall into a broad spectrum and some of them have multiple SRE sites, for example, EGR1 has six and CCN1 has five, even SRF itself has four SRE sequences (Sun et al, 2006). In addition to the well-known immediate early genes (e.g. FOS and EGR1), SRF also controls a long list of muscle-related genes (ACTA2, MYH6, MYH11, SM22α, TNNT1, ATP2A1, etc). In fact, most of the published SRF studies focus on its role in muscular structures including cardiac muscle, smooth muscle and skeletal muscle. Ten years ago, I was a postdoctoral research fellow at Harvard University, walking behind the giants like Greenberg and Treisman and trying to find new meanings for SRF. We created transgenic mice with cardiac-specific overexpression of SRF. The mice died within 6 months after birth due to heart failure. Histological examination revealed severe cardiomyocyte hypotrophy and interstitial fibrosis (Zhang et al, 2001a). The image made to the cover of the **American Journal of Physiology**. From this study, we have learned that too much SRF can drive overexpression of numerous cardiac genes (MYH7, ACTA1, NPPA, etc.) and end up with a bigger and heavier heart than in normal individuals. The heart-to-body weight ratio was almost 4 times greater in transgenic mice compared to non-transgenic littermates. To look at the other side of the coin, we also produced transgenic mice that express a dominant mutant SRF in heart. The mutated SRF gene generated a protein product that was incapable to bind to SRE, and therefore, cardiac genes never had a chance to fully express during embryogenesis. As a result, most embryos died before born, and a few survivors barely made to the second week of their age. Histological examination displayed serious cardiac ventricle dilation and myofiber degeneration (Zhang et al, 2001b). These studies demonstrate that properly functional SRF is essential for both embryonic development and post-natal development. This concept is also supported by the earlier transgenic study showing that complete knockout of SRF was lethal (Arsenian et al, 1998). Similar consequences have been also observed in transgenics of skeletal muscle (Li et al, 2005; Chavret et al, 2006; Lahoute et al, 2008) and smooth muscle (Miano et al, 2004; Werth et al, 2010).

SRE has also been identified in cytoskeletal genes (ACTB, CFL1, DES, DSTN, TTN, KRT17, etc.), another major category with more than 1,000 members, whose protein products form an intracellular network connecting membranous subcellular structures to the cell membrane and the nucleus. Some of these genes are expressed in all types of cells, suggesting that SRF is essential for maintenance of cell shape and locomotion in everywhere of our body. SRF regulates cytoskeletal organization; on the other hand, SRF itself is regulated by the dynamics of actin cytoskeleton. For instance, every time G-actin polymerizes into F-actin, SRF gets activated.

The remaining SRE-containing gene products fall into many diversified categories, such as growth factors (e.g. IGF2, FGF10, FGFR3, TGFB1i1, etc.), ECM proteins (e.g. CCN1, CTGF, etc.), cell adhesion molecules (e.g. ITGA1, ITGA5, ITGB1, etc.), intercellular junctional molecules (e.g. TJP1, CDH5, CDH11, etc.), neuronal receptors (e.g. NR4A1, NR4A2, etc.), and apoptosis regulators (BCL2).

In addition to the hundreds of genes that SRF directly regulates, a growing number of genes that do not contain SRE have been found to respond to SRF activity (Khachigian & Collins, 1997; Miano et al, 2007). From this, one can imagine the influence of SRF on life.

5.4 SRF in ulcer healing

Like any other human diseases, gastric ulcer and gastric ulcer healing have been studied both clinically as well as experimentally. Since the rules and regulations on clinical studies

are extremely strict, most of the mechanistic studies have to be done in animal models complemented by *in vitro* cell culture. Researchers have developed several animal models for gastric ulcer study, which generally can be classified as chemical-induced and surgical-induced. Comparison of all these models reveals striking similarities in the morphological evolution as well as molecular dynamics involved in healing process. Therefore, it is generally accepted that ulcer undergoes common stages of healing, as discussed above, once it develops, regardless the cause (Tarnawski, 2005). Figure 4A shows a typical gastric ulcer developed in rat by topical application of acetic acid on the serosal side of the stomach. This model was initially developed by Japanese researchers and modified and validated by others (Okabe & Pfeiffer, 1972). Briefly, the animal needs to be fasted 12 hours before operation, otherwise, the food in the stomach would interfere ulcer induction. Laparotomy is performed under anesthesia to expose the stomach. Hold the stomach tightly with one hand and apply 50μl of acetic acid to the wall of the glandular stomach with the other hand, through a pipette tip (Ø 4.00mm). Hold for 90 seconds and then clean up the area with saline. In this way, a gastric ulcer can develop within 3-5 days after induction (Chai et al, 2004a; Nguyen et al, 2007).

Immunohistological examination shows that SRF is highly activated in the ulcerated mucosa as well as in underneath connective tissue (Figure 4B). Figure 4C shows a higher magnification of regenerating gastric mucosal glands in the ulcer, and the bright red nuclear stain indicates SRF activation. The similar result can be seen in human gastric ulcer as well (Figure 4D).

To determine what role SRF plays in gastric ulcer healing, we injected SRF expressing plasmid around the ulcer induction site to boost the local level of SRF. As a result, ulcer healing was significantly accelerated by the treatment (Chai et al, 2004a). In particular, re-epithelialization process was speeded up. In addition, a massive amount of smooth muscle α-actin expressing cells were found in the granulation tissue under the ulcer bed, indicating an increase of smooth muscle cells and/or myofibroblasts. *In vitro* overexpression of SRF in gastric mucosal epithelial cells (RGM1) and smooth muscle cells (A7R5) all proved promotions in cell migration and proliferation, as reflected by increased actin polymerization and activations of *c-fos* and *egr-1*, suggesting that the acceleration of ulcer healing by SRF gene therapy is due to SRF-driven cell migration and proliferation.

As we discussed above, no matter re-epithelialization or smooth muscle structure restoration, they all require blood supply provided by angiogenesis, a process that makes sure the newly generated structures during ulcer healing will survive. In order to test what influence SRF has on angiogenesis during ulcer healing, we did same injection around the ulcer, but this time the SRF cDNA in the plasmid was flipped over to become an antisense generator. The idea was to interfere with local SRF expression and to create a local SRF deficiency. As a result, less number of micro-vessels was found in the granulation tissue, indicating that SRF deficiency impairs angiogenesis (Chai et al, 2004b).

This conclusion was also supported by *in vitro* study, which showed that SRF deficiency impaired endothelial cells migration and proliferation capability so that even the most powerful angiogenic factor like VEGF could not stimulate tube formation in Matrigel or collagen gel matrix (Figure 5), a phenomenon called *in vitro* angiogenesis normally observed in the presence of an angiogenic factor (Jones et al, 2001). Further dissection of the mechanisms revealed that VEGF activates SRF through MEK-ERK and Rho signaling, and blocking these pathways interrupts SRF mediated endothelial cell migration and proliferation, and eventually causes failure of angiogenesis.

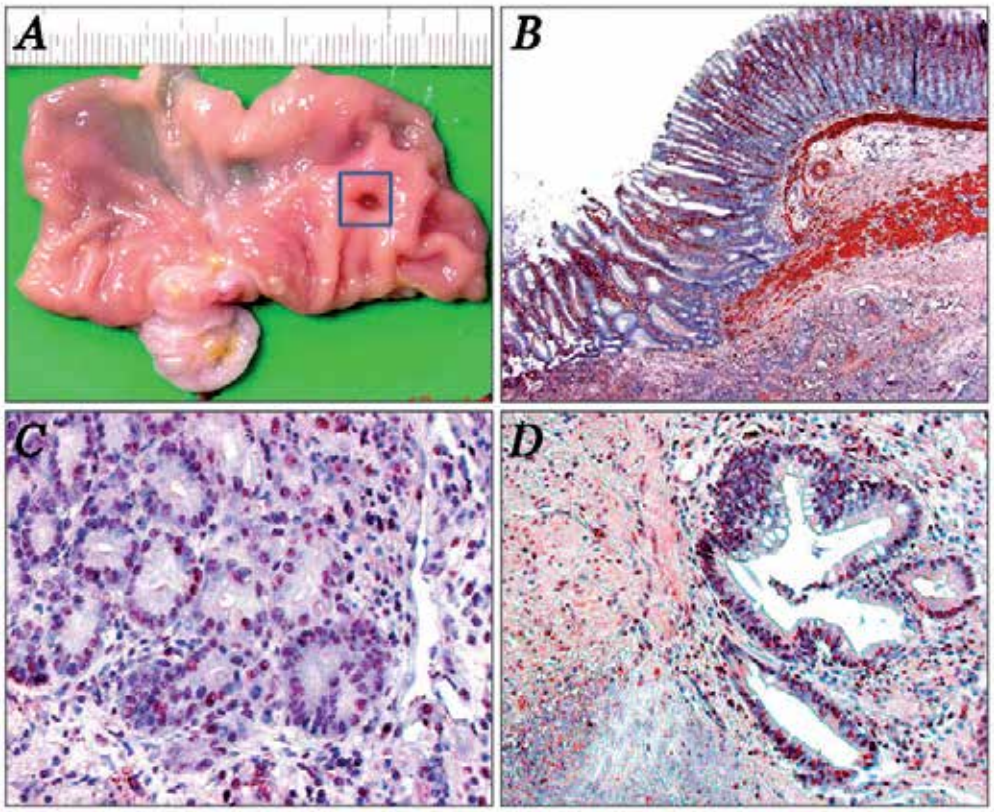

Fig. 4. SRF activation during gastric ulcer healing. *A*. A gastric ulcer induced in rat by acetic acid experimentally. *B*. Immunohistochemistry shows SRF activation in the ulcer region. *C*. SRF activation in the regenerating mucosal glands. *D*. SRF activation in the human gastric ulcer.

In addition to epithelial cells, endothelial cells, and smooth muscle cells, we have also examined another type of cell – myofibroblast. As we discussed above, myofibroblasts play an important role in ulcer healing by producing many growth factors and ECM molecules to mediate the healing process and also by providing contractive force to close the ulcer. We found that ulceration can trigger myofibroblast differentiation from the epithelial cells adjacent to the wound and from the fibroblasts within the ulcer bed (Chai et al, 2007). These cells can be distinguished from their ancestors by their expression of smooth muscle α-actin, and from smooth muscle cells by the absence of smoothelin. Many myofibroblasts were a transient phenotype, once the ulcer was healed, they disappeared. Local increase of SRF level by injecting SRF expressing plasmid into the ulcer greatly boosted the number of cells that express smooth muscle α-actin but not smoothelin, indicating that SRF promotes myofibroblast differentiation. This conclusion was also supported by *in vitro* experiments, which demonstrated that overexpression of SRF in both epithelial cells and fibroblasts induced expression of smooth muscle α-actin (Figure 6).

The involvement of SRF in gastric ulcer healing was also strengthened by the finding of its association with *H. pylori*. In 2001, Japanese researchers found that when gastric cells were

co-cultured with *H. pylori* strain that possesses the cag PAI, SRE promoter activity was increased by 3-6 fold (Mitsuno et al, 2001). Their further investigation showed that when cells were transfected with CagA expressing vector, SRE promoter activity can be increased by 40 fold (Hirata et al, 2002). CagA is one of the cag PAI encoded genes. Upon attaching to the mucosal epithelial cells, cag PAI secretion system transports CagA into the host cells, causing actin cytoskeleton rearrangement into "Hummingbird" phenotype (Backert et al, 2001). These studies link SRF to the main cause of peptic ulcer.

In addition to peptic ulcer, SRF has also been associated with other digestive functions and abnormalities, which has been reviewed elsewhere (Modak & Chai, 2010; Miano, 2010).

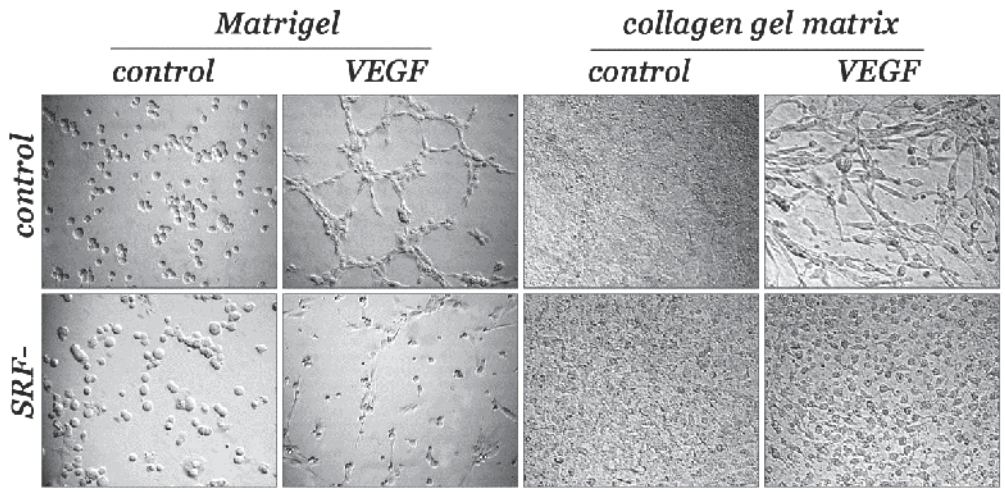

Fig. 5. SRF is required for angiogenesis. Rat gastric microvascular endothelial cells were transfected with either a plasmid expressing antisense SRF (SRF-) or the plasmid vehicle (control). Cells were seeded on Matrigel and collagen gel matrix and treated with either recombinant VEGF at 50ng/ml or vehicle (control). Loss of SRF impaired VEGF-induced tube formation and cell sprouting. Matrigel assay and collagen gel matrix assay are also called 2- and 3-dimensional *in vitro* angiogenesis assay respectively.

6. Serum Response Factor regulated genes in gastric ulcer healing

As we discussed above, hundreds of genes are directly or indirectly regulated by SRF. During ulcer healing, all the damaged parts by ulceration, including mucosal epithelium, muscularis mucosa, connective tissue and microvascular structure, must be repaired or regenerated. Needless to say, molecules constituting these components are definitely involved in the ulcer healing process. For instance, smooth muscle α-actin, smooth muscle γ-actin, smooth muscle myosin heavy chain, smooth muscle calponin, smoothelin, and SM22α are basic molecules of muscularis mucosa; Endothelin 1 and VE-cadherin are essential components of blood vessels; Tight Junction protein 1 and cytokeratins such as CK7, CK8, CK14, CK17, CK18 and CK19 make up epithelium. All of these molecules are direct targets of SRF. In addition to structure molecules, SRF-regulated adhesive and locomotive molecules such as integrin-α1, -α5, -α9, and –β1 and vinculin are involved in cell migration;

Bcl-2 regulates apoptosis; SRF-regulated secreted molecules such as connective tissue growth factor and insulin growth factor 2 are mediators of the healing process. Here we will present a couple of SRF targets that have been studied in detail.

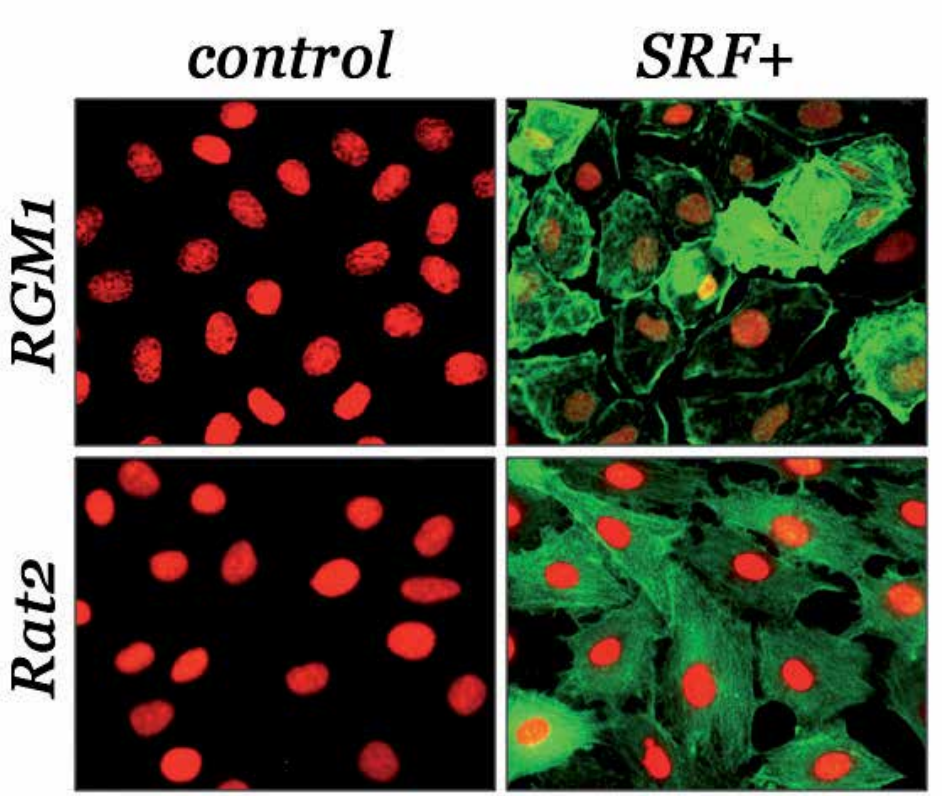

Fig. 6. Overexpression of SRF (SRF+) in rat gastric epithelial cells (RGM1) or fibroblasts (Rat2) can induce myofibroblast phenotype. Cells were stained for smooth muscle α-actin which was identified with a FITC-conjugated secondary antibody. The nuclei were counterstained with propidium iodide.

6.1 Egr-1

Egr-1 is an immediate early gene coded transcription factor that is activated in the early phase of ulceration (Khomenko et al, 2006; Szabo et al, 2000). The fact that its activation requires SRF was first noticed in 1993 when leukemia cells were treated with an anti-leukemia drug called 1-(beta-D-arabinofuranosyl)cytosine (Kharbanda et al, 1993). Egr-1 was found transiently activated by the treatment, but deletion of certain region of the Egr-1 promoter, about 95bp upstream from the initiation site, impaired this activation. Further analysis of this region found six SRE sequences, making Egr-1 a close target of SRF. On the other hand, SRF gene promoter contains Egr-1 binding element, 5′-GCGGGGGCG-3′, suggesting that SRF itself is also regulated by Egr-1 (Spencer et al, 1999). Microarray analysis screened 12,000 gene promoters and found that at least 283 genes have Egr-1 binding sites (Arora et al, 2008). Many of these genes encode proteins (e.g. TGFβ1, bFGF, PDGF, p53, p73,

PTEN, EGFR, BMP4, MMP9, ITGA5, CK16, Egr-2, etc.) that are known to contribute to ulcer or other wound healing. Through regulation of these genes, Egr-1 greatly extends SRF power.

6.2 CCN1

CCN1 (formerly known as Cyr61 or IGFBP10) is another important gene directly regulated by SRF and contains five SRE sites located about 3751bp upstream in the gene promoter. It encodes a matricellular protein that is best known for its angiogenic activity because it stimulates neovascularization in rat corneas and *cyr61*-null mice suffer embryonic death due to vascular defects (Mo et al, 2002). One study demonstrated that intramuscular injection of a CCN1-expression adenovirus in rabbits with ischemic hindlimb improves tissue perfusion even greater than injection of VEGF (Fataccioli et al, 2002). The involvement of CCN1 in wound healing was first known ten years ago in a cutaneous wound model (Chen et al, 2001; Lantinkic et al, 2001). During the experiment, CCN1 was found highly up-regulated in the granulation tissue five days after wounding and remained high for a week till the re-epithelialization was completed. It was shown that CCN1 promotes angiogenesis not only directly but also indirectly through induction of VEGF.

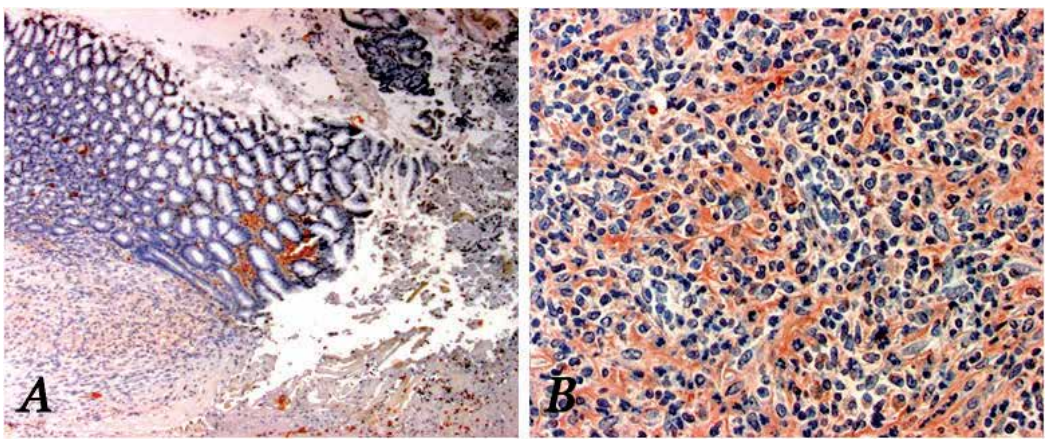

Fig. 7. Gastric ulceration induces CCN1 expression at the ulcer margin (A) and granulation tissue (B).

As a matricellular protein, CCN1 has features intermediate between conventional growth factors and structural ECM molecules; therefore, it can influence tissue remodeling without being an integral element of the structural ECM. In addition to being an angiogenic factor, CCN1 also supports cell adhesion, migration, proliferation, differentiation and survival. Recently, we have found that CCN1 is highly up-regulated in the gastric epithelial cells adjacent to the ulcer and remains high until the wound is healed (Figure 7; Chai et al, 2010b). This was demonstrated by epithelial injury both *in vivo* (gastric ulcer margin) and *in vitro* (gastric epithelial cell culture). Its elevation induces a transient phenotypic change in the mucosal epithelial cells at the ulcer margin and drives the wound closure. These cells lose their epithelial identities and become mesenchymal-like cells. At the molecular level, it shows down-regulation of epithelial markers such as E-cadherin, Occludin and cytokeratins, and up-regulation of mesenchymal markers such as vimentin, N-cadherin and

metalloproteinases. Once the wound is healed, these cells and their progeny can resume their original epithelial phenotype as evidenced both *in vitro* and *in vivo*. However, when CCN1 is knocked down in gastric mucosal epithelial cells, injury-induced EMT is disrupted and wound closure is delayed. We have further dissected the molecular mechanisms of this process and found that CCN1-induced E-cadherin loss is not due to transcriptional repression, which is the main mechanism of E-cadherin loss in many other systems (Zhou et al, 2004; Hayashida et al, 2006; Kang & Massague, 2004), but rather protein degradation caused by the collapse of adherens junctions, which is ignited by β-catenin nuclear translocation. CCN1-activated integrin-linked kinase mediates this event. In addition, our *in vivo* study demonstrated that local injection of recombinant CCN1 protein into gastric ulcers can induce expression of vimentin and smooth muscle α-actin in the mucosal epithelial cells and promote re-epithelialization during ulcer healing, and that local injection of CCN1 antibody neutralizes the effect and delays healing process. We have also found that TGFβ1 up-regulates CCN1 expression in gastric epithelial cells through SRF and it fails to do so when SRF is inhibited by shRNA.

7. Conclusions

SRF is a ubiquitously expressed transcription factor that targets genes containing SRE (Or CArG box). SRE has been found in nearly 1% of total number of human genes and the list is still growing. Some of these genes encode transcription factors (e.g. FOS, FOSB, EGR1, EGR2, EGR4, ELK1, etc.) which have their own specific gene targets. For example, transcription factor Egr-1 has six SRF binding sites in its gene promoter region, indicating a tight control by SRF. It has been shown that Egr-1 is capable to bind to 283 genes, which double the number of genes directly regulated by SRF and extend SRF power to 2% of the human genome. Some other members of SRF targets encode growth factors (e.g. IGF2, TGFB1I1, FGF10, etc.), integrins (e.g. ITGA1, ITGA5, ITGA9, ITGB1, etc.), and matricellular proteins (e.g. CTGF, CCN1, etc.) and all these molecules can transduce signals to influence many other genes. Taken together, SRF influence, including both direct and indirect, can probably reach a quarter of the entire human genome. By now, one can imagine how powerful SRF is.

Ulcer healing is just one of the things SRF does. One can easily find SRF contributions in each phase of ulcer healing: it promotes the production of growth factors and cytokines to mediate inflammation; it regulates formation of actomyosin ring and lamellipodia to promote re-epithelialization; it regulates apoptosis to remove the dead tissue and unnecessary cells; it supports angiogenesis through regulating endothelial cell migration and proliferation; it coordinates tissue remodeling by synchronizing proteases with their antagonists; and much, much more...

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Helicobacter pylori and Peptic Ulcer – Role of Reactive Oxygen Species and Apoptosis

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1. Introduction

Peptic ulcers and gastritis are a serious and growing health problem in the whole world. Ulcers affect about 5 million Americans each year, and more than 40,000 people annually have ulcer-related surgery. Each year, approximately 15,000 people in the Unites States die of ulcer-related complications, the worst of which are an internal bleeding and perforation.

A peptic ulcer is an open sore or lesion in the gastrointestinal mucosa (stomach or duodenum) that extends through the muscularis mucosa. Peptic ulcers occur when the mucous lining of the stomach or duodenum is not sufficient to protect them against the corrosive action of stomach hydrochloric acid, pepsin digestive enzyme, or against other aggressive substances. These aggressive factors can have an endogenous or exogenous origin. The endogenous harmful factors apart from hydrochloric acid and pepsin, are: refluxed bile, leukotrienes and Reactive Oxygen Species (ROS). The exogenous damaging factors include lifestyle factors, such as alcohol abuse, stress, tension and smoking; also, consume of steroidal and nonesteroidal anti-inflammatory drugs (NSAIDs) or drugs which stimulate gastric acid and pepsin secretion. Moreover, it is completely accepted that the bacterium called *Helicobacter pylori* (*H. pylori*) is implicated in the development of gastric ulcers and gastritis.

However, many researchers suggest that both the presence of *H. pylori* and the circumstances related to lifestyle and the consumption of certain drugs are risk factors to develop ulcer, but not the underlying causes, consequently, they add severity to the problem but are not able to cause it. Although these factors are almost certainly of pathogenic relevance, there are majority of people with exposure to them who remain ulcer-free and only a small number of them develop ulcers. In fact, considering the acid-peptic environment of the stomach, the noxious agents both the endogenous and the exogenous that are ingested, and the high prevalence of *H. pylori* infection, ulcers are surprisingly uncommon.

To explain this, it is thought that in gastric mucosa is established a balance between these aggressive factors and other cytoprotective factors, and that gastric ulcer appears when the

balance is lost. The mucosal defense is constituted by mucus-bicarbonate barrier, surface active phospholipid, prostaglandin, mucosal blood flow, cell renewal and migration, antioxidants and antioxidant enzymes, and some growth factors.

2. Free radicals and antioxidant defences

Gastroduodenal disease is associated with a variety of risk factors, including tobacco smoke, hereditary influences, sex, diet, stress and the actions of drugs (NSAIDs) (Marotta & Floch, 1991; Wallace, 1992). There is evidence that free radicals and antioxidants may be important components in the pathophysiology of gastroduodenal disease caused by many of these factors, implicating *H. pylori*. Free radicals have been involved in a wide spectrum of human diseases including other digestive disorders such as inflammatory bowel, toxic liver injury or pancreatic disease (Phull et al., 1995).

ROS can be defined as any chemical species capable of independent existence that contain one or more unpaired electrons in their outer orbital. Considering that the most stable molecular species have the electrons within their outer orbital arranged in pairs, free radicals tend to be unstable and highly reactive.

Oxygen is the most abundant radical in biological systems. The seemingly paradoxical consequences of the beneficial and harmful effects of oxygen (O_2) have been shown for several decades. While more than 95% of the O_2 consumed by the aerobic organisms is fully reduced to water during the process of mitochondrial respiration, a small percentage (<5%) is converted to semireduced species such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). These species are collectively named "Reactive Oxygen Species or ROS". ROS are formed continuously as normal byproducts of cellular metabolism and several of their sources are mitochondrial oxidative phosphorylation, prostaglandin synthesis, non-mitochondrial respiratory burst of neutrophils, and possibly the inhibition of mitochondrial electron transport.

Antioxidant defences: Free radicals play a number of physiological roles, but due to their high reactivity tend to attack the first biochemical component that they encounter inside cells, including macromolecules such as lipids, proteins or nucleic acids and bring about oxidative damage (Reilly et al., 1991). To avoid the oxidative damage, cells have development defence mechanisms to limit or prevent the toxicity caused by an excessive ROS activity. These defenses include non-enzymatic compounds such as vitamin (Vit) A, E and C, and enzymatic substances such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase; the latter two enzymes using glutathione (GSH) as a cofactor. Tissue damage may result from excessive ROS activity, from any deficiency in the defence mechanisms or from both of them.

It is well known that low/moderate concentrations of ROS affect a great number of physiological functions. However, when ROS concentration exceeds the antioxidative capacity of an organism, cells enter a state termed oxidative stress, in which the excess ROS induces oxidative damage on cellular components. So, ROS have been implicated in the pathogenesis of many human sufferings like Parkinson's, Alzheimer, Huntington's diseases and many other neurodegenerative conditions (Halliwell B, 1989). There is ample evidence that free radicals and antioxidants may be important components in the pathophysiology of gastroduodenal disease, in fact, as noted above, an exacerbated synthesis of free radicals has been implicated in inflammatory bowel disease, toxic liver injury or pancreatic disorders.

3. Free radicals, antioxidant defences and gastroduodenal diseases

A growing body of experimental and clinical evidence suggests that gastric mucosal damage by ethanol, NSAIDs and by *H. pylori* (Davies et al., 1994) is mediated through ROS (Phull et al., 1995). The cause could be the excessive synthesis, the deficiency of antioxidants defences or the coexistence of both reasons.

Most of the studies in this area have focused on the link between low intakes of fresh fruit and/or vegetables and gastric cancer. Several data show that exist an increased risk of gastric cancer specifically related to low intakes of Vit C and β-carotene or that β-carotene and Vit E levels were significantly lower in subjects with gastric dysplasia, a precursor of gastric cancer (Haenszel et al., 1985). Lower plasma levels of Vit A, C and E in subjects with chronic atrophic gastritis (Jaskiewicz et al., 1990) has been detected in a cross-sectional and prospectives studies showing that an increased risk of gastric cancer is associated with low plasma α-tocoferol (Knekt et al., 1991), Vit C, β-carotene (Stahelin et al., 1991), retinal levels and with low selenium levels (van den Brandt et al., 1993). Respect to the selenium, some evidence suggest that the diet of dyspeptic patients is deficient in the micronutrient selenium, which is an important constituent of the antioxidant enzyme glutathione peroxidase (GSH-Px).

Involvement of ROS in the pathogenesis of gastric ulceration was first evident from the studies of ischemia-reoxygenation-induced gastric mucosal injury. Many of the works in this area have been performed by Davies et al using a chemiluminiscence assay (Davies et al., 1994). They have shown significantly greater free radical production in duodenal mucosal biopsies from patients with duodenal ulceration and severe duodenitis that in patients with mild duodenitis or controls. This increased free radical activity in duodenal ulcer patients is accompanied by reduced levels of plasma glutathione (GSH). Recent studies show the same conclusions: there is an increased ROS production in gastric ulceration compared with gastric antral mucosa (Peng et al., 2008). Gastric ulceration also appears to be associated with increased plasma free radical activity and lower levels of Vit C and E, but not with superoxide dismutase (SOD) or catalase. ROS also decrease the level of endogenous antioxidants such as GSH, α-tocopherol and ascorbate, and make the mucosa more prone to oxidative damage.

Moreover, ROS may play an important role in gastric ulceration induced by several kinds of stress. The pathogenesis of gastric mucosal lesions by water immersion restraint stress and burn shock in rat is associated with increased lipid peroxidation. Furthermore, cold restraint stress has been shown to alter the level of various damaging and cytoprotective factors of rat gastric mucosa to cause gastric ulceration (Das et al., 1997). It was reported (Yoshikawa et al., 1989) that the gastric mucosal injury induced by ischemia-reperfusion is avoided after administration of SOD and catalase, indicating the role of lipid peroxidation and ROS in the origin of the lesion. Similarly, astaxanthin has been shown to provide protection against naproxen-induced and stress-induced gastric ulceration by reducing the level of lipid peroxides and free radicals indicating again the role of ROS in gastric damage (Oh et al., 2005). The inhibitory effect of diacerein on indomethacin-induced gastric ulceration by inhibition of neutrophil activation (Tamura et al., 2001), and consequently the suppression of ROS production by these cells, leads to similar conclusions.

Furthermore, Davies et al. (Davies et al., 1994) also detected higher levels of ROS in antral mucosa infected with *H. pylori* and they affirmed that there is no evidence for ROS participation in gastric mucosal injury in cases not related to *H. pylori* infection. Other works

show that ROS levels are directly correlated with the infective load of *H. pylori* (Zhang et al., 2007) and lipid peroxidation has been also shown to be increased in *H. pylori*-positive gastric mucosa.

But although it has been established a clear association between bacteria infection and impaired synthesis of ROS, many other clinical data suggest that other factors inherent to host conditions (stress, diet, tobacco, hygiene, genetics...) contribute to the pathogenesis of this infection (Oh et al., 2005). It should be noted that some of these factors, including ingested food and tobacco smoke, directly influence in mucosal oxidative status, since they expose the gastric epithelium to the ROS that they generate within the gastric lumen in a sustained manner.

4. *Helicobacter pylori* and gastroduodenal diseases

4.1 Short history

The evolution over time of the peptic ulcer epidemiology reflects a complex and multifactorial etiology. Peptic ulcers were rare before 1800, so the gastric ulcer pathology was not described until 1835 (J. 1835 Cruveilhier maladies de l'estomac In Anatomy of Human Bailliere Pathologique du Corps, Paris ...). During the nineteenth century, the predominant form was gastric ulcer in young women, while duodenal ulcer was rare until the twentieth century. However, duodenal ulcer was gradually more prevalent until become the more frequent condition in the middle of the century. Moreover, in developed countries the mortality from peptic ulcer has been drastically reduced for the cohorts born after the start of the twentieth century (Sonnenberg, 2007).

It is now clear that the epidemiology of the peptic ulcer is mainly due to external environmental factors, among which are include *H. pylori* infection, the use of NSAIDs and smoking. However, these factors do not explain the whole story of the evolution in time and the birth-cohort effect for peptic ulcer. Specifically, *H. pylori* was a prevalent human infection before 1800, so that infection per se cannot explain the increased prevalence of the ulcer after this time nor the shift from gastric ulcer to duodenal ulcer (Graham, 2003). Interest in understanding the role of *H. pylori* in gastroduodenal diseases was started in the 1970s with the visualization of bacteria in the stomach of gastric ulcer patients, although the more important researches about it were carry out by the Australian pathologist Berry Marshal in 1979 in collaboration with J Robin Warren from 1981 (Warren JR, 1983). Almost by accident, successful culture of *H. pylori* in 1982 occurred. Marshall frustrated by not being able to get a good animal model of infection, ingested these bacteria and he became ill, developed inflammation and ulcer of stomach, and he was able to culture the bacteria from his own ulcer, proving *H. pylori* to be the cause of ulcers. They published the results of self-induced infection in 1985 (Marshall et al., 1985) and initially, the isolated bacteria was termed *Campylobacter pyloridis* but it was re-named *Helicobacter pylori* (*H. pylori*) when biochemical and genetic characterization of the organism showed that it was not a member of the *Campylobacter* genus (Tan & Wong, 2011).

4.2 Morphological and biochemical characteristics

H. pylori is a spiral-shaped, flagellated, microaerophilic Gram-negative bacillus. It inhabits various areas of stomach and duodenum. Stomach is normally a hostile environment to the survival of viruses, bacteria and other micro-organisms due to its low pH. However *H. pylori* has evolved to be uniquely suited to thrive in the harsh stomach environment (Fig. 1).

The bacterium secretes urease, a special enzyme that converts urea to ammonia. Ammonia reduces the acidity of stomach, making it more hospitable home for *H. pylori* (Pandey et al., 2010).

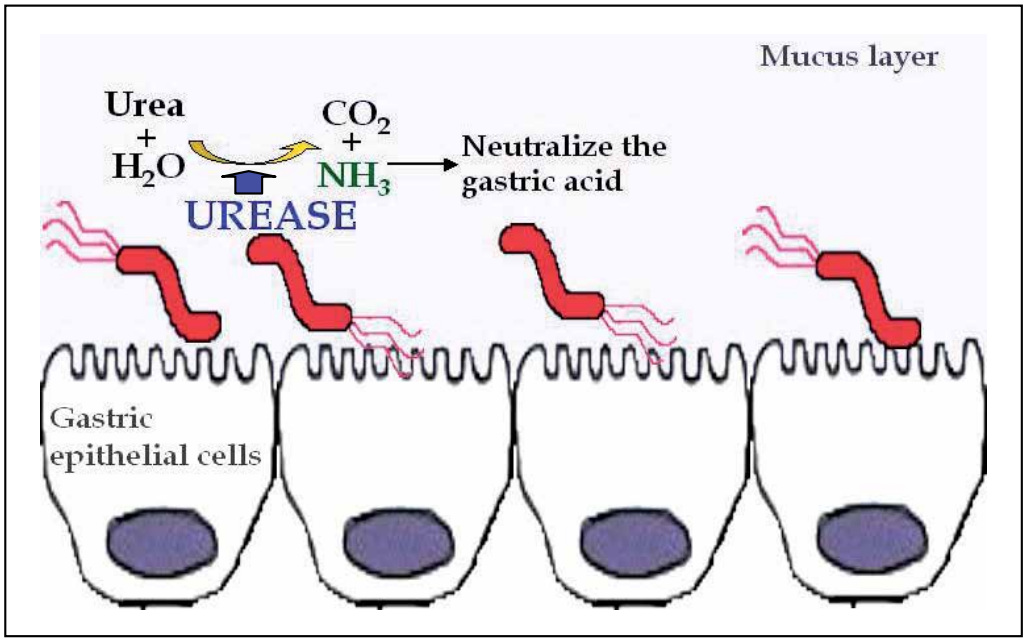

Fig. 1. *H. pylori* localization in the gastric mucosa and ammonia production.

H. pylori exerts a trophic influence on the gastric epithelium, but before it can attach to the epithelium surface it has first to cross the thick mucus layer by adhering to the mucosal surface. The presence of unipolar flagella helps to establish the *H.pylori* colonization of the stomach and its attachment to the epithelial surface of mucosa in the stomach despite the host attempts to rid itself of the bacterial infection. It is of interest that mutant *H. pylori* strains that are non-motile are unable to colonize the stomach of gnotobiotic piglets (Eaton et al., 1989).

4.3 Time, mode and geographic distribution of infection

H. pylori colonizes the gastric mucosa of more than 50% of human population. Infection is usually acquired in childhood while natural acquisition in adults is rare. The major risk factor for *H. pylori* infection are the socio-sanitary conditions lived during childhood, particularly at home, being important factors the level of sanitation, hygiene, and number of people in the household (the overcrowding). Studies in Kazakhstan (Nurgalieva et al., 2002) and Peru (Klein et al., 1991), have confirmed that high *H. pylori* infection prevalence in children in these countries, is related to these factors besides with the use of contaminated water with bacteria. These data determine that the water could be a reservoir and a transmission route for the bacteria.

Moreover, although it has been demonstrated a family association for infection (Nam et al., 2011), the transmission mode of *H. pylori* between individuals and within families remains to be elucidated and several interesting myths related to oral-oral transmission have been

debunked when a study of couples without children revealed a low concordance of *H.pylori* infection (Perez-Perez et al., 1991); currently favored mechanisms of transmission appear to be gastro-oral and faecal-oral routes (Xia & Talley, 1997). Genetic susceptibility also appears to be significant in the acquisition of *H.pylori* infection as well as its clearance (Malaty et al., 1994).

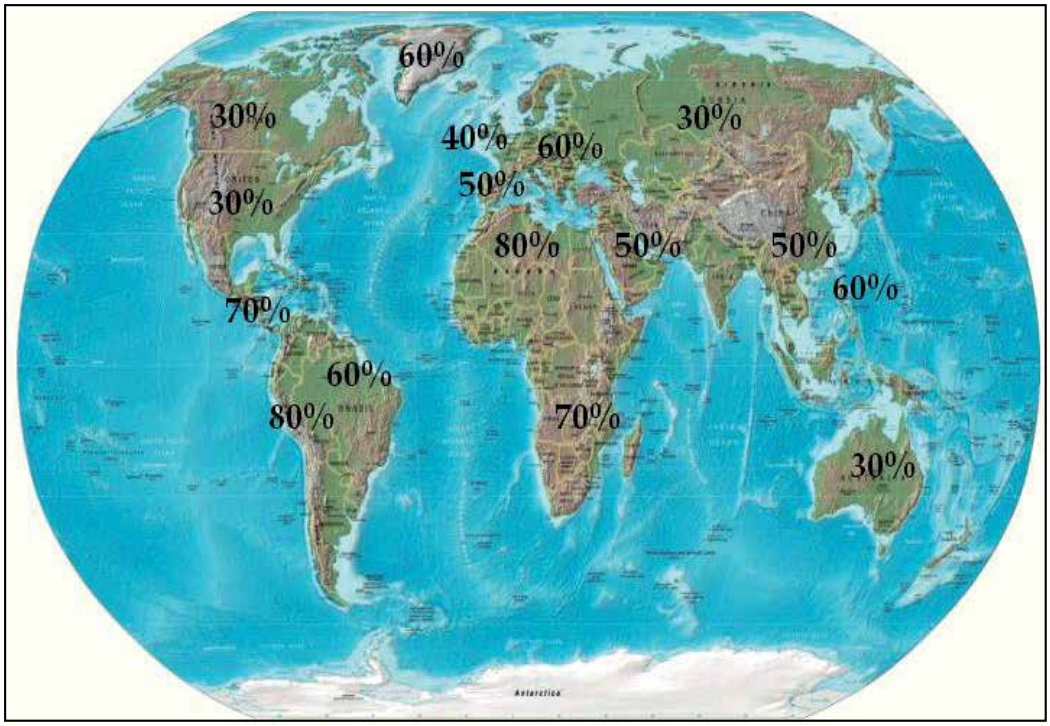

Fig. 2. *H. pylori* world distribution

Although generally speaking a rate of infection worldwide by about 50%, prevalences varying widely within countries (Fig. 2), and even between geographical areas of the same country (Krejs, 2010). Thus, in developing countries such as the eastern regions of Asia and in some parts of Latin America, the infection prevalence is characterized by a rapid rate of acquisition, usually in childhood, so that about 80% of the population is infected by the age of 20 (Graham et al., 1991).

In contrast, in developed countries such as Spain, USA, UK or Australia, the prevalence of *H. pylori* infection in children is low for ages below 10 years, and peaks of 40% aproximately, occur about to 30-40 years of age (Lehours & Yilmaz, 2007).

4.4 General features of *Helicobacter pylori* infection

In all infected subjects by *H. pylori*, the basic process that mediates the mucosa damage is the development of gastritis, whose extent and distribution will determine the clinical outcome (Amieva & El-Omar, 2008).

The arrival of lymphocytes and plasma cells in the mucosa signals augmentation of the acute inflammatory response by the production of cytokines and specific anti-*H. pylori*

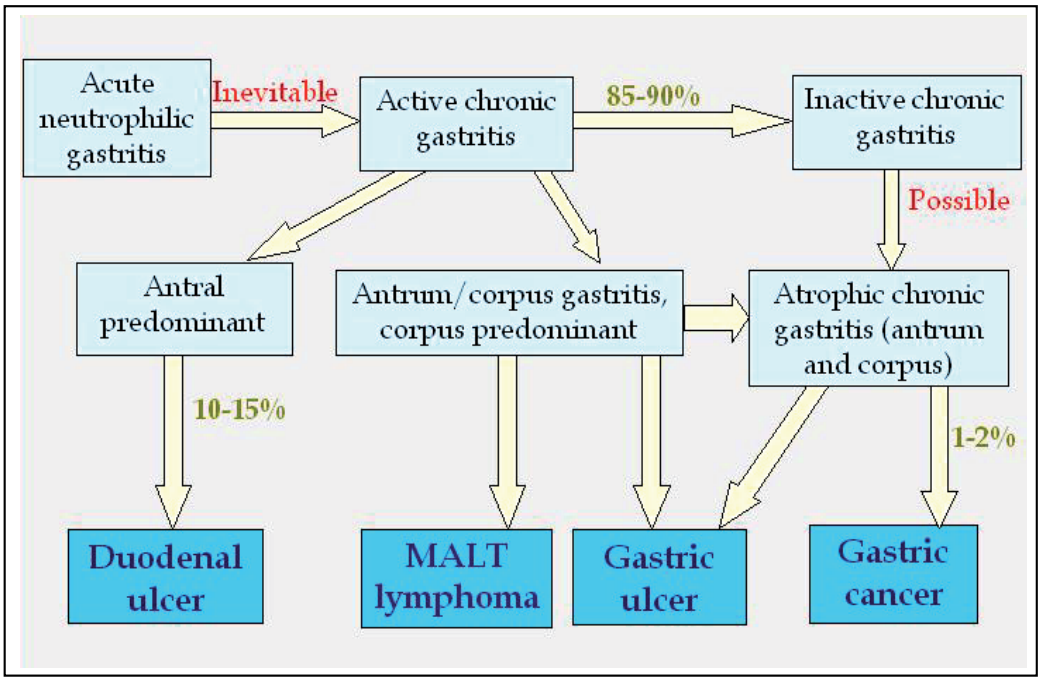

Fig. 3. *H. pylori*, gastritis and disease. During infection with *H. pylori* a number of inflammation patterns are associated with different disease stages.

antibodies. However, this vigorous response fails to eliminate infection, and the continued presence of *H. pylori* leads to the development of a second arm of the immune response more specifically aimed at preventing the damaging effects of intraluminal pathogens.

The initial acute phase of infection is subclinical in the great majority of subjects. This phase is short lived, and histologically results in a neutrophilic gastritis followed by a gradual infiltration of the gastric mucosa by inflammatory cells, and is coupled with a transient hypochlorhydria (Egan et al., 2007).

In a small minority of people, and particularly in childhood, the bacteria may be spontaneously cleared, the cellular infiltrate resolves, and the mucosa return to normal appearance. However, in the majority of subjects although *H. pylori* stimulates a robust inflammatory and immune response, the host fails to eliminate the infection and over the next 3 or 4 weeks there is a gradual accumulation of chronic inflammatory cells that come to dominate the histological picture. An acute neutrophilic gastritis gives way to an active chronic gastritis or "bening gastritis" characterized by mild pangastritis with little disruption of gastric acid secretion. This phenotype is exhibited by the asymptomatic subjects and those who do not develop serious gastrointestinal alterations (Amieva & El-Omar, 2008). In fact, it is commonly accepted that *H. pylori* infection, followed by the induction of inflammatory changes in gastric mucosa, may persist for decades without causing any gastric disturbances (Konturek et al., 2009).

Chronic gastritis is a common denominator linking peptic ulceration, gastric carcinoma, and lymphoma, and the histological picture encompassed chronic inflammation, atrophy, and intestinal metaplasia and finally adenocarcinoma (Dixon, 2001) (Fig. 3). Once chronic gastritis is established, it can progress mainly towards two topographic patterns that are

related to different clinical outcomes. The first pattern named "duodenal ulcer" phenotype is an antral predominant gastritis, accounts for up 15% of infected subjects and is characterized by inflammation mostly limited to the antrum. Subjects with this phenotype have high antral inflammatory scores, high gastrin, relatively healthy corpus mucosa, and very high acid output (Graham & Yamaoka, 1998). These subjects also have defective inhibitory control of gastric acid secretion.

This combination of pathophysiologic abnormalities contributes to the development of peptic ulcers, and confers protection against gastric cancer. Conversely, gastric ulcers are thought to be initially associated with a chronic non-atrophic gastritis which progresses to chronic atrophic gastritis involving both corpus and, invariably, the antrum and decreased acid output.

The second pattern, termed "gastric cancer" phenotype, is one of progressive pan-gastritis or multifocal atrophic gastritis, and hypo- or achlorhydria, characterized by active infection of both the gastric corpus and antrum with progressive development of gastric atrophy and intestinal metaplasia. These abnormalities affect at 1% of *H. pylori*-infected individuals who presented an increased risk of development gastric carcinoma (Amieva & El-Omar, 2008).

It is clear that *H. pylori* infection can lead to several divergent clinical outcomes. Explaining this apparent paradox is essential for understanding the pathogenesis of *H. pylori*-related disease because the mechanisms underlying the differences in outcome of *H. pylori* infection are currently poorly understood. It appears that the host reaction to the infection is very complex and it has been hypothesized that many sequential events and several mechanisms of tissue injury participate in the process. Between these pathogenic mechanisms of *H. pylori* are identified the induction of gastric inflammation, the disruption of the gastric mucosal barrier and the altered gastrin-gastric acid homeostasis (Dunn et al., 1997). Also, these different clinical manifestations of the infection are influenced by the host characteristics, the environmental factors and the bacterial genetic. Host factors are mainly related to the recognition of *H. pylori* by the immune system, variations in the level of cytokine response, sex and hereditary influences. Environmental data include tobacco smoke, diet, stress and the action of drugs such as NSAIDs. Bacterial factors may increase the risk of more severe disease, causing increased proinflammatory cytokine release. Strains possessing the "cag pathogenicity island" are more likely to be associated with peptic ulceration or gastric adenocarcinoma than strains lacking it. Another genes and proteins, such as iceA, vacA, OipA, BabA, have been analyzed but different studies were unable to show an association between them and the pattern of gastritis (Egan et al., 2007).

4.5 Toxicity of *Helicobacter pylori* on gastric mucosa (ROS)

In the early stages of infection, *H. pylori* induces secretion of chemokines (RANTES, GROα, MIP-1α, ENA-78, MCP-1, and IL-8), as well as of proinflammatory cytokines (IL-1, IL-6 and TNF-α, mainly) (Ibraghimov & Pappo, 2000). These molecules provoke recruitment of cells (macrophages, PMN, mast cells, T and B lymphocytes) to the infected gastric tissue, being neutrophils the initial inflammatory component of the response to the pathogen (Naito & Yoshikawa, 2002). Recruited PMNs, in turn, secrete more inflammatory mediators that amplify the primary signal and mediates directly the influx of more PMN to the gastric mucosa. Furthermore, soluble proteins of *H. pylori* can also function as chemoattractants for neutrophils. PMNs into gastric tissue induce oxidative burst responses in phagocytes being these activities typical in the development of gastric disease. It is demonstrated that *H. pylori*

isolates which induce a strong and rapid oxidative burst in neutrophils are associated with higher inflammation scores in gastric ulcer patients and with histological mucosal damage (Louw et al., 1993). Furthermore, IL-8 levels secreted by mucosa cells positively correlate with infiltration of PMN and mononuclear cells, and with higher production of ROS in *H. pylori*-infected antral gastric mucosa (Danese et al., 2001).

ROS production in *H. pylori* infection is catalyzed by nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase; Nox) on the cell membrane (Lambeth, 2004). It is also produced superoxide anion ($O_2^{\cdot-}$), a precursor of microbicidal oxidants. This $O_2^{\cdot-}$ is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) catalysis, or by non-enzymatic dismutation in the phagosome. H_2O_2 can passively permeate cell membranes and is converted to hypochlorous acid (HOCl), which is 100 times more toxic than H_2O_2. This conversion is mediated by myeloperoxidases (MPO) released by Azur granules in phagocytes in the presence of chloride ions (Cl-). H_2O_2 also reacts nonenzymatically with $O_2^{\cdot-}$ to form hydroxyl radicals ($^{\cdot}OH$) in the presence of ferrous (Fe2+) or cuprous (Cu+) ions. In general, these highly reactive ROS (i.e., HOCl and $^{\cdot}OH$) are used by the phagocyte to kill pathogenic bacteria (Fig.4).

However, in *H. pylori*-infected gastric mucosa, these ROS cannot eradicate *H. pylori* and this excessive production is believed to be a major cause of gastric mucosal damage, inducing oxidative stress to the gastric mucosa cells (Handa et al., 2010).

Thus, the role of oxidative stress on gastric mucosa is multi-functional. For neutrophils, it is the result of excessive defense reactions of the body against *H. pylori* intrusion, and for *H. pylori*, it is a convenient tool for invading the human gastric mucosa.

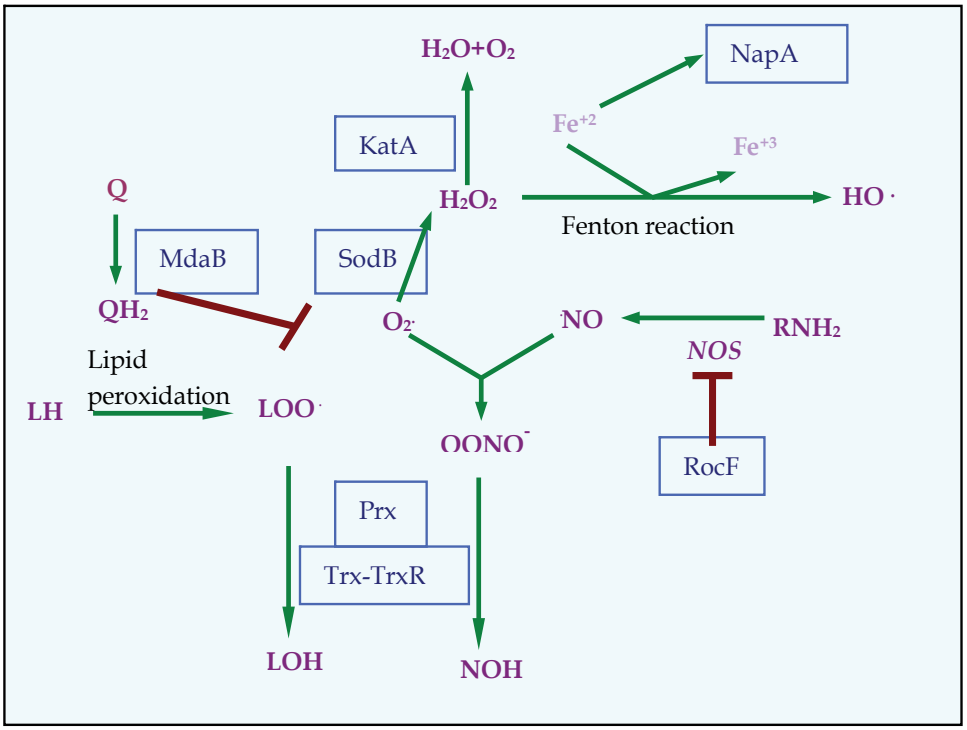

Fig. 4. Sources of ROS and the detoxification systems in *H. pylori*

As noted above, some factors that may further aggravate the pathogenicity caused by *H. pylori* are the genetic characteristics of bacteria. In fact, the possession of "cytotoxin-associated gene (cagA) or pathogenicity island" is associated with a high prevalence of peptic ulcer disease. And also, according to the authors Danese et al (Danese et al., 2001) and others, the gastric mucosa of patients infected by CagA (+) strains are characterized by increased ROS generation and neutrophil counts greater than that observed in CagA (-) subjects.

4.6 *Helicobacter pylori*, ROS and cellular apoptosis

Several studies have shown a relationship between *H. pylori* infection and an increased apoptosis rate in gastric lesions such as chronic gastritis, generally accompanied by glandular atrophy, gastric ulcer and intestinal metaplasia. Specifically, studies in patients with gastric ulcer determine a higher apoptotic index in this type of gastric lesion with *H. pylori* infection compared to *H. pylori*-negative normal gastric mucosa (Targa et al., 2007). Results of Satoh et al (Satoh et al., 2003) showed that apoptosis, not only of surface epithelial cells but also of glandular cells in the upper portion of fundic glands, is increased in *H. pylori*-positive patients with gastric ulcers and decreased to normal levels after eradication of *H. pylori*. Recent studies are intended to avoid triggering apoptotic processes caused by *H. pylori* (Hsu et al., 2010). In these experiments, authors use substances capable of inhibiting the activation of caspase cascade (extract solanum lyratum in this study). This could be a new approach for the treatment of infection with *H. pylori*.

It is known that some of the major stimuli that can induce apoptosis are: ionising radiation, viral infection, growth factor depletion, cytoplasmic stress, serum starvation, presence of hormones such as corticosteroids, the damage to genetic material and an excessive presence of free radical. Given that one of the factors that triggers cellular apoptosis is the excess of ROS, it is reasonable to think that the apoptosis observed in gastric mucosal cells infected by *H. pylori* may have this origin.

Apoptosis was first described in 1972 by Currie and colleagues (Kerr et al., 1972). It plays a pivotal part in many physiological settings, including the embryonic and post-embryonic development of multicellular organisms, tissue homeostasis and the removal of damaged and/or infected cells. It is delicately regulated and balanced in a physiological context. Failure of this regulation results in pathological conditions such as developmental defects, autoimmune diseases, neurodegeneration or cancer (Thompson, 1995). Apoptosis is by far the best-characterized mode of programmed cell death that is associated to morphological features that had been repeatedly observed in various tissues and cell types. Apoptotic cells display typical morphological features such as nuclear fragmentation, chromatin condensation and cell shrinkage, and eventually break down into apoptotic bodies. These morphological changes are accompanied by ATP-dependent biochemical changes that lead to the cells to various functional alterations. The results of these changes are reflected in an asymmetry of the cytoplasmic membranes by translocation of phosphatidylserine to the surface, breaking of DNA in multiples fragments (ladder pattern), release of pro-apoptotic proteins (cytochrome c (cyt c), AIF,…) from the mitochondria to cytosol, activation of caspases cascade, and finally, cell death.

Apoptotic cascade appears to have many regulatory “switch points’’ between proapoptotic and antiapoptotic forces. The imbalance of these forces has led to several key concepts regarding the importance of apoptosis in health and disease. Thus, atrophy of an organ with

a decrease in cell numbers could be related to an increase in cellular apoptosis, whereas tissue proliferation may be associated with molecular inhibition of apoptosis; in some diseases, internal or external factors could trigger the proapoptotic machinery within the cell causing cell death and tissue destruction; or cellular malignant transformation may occur because of a failure to activate apoptosis and delete cells with genetic damage (i.e., oncogenic mutations). These concepts suggest the possibility that several treatment strategies are useful in certain clinical conditions. Thus, inhibition of apoptosis could facilitate tissue repair processes by promoting cellular proliferation, and tissue regeneration, and, moreover, induction of apoptosis could be proven useful in treating malignant carcinomas (Que & Gores, 1996).

In relationship with the gastrointestinal tract, apoptosis plays an important role in the regulation of epithelial cell numbers, being the deregulation of the apoptotic pathway implicated in a number of disease processes in the gastrointestine. In *H. pylori*-induced chronic gastritis, cell loss by apoptosis is excessive compared with proliferation, suggesting that infection with the bacteria triggers the acceleration of apoptosis, fact that has been proven in *in vivo* experiments (Hall et al., 1994).

Two distinct, but partially overlapping, pathways are known to lead to apoptosis (Fig.5):

- the extrinsic (receptor mediated pathway), and
- the intrinsic (mitochondrial pathway).

The extrinsic pathway is activated by apoptotic stimuli comprising external signals such as the binding of death inducing ligands to cell surface receptors. Among death inducing ligands more studied are Fas, tumor necrosis factor receptor or TRAIL receptors.

Death ligand stimulation results in oligomerization of the receptors and recruitment of the adaptor protein Fas-associated death domain (FADD) and caspase-8, forming a death-inducing signalling complex (DISC). Autoactivation of caspase-8 at the DISC is followed by activation of effector caspases, including caspase-3, -6 and -7, which function as downstream effectors of the cell death program (Ashkenazi & Dixit, 1998). Fas has a central role in the physiological regulation of apoptosis and has been implicated in the pathogenesis of various malignancies as well as in diseases of the immune system. Fas is involved in cytotoxic T-cell mediated killing of cells (for example, CTL-mediated killing of virus-infected cells), destruction of inflammatory and immune cells in immune-privileged sites, deletion of self-reacting B cells and activated T-cells at the end of an immune response (Jin & El-Deiry, 2005).

Some *in vitro* studies reveal that *H. pylori* stimulates apoptosis of gastric epithelial cells in association with the enhanced expression of the Fas receptor, indicating a role for Fas-mediated signaling in the programmed cell death that occurs in response to *H. pylori* infection (Jones et al., 1999). Wang et al. (Wang et al., 2000) also demonstrated that local Th1 cells (cellular subtype that is mainly recruited to the gastric mucosa during the infection) may contribute to the pathogenesis of gastric disease during *H. pylori* infection by increasing the expression of Fas on gastric epithelial cells and inducing apoptosis through Fas/FasL interactions. Moreover, *H. pylori* can sensitize human gastric epithelial cells and enhance susceptibility to TRAIL-mediated apoptosis (Wu et al., 2004).

Intrinsic apoptotic pathway is initiated inside cells. The most important turning point in the course of the intrinsic apoptotic process occurs in the mitochondria. Their structure and compartmentalization are highly related to a perfect performance of their functions, being the most relevant, in eukaryotic cells, energy production in ATP form molecules (Chinnery & Schon, 2003).

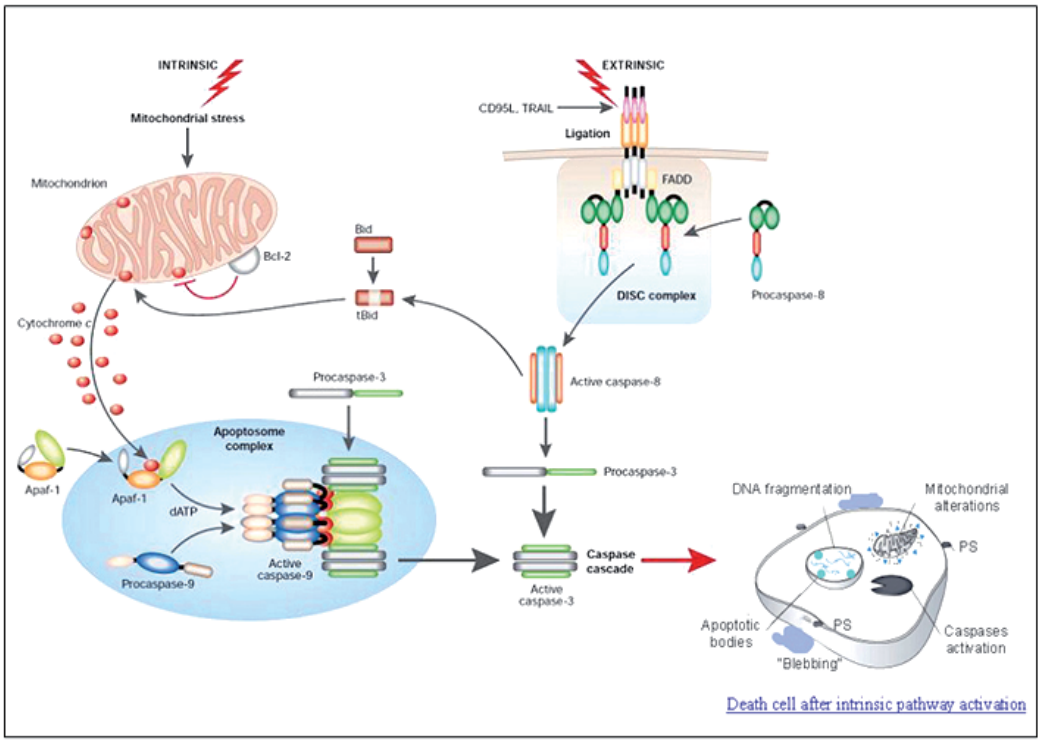

Fig. 5. Apoptotic pathways: the extrinsic pathway involves so-called death receptors, the intrinsic one involves mitochondrial alterations. Both pathways converge at caspase-3 activation, where classic biochemical and morphological changes in association with the apoptotic phenotype are originated

Every mitochondrion has a double lipid envelope that delimites the matrix, located within the inner mitochondrial membrane (IMM) and the intermembrane space located, in turn, between the IMM and the outer mitochondrial membrane (OMM). IMM cristae or invaginations increase the area where specific mitochondrial processes develop (electron transport and oxidative phosphorylation) (Fig. 6). The efficacy of these processes greatly depends on the bilayer's appropriate composition and structure, which relies on the role of the phospholipid cardiolipin (CL). CL is a specific component of IMM and the most abundant in it. On the CL are anchored many proteins such as respiratory chain I-IV complexes and cytochrome c (cyt c) (Calvino Fernandez & Parra Cid, 2010). Particularly relevant is the electrostatic binding between CL and cyt c in the IMM since this limits the amounts of cyt c that can be released during apoptosis (Iverson & Orrenius, 2004; Ott et al., 2002).

The crucial step in mitochondrion regulated apoptosis is the permeabilization of the OMM, often considered as the "point of no return" in apoptosis signalling (Orrenius et al., 2003), accompanied by the loss of mitochondrial membrane potential ($\Delta\Psi m$) and mitochondrial transition pores (MTP) opening. OMM permeabilization is followed by the release of caspase activating proteins such as cyt c and second mitochondrion-derived activator of caspase (SMAC; also known as DIABLO) into the cytosol.

The past 10 years have seen considerable efforts to decipher the molecular pathways leading to permeabilization of the OMM, and it was early recognized that the Bcl-2 family of

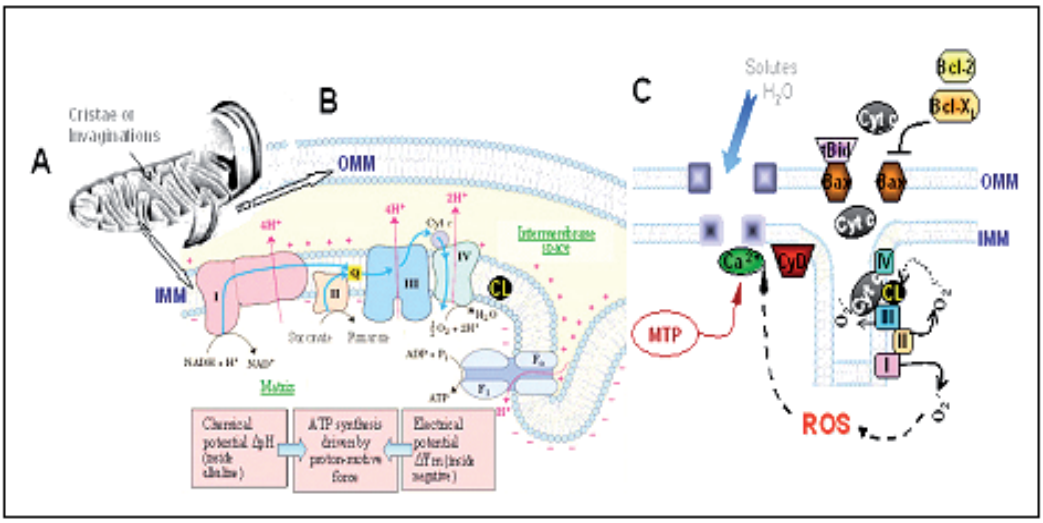

Fig. 6. Mitochondria: structure and function. **A)** Mitochondrial structure. **B)** Mitochondrial membranes: relationship between electron transport chain complexes, cytochrome c, and cardiolipin. **C**) Mitochondrial transition pore formation: ruptured links between protein complexes and cardiolipin, and release of cytochrome c into the cytosol . OMM: outer mitochondrial membrane; IMM: inner mitochondrial membrane; CL: cardiolipin; cyt c: cytochrome c; MTP: mitochondrial transition pores.

proteins play a prominent role in the regulation and execution of this process. As a result of this work, three different types of Bcl-2 family proteins have been identified: (a) the pro-apoptotic mediators, namely Bax and Bak; (b) the anti-apoptotic effectors, notably Bcl-2, Bcl-XL and Mcl-1; and (c) a host of Bcl-2 homology domain 3 (BH3-only) proteins which control either both the pro- and anti-apoptotic family members or only a specific member of one group (Youle & Strasser, 2008). The exact mechanisms of action and interplay of all these proteins are still a matter of vibrant debate. But there is general agreement that Bax and Bak are the terminal mediators of OMM permeabilization. As a result of Bax insertion /oligomerization into the OMM (promoted, i.e., by the cleavage of the BH3-only protein, Bid), pores are formed that mediate the release of pro-apoptotic proteins from the intermembrane space into the cytosol. The molecular nature of such pores, that is whether they are proteinaceous or lipidic, is currently not known (Ott et al., 2009).

Recently, apoptosis by mitochondrial pathway in *H. pylori* infection has been thoroughly studied (Calore et al., 2010; Calvino-Fernandez et al., 2008; Chiozzi et al., 2009; Domanska et al., 2010; Kim et al., 2010; Kim et al., 2007; Matsumoto et al., 2010; Yamasaki et al., 2006; Zhang et al., 2007). Thus, in this research line we can find papers that evidence how VacA, one of the major pathogenic products of *H. pylori* which induces large vacuoles within gastric epithelial cells, stimulates apoptosis via a mitochondria-dependent pathway (Chiozzi et al., 2009; Domanska et al., 2010; Galmiche et al., 2000; Kim et al., 2010). Some authors say that it interferes with mitochondrial permeability and reduces $\Delta\Psi m$, followed by cyt c release. It is suggested that VacA may not act directly to induce cyt c release from mitochondria, instead Bax and Bcl-2 homologous antagonist/killer (Bak), were activated and relocated to mitochondria, promoting cyt c release (Calore et al., 2010; Matsumoto et al., 2010; Yamasaki et al., 2006).

Not only VacA has been seen to be involved in *H. pylori*-induced mitochondria pathway apoptosis. Apurinic/apyrimidinic endonuclease-1 (APE-1) regulates transcriptional activity of p53, and *H pylori* (and ROS) induce APE-1 expression in human gastric epithelial cells, increasing intracellular calcium ion concentration of these cells which induces APE-1 acetylation. This *H. pylori*-mediated acetylation of APE-1 suppresses Bax expression, preventing p53-mediated apoptosis when *H. pylori* infects gastric epithelial cells (Bhattacharyya et al., 2009; Chattopadhyay et al., 2010).

In vivo studies indicate that *H. pylori*-induced apoptosis is associated with an increase in Bak expression in gastric biopsies from patients colonized by the bacterium. Konturek et al. reported induction of apoptosis with evidence of Bax up-regulation and Bcl-2 down-regulation in duodenal ulcer patients with *H. pylori* infection (Konturek et al., 1999).

It is known that mitochondria are one of the possible targets and the major intracellular source of free radicals (ROS), since it is estimated that 1–2% of the oxygen consumed by mitochondria (and they consume 85% of all body oxygen) in the electron transport chain is converted to O_{2^-} (Shigenaga et al., 1994). Elevated amounts of O_{2^-} could have detrimental effects on nearby molecules, modifying several proteins of the mitochondrial membrane, lipids or even mitochondrial DNA (which has limited protection because of its lack of histones). In physiological conditions, mitochondria have several enzymes (manganese-dependent superoxide dismutase, glutathione peroxidase) and non-enzimatic systems (NADPH, Vit C and E) that maintain O_{2^-} concentrations at very low levels. But when some events cause an overproduction of free radicals, these systems are not able to eliminate the excess. Therefore, overproduction of ROS may also reduce the antioxidant defenses.

This oxidative stress may damage cellular components (Fig. 7), including polyunsaturated fatty acids, carbohydrates, structural and regulatory proteins, and DNA (Baik et al., 1996; Calvino-Fernandez et al., 2008; Jacobson, 1996).

It could well play a role in epithelial proliferation, apoptosis, and oxidative DNA damage.

Lipids of cellular membranes, such as CL, are particularly susceptible to oxidation due to the amount of double bonds in their structure, and it is expected that structural changes would be deleterious to normal mitochondrial function (Calvino-Fernandez et al., 2008; Calvino Fernandez & Parra Cid, 2010).

It is widely known that *H. pylori* infection increases epithelial apoptosis in gastric mucosa, which may play an important role in gastric carcinogenesis (Xia & Talley, 2001), and *H. pylori*-induced apoptosis may stimulate compensatory hyperproliferation which results in potential preneoplastic changes in chronic *H. pylori* infection (Moss et al., 1996). Besides, as noted above, higher levels of apoptosis have been detected in ulcer lesions (Satoh et al., 2003; Targa et al., 2007) in patients infected with bacteria, and also, this increased apoptosis disappears when *H. pylori* is eradicated. In both clinical alterations, ulcer and cancer, are also shown that there is an increase of oxidative stress.

With all these data, it is reasonable to assume that whether *H. pylori* causes oxidative stress with an increase of free radicals production, these ROS could directly affect mitochondria and trigger the apoptosis by a similar way shown in Fig.7 (Calvino Fernandez & Parra Cid, 2010).

An indisputable proof that these two processes (ROS and apoptosis) are related to, is the inhibition of apoptosis by treatment with antioxidants. In *in vitro* experiments (Calvino-Fernandez et al., 2008), *H. pylori* caused apoptosis in gastric epithelial cells. Simultaneously alterations in several structural and functional characteristics of mitochondria (high O_{2^-} synthesis, decreased levels of antioxidant enzymes, cardiolipin oxidation, loss of membrane potential, large amounts of cytosolic cit c, higher levels of Bax and caspases,...) were

detected. All these changes were eliminated by incubating the cells with Vit E during the period of infection. The antioxidant, also prevented that the cells died by apoptosis.

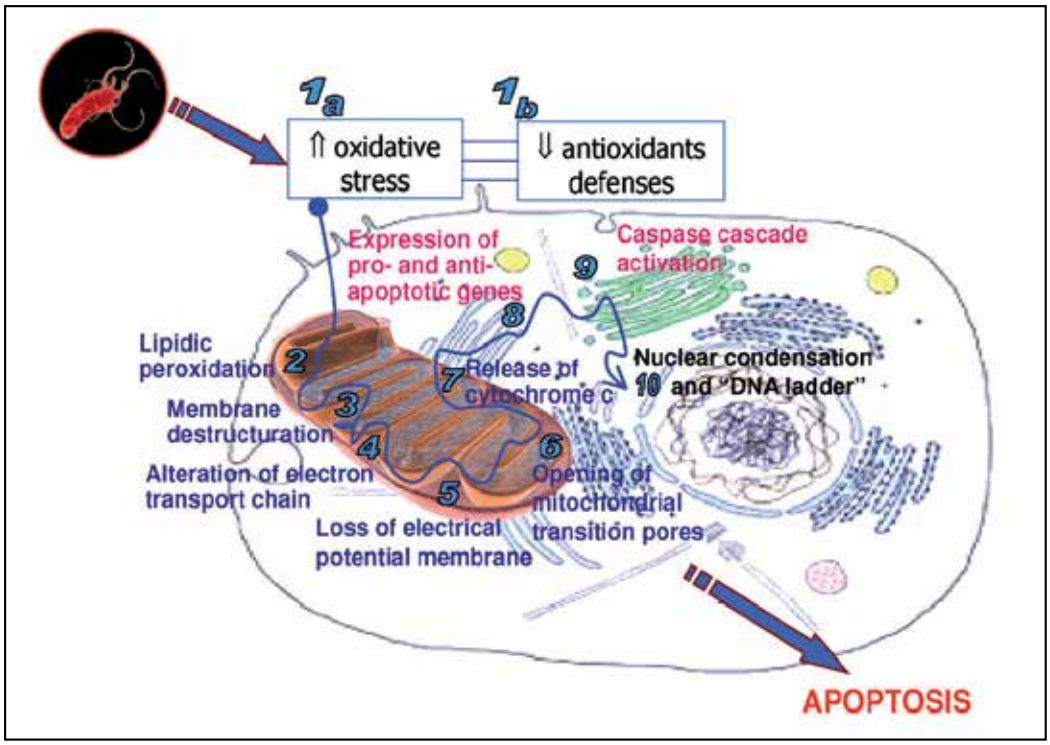

Fig. 7. Morphologic and functional alterations caused by *H. pylori* in epithelial gastric cells. ROS can directly affect mitochondria and trigger apoptotic process.

Solanum lyratum extract (SLE) was also able to suppress *H. pylori*-induced apoptosis (Hsu et al., 2010). SLE inhibited caspase-8 activation, thereby preventing the release of cyt c from mitochondria and activation of the subsequent downstream apoptotic pathway. Thus, SLE may offer a new approach for the treatment of *H. pylori* by down-regulation of apoptosis in the *H. pylori* infected gastric epithelium. As it does not directly target bacteria, SLE treatment might not cause development of resistant strains.

5. Conclusions

General knowledge of scientific advances is necessary not only to read and interpret the literature but also to diagnose and treat diseases appropriately. The biological phenomena contributing to oxidative stress and cell death, fundamental processes in many gastrointestinal diseases, are important in the science and practice of gastroenterology. Currently a scientific revolution is ongoing in the understanding of cell death by a process referred to as "apoptosis or programmed cellular death", and the underlying causes of apoptosis are being the subject of many studies.

H. pylori infection is associated with chronic gastritis, peptic ulcers, and gastric cancer. Extensive scientific evidence shows that in these alterations are involved oxidative stress

(exacerbated synthesis of ROS) caused by the bacteria by different mechanisms. In addition, a common feature of all these pathologies is the activation of apoptosis, either by extrinsic or, as it is more accepted today, for intrinsic pathway.

Novel therapeutic strategies that may be useful in the prevention or treatment of disorders of the stomach and duodenum caused by bacteria, should have as target the inhibition of the synthesis of ROS and/or apoptosis. Whereas the excess of free radicals is the origin of these alterations, the first step to take would be to restore the balance between oxidants and antioxidant systems. This would eliminate the oxidative stress of the gastric mucosa.

With respect to ROS, the actions may be aimed to the inhibition of its exacerbated synthesis, which serve to mitigate the toxic effects caused by the observed deficiencies in the antioxidant levels. Another strategy could be external supplementation of scavengers to remove this excess of free radicals. The antioxidant vitamins are the logical first choice as therapeutic agents, because of the large amount of data available on their role in *H. pylori* infection, gastroduodenal disease and gastric cancer.

Other new approach for the treatment of *H. pylori* could be the down-regulation of apoptosis in infected gastric epithelium. This might be possible by administering substances that inhibit the activation of caspases. Thus, it would prevent the release of cyt c from mitochondria and as a result, the subsequent downstream apoptotic pathway.

These actions are directed against the toxic manifestations caused by infection, while the traditional treatments with antibiotics have as target the bacterium itself. Consequently, these strategies would also prevent the development of resistant strains, an increasingly common problem due to the usual antibiotic treatments.

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10

Role of New Appetite Hormones Ghrelin, Orexin-A and Obestatin in the Mechanism of Healing of Chronic Gastric Ulcers

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1. Introduction

The regulation of appetite is regulated by complex mechanism involving brain-gut axis and hormonal and non-hormonal factors acting through the activity of satiety and hunger centers located in the hypothalamus [Wren & Bloom, 2007]. Among hormones controlling appetite are gastrointestinal hormones such as cholecystokinin, PYY, pancreatic polypeptide, glucagon-like peptide 1, oxyntomedulin, the endocrine pancreatic hormones including insulin and glucagon and endocannabinoids [Wren &Bloom, 2007]. Recent discoveries of ghrelin and orexin-A gave a new insight into the understanding of mechanism of appetite control, satiety and obesity. Ghrelin is a novel 28-amino acid peptide that has recently been discovered in rat and human gastrointestinal tract, particularly in gastric mucosa, as an endogenous ligand for growth hormone (GH) secretagogue receptor (GHS-R) [Kojima et al., 1999]. Ghrelin stimulates food intake and body weight gain exerting a modulating effect on energy expenditure acting through afferent nerves and directly on hypothalamic feeding centers [Kojima et al., 1999; Tomasetto et al., 2000]. This peptide was also shown to enhance the gastric motility and gastric secretion [Tschop et al., 2000; Date et al., 2001]. Ghrelin is considered as an orexigenic peptide produced by the stomach, acting as a meal initiator [Masuda et al., 2000; Ariyasu et al., 2001]. Little is known about other factors that might affect ghrelin secretion in the stomach and its influence on gastrointestinal integrity has not been fully elucidated. A recent study in humans revealed that stomach is a major source of circulating ghrelin and that gastrectomy produced dramatic fall in the plasma ghrelin levels, whereas fasting and *anorexia nervosa* was accompanied by elevated plasma ghrelin levels [Ariyasu et al., 2001].
The orexins also called hypocretins, are neuropeptides novel originally discovered in a small group of neurons in the hypothalamic area (LHA) [Sakurai et al., 1998; de Lecea et al., 1998]. identified two peptides that were endogenous ligands to orphan receptor HFGAN72 [Sakurai et al., 1998]. They found that these peptides after i.c.v. injection stimulated food

intake and their expression was upregulated upon fasting. Therefore, the group of these peptides has been named "orexins" after the Greek word "orexis" meaning appetite [Komaki et al., 2001]. The orexin group of peptides was originally identified as regulators of food intake and of sleep behavior and act at one or the other of 2 G protein-coupled receptors, the orexin-1 (OX-R1) and orexin-2 (OX-R2) receptors [Sakurai et al., 1998; Mondal et al., 1999]. OX-A is a 33-residue peptide possessing the identical sequence in humans and rodents, whereas human and rat OX-B differ by two residues. OX-A and OX-B activate two G protein-coupled receptors known as OX-1 and OX-2, the OX-1 receptor having a greater affinity for OX-A over OX-B, whereas the OX-2 receptor has similar affinity for both ligands [Sakurai, 2003]. Besides the presence in the brain, orexin-like immunoreactivity was found in the ENS of different species including guinea pig, rat, mouse and humans [Kirchgessner & Liu, 1999]. Interestingly, the prepro-orexin, orexin A and orexin receptor mRNA expression were demonstrated in the myenteric plexus and afferent nerves co-localizing with VIP-ergic nerves and in the gastrin-producing cells of the rat stomach [Kirchgessner & Liu, 1999]. Orexin-A has been linked with the peripheral energy balance and central nervous system mechanisms that coordinate sleep-wakefulness and motivated behaviors such as food seeking, especially in physiological state of fasting stress [Mondal et al., 1999; Sakurai, 2003]. Recent studies in humans revealed that the plasma orexin-A concentrations are increased during fasting in humans [Komaki et al., 2001].

The question remains as to whether these newly discovered peptides could play a role in the mechanism of gastric integrity and gastric mucosal defense has not been fully explored. Both, ghrelin and orexin-A were implicated in the mechanism of gastroprotection but their role in the healing process of chronic gastric ulcers has been little investigated. Previous studies by [Sibilia et al., 2003] and our group [Brzozowski et al., 2004; Brzozowski et al., 2006 b.; Konturek et al., 2004] revealed that that central and peripheral administration of ghrelin reduced the formation of acute gastric lesions induced by ethanol and cold stress. It was proposed that NO and sensory neuropeptides mediate these gastroprotective effects of ghrelin because the blockade of NO-synthase (NOS) activity with L-NAME and the functional ablation of sensory afferent nerves with capsaicin attenuated the protection and gastric hyperemia induced by ghrelin [Brzozowski et al., 2004; Brzozowski et al., 2006a.; Dimitrova et al., 2010]. In contrast to prevention of acute damage to the mucosal structure, the healing of chronic gastric ulcers lasts days and weeks and may involve restoration of gland architecture, angiogenesis and scar formation and it remains unknown whether appetite hormones may influence these processes.

Recently, endogenous prostaglandins (PG) have been implicated in the control of food intake and appetite [Lugarini et al., 2002; Scholz, 2003] but the possibility that these arachidonate metabolites may play any important role in the mechanism of ghrelin or orexin-A affecting ulcer healing has not been explored. This prompted our interest in endogenous PG because these arachidonate metabolites play an essential role in the mechanism of gastric defense and ulcer healing [Brzozowski et al., 2001] but the importance of PG derived from cyclooxygenase (COX-1) and COX-2 in the possible ulcer healing effects of orexigenic peptides has not been so far determined.

This study was designed to compare the effects of daily intraperitoneal (i.p.) administration of vehicle, ghrelin, orexin-A and obestatin on healing of preexisting gastric ulcers and accompanying changes in the gastric blood flow (GBF) at ulcer margin and the generation of PGE_2 in the gastric mucosa. An attempt was made to examine the effect of antagonism of

GHS-R1a and OX-A1 receptors with D-lys^{3}GHRP-6 and SB334867 [Peeters, 2005; Smart et al., 2001] respectively, on spontaneous ulcer healing and the alteration in ulcer size induced by ghrelin and orexin-A. We also determined the effects of PG inhibition with a non-selective (indomethacin) and selective cyclooxygenase (COX)-1 (SC-560) and COX-2 (rofecoxib) inhibitors and blockade with L-NNA, the NO-synthase inhibitor on healing of these ulcers and accompanying changes in the GBF in rats without and with ghrelin or orexin-A administration. In addition, the expression of ghrelin mRNA at the ulcer margin and that in non-ulcerated gastric mucosa was assessed in rats with gastric ulcer. Finally, ghrelin rats were concomitantly treated with obestatin to check whether obestatin, recently considered as physiological opponent of ghrelin regarding food intake and appetite control [Zhang et al., 2005], could influence the ulcer healing itself and affect the healing and hyperemic effects of ghrelin.

2. Material and methods

Male Wistar rats, weighing 200-250 g and fasted for 24 h were used in all studies. All experimental procedures were run according to Helsinki Declaration and approved by the Jagiellonian University Institutional Animal Care and Use Committee.

2.1 Production of gastric ulcers

Gastric ulcers were produced in rats using our modification (19) of acetic acid method originally proposed by Okabe et al. (1971) with our group modification [Konturek et al., 1987]. Animals were anesthetized with ether, the stomach was exposed and 75 μl of acetic acid was poured through the plastic mold (6 mm diameter) onto serosal surface of anterior wall of the stomach just proximal to the antral gland area for 25 s. This produced an immediate necrosis of the entire mucosa and submucosa (but not serosa) within the area where the acetic acid was applied, i.e., about 28 mm^2. The excess of acetic acid was then removed and the serosa was gently washed out with saline. Our previous studies documented that these ulcers became chronic within 2-3 days and healed completely within 2-3 weeks without perforation or penetration to the surrounding organs as described in original technique [Konturek et al., 1987; Okabe et al., 1971]. After the application of acetic acid the animals were allowed to recover from anesthesia and received only water at the day of operation (day 0). Then, they were divided into various groups and received normal chow and water *ad libitum* for the next 9 days and then were sacrificed.

2.2 Effect of orexin-A, ghrelin without and with the antagonists of ghrelin and orexin-A receptors on ulcer healing and the alterations in GBF at ulcer margin

Several groups of rats with gastric ulcers, each consisting of 6-8 animals, were treated daily either with 1) vehicle (saline), 2) ghrelin or orexin-A (2.5-30 μg/kg-d i.p.), 3) D-Lys^3-GHRP-6 (200 μg/kg i.p.), the ghrelin receptor antagonist (Peeters, 2005), or SB 334867 which is a selective non-peptide OX-R1 receptor antagonist [Smart, *et al.*, 2001; Holland, *et al.*, 2006] administered alone or in the combination with ghrelin and orexin-A (standard dose of 30 μg/kg-d i.p.), respectively. SB 334867 was dissolved in DMSO and kept in a 20 mM solution as recommended by manufacturer [Tocris Cookson Ltd., Bristol, UK]. The dose of OX-R1 antagonist used in this study was selected on the basis of our earlier studies (unpublished

observation) with dose-dependent inhibition of orexin-A protection against WRS damage obtained with this orexin-A antagonist.
In another group of animals with gastric ulcers and treated with COX-1 and COX-2 inhibitors with or without ghrelin, orexin-A and obestatin administration, the effect of prostaglandin replacement therapy using 16,16 dimethyl PGE_2 [Upjohn, Kalamazoo, MI, USA] applied i.g. in a dose of 5 μg/kg-d was examined. This dose of dimethyl PGE_2 was found in our preliminary study to be without any influence on ulcer healing and accompanying fall in GBF at ulcer margin. For this reason, synthetic PGE_2 analog was administered together with each COX-1 or COX-2 inhibitor with or without ghrelin, orexin-A and obestatin administration.
Upon the termination of experiment, the animals were anesthetized with pentobarbital, their abdomen was opened by midline incision and the stomach exposed for measurement of GBF by means of the H_2-gas clearance technique as described previously [Brzozowski et al., 2000 a]. For this purpose two electrodes of an electrolytic regional blood flow meter (Biotechnical Science, Model RBF-2, Osaka, Japan) were inserted into the gastric mucosa. The measurements were made in three areas of the oxyntic mucosa at ulcer margin and the mean values were calculated and expressed as percent changes of those recorded in the control animals. After GBF measurement, the stomach was removed, rinsed with saline and pinned open for macroscopic examination. The area of gastric lesions was determined by computerized planimetry (Morphomat, Carl Zeiss, FRG) [Brzozowski et al., 2000 b] by a researcher blind to the experimental grouping.
Samples of the oxyntic gland area from the ulcer margin were taken by biopsy (about 100 mg) immediately after the animals were sacrificed to determine the mucosal generation of PGE_2 by specific radioimmunoassay (RIA) as described previously [Konturek et al., 1995; Brzozowski et al., 2000]. PGE_2 was measured in duplicate using RIA kits (New England Nuclear, Munich, Germany). The mucosal generation of PGE_2 was expressed in nanograms per gram of wet tissue weight.

2.3 Effect of ghrelin and orexin-A on the healing of acetic acid ulcers and accompanying changes in the GBF at ulcer margin. Involvement of the COX-PG and NO-NOS systems in ghrelin-induced acceleration of ulcer healing

The involvement of the COX-PG system in the ulcer healing effects of ghrelin was studied in rats treated with (or without) indomethacin, a non-selective COX-1 and COX-2 inhibitor, and with SC-560, a selective COX-1 inhibitor or rofecoxib, a specific COX-2 inhibitor [Brzozowski et.al., 1999]. In the experimental protocol, the following groups of rats, each consisting of 6-8 animals, were used: 1) vehicle (saline 1 ml i.p.); 2) ghrelin, orexin-A or obestatin, each applied in a standard dose of 30 μg/kg-d i.p.; 2) indomethacin (5 mg/kg i.p.), SC-560 (5 mg/kg-d i.g.) and rofecoxib (10 mg/kg-d i.g.), each applied alone or co-administered with ghrelin, orexin-A or obestatin (30 μg/kg-d i.p.). The doses of SC-560 and rofecoxib were selected on the basis of previous studies showing that these agents almost completely suppress PGE_2 generation in exudates of air-pouch inflammation and inhibit gastric PGE_2 production in mucosa with preexisting gastric ulcer [Futaki et al., 1993; Lesch et al., 1998]. SC-560 (Cayman Chemical Co., Ann Arbor, Michigan, USA) was first dissolved in absolute ethanol to obtain a stock solution of 50 mg/ml and then diluted to the desired concentration with isotonic saline. Rofecoxib [Sharp & Dhome, Warsaw, Poland] was first dissolved in methanol to obtain a stock solution 75 mg/ml and then diluted to the desired

concentration with isotonic saline. Control rats received the corresponding vehicle. Our preliminary studies (data not included) showed that none of the COX inhibitors used in this study produced by itself any gastric lesions at the doses tested. At the dose used in the present study, indomethacin has been shown previously to inhibit gastric PGE_2 generation by ~ 90 % without itself causing any mucosal damage [Konturek et al., 1987].
In another group of animals with gastric ulcers and treated with COX-1 and COX-2 inhibitors with or without ghrelin, orexin-A and obestatin administration, the effect of prostaglandin replacement therapy using 16, 16 dimethyl PGE_2 (Upjohn, Kalamazoo, MI, USA) applied i.g. in a dose of 5 μg/kg-d was examined. This dose of dimethyl PGE_2 was found in our preliminary study to be without any influence on ulcer healing and accompanying fall in GBF at ulcer margin. For this reason, synthetic PGE_2 analog was administered together with each COX-1 or COX-2 inhibitor with or without ghrelin, orexin-A and obestatin administration.
Upon the termination of experiment, the animals were anesthetized with pentobarbital, their abdomen was opened by midline incision and the stomach exposed for measurement of GBF by means of the H_2-gas clearance technique as described previously [Brzozowski et al., 2000 a]. For this purpose two electrodes of an electrolytic regional blood flow meter (Biotechnical Science, Model RBF-2, Osaka, Japan) were inserted into the gastric mucosa. The measurements were made in three areas of the oxyntic mucosa at ulcer margin and the mean values were calculated and expressed as percent changes of those recorded in the control animals. After GBF measurement, the stomach was removed, rinsed with saline and pinned open for macroscopic examination. The area of gastric lesions was determined by computerized planimetry (Morphomat, Carl Zeiss, FRG) [Brzozowski et al., 2000b] by a researcher blind to the experimental grouping.
Samples of the oxyntic gland area from the ulcer margin were taken by biopsy (about 100 mg) immediately after the animals were sacrificed to determine the mucosal generation of PGE_2 by specific radioimmunoassay (RIA) as described previously [Konturek et al., 1995]. PGE_2 was measured in duplicate using RIA kits (New England Nuclear, Munich, Germany). The mucosal generation of PGE_2 was expressed in nanograms per gram of wet tissue weight.

2.4 Determination of plasma ghrelin, orexin-A and obestatin levels by radioimmunoassay

At the termination of some experiments with obestatin applied i.p. alone or co-administered with ghrelin, the rats were anesthetized with pentobarbital and blood samples (about 3 ml) taken from the *vena cava* for the measurement of plasma ghrelin, orexin-A and obestatin levels by RIA. Intact rats fasted overnight and given only vehicle (saline) i.p. were measured similarly in order to determine control values for plasma ghrelin, orexin-A and obestatin concentration. Blood samples were collected in heparin coated polypropylene tubes and centrifuged at 3000 rpm for 20 minutes at 4°C. The supernatant was then stored at -80°C until measurement of plasma ghrelin, orexin-A and obestatin levels using an RIA-kit for rat ghrelin, orexin-A and obestatin (Phoenix Peptide, Belmont, CA, USA) [Brzozowski et al., 2004; Brzozowski et. al., 2006 b]. Briefly, the ghrelin, orexin-A and obestatin RIA involved the competition of each rat peptide sample with ^{125}I-rat ghrelin, orexin-A and obestatin tracers for binding to a specific rabbit anti-ghrelin-, anti-orexin-A- and anti-obestatin polyclonal antibody. The limit of assay sensitivity for ghrelin, orexin-A and

obestatin were 3 pg, 6pg and 5 pg per tube, the intra-assay variation was less than 8%,7% and 9%, and the interassay variation less than 5%, 4% and 7%, respectively.

2.5 Reverse-transcriptase-polymerase chain reaction (RT-PCR) for detection of messenger RNA (mRNA) for ghrelin and proinflammatory cytokines IL-1β and TNF-α in rats without and with gastric ulcers

The stomachs were removed from vehicle (control) rats for the determination of ghrelin mRNA expression using specific primers by RT-PCR. Gastric mucosal specimens were scraped off from oxyntic mucosa using a slide glass and immediately snap frozen in liquid nitrogen and stored at -80°C until analysis. Total RNA was extracted from mucosal samples by a guanidium isothiocyanate/phenol chloroform method using a kit from Stratagene® (Heidelberg, Germany). Single stranded cDNA was generated from 5 μg of total cellular RNA using StrataScript reverse transcriptase and oligo-(dT)-primers (Stratagene, Heidelberg, Germany). The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Perkin-Elmer-Cetus, Norwalk, CT) in an area set aside for performing the PCR reaction. The nucleotide sequences of the primers for ghrelin and β-actin were selected on the basis of the published cDNA encoding ghrelin and β-actin, respectively [Konturek et al., 2004; Brzozowski et al., 2006 b]. The sense primer for ghrelin was 5′-TTGAGCCCAGAGCACCAGAAA-3′, and the antisense primer was 5′-AGTTGCAGAGGAGGCAGAAGCT-3′. The IL-1β primer sequences were designed according to the published cDNA sequence for primer sequences and were as follows: up-stream, 5' GCT ACC TAT GTC TTG CCC GT; downstream, 3' GAC CAT TGC TGT TTC CTA GG. The expected length product was 543 bp. The TNF-α primer sequences were as follows: up-stream, 5'TAC TGA ACT TCG GGG TGA TTG GTC C; downstream, 3' CAG CCT TGT CCC TTG AAG AGA ACC. The expected length product was 295 bp [Konturek et al., 2010]. The oligonucleotide sequences for β-actin were TTG TAA CCA ACT GGG ACG ATA TGG (sense) and GAT CTT GAT CTT CAT GGT GCT AGG (antisense). The primers were synthesized by GIBCO BRL/Life Technologies, Eggenstein, Germany. The signals for ghrelin mRNA was standardized against the β-actin signal for each sample and results were expressed as the ratio of ghrelin mRNA to β-actin mRNA.

3. Results

3.1 Effect of treatment with orexin-A, ghrelin and obestatin on ulcer healing and the accompanying changes in the GBF at ulcer margin

The effect of i.p. administration of orexin-A applied in graded doses ranging from 1 μg/kg-d up to 30 μg/kg-d and ghrelin administered in a dose of 30 μg/kg-d (i.p.) on acetic acid-induced gastric ulcers are shown in Fig.1. Orexin-A dose-dependently reduced the ulcer area, the dose inhibiting this area by 50% (ED_{50}) was 15 μg/kg.

Almost double reduction of the ulcer area was obtained with orexin-A administered at the dose 30 μg/kg as compared with control rats injected i.p. with vehicle (saline). This reduction in ulcer area achieved with orexin-A was accompanied by the dose-dependent increase in GBF. Ghrelin administered in a dose of 30 μg/kg-d i.p. significantly reduced the ulcer area and significantly increased GBF with the extent similar to that observed with orexin-A applied in the highest dose. Eight days of i.p. administration of obestatin applied in graded doses ranging from 2.5 μg/kg up to 30 μg/kg-d, dose-dependently reduced the

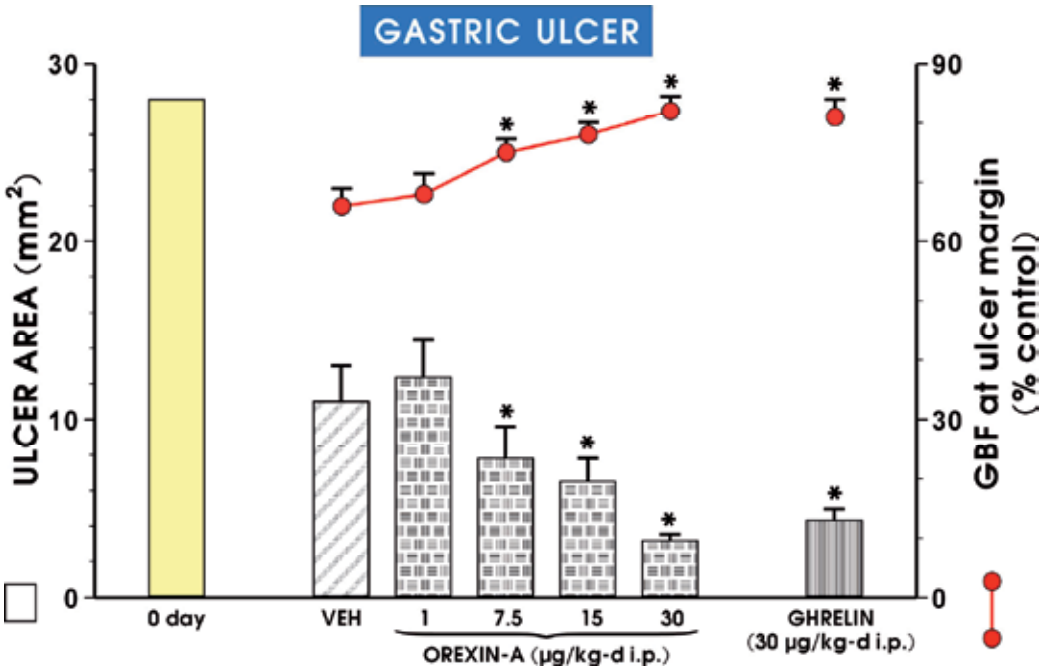

Fig. 1. The area of gastric ulcer induced by acetic acid and gastric blood flow (GBF) at ulcer margin at 8 day upon ulcer induction in rats treated with vehicle (saline) or with various doses of orexin-A (1 - 30 µg/kg-d i.p.) or ghrelin (30 µg/kg-d i.p.). Means ± SEM of 6-8 rats. Asterisk indicates a significant change ($p<0.05$) as compared to the vehicle-control values.

area of gastric ulcer and significantly increased the GBF at ulcer margin starting from the dose of 5 µg/kg (Fig. 2). The maximal reduction of ulcer area accompanied by a significant rise in the GBF at ulcer margin was recorded at the dose of 30 µg/kg of obestatin where 35% reduction of ulcer area and an increase by about 19% of GBF at ulcer margin were observed as compared to the respective values in vehicle-treated animals.

3.2 Role of cyclooxygenase (COX)-1 and COX-2 inhibition and the blockade of nitric oxide (NO) synthase on ulcer healing activity and alterations in the GBF at ulcer margin in rats treated with ghrelin, orexin-A and obestatin

As shown in Fig. 3, ghrelin and orexin-A given i.p. in dose 30 µg/kg-d significantly decreased ulcer area and enhanced GBF as compared to those observed in vehicle. Treatment with D-Lys^3-GHRS-6 (200 µg/kg i.p.), the ghrelin GHS-R1a receptor antagonist, by itself failed to affect the area of gastric ulcers and GBF at ulcer margin at day 8 upon ulcer induction. The decrease in the area of these ulcers and accompanying rise in the GBF at ulcer margin induced by ghrelin and orexin-A were significantly attenuated by concurrent treatment with ghrelin and orexin-A receptor antagonists, D-Lys^3-GHRS-6 and SB334867. The administration of D-lys^{3}GHRP, which is a specific ghrelin receptor antagonist or SB334867, an antagonist of orexin-A OXR-1 receptors when applied alone, failed to affect the area of gastric ulcer and the GBF at ulcer margin as compared to those measured in vehicle-treated rats. Treatment with D-lys^{3}GHRP significantly attenuated ghrelin-induced acceleration of ulcer healing and the accompanying increase in GBF at ulcer margin.

Concomitant treatment of orexin-A with its receptor antagonist, SB334867 (5 mg/kg-d s.c.), completely abolished the reduction in ulcer area induced by orexin-A. This antagonist of orexin-A receptors reversed also an increase in GBF at ulcer margin caused by orexin-A.

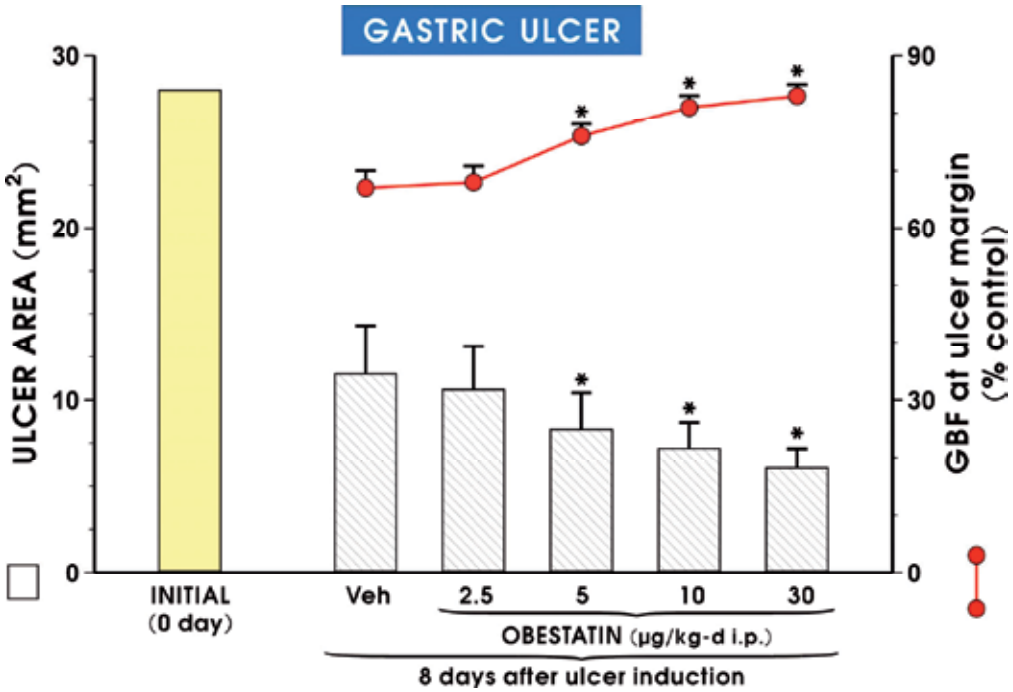

Fig. 2. The area of gastric ulcers and the alterations in the GBF at ulcer margin in rats treated for 8 days with vehicle (Veh; control) or obestatin applied i.p. in graded doses ranging from 2.5 µg/kg-d up to 30 µg/kg-d. Mean ± SEM of 6-8 rats. Asterisk indicates a significant change ($p<0.05$) compared to the vehicle-pretreated controls.

As shown in Fig. 4, treatment with ghrelin, orexin-A and obestatin (30 µg/kg-d i.p.) resulted in a similar attenuation in the area of gastric ulcer and a similar rise in GBF as that shown in Figs. 1 and 2. The mucosal generation of PGE_2 was significantly increased at ulcer margin as compared to the respective value in the non-ulcerated control (128±9 vs. 189±12 ng/g wet tissue weight, $p<0.05$). Ghrelin, orexin-A and obestatin applied i.p. resulted in a significant increase in the PGE_2 generation at ulcer margin as compared to vehicle-treated animals with gastric ulcer (189±12 vs 234±19 ng/g, 189±12 vs 228±9 ng/g and 189±12 vs 219±8 ng/g, respectively, $p<0.05$). Indomethacin (5 mg/kg i.p.), which by itself significantly increased the ulcer area, suppressed the generation of PGE_2 by about 85% ($p<0.02$) and produced a significant fall in GBF as compared to vehicle-pretreated animals (Fig. 4). Treatment with this non-selective COX-1 and COX-2 inhibitor completely abolished the reduction in the area of gastric ulcers and the accompanying rise in GBF evoked by ghrelin, orexin-A and obestatin. The decrease in the area of ulcer and accompanying increase in GBF caused by ghrelin, orexin-A and obestatin as well as the rise in the PGE_2 generation they induced were also significantly attenuated by pretreatment with rofecoxib, the selective COX-2 inhibitor (Fig. 4). SC-560 (5 mg/kg-d i.g.), which by itself significantly reduced the PGE_2 generation (not shown), significantly attenuated the ghrelin-, orexin-A- and obestatin-induced decrease in ulcer area

and the accompanying rise in GBF at ulcer margin (Fig. 4). Concurrent treatment with a minute amount of synthetic dimethyl analog of PGE_2 (5 μg/kg-d i.g.) in addition to ghrelin, orexin-A and obestatin restored the decrease in the ulcer area and the increase in GBF at ulcer margin in rats treated with indomethacin, SC-560 or rofecoxib (Fig. 4).

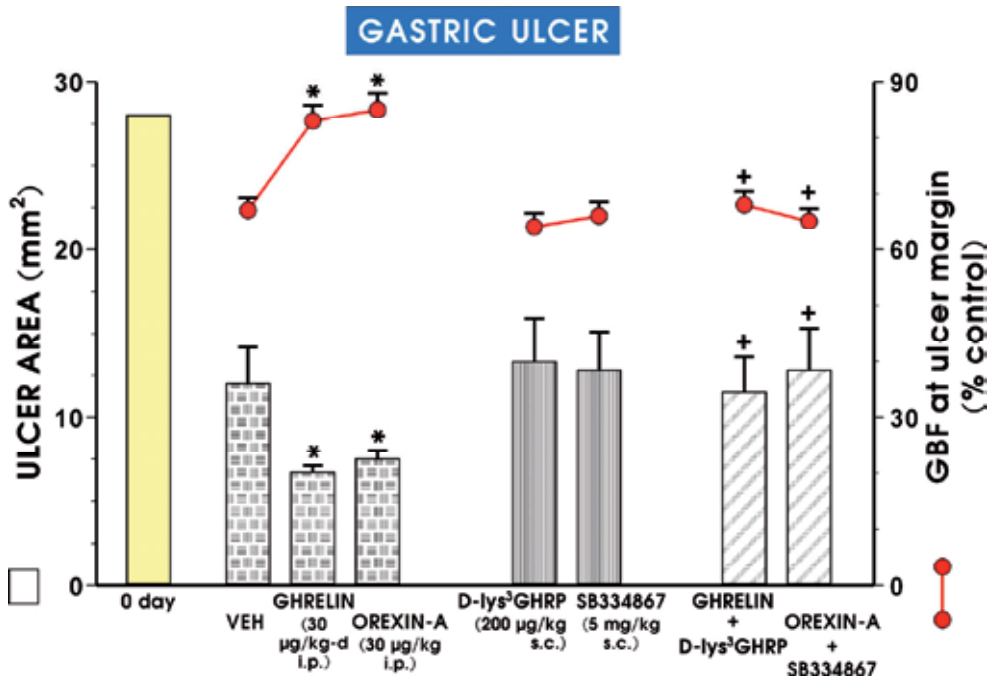

Fig. 3. The area of gastric ulcers and the accompanying changes in the GBF at ulcer margin in rats with or without ghrelin or orexin-A (both at a dose of 30 μg/kg-d i.p.) applied without or with the ghrelin receptor antagonist D-Lys3-GHSR-6 (200 μg/kg-d i.p.) or orexin-A receptor antagonist SB334867 (200 μg/kg-d i.p.). Mean ± SEM of 6-8 rats. Asterisk indicates a significant change ($p<0.05$) compared to the vehicle-pretreated controls. Cross indicates a significant change ($p<0.05$) compared to the values obtained in rats treated with ghrelin and orexin-A without the concurrent treatment with GHS-R1a receptor or OXR-1 receptor antagonists.

3.3 Expression of ghrelin and proinflammatory cytokines in ulcer area during ulcer healing without and with ghrelin treatment

The effect of eight day administration of L-NNA (20 mg/kg-d i.p.), the NO-synthase inhibitor on ulcer area and the changes in GBF at ulcer margin in rats treated without and with vehicle, ghrelin, orexin-A and obestatin, each applied in a dose of 30 μg/kg-d i.p. is presented in Fig. 5. Treatment with ghrelin, orexin-A and obestatin significantly reduced the ulcer area and increased the GBF at ulcer margin as shown in Fig. 4. Treatment with L-NNA, which by itself significantly enhanced ulcer area also significantly decreased GBF at ulcer margin as compared to respective values in vehicle-control. Concurrent treatment with L-NNA together with ghrelin, orexin-A and obestatin reversed the ghrelin-, orexin-A- and obestatin-induced decrease in ulcer area and the accompanying rise in the GBF at ulcer margin.

Fig. 6 shows that ghrelin was expressed in intact non-ulcerated gastric mucosa and that with gastric ulcer. Ghrelin mRNA expression was detected as the strong signal in intact gastric mucosa and vehicle-treated gastric mucosa with gastric ulcer. Ratio of mRNA for ghrelin over β-actin revealed that this ratio was significantly higher in ulcerated gastric mucosa as in case of that in intact animals without an ulcer (Fig. 6, left panel).

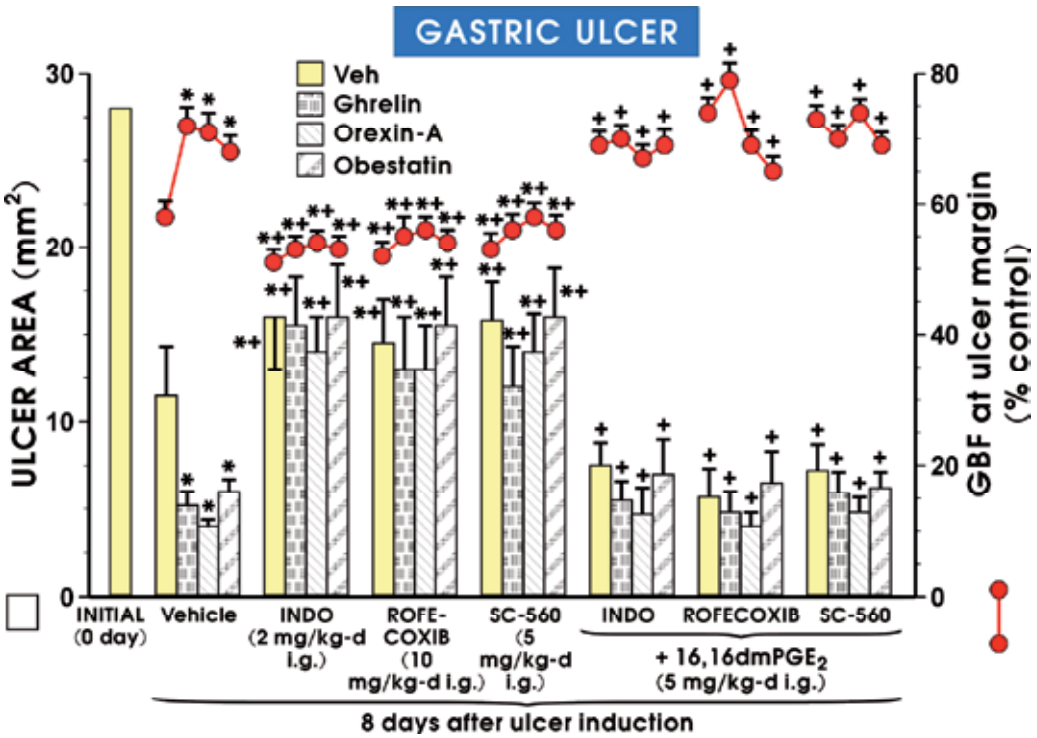

Fig. 4. The area of gastric ulcers and the accompanying changes in the GBF at ulcer margin in rats treated for a period of 8 days with vehicle (saline) and ghrelin, orexin-A and obestatin (each given in a dose of 30 μg/kg-d i.p.) with or without the concurrent treatment with indomethacin (5 mg/kg-d i.p.), rofecoxib (10 mg/kg-d i.g.) or SC-560 (5 mg/kg-d i.g.) applied alone or combined with synthetic 16,16 dimethyl (dm) PGE_2 analog (5 μg/kg-d i.g.). Mean ± SEM of 6-8 rats. Asterisk indicates a significant decrease ($p<0.05$) as compared to the value obtained in vehicle-controls. Asterisk and cross indicate a significant increase ($p<0.05$) as compared to vehicle-treated controls administered without or with ghrelin, orexin-A or obestatin. Cross indicate a significant change ($p<0.02$) as compared to respective values obtained in animals treated with indomethacin, SC-560 or rofecoxib without the concurrent treatment with PGE_2 analog.

As shown in Fig. 7, the signal of mRNA for IL-1β and TNF-α mRNAs was faintly expressed in intact gastric mucosa but it was observed as strong signal in vehicle-control mucosa of animals with gastric ulcer. Ratio of mRNA for ghrelin over β-actin indicated that expression of mRNA for IL-1β and TNF-α was significantly increased in vehicle-treated rats with gastric ulcer over that recorded in intact gastric mucosa. In contrast, in rats treated with ghrelin applied i.p. at the doses of 15 μg/kg and 30 μg/kg, the signal for IL-1β and TNF-α

mRNAs was significantly decreased in gastric mucosa with gastric ulcer as compared to that in vehicle-control animals. Ratio of mRNA for IL-1β and TNF-α mRNA over β-actin mRNA revealed that this ratio was significantly decreased in gastric mucosa of rats treated with ghrelin administered at the dose of 15 μg/kg. The ratio of IL-1β and TNF-α was significantly decreased in animals treated with ghrelin applied i.p in a higher dose of 30 μg/kg than in those treated with ghrelin administered in a lower dose of 15 μg/kg of this peptide (Fig. 7).

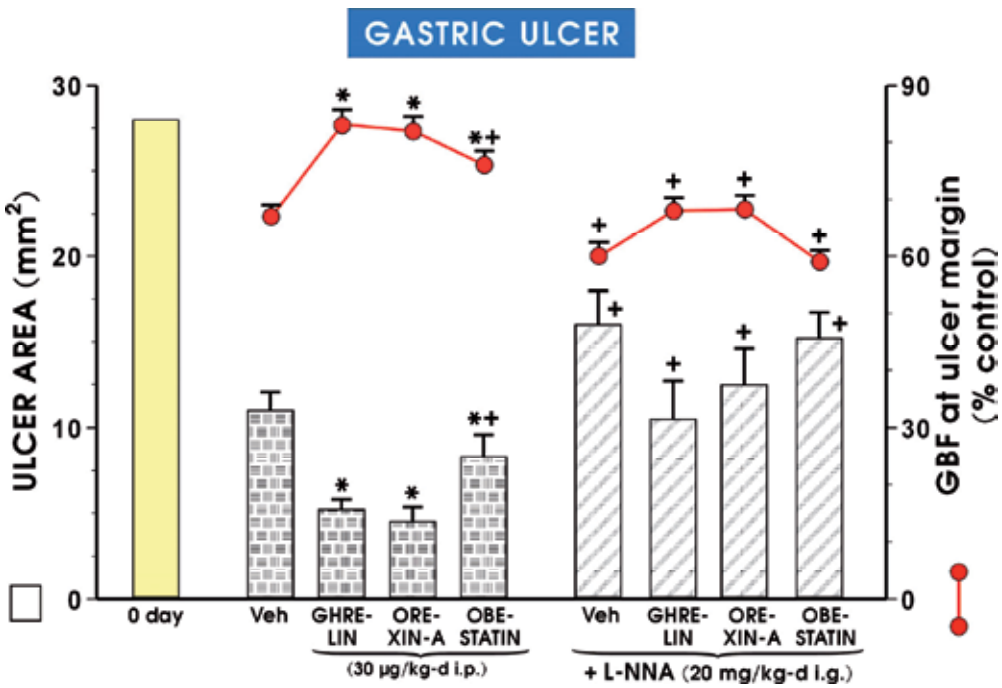

Fig. 5. The area of gastric ulcers and the accompanying changes in the GBF at ulcer margin in rats treated with vehicle (Veh; saline-control), ghrelin, orexin-A and obestatin (each administered in a dose of 30 μg/kg-d i.p.) applied without or with the NO-synthase inhibitor L-NNA (20 mg/kg-d i.p.). Mean ± SEM of 6-8 rats. Asterisk indicates a significant change ($p<0.05$) compared to the vehicle-treated controls. Asterisk and cross indicate a significant change ($p<0.05$) compared to the values obtained in rats treated with vehicle. Cross indicates the significant change ($p<0.02$) compared to the values obtained in ghrelin, orexin-A and obestatin without the concurrent treatment with L-NNA.

3.4 Effect of treatment with obestatin on ulcer healing in rats with of ghrelin administration

Fig. 8 shows the effects of eight day of concomitant administration of ghrelin, the appetite stimulating peptide, and obestatin which is opponent of ghrelin acting as the natural ligand of the GPR39 receptor, on the alterations in area of gastric ulcer and accompanying changes in GBF at ulcer area and plasma ghrelin and obestatin levels. Ghrelin and obestatin given i.p. in the same comparable dose of 30 μg/kg-d significantly reduced the area of gastric ulcer, however the significantly greater reduction in ulcer area was observed in ghrelin-

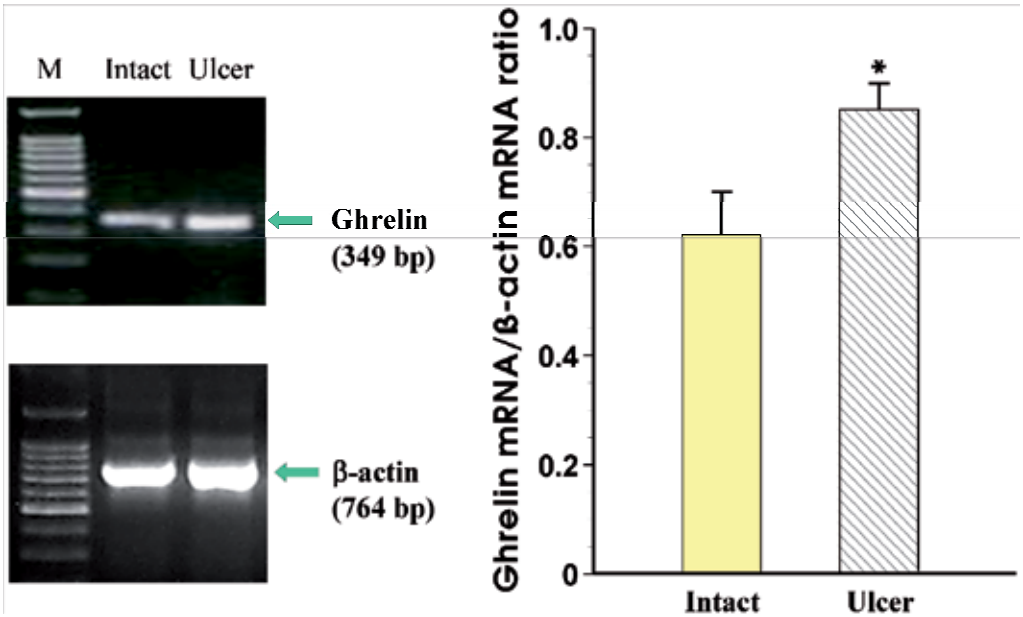

Fig. 6. Determination of expression of β-actin and ghrelin mRNAs (left panel) by RT-PCR and the ratio of ghrelin to β-actin mRNA (right panel) in non-ulcerated (intact) gastric mucosa (lane Intact) and in gastric mucosa at ulcer margin (lane Ulcer), M - DNA size marker. Mean ± SEM of 4-6 rats. Asterisk indicates a significant change ($p<0.05$) as compared to the value obtained in non-ulcerated gastric mucosa.

treated rats as compared with those treated with obestatin. Plasma ghrelin level increased after i.p. administration of exogenous ghrelin but remained unchanged after combined treatment of ghrelin with obestatin as compared with those treated with ghrelin alone. The plasma obestatin concentration which was significantly increased in obestatin-treated rats with gastric ulcer was not significantly affected by the concurrent ghrelin administration as compared to vehicle-treated rats. The combined treatment of obestatin co-administered with ghrelin failed to significantly the affect the ghrelin-induced attenuation of ulcer area and the accompanying rise in plasma ghrelin level which reached similar values, as in case of ghrelin administered alone.

4. Discussion

This study shows that appetite hormones such as ghrelin, orexin-A and obestatin besides their well recognized action in the control of food intake and energy expenditure, exhibit ulcer healing and hyperemic activities as documented by an acceleration of ulcer healing by these peptides accompanied by an increase in the GBF at ulcer margin. These healing and hyperemic effects of ghrelin and orexin A seem to be very specific because both hormones induced acceleration of the healing and hyperemia at ulcer margin were reversed by ghrelin receptor antagonist D-Lys^3-GHRP-6, and orexin-A receptor antagonist SB 334867, respectively, indicating that ghrelin- and orexin A-induced ulcer healing promoting and hyperemic effects are mediated by the functionally active form of GHS-R1a receptor and orexin (OX-R1) receptors. These receptors GHS-R1a and OX-R1 have been shown to bind

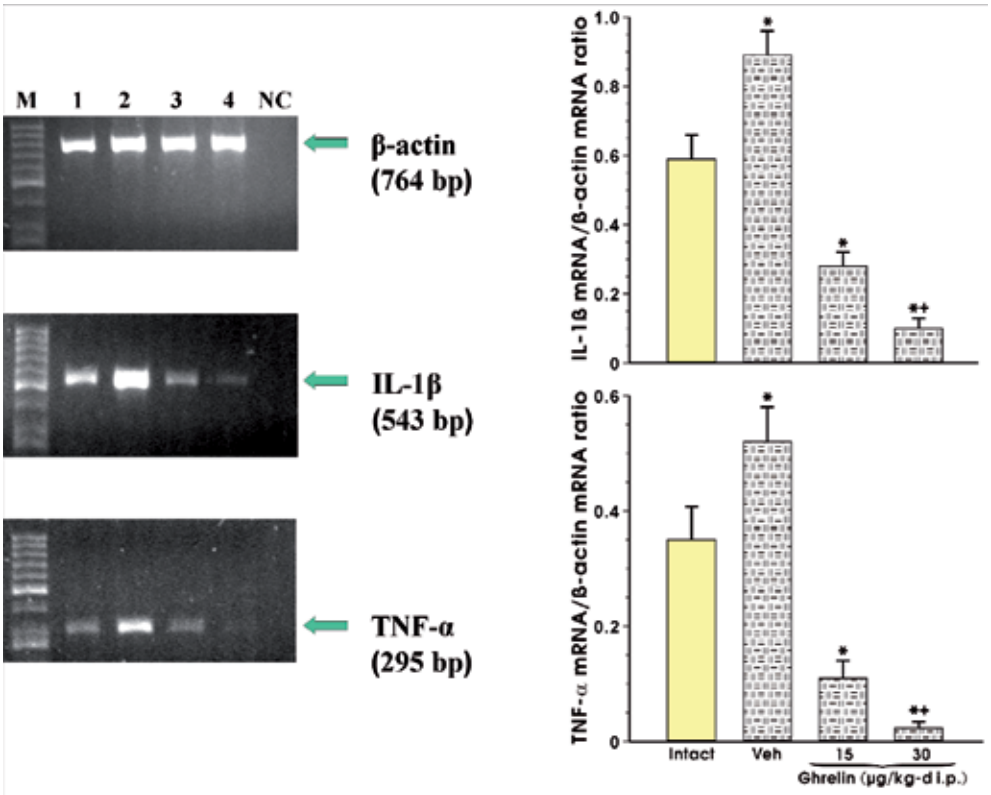

Fig. 7. Determination of expression of β-actin, IL-1β and TNF-α mRNAs (left panel) by RT-PCR and the ratio of mRNA for ghrelin, orexin-A and obestatin (each administered at the dose of 30 μg/kg-d i.p.) to β-actin mRNA (right panel) in intact non-ulcerated gastric mucosa and in those with acetic acid-induced gastric ulcer treated with vehicle (saline) or ghrelin applied i.p. in a dose of 15 μg/kg or 30 μg/kg, M - DNA size marker. Mean ± SEM of 4-6 rats. Asterisk indicates a significant change ($p<0.05$) as compared to the value obtained in intact non-ulcerated gastric mucosa. Asterisk and cross indicate a significant change ($p<0.05$) as compared to the value obtained in animals treated eight days with ghrelin administered daily i.p. at the dose of 15 μg/kg-d.

acylated ghrelin and orexin A, respectively. Moreover, we found that ghrelin, orexin A and to lesser extent also obestatin increased the PGE_2 generation at ulcer margin and that these peptides ulcer healing promoting and hyperemic effects and an increase in PGE_2 generated at ulcer margin were significantly attenuated by non-selective (indomethacin), selective COX-1 (SC-560) and selective COX-2 inhibitors (rofecoxib). These findings indicate that endogenous PG derived from both COX-1 and COX-2 enzymatic pathways are involved in the mechanism of ulcer healing by these hormones. Obestatin which was originally claimed to act as ghrelin opponent in the regulation of appetite behaviour [Zhang et. al., 2005], also accelerated ulcer healing and increased the GBF at ulcer margin in our study though these effects were less pronounced as compared with those of ghrelin and orexin-A.

Ghrelin is a recently described 28-amino acid peptide that has been discovered in rat and human gastrointestinal tract, particularly in gastric mucosa, as an endogenous ligand for

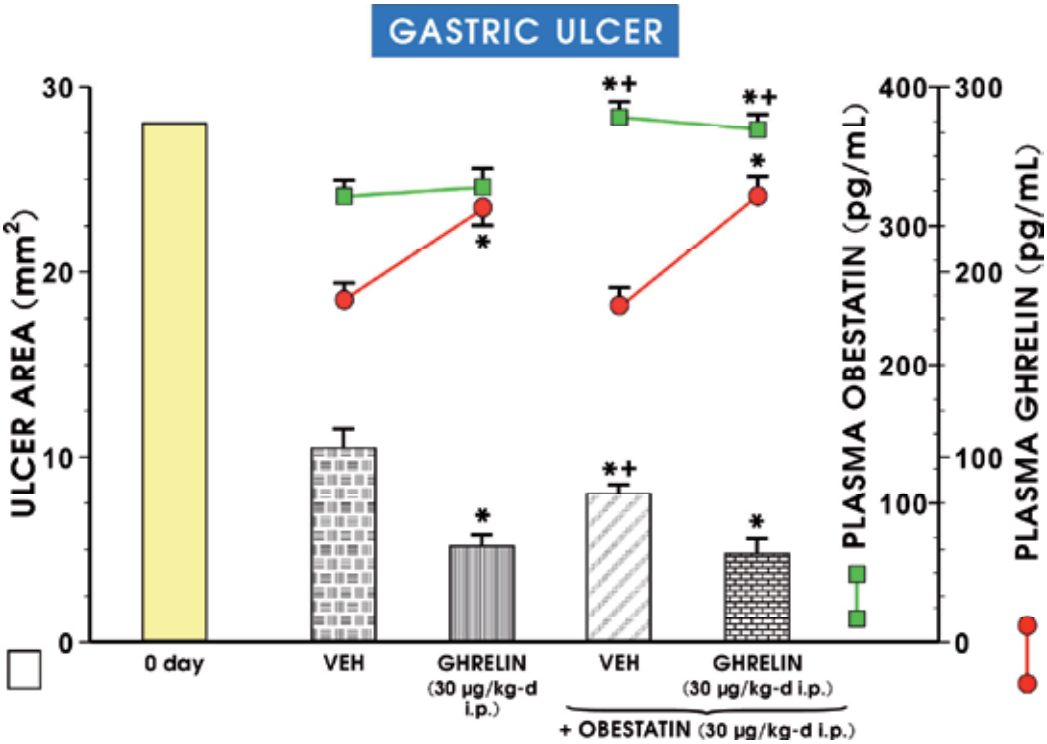

Fig. 8. The area of gastric ulcers and the alterations in the GBF at ulcer margin in rats treated for 8 days with vehicle (Veh, saline) or ghrelin (30 µg/kg-d i.p.) without or with concurrent treatment with obestatin (30 µg/kg-d i.p.). Mean ± SEM of 6-8 rats. Asterisk indicates a significant change ($p<0.05$) compared to vehicle-control- or obestatin-treated rats. Asterisk and cross indicate a significant change ($p<0.05$) compared to rats treated with ghrelin.

growth hormone secretagogue receptor (GHS-R) [Kojima et al., 1999, Hosoda et. al., 2000]. Previous studies revealed that two GHS-R subtypes are generated by alternative splicing of a single gene: the full-length type 1a receptor (GHS-R1a) and a carboxyl-terminally truncated GHS-R type 1b (GHS-R1b) [Peeters , 2005; Brzozowski et al., 1999]. The GHS-R1a is the functionally active, signal transducing form of the GHS-R, while the GHS-R1b is devoid of high-affinity ligand binding and signal transduction activity. Ghrelin molecules, produced by endocrine cells of gastric glands exist in two major molecular forms, ghrelin and des-n-octanoyl ghrelin (des-acyl ghrelin) [Hosoda et al., 2000; Fukuhara et al., 2005]. The acylation by n-octanoic acid of the hydroxyl group of their third residue, which is either serine or threonine, is essential for binding of ghrelin to the GHS-R1a [Hosoda et al., 2000]. We attempted in this study to determine whether the expected ulcer healing activity of ghrelin and orexin A is due to a direct activation of GHS-1a and orexin A receptors or involves other mediators or receptors, as yet uncharacterized and distinct from the GHS-R and orexin A. We have found that the acceleration of healing and the functional features of this healing such as an improvement in the GBF at ulcer area, the crucial mechanism involved in the healing of gastric ulcer, were mediated by specific ghrelin and orexin-A receptors.

Ghrelin was originally reported to exhibit gastroprotective activity against mucosal lesions induced by corrosive substances such as ethanol as well as against damage induced by

stress and ischemia-reperfusion [Sibilia et al., 2003; Brzozowski et al., 2004; Konturek et al., 2004; Brzozowski et al., 2006]. These effects were associated with a significant increase in plasma ghrelin concentration as well as marked attenuation of the fall in GBF provoked by stress and I/R but remained independent on gastric acid secretory activity of this peptide [Brzozowski et al., 2006]. This study attempted to determine the effects of graded doses of exogenous orexin-A and obestatin compared with ghrelin on healing of acetic acid ulcers and GBF at ulcer margin and PGE_2 generation in the gastric mucosa. We found that peripherally administered orexin-A dose-dependently accelerated ulcer healing, elevated mucosal generation of PGE_2 and raised GBF at ulcer margin. Ghrelin and obestatin also accelerated ulcer healing with the magnitude similar to orexin-A while causing an increase in GBF and PGE_2 generation in the ulcerated gastric mucosa. The key finding of this study is the demonstration that ghrelin mRNA is upregulated in the margin of gastric ulcer suggesting that endogenous ghrelin might reduce the size of the ulcer and improve the microcirculation around the ulcer bed. Interestingly, ghrelin mRNA was also increased in the gastric mucosa exposed to ischemia-reperfusion suggesting that enhanced expression of transcripts of ghrelin may limit the extent of gastric damage provoked by I/R. Thus, our study is in partial agreement with previous observations that plasma ghrelin is increased following stress-induced gastric lesions, and that its enhanced immunoreactivity is associated with duodenal ulcerations induced by cysteamine in rats [Brzozowski et al., 2006; Fukuhara et al., 2005].

Since endogenous prostaglandins (PGs) have been implicated in the control of food intake and appetite [Eberhart, E.C, 1995; Scholz, H, 2003] we hypothesized that these cytoprotective arachidonate metabolites could play an important role in the healing effect of ghrelin. This prompted our interest in endogenous PGs because their role as well as the importance of expression of cyclooxygenase (COX-1) and COX-2 at ulcer margin in the possible ulcer activity of appetite hormones such as ghrelin, orexin-A and obestatin have not been studied. The present study supports the notion that the ulcer healing and hyperemic activities of ghrelin, orexin-A and obestatin during the time-course of ulcer healing involve an increase in mucosal generation of endogenous PGs. Previous studies have established that PG synthesis depends upon the activity of cyclooxygenase (COX), a rate limiting enzyme in the synthesis of eicosanoids from arachidonic acid [Eberhart CE & Dubois RN, 1995]. In contrast to COX-1, COX-2 is not constitutively expressed in most of tissues, but is dramatically upregulated during inflammation and ulcer healing [Takeuchi et al., 2004; Brzozowski et al., 2006] and following inhibition of mucosal COX-1 activity [Davies et al., 1997; Tanaka et al., 2002; Takeuchi et al., 2004]. First, we found that all hormones tested that is mean ghrelin, orexin A and obestatin enhance the PGE_2 generation at ulcer margin as compared with that of vehicle-control at day 8 upon ulcer induction. Second, indomethacin almost completely abolished, while SC-560, the selective COX-1 inhibitor and rofecoxib, the selective COX-2 inhibitor, greatly attenuated the ulcer healing and hyperemic effects of ghrelin, orexin A and obestatin, indicating that endogenous PGs, potentially derived from the activities of both COX-1 and COX-2, are responsible for the putative beneficial effects of this peptide on ulcer healing. It is of interest that COX-1 and COX-2 inhibitors by themselves delayed ulcer healing and abolished the ulcer healing promoting activity of ghrelin, orexin A and obestatin and counteracted their accompanying rise in GBF at ulcer margin suggesting that endogenous PG might be considered as primary mediators involved in the mechanism of ulcer healing by these peptides.

It is not excluded that the beneficial effect of these hormones depend also upon the activation of NO/NOS system by these hormones, which is an important metabolic pathway involved in the mechanism of mucosal defense and ulcer healing. The involvement of cNOS/iNOS-NO system in ulcer healing and the possible role of ghrelin in ulcer healing are not quite clear from this and other studies [Sibillia et al., 2009]. Although the non-specific suppression of cNOS/iNOS-NO system by L-NNA was found in our study to delay ulcer healing, little is known whether appetite hormones could affect the expression and activity of cNOS or iNOS or both. Indeed, the concurrent treatment with L-NNA not only markedly reduced the acceleration of ulcer healing but also the accompanying hyperemia at ulcer margin caused by these hormones. This finding suggests that NO could be considered as one of the essential mediator of both, the ulcer healing and the hyperemic activity of ghrelin, orexin A and obestatin. Thus we propose that NO possibly derived from constitutive cNOS rather than inducible iNOS expression and activity, plays an important role in the ghrelin-, orexin A and obestatin induced acceleration of the ulcer healing process. However, factors other than endogenous PGs and NO, possibly also sensory neuropeptides such as CGRP could also contribute to this effect, and therefore, their involvement in ulcer healing activity of ghrelin, orexin A and obestatin should be addressed in future studies. The finding that COX-1 and COX-2 inhibitors greatly attenuated the ulcer healing activity of ghrelin, is in keeping with the observation that ghrelin is ineffective in the protection against indomethacin-induced gastric ulcers [Sibillia et al., 2004], where the mucosal generation of PGE_2 is greatly suppressed.

Interestingly, the overexpression of TNF-αa and IL-1β was detected in vehicle treated control rats with gastric ulcer comparing to intact gastric mucosa , and this effect was probably caused by severe tissue ischemia, resulting from the application of acetic acid. This overexpression of proinflammatory cytokines such as TNF-α and IL-1β, likely contributed to early tissue damage and formation of ulceration. As reported by our group recently [Brzozowski et al., 2006] vascular injury leading to ischemia is the major factor associated with early induction of acute mucosal damage and also involved in the time-course of chronic ulceration in the stomach, where the acetic acid was applied. [Guo et al., 2003] reported an early rise in the expression of iNOS, suggesting that this expression accompanied by excessive generation of NO possibly forming peroxynitrate, was probably responsible for the enlargement of ulcer crater observed at early first days upon ulcer induction by acetic acid in rats. Our results seem to support this notion by overexpression of mRNA for proinflammatory cytokines, TNF-α and IL-1β, that could be responsible for the formation of focal tissue damage caused by acetic acid application. Treatment with ghrelin markedly attenuated the expression of IL-1β and TNF-α in gastric mucosa around the ulcer suggesting that the acceleration of ulcer healing by ghrelin could be, at least in part, due to the potent anti-inflammatory activity of this hormone.

We reported before that the induction of gastric ulcer by serosal application of acetic acid is accompanied by the co-expression of gastroprotective COX-2-PG system with the increased expression and release of vasoactive, proliferative and trophic gastric hormones such as gastrin and ghrelin [Konturek et al., 2008]. Ghrelin was shown to exhibit gastroprotective activity and our present study confirming that this hormone can accelerate ulcer healing, suggests that the upregulation in the ghrelin gene expression could play the central event in the mechanism of gastric ulcer healing. These protective and trophic hormonal responses of gastric mucosa to ulceration, as manifested by increased expression of ghrelin co-expressed

with COX-2 [Konturek et al., 2008] possibly triggered by an overexpression of IL-1β and TNF-α, seem to play a crucial role in the restoration cellular and glandular structure of the mucosa and the quality of ulcer healing.

Interestingly, obestatin, which is encoded by the same gene as ghrelin has recently been reported to counteract physiological effects of ghrelin [Zhang et al., 2005]. Intraperitoneal and intracerebroventricular treatment with obestatin suppressed food intake in a time-dependent and dose-dependent manner. This was supported by observation that obestatin effectively blunted the hunger caused by short-term starvation and inhibited feeding or body weight in rats [Sibillia et al., 2009] and food consumption in mice [Lagaut et al., 2007]. However, the vast majority of studies indicates that obestatin exerts little or no effects on food intake and body weight and failed to modify the CCK-induced decrease in food intake [Moechrs et al., 2006; Nogueiras et al., 2007; Tremblay et al., 2007]. Our present findings are in keeping with these latter observations because obestatin by itself accelerated ulcer healing but when administered together with ghrelin, remained without influence on ghrelin induced promotion of the ulcer healing and an increase in the GBF at ulcer margin and plasma ghrelin levels. This suggests that obestatin might not be considered as physiological opponent of ghrelin with the respect to ulcer healing activity which clearly was enhanced by this peptide.

5. Conclusion

In summary, these results demonstrate for the first time, that administration of exogenous ghrelin dose-dependently accelerates healing of chronic gastric ulcers and that other hormones involved in the control of appetite and food intake such as orexin-A and obestatin can also exert beneficial effect on the speed of ulcer healing. An evidence was provided that these ulcer healing effects of orexin-A, ghrelin and obestatin may depend upon endogenous PGs derived from COX-1 and COX-2 activity and an enhancement of gastric microcirculation around the ulcer area, possibly mediated by NO. Our finding that ghrelin is expressed in gastric mucosa, particularly at ulcer margin during ulcer healing suggests that this hormone may act locally by paracrine pathway to activate the vasoactive compounds such as PG and NO that may lead to an acceleration of the ulcer healing and the suppression of the mucosal expression and the release of proinflammatory cytokines TNF-α and IL-1β.

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11

Activation of the Hypothalamic-Pituitary-Adrenocortical Axis as a Gastroprotective Component of Stress Response

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1. Introduction

Gastric ulcer disease remains widespread; a stressful lifestyle and non-steroidal anti-inflammatory drugs (NSAIDs) make significant contributions to this pathological situation (Glavin et al., 1991; Hawkey, 2000; Laine et al., 2008). Despite indubitable advances in elucidation of the pathogenesis of gastric ulceration, there are gaps in our understanding of ulcerogenesis, particularly the role of key hormonal system of adaptation: the hypothalamic-pituitary-adrenocortical (HPA) axis.

2. Glucocorticoid hormones and gastric ulceration

Glucocorticoid hormones and gastric ulceration have been discussed in many contexts. The action of acute and chronic treatment of patients or experimental animals with glucocorticoids as well as the effects of basal and stress-induced glucocorticoid production on the gastric mucosa has been considered. Although there is a long-standing debate over whether glucocorticoid therapy by itself leads to peptic ulcer disease in human (Luo et al., 2009; Olsen et al., 2010), it is established that administration of glucocorticoids to experimental animals can result in an acute gastric erosion (Bandyopadhyay et al., 1999, as cited in Filaretova et al., 2009b; Black, 1988, as cited in Filaretova et al., 1998; Takeuchi et al., 2008). In the same time, in some cases administration of glucocorticoids to animals can attenuate gastric erosion (Derelanko & Long 1982; Filaretova et al., 2009b; McCafferty et al., 1995). It is also known that basal glucocorticoid production contribute to the maintenance of the gastric mucosal integrity (Takeuchi et al., 1989). The glucocorticoids may modulate the cytoprotective effect of adrenal catecholamines (Hernandez et al., 1984). They may have a permissive role in allowing gastroprotective mechanisms to exert their full potential. A permissive role was suggested in gastric mucosal protection induced by prostaglandins (PGs), sulfhydryls, cimetidine (Szabo et al., 1983) or interleukin-1 (Perretti et al., 1992).
The most controversial question is the question about the action of stress-produced glucocorticoids. Based on the notion that exogenous glucocorticoids used at pharmacological doses have ulcerogenic properties, the increase of glucocorticoids during stress was also considered to be an ulcerogenic factor. In the same time, it is known that

glucocorticoid hormones released during acute stress-induced activation of the HPA axis help the body overcome negative effects of stress stimuli (Munk et al., 1984, as cited in Filaretova et al., 1998). Despite this knowledge, it has been generally accepted for several decades that stress-produced glucocorticoids cause an ulcerogenic response in the stomach, and stress-induced activation of the HPA axis is considered a pathogenic component of this response.

As the widely held view about the ulcerogenic role of glucocorticoids released during stress is difficult to reconcile with the adaptive role of the HPA axis hormones, we designed experiments in rats to clarify the validity of this view. The results obtained do not support the traditional paradigm and suggest that glucocorticoids released during acute activation of the HPA axis are important gastroprotective factors. In the chapter, we review our results on the role of glucocorticoids in gastroprotection.

2.1 Gastroprotective action of stress-produced glucocorticoids

Various stressful stimuli activate the HPA axis, and consequently, the production of glucocorticoids, and severe stress stimuli may also induce gastric erosion, called “stress ulcers”. Hans Selye, the "Father" of the field of research into stress, attracted attention to these signs of stress. His greatest contributions were the demonstration of the stress triad (gastrointestinal ulceration, thymico-lymphatic atrophy, and adrenal hypertrophy) and of the role of the hypothalamus in activating the hypophysis, which, in turn, stimulates the adrenals to produce corticoids (Selye, 1967). From the very outset, researchers have focused on the idea that stress-generated glucocorticoids are causally related with gastric ulcerogenesis. This possibility was also investigated in hypophysectomized and adrenalectomized animals by Selye himself, who observed that although stress-induced thymico-lymphatic atrophy was inhibited in these animals, “stress ulcers” were not prevented, and concluded that the formation of “stress ulcers” depends on not only the pituitary-adrenal axis but other factors as well. He also proposed that neurostimulators play a major role in stress-induced ulcerogenesis, although high levels of corticoids in blood could be a sensitizing factor (Selye, 1967). Weiss (Weiss, 1971, as cited in Filaretova et al., 1998) found in rats that the severity of stress-induced ulceration is positively correlated with the level of corticosterone in plasma and proposed that "steroids, in quantities that the animal is capable of secreting, may contribute to the production of ulcers". Further support for this idea came from the observation that animals with hippocampal lesions had increased levels of plasma corticosterone and developed more gastric ulcers during stress (Murphy et al., 1979, as cited in Filaretova et al., 1998). One approach used to support the view that stress-generated glucocorticoids are ulcerogenic was a groundless extrapolation of the ulcerogenic properties of exogenous glucocorticoids observed at high pharmacological doses to the properties of endogenous glucocorticoids released during stress.

From the beginning (Filaretova, 1990), we have focused on the idea that glucocorticoids released during acute stress also have an adaptive effect on the stomach and, therefore, are gastroprotective rather than ulcerogenic. To test this hypothesis, we examined the effect of glucocorticoid deficiency or the glucocorticoid receptor antagonist RU-38486 on water and immersion-restraint-induced or cold-restraint-induced gastric erosion in rats (Filaretova, 1990, 2006; Filaretova et al., 1998). Different approaches were used to inhibit the stress-induced release of corticosterone: the inhibition of corticotropin-releasing hormone synthesis in the hypothalamic paraventricular nucleus by intrahypothalamic implantation of

dexamethasone, the immunoneutralization of ACTH by pretreatment with ACTH antiserum, and the inhibition of the HPA axis at the hypothalamic and the pituitary levels by pretreatment with a pharmacological dose of cortisol one week before stress. Corticosterone replacement, that is, the injection of corticosterone at a dose mimicking the stress-induced rise in corticosterone (4 mg/kg) 15 min before stress, was used in our experiments.

Intrahypothalamic dexamethasone implantation significantly decreased the stress-induced increase in corticosterone and markedly provoked the gastric erosion caused by stress. Corticosterone replacement prevented the aggravating effect of dexamethasone on the ulceration. ACTH antiserum administered shortly before cold-restraint stress decreased the release of corticosterone in response to stress and enhanced the severity of the gastric erosion (Filaretova et al., 1998). Pretreatment with glucocorticoid (cortisol) at a pharmacological dose caused an inhibition of the HPA axis at the hypothalamic and pituitary levels via a negative feedback mechanism and resulted in a long-lasting decrease in the stress-induced rise in corticosterone levels (Filaretova, 2006; Filaretova et al., 2001a). It is important to emphasize that animals were stressed one week after the treatment with cortisol when the exogenous hormone had already been eliminated but the corticosterone response to stress was still inhibited. The cortisol pretreatment increased the ulcerogenic action in both models of stress, and acute corticosterone replacement that mimicked the stress-induced corticosterone response reduced gastric erosion in rats with an inhibited HPA axis (Filaretova, 1990, 2006). These results support the idea that the gastric ulcerogenic response to stress is potentiated by a reduction of stress-induced glucocorticoid production.

Glucocorticoid antagonists offer another way to demonstrate the role of the stress-induced rise in corticosterone in the gastric ulcerogenic response to stress. The specific progesterone/glucocorticoid receptor antagonist RU-38486 is known to bind with a high affinity to type II glucocorticoid receptors and may influence peripheral as well as central glucocorticoid receptors (Moguilewski & Philibert, 1984, as cited in Filaretova et al., 1998). In the simultaneous presence of glucocorticoids and the antagonist in vivo, glucocorticoid receptors are predominantly occupied by the antagonist (Alexandrova, 1994). The RU-38486-glucocorticoid receptor complex is incapable of nuclear translocation and does not produce a biological effect (Moguilewski & Philibert, 1984, as cited in Filaretova et al., 1998). For this reason the glucocorticoid receptor antagonist RU-38486 can be a tool for investigating modes of glucocorticoid action. It was found that the occupation by RU-38486 of glucocorticoid receptors during cold-restraint stress aggravates the stress-induced gastric erosion (Filaretova et al., 1998). We believe these results support the gastroprotective role of glucocorticoids released during stress. These data also suggest that gastroprotective action of glucocorticoids may occur, at least partly, through the classical genomic mechanism. There is also evidence that glucocorticoids can act through nongenomic pathways (Whitehouse, 2011). We do not rule out that nongenomic mechanisms may also be involved in gastroprotective action of glucocorticoids.

To investigate contribution of glucocorticoids to the maintenance of gastric mucosal integrity during stress we predominantly used ulcerogenic stress models, although in some cases we also used non-ulcerogenic stress models. Using the ulcerogenic models we demonstrated that glucocorticoids released in response to the ulcerogenic stimuli attenuated their harmful action on the gastric mucosa. Our data obtained from the non-ulcerogenic

models suggests that mild stress stimuli don't damage the gastric mucosa due to physiologic gastroprotective action of glucocorticoids released in response to these stimuli (Filaretova, 1990; Filaretova et al., 2001a). Indeed, we showed that in rats with glucocorticoid deficiency normally non-ulcerogenic stress stimulus turns into an ulcerogenic one (Filaretova, 1990). Another striking demonstration of the physiological gastroprotective role of glucocorticoids is the participation of glucocorticoids in gastroprotective effects of preconditioning stress (Filaretova et al., 2008). Preconditioning mild stress may attenuate gastric injury caused by severe stress and this effect is known to be mediated by PGs (Tanaka et al., 2007). It is known that mild stressors induce an increase in glucocorticoid production, however, it remained unknown whether glucocorticoids released during preconditioning mild stress contribute to the gasroprotective effect of mild stress against severe stressors. The contribution of glucocorticoids to the protective effect of preconditioning mild stress on gastric mucosa has not been investigated previously, apparently due to the prevailing traditional point of view on ulcerogenic action of glucocorticoids released during stress. Our findings about gastroprotective role of glucocorticoids produced during ulcerogenic stress allowed us to hypothesize that glucocorticoids contribute to gastroprotective effect of preconditioning non-ulcerogenic stress. To verify the hypothesis we compared the effects of mild stress on gastric erosion caused by severe stress in rats with normal and deficient corticosterone response to preconditioning mild stress. To inhibit glucocorticoid synthesis during mild stress metyrapone was injected shortly before the onset of mild stress. Metyrapone pretreatment caused a fast inhibition of corticosterone response to mild stress and prevented its protective effect on gastric ulceration induced by severe stress. The results obtained argue for a participation of glucocorticoids in the protective influence of preconditioning mild stress on gastric mucosa (Filaretova et al., 2008). We consider this fact as a further support for the point of view that glucocorticoids released during an acute stress are naturally occurring gastroprotective factors.

Therefore, our data allows us to conclude that an acute stress-induced increase of glucocorticoids has gastroprotective action against stress-induced gastric damage. It should be emphasized that our studies on the mode of gastroprotection by glucocorticoids have been performed on animals during acute ulceration. The effects of glucocorticoids on the gastric mucosa during chronic stress conditions may be different from those observed in acute experiments.

2.2 Gastroprotective action of glucocorticoids during treatment with non-steroidal anti-inflammotary drugs

According to our results (Filaretova et al., 2001b, 2002a), NSAID treatment, similar to stress, may activate the HPA axis. Administration of both indomethacin and aspirin induced a release of corticosterone, which in turn may help to protect the gastric mucosa against NSAIDs. Indeed, adrenalectomy prevented NSAID-induced corticosterone release and markedly worsened the gastric erosion caused by NSAIDs. Acute corticosterone replacement, mimicking the indomethacin- and aspirin-induced rise in corticosterone, also prevented the aggravation of gastric ulcers generated by adrenalectomy (Filaretova et al., 2002a). The aggravation of NSAID-induced gastric erosion was also demonstrated in another model of glucocorticoid deficiency where the NSAID-induced corticosterone rise was prevented by pharmacological blockade of the HPA axis (Filaretova et al., 2001b, 2005).

Likewise, pretreatment of the animals with RU-38486, the glucocorticoid receptor antagonist, significantly aggravated the severity of gastric erosion induced by indomethacin as well as aspirin (Filaretova et al., 2002a). It is thus assumed that endogenous glucocorticoids released during NSAID treatment increase the resistance of the gastric mucosa to NSAID-induced injury.

The gastric ulcerogenic properties of NSAIDs limit the use of these drugs for the treatment of chronic inflammatory disorders, and it has been considered that combined treatment with therapeutic doses of glucocorticoid increases the risk of gastric ulceration (Hawkey, 2000). The results obtained in our studies (Filaretova et al., 2001b, 2002a, 2005) suggest that the increased risk of adverse gastric reactions should be considered when NSAIDs are used in patients with impaired glucocorticoid production.

Endogenous glucocorticoids may have a permissive role in allowing gastroprotective mechanisms against NSAID-induced injury to exert their full beneficial potential. This action was suggested in gastric mucosal protection against aspirin-induced erosion induced by cimetidine (Szabo et al., 1983) or interleukin-1 (Perretti et al., 1992). Likewise, a normal basal production of glucocorticoids is also important for the gastric mucosa to resist indomethacin- (Takeuchi et al., 1989) or aspirin-induced damage (Perretti et al., 1992). Furthermore, both aspirin and indomethacin at ulcerogenic doses stimulate glucocorticoid production to cause an acute elevation of glucocorticoid content in the physiological range, which in turn protects against gastric damage induced by these NSAIDs.

These data together with our previous findings support the point of view that glucocorticoids released during acute activation of the HPA axis caused by stress or NSAIDs as well as other ulcerogenic stimuli (Filaretova et al., 2001a) act as gastroprotective hormones. From the beginning (Filaretova, 1990), we have focused on the hypothesis that glucocorticoids released during stress also have an adaptive effect on the stomach. The results obtained in our studies confirm this hypothesis and furthermore demonstrate that glucocorticoids released in response to NSAIDs or other ulcerogenic stimuli also have an adaptive effect on the stomach. In turn, it means that an acute HPA axis activation is a physiologic gastroprotective component of acute stress response.

It is known that both humoral and neuronal factors, such as PGs, nitric oxide (NO), and capsaicin-sensitive afferent neurons, play a pivotal role in the defense against gastric mucosal injury (Holzer, 1998; Wallace, 1997). They contribute to gastroprotection by modulating mucosal blood flow, mucus secretion, and repair of injured gastric mucosa. We showed that glucocorticoids released in response to ulcerogenic stimuli are naturally occurring gastroprotective factors and exert many of the same actions in the stomach as PGs, NO, and capsaicin-sensitive afferent neurons. This has prompted us to consider the interaction between glucocorticoid hormones and other protective factors in the maintenance of gastric mucosal integrity.

We compared the effects of the drug-induced inhibition of PG or NO production or the desensitization of capsaicin-sensitive sensory neurons on the gastric mucosa in rats with deficient or with normal glucocorticoid production, under normal or ulcerogenic conditions. Indomethacin at 35 mg/kg (s.c.) was used as an ulcerogenic stimulus. The glucocorticoid deficiency was caused by adrenalectomy one week before the experiment. Two kinds of corticosterone replacement were used in adrenalectomized rats. Indomethacin at a nonulcerogenic dose (5 mg/kg i.p.) or L-NAME (50 mg/kg s.c.) was acutely given to inhibit PG and NO production, respectively. For the desensitization (functional ablation) of

capsaicin-sensitive afferent neurons, rats were given subcutaneous injections of capsaicin in 3 consecutive doses of 20, 30, and 50 mg/kg (Bobryshev et al., 2005; Filaretova et al., 2007). Adrenalectomy by itself did not cause damage in the stomach. Neither inhibition of PG or NO, nor sensory deafferentation by itself provoked any damage in the gastric mucosa of sham-operated rats. However, each of these treatments damaged the gastric mucosa in adrenalectomized rats, and all of these responses were prevented by corticosterone in drinking water at a concentration mimicking the basal corticosterone level in normal rats (Bobryshev et a., 2005; Filaretova et al., 2007).

Indomethacin-induced gastric erosion was aggravated to a similar extent by adrenalectomy, inhibition of NO production, or desensitization of capsaicin-sensitive afferent neurons. These data suggest that the role of glucocorticoid hormones in protection of the gastric mucosa against indomethacin is no less significant than that of NO or capsaicin-sensitive afferent neurons. The combination of adrenalectomy with inhibition of NO production or sensory deafferentation markedly potentiated the aggravating effect of these treatments by themselves on indomethacin-induced gastric erosions: the mean erosion area was increased approximately 5 or 10 times, respectively. Corticosterone at a dose mimicking the indomethacin-induced corticosterone rise totally prevented the aggravating effect of adrenalectomy in these experiments (Bobryshev et al., 2005; Filaretova et al., 2007). These results demonstrate that the effect of inhibition of NO production or sensory deafferentation on indomethacin-induced gastric erosion is significantly modified by glucocorticoid deficiency. This, in turn, suggests the important role of glucocorticoid hormones in the maintenance of gastric mucosal integrity under adverse conditions when the gastroprotective action of NO or capsaicin-sensitive neurons is impaired.

Thus, our data demonstrates a pivotal compensatory role of glucocorticoids in the maintenance of gastric mucosal integrity in the case of impaired gastroprotective mechanisms provided by PGs, NO and capsaicin-sensitive afferent neurons. The compensatory gastroprotective role of glucocorticoids during PG deficiency (Filaretova et al., 2002a) or desensitization of capsaicin-sensitive afferents (Bobryshev et al., 2005) may be provided through enhancement of their production in these situations. We also showed that glucocorticoid deficiency, in turn, induces a compensatory enhancement in PG production in the stomach through COX-2 expression, which contributes to maintain the gastric mucosal integrity (Filaretova et al., 2002b). These date allowed us to conclude that there is some cooperative interaction between glucocorticoids and PGs in gastroprotection, in a way that a deficiency of one protective factor can lead to an apparently compensatory increase of the other. The gastric mucosa becomes more susceptible to injury during deficiency of both glucocorticoids and PGs (Filaretova et al., 2002b). This finding is important for clinical practice, especially for NSAID users. This further supports a warning that the increased risk of adverse gastric reactions should be considered when NSAIDs are used in patients with impaired glucocorticoid production.

It has been suggested that “PGs, NO, and sensory neuropeptides act in concert in the maintenance of mucosal viability” (Whittle et al., 1990). The suggestion was confirmed and reinforced by other investigations. Our data adds new information to such a “concerted” modulation of the gastric mucosal integrity and suggests that glucocorticoids are also important participants in this modulation. According to the data the ability of glucocorticoids protect the gastric mucosa seems especially important for the maintenance of gastric mucosa when the protective mechanism provided by PGs or NO or capsaicin-

sensitive afferent neurons is impared. We consider this fact as a striking manifestation of adaptive role of glucocorticoids.

2.3 Mechanisms of gastroprotective action of glucocorticoids: the maintenance of gastric mucosal integrity through the maintenance of general body homeostasis

There may be multiple targets for glucocorticoids to exert their beneficial influence on the gastric mucosa. We demonstrated that the gastroprotective action of glucocorticoids is provided by the maintenance of gastric mucosal blood flow, mucus secretion and repair processes as well as the attenuation of pathogenic elements such as the enhanced gastric motility (Filaretova et al., 1999, 2001b, 2002b,c, 2004). Anti-inflammatory properties of glucocorticoids may also contribute to their gastroprotective action. Glucocorticoids as anti-inflammatory hormones may contribute to gastroprotection by inhibition of neutrophil adherence (Wallace, 1997) and attenuation of microvascular permeability (Filaretova et al., 2002c).

Because the glucocorticoid receptors are expressed ubiquitously it is possible that glucocorticoids may act directly on local gastric targets as well as using a more general mechanism. The contribution of glucocorticoids to the maintenance of gastric mucosal integrity may be closely related with their contribution to general body homeostasis (Filaretova et al., 2006). General homeostasis's various links can be primary targets of glucocorticoid action and, therefore, the maintenance of general body homeostasis by glucocorticoids may be the base for their action on the maintenance of gastric mucosal integrity. The following facts support this statement.

Glucocorticoids participate in maintaining normal blood glucose level that is especially important for the brain. There is a close relationship between HPA axis activity and blood glucose regulation. Hypoglycemia is the major trigger that activates the HPA axis and leads to enhancement of glucocorticoid production (Erturk et al., 1998, as cited in Filaretova et al., 2006). Glucocorticoids increase hepatic gluconeogenesis, inhibit glucose uptake in adipocytes and fibroblasts, sensitize the liver to glucagon and epinephrine, decrease the hepatic sensitivity to insulin and, as a result, they increase blood glucose level (Chan et al., 2002). In turn, maintaining the normal blood glucose level is important for the maintenance of gastric mucosal integrity. Insufficient supply of glucose may stimulate hypothalamic glucose-sensitive neurons (Mobbs et al., 2001, as cited in Filaretova et al., 2002c), resulting in a vagally-mediated increase in gastric motility and secretion (Shiraishi, 1988, as cited in Filaretova et al., 2002c) that are well-known pathogenic elements in various gastric ulcerogenic models. Exogenous glucose reverses the hypoglycemia-induced stimulation of hypothalamic glucose-sensitive neurons (Oomura et al., 1974, as cited in Filaretova et al., 2002c), inhibits vagally-mediated gastric hypermotility (Barnett & Owyang, 1988, as cited in Filaretova et al., 2002c) and attenuates gastric ulceration (Takeuchi et al., 1990, as cited in Filaretova et al., 2002c). It was reasonable to assume that the maintenance of glucose homeostasis by glucocorticoid hormones could be fundamental to their beneficial actions on local gastric targets.

The data obtained from the model of indomethacin-induced ulceration demonstrates that the maintenance of glucose homeostasis by glucocorticoids is responsible for their beneficial actions on gastric motility. Although the mechanisms by which indomethacin induces gastric injury involves multiple, closely interacting elements such as depletion of PGs, gastric hypermotility, microcirculatory disturbances, neutrophil-endothelial cell interactions

and superoxide radicals (Takeuchi et al., 1991; Wallace, 1997), gastric hypermotility may be a key element in the pathogenesis of indomethacin-induced gastric damage (Takeuchi et al., 1989). The glycoprivic response is involved in the mechanism of gastric hypermotility induced by NSAIDs (Mersereau & Hinchey, 1982, as cited in Filaretova et al., 2002c). To understand the mechanisms underlying glucocorticoids' gastroprotective actions against indomethacin-induced injury, we investigated the effect of adrenalectomy, with or without corticosterone replacement, on gastric motility and blood glucose level 4 hours after administration of indomethacin at the ulcerogenic dose. We confirmed (Filaretova et al., 2002c) that indomethacin significantly enhanced gastric motility in sham-operated rats, and this hypermotility response was significantly aggravated in adrenalectomized rats, in parallel with an increase in gastric lesion score (Takeuchi et al., 1989). These results support a causal relationship between gastric hypermotility and lesion formation following administration of indomethacin. Blood glucose levels were low in adrenalectomized rats and decreased further after administration of indomethacin. This suggests a relation between low blood glucose levels, enhanced gastric motility and ulcerogenic responses to indomethacin. Indeed, adrenalectomized rats (with deficiency of corticosterone) given indomethacin showed minimum blood glucose levels, maximum gastric motility index values and maximum gastric lesion score when compared to sham-operated indomethacin-treated group (Filaretova et al., 2002c). A single injection of corticosterone to adrenalectomized animals, at the dose imitating the indomethacin-induced rise in corticosterone, restored blood glucose levels and significantly reduced gastric hypermotility and ulcerogenic responses to indomethacin, whereas these beneficial effects of corticosterone was attenuated by a glucocorticoid receptor antagonist RU-38486 (Filaretova et al., 2002a,c). The findings suggest that there is close relationship between the gastroprotective action of glucocorticoids and their attenuation of gastric hypermotility through maintaining blood glucose level.

The contribution of glucocorticoids to general body homeostasis involves their beneficial influences on cardiovascular system. The major actions of glucocorticoids on cardiovascular system are to enhance vascular reactivity to other vasoactive substances and to maintain systemic blood pressure (Darlington et al., 1989, as cited in Filaretova et al., 2006; Grunfeld & Eloy, 1987). Glucocorticoid deficiency is associated with reduced response to vasoconstrictors such as norepinephrine and angiotensin II. The latter stage of glucocorticoid deficiency is associated with cardiovascular collapse and heart failure in mammals after various stressors (Cleghorn, 1983, as cited in Filaretova et al., 2006). Glucocorticoid replacement is crucial in the treatment of patients with adrenal crisis, including the patients with Addison's disease (Darlington et al., 1989, as cited in Filaretova et al., 2006). On the contrary, glucocorticoid excess induces hypertension in human and rats. Hypertension is seen in patients with excessive glucocorticoid secretion, occurring in most patients with Cushing's syndrome and in patients with glucocorticoid treatment (Nieman et al., 1985, as cited in Filaretova et al., 2006). It was reported that a patient with Cushing's syndrome was treated successfully with glucocorticoid receptor antagonist RU-38486 at high dose (Nieman et al., 1985, as cited in Filaretova et al., 2006). Experimental data obtained in adrenalectomized animals with or without glucocorticoid replacement (Darlington et al., 1989; Darlington & Tehrani, 1997, as cited in Filaretova et al., 2006) as well as in rats with occupation of glucocorticoids receptors by RU-38486 (Grunfeld & Eloy, 1987, as cited in Filaretova et al., 2006) confirm and further develop clinical observations about the important contribution of glucocorticoid hormones to the regulation of blood pressure.

Under certain conditions, maintaining blood pressure is especially important for the maintenance of gastric mucosal integrity. There is evidence showing the linear correlation between the graded systemic hypotension and the mucosal blood flow as an important defensive factor (Guth, 1992). It was hypothesized that the blood flow to the stomach, a nonessential organ, decreases more rapidly and at an early stage of graded hypotension, in order to maintain blood flow to the essential organs such as the brain and kidney. The decrease in submucosal and mucosal blood flow during stress is an important factor, leading to mucosal ischemia, impairment in tissue resistance, and subsequent ulceration in stressed animals (Guth, 1992; Tarnasky et al., 1990). These facts allow us to assume that maintaining systemic blood pressure by glucocorticoids may be fundamental to their beneficial action on gastric blood flow and, consequently, on gastric mucosal integrity.

Utilizing an in vivo microscopy technique for the direct visualization of the gastric microcirculation (Filaretova et al., 1999) as well as methods creating the alterations in glucocorticoid supply, we examined whether gastric microcirculation and arterial blood pressure are involved in the mechanism of gastroprotective action of glucocorticoids during 3 hour water immersion-restraint stress. To this end the effects of deficiency of glucocorticoid production followed by corticosterone replacement on the stress-induced gastric microcirculation, systemic arterial pressure and gastric erosion were investigated. The stress-induced glucocorticoid production was inhibited by a single high dose of cortisol injected one week before stress. Gastric microcirculation was evaluated by measurement of the blood flow velocity in submucosal and mucosal microvessels (Filaretova et al., 1999).

Water immersion-restraint stress caused decrease in blood flow velocity in submucosal and mucosal gastric microvessels. The deficiency of glucocorticoids during water immersion-restraint stress promoted the stress-induced decrease of blood flow velocity in submucosal and mucosal microvessels, and corticosterone replacement prevented this effect (Filaretova et al. 1999, 2004). The results suggest that glucocorticoids released during water immersion-restraint stress maintain gastric blood flow during the stress. Our data also confirms that the decrease in gastric blood flow is associated with the reduction in systemic blood pressure. Mean systemic blood pressure in stressed rats with glucocorticoid deficiency was about 60 mm Hg, and these animals had a very low gastric blood flow velocity and large erosion area. Corticosterone replacement increased both systemic blood pressure and gastric blood flow and, as a result, improved the resistance of gastric mucosa to ulcerogenic stress action. It means that the improvement of gastric blood supply by glucocorticoids is provided, at least partly, through their beneficial action on systemic blood circulation. The data suggests that the gastroprotective actions of glucocorticoids during water immersion-restraint stress may be provided by the maintenance of gastric blood flow that may be brought about by their beneficial effect on arterial blood pressure (Filaretova et al. 1999, 2004).

Thus, glucocorticoids released during activation of the HPA axis may contribute to protection of the gastric mucosa by maintaining general body homeostasis, including glucose levels and systemic blood pressure, which could be a basis for their beneficial influence on gastric mucosal integrity.

2.4 How gastroprotective action of glucocorticoids may be transformed to proulcerogenic one

Thus, in general glucocorticoid hormones may have dual action on the stomach: physiological gastroprotective and pathological proulcerogenic one. In al physiologic

conditions, even in acute stress situations, glucocorticoids have an adaptive effect on the stomach and, therefore, are gastroprotective, while in some situations their action on the gastric mucosa may become proulcerogenic. It is important to understand how physiological gastroprotective action can be transformed to pathological proulcerogenic effect.

Because the maintenance of glucose homeostasis by glucocorticoids could be fundamental to their gastroprotective action (Filaretova et al., 2002c, 2006), it was reasonable to assume that glucocorticoid-induced disturbance of glucose regulation, observed in clinical and experimental situations (Subramanian & Trence, 2007, as cited in Filaretova et al., 2009a), may contribute to ulcerogenic action of glucocorticoids on the gastric mucosa. We supposed that short-term maintenance of blood glucose level provides the gastroprotective action of glucocorticoids, while long-lasting maintenance of blood glucose level or long-lasting hyperglycemia through a disturbance of carbohydrate regulation may account, at least partly, for the ulcerogenic action of glucocorticoids. Thus, we hypothesized that glucocorticoid-induced long-lasting maintenance of blood glucose level accompanied by their catabolic effects may be responsible for the transformation of gastroprotective action of glucocorticoids to their proulcerogenic effect.

We verified the hypothesis investigating the effects of exogenous glucocorticoid and dexamethasone was selected for this aim as synthethic long-acting glucocorticoid. Stress and indomethacin were used as ulcerogenic stimuli because both of them are considered as most significant ulcerogenic factors in human (Laine et al., 2008). It is important to note that both stimuli were applied to rats after 24 hour fasting. Taking into consideration that action of exogenous glucocorticoids on the gastric mucosa is depended on the dose (Laine et al., 2008) first, we investigated dose-dependent effects of dexamethasone. Surprisingly, dexamethasone, even at pharmacological dose 10 mg/kg protected the gastric mucosa against stress- and indomethacin-induced injury, at least, during first hour of its action (Filaretova et al., 2009a,b).

Because dexamethasone at the dose of 1 mg/kg decreased the gastric erosion area and maintained blood glucose level in fasted stressed or indomethacin-pretreated rats (in the case of its injection 1 h before the onset of cold-restraint or indomethacin administration) this dose has been selected for the next step, time-dependent study. The results obtained demonstrate that single injection of dexamethasone at a dose of 1 mg/kg may attenuate or aggravate both cold-restraint- and indomethacin-induced gastric erosion depending on the duration of its action before the onset of the stress or indomethacin, respectively. Short-lasting (1-12 hours) action of dexamethasone attenuated cold-restraint- and indomethacin-induced gastric ulceration. However long-lasting (21-24 hours) dexamethasone action resulted in an aggravation of cold-restraint- and indomethacin-induced gastric erosion (Filaretova et al., 2009a,b). The findings suggest that manifestation of gastroprotective or ulcerogenic action of glucocorticoids used at the same dose may be dependent very much on the time interval between the hormonal injection and onset of ulcerogenic stimulus. Prolongation of dexamethasone action may lead to enhancement of gastric mucosal susceptibility to ulcerogenic action of cold-restaint or indomethacin.

Both short- and long-lasting dexamethasone actions resulted in maintenance of blood glucose level in fasted stressed and indomethacin-treated rats. Dexamethasone-induced long-lasting maintenance of blood glucose level accompanied with the signs of catabolic effects. It should be note that dexamethasone-induced increase in the lost of body weight

during fasting preceded the appearance of its ulcerogenic action. Thymus weight was used as another marker of dexamethasone-induced catabolic effects. It is known that glucocorticoids at pharmacological doses tend to kill off many of the thymus cells. This phenomenon is the basis for the immunosuppressive use of glucocorticoids (Young et al., 1981, as cited in Filaretova et al., 2009a). It was shown that dexamethasone accelerates the rate of apoptosis in thymocytes (de Belle et al., 1994, as cited in Filaretova et al., 2009a). According to our data in distinguish from the body weight changes the thymus weight changes started earlier. These findings are in agreement with the data of literature showed that metabolic glucocorticoid effects in thymus cells evolve more rapidly comparing those in other target cells. The most prominent effect of glucocorticoid in thymus cells is a large inhibition of glucose transport that reaches 25-30% by about 30 min after the hormone addition. The metabolic inhibitions followed by cell destruction (Young et al., 1981, as cited in Filaretova et al., 2009a).

Dexamethasone treatment inhibited cold-restaint- and indomethacin-induced corticosterone production. Because according to our data deficiency of corticosterone aggravates cold-restaint- and indomethacin-induced gastric erosion, it is quite possible that simultaneous corticosterone deficiency and consequences of disturbances of carbohydrate regulation contributed together to proulcerogenic effect of long-lasting dexamethasone treatment.

We prolonged our study till the 7th day to clarify the questions how long dexamethasone effects may be continued and whether they are reversible. It was found that the dexamethasone-induced proulcerogenic action was continued till the 5th day and then, on the 7th day was disappeared. The restoration of stress- or indomethacin-induced corticosterone production, which preceded the restoration of normal susceptibility of the gastric mucosa to ulcerogenic action of cold-restraint, may contribute to this event. The gradual restoration of normal body and spleen weight is a good symptom of reversibility dexamethasone-induced catabolic effects. Disappearance of dexamethasone-induced maintenance of blood glucose level preceded the restoration of normal body and spleen weight.

In our experimental situations the transformation of gastroprotective action of dexamethasone to proulcerogenic one occurred 18 h after its administration, but it is clear that in general this time interval depends on many factors, including a kind of glucocorticoid and its dose, a specificity of situation. As far back as 1950 it was noted on the base of clinical observations that it needs at least 5-7 days of corticosteroids use before appearance of ulcer symptoms (Sandweiss, 1954, as cited in Filaretova et al., 2009a). One of the principles for minimizing undesirable side effects of glucocorticoid therapy is “keep treatment as short as possible, since treatment lasting 5 to 7 days shows fewer side effects” (Longui, 2007, as cited in Filaretova et al., 2009a). It is more often glucocorticoid-induced ulcer symptoms appeared after much more long hormonal treatment. Our results allow us to speculate that glucocorticoid-induced disturbance of carbohydrate regulation, which needs time for developing, contributes to appearance of ulcer symptoms after long-lasting hormonal therapy. It means that control of glucose regulation and its correction in case of need may be considered as useful approach minimizing ulcerogenic side effect of glucocorticoid therapy.

In conclusion, the data obtained so far suggest that short-lasting maintenance of blood glucose levels may be responsible for the gastroprotective action of glucocorticoids, while

glucocorticoid-induced long-lasting maintenance of blood glucose levels accompanied with the signs of their catabolic effect and glucocorticoid-induced corticosterone deficiency may be responsible, at least partly, for the transformation of gastroprotective action of glucocorticoids to their proulcerogenic effect (Filaretova et al. 2009a, 2009b). Further investigation of detailed mechanisms underlying proulcerogenic glucocorticoid action is the task of our future studies. We take into consideration other, additional, possibilities for explanation the question how physiological gastroprotective action can be transformed to pathological proulcerogenic effect.

3. Conclusion

According to our data an acute stress-induced increase of glucocorticoids has a gastroprotective action against stress-induced gastric injury but is not ulcerogenic, as it has generally been considered for some decades. Beneficial action of high levels of endogenous glucocorticoids released during acute stress on the stomach is opposite to the harmful actions of exogenous glucocorticoids at pharmacological doses used as a hormonal therapy. NSAIDs as well as other ulcerogenic stimuli, similar to stress, induce an increase in glucocorticoid production that in turn helps the gastric mucosa to resist the harmful actions of these stimuli. It is assumed that the adaptive action of glucocorticoids released during acute activation of the HPA axis may be applied to the gastric mucosa. Glucocorticoids exert gastroprotective actions in co-operation with PGs, NO and capsaicin-sensitive sensory neurons: their compensatory gastroprotective action is observed when the protective mechanism provided by either of these factors is impared. Gastroprotective effects of glucocorticoids may be mediated by multiple actions, including maintenance of gastric mucosal blood flow, mucus production, and attenuation of enhanced gastric motility and microvascular permeability. The contribution of glucocorticoids to gastroprotection is tightly related with their contribution to general body homeostasis. Glucocorticoids released during activation of the HPA axis may contribute to protection of the gastric mucosa by maintaining general body homeostasis, including glucose levels and systemic blood pressure, which could be a basis for their beneficial influence on gastric mucosal integrity. These findings further support idea that gastroprotective action of glucocorticoids is an essential element of their general adaptive action. In conclusion, the results obtained in our studies suggest that glucocorticoids released during acute activation of the HPA axis are naturally occurring protective factors that play an important role in maintenance of the gastric mucosal integrity. In turn, it means that acute activation of the HPA axis is a gastroprotective component of stress response.

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12

Helicobacter pylori Suppresses Serum Immunoglobulin Levels in Smokers with Peptic Ulcer: Probable Interaction Between Smoking and *H. pylori* Infection in the Induction of Th1 Predominant Immune Response and Peptic Ulceration

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1. Introduction

1.1 T helper cell subset

Helicobacter pylori (*H. pylori*) and smoking are well known risk factors for gastric ulcer, and both are classified as definite carcinogens. Interestingly, the two also reportedly share common immune response features.

With recent advances in immunology, various functions of T lymphocytes (T cells) have been discovered. T cells are divided into suppressor and helper on the basis of immunological functionings, and T helper cells are now known to consist of two distinct groups, as demonstrated using mouse models in the 1980s [Reiner, 2008; Mosman & Coffman, 1989]. Both groups are derived from naïve T cells: interleukin-12 (IL-12) causes naïve T helper cells to differentiate into type 1 (Th1) cells, while augmentation of IL-4 around naïve T cells leads to Th2 differentiation. Th1 cells produce IL-2 and interferon- γ (IFN-γ) to maintain cell mediated immunity against intracellular organisms such as viruses and mycobacteria, and Th2 cells produce IL-4 and IL-13 promoting the differentiation of B cells to plasma cells and the induction of class-switching resulting in IgE production. Differentiated plasma cells produce immunoglobulins which participate in mucosal defense against extracellular organisms including *H. pylori*. Groundbreaking research inspired subsequent studies which finally led to the discovery of Th17 cells [Steinman, 2007] and T regulatory cells (Treg) [Sakaguchi *et al.,* 1995, 2008]. As a consequence of this pioneering research, T helper cells, at present, are sub-grouped into 4 types according to the differences in their cytokine productions (Table 1).

Table 1. T helper subsets

Inductive, Selective T helper subset	Cytokines	Secreted Cytokines
Th1*	IL-12, IFN- γ	IL-2, TNF, IFN- γ
Th2**	L-4, IL-33	IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-25
Th17	TGF-β, IL-6, IL-23	IL-17, IL-6, IL-22, TNF
Treg	TGF- β, IL-10	TGF- β IL-10, CTLA4

Table 1a. Subsets of T helper cells and related cytokines

T helper subset	Protection	Pathology
Th1*	Defense against intracellular organisms	Inflammation
Th2**	Defense against extracellular bacteria at mucosal and epithelial surface	Allergy
Th17	Defense against extracellular bacteria	Autoimmunity Cancer
Treg	Suppression of immune response	Anti-inflammation

Concept of Th1 and Th2 cells originate from the cytokine production pattern of murine T cells. Therefore attention such differences is necessary when considering human immunity.
*Exert cell mediated immunity
**Exert humoral immunity
CTLA4: Cytotoxic T lymphocyte antigen 4
IFN: Interferon
IL: Interleukin
TGF: Tumor growth factor
TNF: Tumor necrosis factor
Table 1: Table by Reiner modified by authors (ref. 1 p412)

Table 1b. Roles of each T helper subset

Th1 cells produce IL-2, IFN- γ, tumor growth factor-β(TGF- β) and so on, thereby exerting cell mediated immunity mainly through IFN- γ.
Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and so on, thereby up-regulating humoral immunity against extracellular pathogens and inducing allergy mainly through IL-4.
Th17 cells produce IL-17A, IL-17F, IL-22 and so on, thereby eradicating bacterial/fungal infections and might be related to autoimmunity and cancer. Th17 cells produce inflammatory cytokines, and over-expression of such cytokines is associated with autoimmune diseases such as type 1 diabetes, inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis. IL-6 and TGF- β are key cytokines for differentiation into Th17.
Treg produces TGF- β, IL-10, and cytotoxic T lymphocyte antigen 4 (CTLA4), and suppress activated T cells/dendritic cells. Treg can suppress Th1, Th2, and Th17 to terminate immune responses/inflammation and also plays crucial roles in immune tolerance [Sakaguchi *et al.*, 1995, 2008].

As stated above, Th1 cells can down-regulate immunoglobulin production through secretion/production of IFN-γ whereas Th2 cells IL-4-dependently up-regulate immunmoglobulin secretion/production.

1.2 Th1 response in patients with peptic ulcer

As to gastric and duodenal ulcers, *H. pylori* infection has increasingly been reported to exert a Th response on type 1 (Th1 cells) [Hida *et al.,* 1999; Holck *et al.;* 2003; D'Elios *et al.,* 1997, 2003,2005; Itoh *et al.,* 2005; Goll *et al.,* 2007; Ayada *et al.,* 2009; Mohammadi *et al.,* 1996; Fan *et al.,* 1998; Bamford *et al.,* 1998; sommer *et al.,* 1998; Lindholm *et al.,* 1998; Ihan *et al.,* 2000; Smythies *et al.,* 2000; Akhiani *et al.,* 2002; Guiney *et al.,* 2003; Amedei *et al.,* 2006; Taylor *et al,* 2006], and peptic ulcer disease has also been increasingly reported to produce Th1 skew [Hida *et al.,* 1999; D'Elios *et al.,* 2007; Goll *et al.,* 2008; Codolo *et al.,* 2008; Del Prete *et al.,* 2008; Shimada *et al.,* 2008; Watanabe *et al.,* 2010; Hosseini *et al.,* 2010]. In addition, a unique study conducted by Itoh *et al.* suggested Th1 polarization of gastric T cells in the antrum of dyspeptic pateints, irrespective of *H. pylori* infection [Itoh *et al,* 1999].

1.3 Th1 response in patients with *H. pylori* infection

H. pylori has increasingly been reported to show Th1 predominance [Mohammadi *et al.,* 1996; D'Elios *et al.,* 1997, 2003,2005; Bamford *et al.,* 1998; Fan *et al.,* 1998; Lindholm et al., 1998; Sommer *et al.,* 1998; Hida *et al.,* 1999; Ihan et al., 2000; Smythies et al., 2000; Akhiani et al., 2002; Guiney *et al.,* 2003; Holck *et al.;* 2003; Itoh *et al.,* 2005; Amedei *et al.,* 2006; Taylor *et al,* 2006; Goll *et al.,* 2007; Ayada *et al.,* 2009] with only a few studies obtaining opposing results [Bergman *et al.,* 2004; Campbell *et al.,* 2004; Kayhan *et al.,* 2008; Kido *et al.,* 2010]. Therefore, *H. pylori* is presumed to down-regulate immunoglobulin production/secretion.

1.4 Influence of smoking on serum immunoglobulins and Th response

Smoking has been reported to suppress serum immunoglobulin levels [Andersen *et al.,* 1982; Tollerud *et al.,* 1995; Barbour *et al.,* 1997; Gonzalez-Quintela *et al.,* 2007] and some studies have indicated a Th1 skew in smokers [Hallquist *et al.,* 2000; Whetzel *et al.,* 2007; Kikuchi *et al.,* 2008], although controversial data have also been reported [Hagiwara *et al.,* 2001; Zeidel *et al.,* 2002; Cozen *et al.,* 2004].

1.5 Possible mechanisms by which *H. pylori* infection induces Th1 skew

Although a Th1 skew in *H. pylori*-infected patients is suggested by the vast majority of research conducted on this subject, the precise mechanism by which Th1 differentiation is induced has yet to be elucidated. However, some investigators have conducted crucial studies that may explain this phenomenon: Eaton *et al.* reported CD4+ T cells to be essential for the development of *H. pylori*-induced gastritis [Eaton *et al.,* 2001], and Nagai *et al.* showed the coccoid form of *H. pylori* to reach to Peyer's patches and then be phagocytosed by dendritic cells thereby sensitizing CD4+ T cells, and that these sensitized CD4+ T cells homed to the lamina propria of the gastric mucosa [Nagai *et al.,* 2007]. Finally, such dendritic cells produce IL-12 which promotes Th1 differentiation after phagocytosis of *H. pylori* [Codolo *et al.,* 2008].

In addition to mentioned above, a number of investigators have demonstrated a Th1 skew in patients with peptic ulcers, as compared to those with gastritis or gastric cancer [Hida *et al.,* 1999; D'Elios *et al.,* 2007; Goll *et al.,* 2008; Codolo *et al.,* 2008; Del Prete *et al.,* 2008; Shimada *et al.,* 2008; Hosseini *et al.,* 2010; Watanabe *et al.,* 2010].

We therefore conducted the current study to assess the influence of both *H. pylori* and smoking on serum immunoglobulin levels for the purpose of evaluating the presence of Th1 skew in patients with peptic ulcers.

2. Patients and method

2.1 Study design

Study 1. Effects of current smoking on levels of serum immunoglobulins
To evaluate the influences of smoking on serum immunoglobulin levels, serum IgG, IgA, and IgM levels were measured in both peptic ulcer and non-ulcer gastritis patients with and without *H. pylori* infection.
Study 2. Effects of *H. pylori* infection on levels of serum immunoglobulins in peptic ulcer patients
To evaluate the influences of *H. pylori* infection on serum immunoglobulin levels, serum IgG, IgA, and IgM were measured in peptic ulcer patients, both current smokers and non-smokers.
Study 3. As a control for study 2, serum IgG, IgA, and IgM levels were measured in non-ulcer gastritis patients with and without current smoking.

2.2 Patients

Dyspeptic patients and those recommended to undergo fiberscopic examination received gastroduodenoscopic examinations. Those endoscopically diagnosed as having gastric or duodenal ulcers were included in the current study. Following informed consent to check *H. pylori* status and immunohematologic parameters, dyspeptic patients underwent gastrofiberscopic examination. Patients with hematologic, immunologic, rheumatic, malignant, and infectious diseases were excluded. Those taking corticosteroids, antibiotics, and/or immunosuppressive drugs were also excluded. Because non-steroidal anti-inflammatory drugs (NSAIDs) [Franch *et al,* 1994; Mazzeo *et al.,* 1998; Yamaki *et al.,* 2003, 2005; Andreone *et al.,* 2004; Mored *et al.,* 2004] and proton-pump inhibitors (PPIs) [Tsutsumi *et al.,* 2005; Matsukawa *et al.,* 2007] have increasingly been reported to skew the T helper response toward type 2, patients taking these drugs were also excluded. Both smokers and non-smokers with endoscopically diagnosed non-ulcer gastritis were also evaluated as control groups.

2.3 Methods

Following informed consents to measure titers of serum anti-*H. pylori* IgG antibody, serum immunoglobulins and complete blood cell counts, patients with gastric or duodenal ulcer was diagnosed according to the classification of Sakita and Miwa [Matsukawa *et al.,* 1997], and those with non-ulcer gastritis did according to the updated Sydney System [Dixon *et al.,* 1996] under gastrofiberscopic observation. To evaluate *H. pylori* status, biopsy specimens were obtained from the antrum and lower body of the greater curvature in the stomach and from the major lesions. The samples from the antrum and lower body were placed in rapid urease test (RUT) kits, and the results were evaluated 24 hr later. These samples were also prepared for pathologic evaluation. Immediately after completion of the procedure, blood samples were collected to measure IgG, IgA, IgM, and anti-*H. pylori* IgG antibodies. Serum levels of IgG, IgA, and IgM were measured by an automated turbidimetric immunoprecipitation method [Matsukawa *et al., 1997*], and the anti-*H. pylori* antibody was measured by a commercially available ELISA kit. Confirmed *H. pylori* infection required

both RUT and anti-*H. pylori* IgG antibody to be positive. Smoking status was ascertained on the day of the endoscopic examination.

2.3.1 Statistical analysis

Data were expressed as means+/-SD. The statistical significance of differences was anlyzed employing the Student unpaired *t-test* and the χ-square test. We evaluated statistical differences using Macintosh StatView version 4, and p values less than 0.05 were accepted as statistically significant.

3. Results

3.1 Recruited patients and controls

Table 2. Profiles of patients and controlls

Sex	Female	Male
Number	90	146
Age (years)**	60.0+/-11.5	53.4+/-13.6
*H. pylori**	66 (73.3%)	127 (87.0%)
Smokers*	24 (37.5%)	82 (56.2%)

Table 2a. Profiles of patients with peptic ulcer

Sex	Female	Male
Number	408	312
Age (years)	56.6+/-14.3	55.8+/-13.3
*H. pylori**	229 (56.1%)	192 (61.1%)
Smokers*	44 (10.8%)	106 (34.0%)

Smokers: peptic ulcer>non-ulcer gastritis (female.=0001 and male <.0001)
H. pylori prevalence: peptic ulcer>non-ulcer gastritis (<.0001 for females and males)
*P<.0001 **P=.0001

Table 2b. Profiles of patients with non-ulcer gastritis

There were 146 patients with gastric ulcer, 58 with duodenal ulcer, and 32 with both types (Table 3). There were no differences in these lesions between smokers and non-smokers.

Smokers (F:M)			
Body	Angle/Antrum	Duodenum	Multiple
33 (7:26)	30(10:20)	28 (2:26)	15 (5:10)
Non-smokers (F:M)			
Body	Angle/Antrum	Duodenum	Multiple
51 (32:19)	32(15:17)	30 (15:15)	17 (5:12)
Total			
Body	Angle/Antrum	Duodenum	Multiple
84 (39:45)	62(25:37)	58 (17:41)	32 (10:22)

Table 3. Ulcer location (F:M)

Table 2 presents the profiles of both patients with peptic ulcer and those with non-ulcer gastritis serving as controls. In total, 236 patients (F:M=90:146) were diagnosed as having gastric and/or duodenal ulcers and were enrolled in this study. There was an age difference

between female and male patients (F:M=60.0+/-11.5 vs. 53.4+/-13.6 years, p=.0001) (Table 2a). Patients with non-ulcer gastritis consisted of 408 females and 312 males, and there was no difference in age between genders (56.6+/-14.3 vs. 55.8+/-13.3 years) (Table 2b).
Patients with peptic ulcer had higher prevalences of both *H. pylori* infection and smoking, as compared to those with non-ulcer gastritis: p=.0001 for smoking in females and <.0001 for smoking in males, while p<.0001 for *H. pylori* infection in both females and males (Tables 2a and 2b).

3.2 Serum levels of IgG, IgA, and IgM in the current study

3.2.1 The results of study 1

Tables 4 and 5 show the results of study 1, examining the effects of current smoking on serum immunoglobulin levels in patients with petic ulcer and non-ulcer gastritis. There was age difference between smokers and non-smokers with *H. pylori* infection among ulcer patients (p=.0019). Smoking was associated with definite suppressions of serum IgG, IgA, and IgM levels in *H. pylori*-infected patients with peptic ulcer (p<.0001, .0006, and .0009, respectively), whereas ulcer patients without *H. pylori* infection showed no such tendency. Table 5 presents the effects of current smoking on serum immunoglobulin levels in non-ulcer patients with gastritis. There was an age difference between smokers and non-smokers (p<.0001). Among patients with non-ulcer gastritis, smokers had suppressed serum IgG (p<.0001), IgA (p<.05), and IgM levels, although the reduction of IgM in patients with *H. pylori* infection failed to reach statistical significance. Like those with *H. pylori* infection, non-ulcer patients without *H. pylori* infection also showed suppression of both IgG and IgM (p<.05, respectively).
Table 4. Influence of smoking on serum immunoglobulin levels in peptic ulcer patients

	Smokers	Non-smokers	P
N	92	101	
Age	52.2+/-12.2	58.0+/-13.2	.0019
IgG	1178.0+/-250.3	1376.6+/-343.2	<.0001
IgA	218.4+/-98.2	271.4+/-109.1	.0006
IgM	93.8+/-41.9	123.1+/71.1	.0009

Table 4a. Serum immunoglobulin levels in *H. pylori*-infected patients

	Smokers	Non-smokers	P
N	14	29	
Age	56.0+/-13.7	60.7+/-13.7	NS
IgG	1348.6+/-254.7	1463.7+/-286.3	NS
IgA	295.1+/-113.2	253.2+/-99.3	NS
IgM	105.8+/-49.8	123.5+/47.9	NS

IgG: Immunoglobulin G (mg/dl)
IgA: Immunoglobulin A (mg/dl)
IgM: Immunoglobulin M (mg/dl)
N: Number of patients
NS: Not significant
P: Probability

Table 4b. Serum immunoglobulin levels in patients without *H. pylori* infection

Table 5. Effects of smoking on serum immunoglobulin levels in non-ulcer gastritis patients

	Smokers	Non-smokers	P
N	93	325	
Age	53.5+/-11.6	60.0+/-11.4	<.0001
IgG	1224.1+/-264.3	1392.1+/-278.6	<.0001
IgA	236.6+/-97.0	264.0+/-112.7	.0384
IgM	104.7+/-58.6	112.4+/63.5	NS

Table 5a. Serum immunoglobulin levels in *H. pylori*-infected patients

	Smokers	Non-smokers	P
N	57	247	
Age	51.9+/-13.4	57.8+/-13.9	.0071
IgG	1205.5+/-278.6	1295.0+/-237.7	.0228
IgA	254.7+/-118.3	251.0+/-89.7	NS
IgM	83.4+/-42.9	104.8+/69.0	.0408

IgG: Immunoglobulin G (mg/dl)
IgA: Immunoglobulin A (mg/dl)
IgM: Immunoglobulin M (mg/dl)
N: Number of patients
NS: Not significant
P: Probability

Table 5b. Serum immunoglobulin levels in patients without *H.* pylori infection

3.2.2 The results of study 2

Table 6. Effects of *H. pylori* infection on serum immunoglobulin levels in peptic ulcer patients

H. pylori	Positive	Negative	P
N	92	14	
Age	52.6+/-12.2	56.0+/-13.7	NS
IgG	1177.9+/-250.3	1348.6+/-254.7	.0197
IgA	218.4+/-98.2	295.1+/-113.2	.0092
IgM	93.8+/-41.9	105.8+/-49.8	NS

Table 6a. Serum immunoglobulin levels in smokers

H. pylori	Positive	Negative	P
N	101	29	
Age	58.0+/-13.2	60.7+/-13.7	NS
IgG	1376.6+/-343.3	1463.7+/-286.3	NS
IgA	271.4+/-109.1	253.2+/-99.3	NS
IgM	123.1+/-71.1	123.5+/-47.9	NS

IgG: Immunoglobulin G (mg/dl)
IgA: Immunoglobulin A (mg/dl)
IgM: Immunoglobulin M (mg/dl)
N: Number of patients
NS: Not significant
P: Probability

Table 6b. Serum immunoglobulin levels in non-smokers

Table 6 presents the results of study 2, examining the effects of *H. pylori* infection on serum levels of immunoglobulins in peptic ulcer patients. As a whole, patients with peptic ulcer showed decreases in serum IgG, IgA, and IgM levels, although only the decrease in IgG reached statistical significance (data not shown). Among those with peptic ulcer, smokers with *H. pylori* infection showed decreases in both IgG and IgA (p<.0197 and .0092, respectively), whereas the difference in IgM did not reach statistical significance (Table 6a). In contrast to smokers, among patients with peptic ulcers, non-smokers with *H. pylori* infection showed no difference in IgG, IgA, or IgM levels.

3.2.3 The results of study 3

Table 7 presents the results of study 3, the control for study 2, examining the effects of *H. pylori* infection on serum levels of immunoglobulins in non-ulcer gastritis patients. As to the effect of *H. pylori* infection, patients with non-ulcer gastritis showed a phenomenon opposite to that in peptic ulcer patients, except for IgA in smokers. Patients with *H. pylori* infection had increased serum IgG, IgA, and IgM levels regardless of smoking status, although only the IgG difference in non-smokers (p<.0001) and the IgM difference in smokers (p=.0288) were statistically significant. Compared to patients with peptic ulcer, *H. pylori* infection, at minimum, did not suppress serum immunoglobulin levels regatdless of smoking status. *H. pylori* infection appeared to up-regulate serum immunoglobulin levels in non-ulcer patients with gastritis.

Table 7. Effect of *H. pylori* infection on serum immunoglobulin levels in patients with non-ulcer gastritis

H. pylori	Positive	Negative	P
N	93	57	
IgG	1234.5+/-264.3	1205.5+/-278.6	NS
IgA	236.6+/-97.0	254.7+/-111.8	NS
IgM	104.7+/-58.6	83.4+/42.9	.0288

Table 7a. Serum immunoglobulin levels in smokers

H. pylori	Positive	Negative	P
N	325	247	
IgG	1392.1+/-288.7	1295.0+/-237.7	<.0001
IgA	264.0+/-112.7	251.0+/-89.7	NS
IgM	112.4+/-63.5	104.8+/69.0	NS

IgG: Immunoglobulin G (mg/dl)
IgA: Immunoglobulin A (mg/dl)
IgM: Immunoglobulin M (mg/dl)
N: Number of patients
NS: Not significant
P: Probability

Table 7b. Serum immunoglobulin levels in non-smokers

4. Discussion

We initially showed definite suppression of serum immunoglobulin levels in current smokers with *H. pylori*-associated peptic ulcer (Tables 4a), and this suppression was observed even in patients without *H. pylori* infection, although the difference did not reach statistical significance possibly due to our small sample size (Table 4b). In contrast to patients with peptic ulcer, those with non-ulcer gastritis showed suppressed levels of serum immunoglobulins, regardless of *H. pylori* status. These observations support the notion that smoking causes a skewed Th1 response in current smokers, regardless of whether or not *H. pylori* infection or peptic ulceration is present. As to the Th skew in smokers, there are conflicting reports, with some reporting a Th2 skew in smokers [Hagiwara *et al.,* 2001; Zeidel *et al.,* 2002; Cozen *et al.,* 2004]. However, two noteworthy studies conducted recently have challenged this concept. Whetzel *et al*. reported elevated peripheral IFN-γ levels, especially in female smokers, and in surgically resected specimens from the colon of smokers [Whetzel *et al.,* 2007], and Kikuchi *et al*. showed that nicotine exerted a Th1-dominant effect via nicotinic acetylcholine receptors in the intestine [Kikuchi *et al.,* 2008].

As stated in the introduction, *H. pylori* infection is known to skew T helper differentiation toward type 1 (Th1) properties (production of IL-2, IFN- γ, and TNF)- thereby counteracting Th2-dependent processes. Th1 differentiation may reduce humoral immunity by down-regulating immunoglobulin production resulting in suppressions of serum IgG, IgA, and IgM levels. *H. pylori*, therefore, is presumed to down-regulate serum immunoglobulin levels in infected individuals. On the contrary, extracellular bacterial infections usually up-regulate IgM initially, and then IgG. Because *H. pylori* extracellularly colonizes the gastric mucosa, it should induce a Th2 response because such ubiquitous bacterium would be expected to colonize the mucosa (Table 1b). In accordance with this theory, Mohammadi *et al.* reported the presence of a Th2 response to effectively reduce the bacterial load in a mouse model of *H. pylori* infection: Th1 cells enhance gastritis and Th2 cells reduce bacterial load [Mohammadi *et al.,* 1997]. The current data from the control group in study 3 are also in accordance with this theory, i.e., *H. pylori* infection raises levels of serum immunoglobulins in both smokers (IgM) and non-smokers (IgG) with non-ulcer gastritis. This differs from the situation in patients with peptic ulcer, in whom *H. pylori* infection did not suppress serum immunoglobulin levels, of non-ulcer patients suggesting the unique phenomenon of Th1 skew seen only in patients with peptic ulcer (Table 7). Taking our current observations together, suppression, i.e., a lack of upregulation of serum immunoglobulins appears to be a unique feature of smokers with both peptic ulcer and *H. pylori* infection. Th1 skew observed in *H. pylori*-infected patients with peptic ulcer appeared to exceed the expected Th2 skew in patients infected with extracellular bacteria such as *H. pylori* itself, especially in smokers. In addition, vast majority of gastric T cells may be already polarized to produce Th1 cytokine even in the absence of *H. pylori* infection [Itoh, et al., 1999]. We therefore stress that the Th1 skew induced by *H. pylori*, smoking, and the presence of peptic ulceration may synergistically exert a Th1 response which prevails over the expected Th2 skew, i.e., up-regulation of serum immunoglobulin levels induced by the presence of extracellular bacterial infection by *H. pylori* itself.

The Th1 skew observed in patients with *H. pylori* infection indicated a Th1-polarized response to be associated with mucosal damage that can induce peptic ulcer, while a mixed Th1 and IL-4-drived Th2 polarized response appeared to be associated with a low degree of gastric inflammation and reduced bacterial load resulting in the prevention of ulcer

formation [D'Elios *et al.,* 1997, 2003, 2005; Mohammadi *et al.,* 1997; Holck *et al*., 2003]. Th2 drive therefore may be preferable to hasten ulcer healing in such patients. However, mixed or dysregulated Th responses may trigger T cell-dependent B cell activation involved in the development of low grade B cell lymphoma associated with *H. pylori* [D'Elios *et al.,* 2003, 2005].

5. Conclusion

As shown herein, current smoking is consitently associated with suppressed serum immunoglobulin levels (study 1), and *H. pylori* infection definitely reduced these levels in smokers with peptic ulcer (study 2). Furthermore *H. pylori* infection up-regulated IgG, IgA, and IgM in the absence of peptic ulcer. Current smoking, *H. pylori* infection, and the presence of peptic ulceration may interact to suppress the levels of serum immunoglobulins as a result of a Th1 shift which overwhelms the Th2 shift expected with extracellular bacterial infection.

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13

Helicobacter pylori and Host Response

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1. Introduction

Helicobacter pylori, a pathogen infecting the gastric antrum of half of the adult population worldwide, is thought to be the major cause of acute and chronic gastroduodenal pathologies, including gastric and duodenal ulcer, gastric cancer and gastric B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) (Marshall et al., 1994; Parsonnet et al., 1991; Wotherspoon et al., 1991). Despite a vigorous humoral response against *H. pylori* antigens, most of infected subjects fails to eliminate the pathogen spontaneously. As in other infectious diseases, besides the virulence of the pathogen, both the natural and the specific immune responses of the host are crucial for determining the outcome of the infection. The immune system has evolved different defence mechanisms against pathogens. The first defensive line is provided by 'natural' immunity, including phagocytes, T cell receptor (TCR) γδ+ T cells, natural killer (NK) cells, mast cells, neutrophils and eosinophils, as well as complement components and pro-inflammatory cytokines, such as interferons (IFNs), interleukin (IL)-1, IL-6, IL-12, IL-18 and tumor necrosis factor (TNF)-α. The more specialized TCR αβ+ T lymphocytes provide the second defence wall. These cells account for the specific immunity, which results in specialized types of immune responses which allow vertebrates to recognize and clear (or at least control) infectious agents in different body compartments. Viruses growing within infected cells, are faced through the killing of their host cells by CD8+ cytotoxic T lymphocytes (CTL). Most of microbial components are endocytosed by antigen-presenting cells (APC), processed and presented preferentially to CD4+ T helper (Th) cells. Th cells co-operate with B cells for the production of antibodies which opsonize extracellular microbes and neutralize their exotoxins. This branch of the specific Th cell-mediated immune response is known as humoral immunity. Other microbes, however, survive within macrophages in spite of the unfavorable microenvironment and antigen-activated CD4+ Th cells are required to activate macrophages, whose reactive metabolites and TNF-α finally lead to the destruction of the pathogens. This branch of the specific Th cell-mediated response is known as cell-mediated immunity (CMI).

Most of successful immune responses involve both humoral and cell-mediated immunity. CD4+ Th cells can develop different polarized patterns of cytokine production, such as type-1 or Th1, type-2 or Th2, type-17 or Th17 (Mosmann et al., 1986; Del Prete et al., 1991; Korn et al., 2009).

Th1 cells produce IFN-γ, IL-2 and TNF-β, elicit macrophage activation and delayed-type hypersensitivity (DTH) reactions, whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which act as growth/differentiation factors for B cells, eosinophils and mast cells and inhibit several macrophage functions (Del Prete, 1998). A new subset of Th cells, named Th17 cells, producing IL-17 alone or in combination with IFN-γ, has been identified recently (Weaver et al., 2006). Th17 cells play a critical role in protection against microbial challenges, particularly extracellular bacteria and fungi (Bettelli et al., 2007).

However, most of T cells do not express a polarized cytokine profile; such T cells (coded as Th0) represent a heterogenous population of partially differentiated effector cells consisting of multiple subsets which secrete different combinations of both Th1 and Th2 cytokines. The cytokine response at effector level can remain mixed or further differentiate into the Th1 or the Th2 pathway under the influence of polarizing signals from the microenvironment. Human Th1 and Th2 cells e.g. also differ for their responsiveness to cytokines. Both Th1 and Th2 cells proliferate in response to IL-2, but Th2 are more responsive to IL-4 than Th1; on the other hand IFN-γ tend to inhibit the proliferative response of Th2 cells (Del Prete et al., 1993). Th1 and Th2 cells substantially differ for their cytolytic potential and mode of help for B-cell antibody synthesis. Th2 clones, usually devoid of cytolytic activity, induce IgM, IgG, IgA, and IgE synthesis by autologous B cells in the presence of the specific antigen, with a response which is proportional to the number of Th2 cells added to B cells. In contrast, Th1 clones, most of which are cytolytic, provide B-cell help for IgM, IgG, IgA (but not IgE) synthesis at low T-cell/B-cell ratios. At high T-cell/B-cell ratios there is a decline in B-cell help related to the Th1-mediated lytic activity against antigen-presenting autologous B-cells (Del Prete et al., 1991b). Th1 and Th2 cells exhibit different ability to activate cells of the monocyte-macrophage lineage. Th1, but not Th2, help monocytes to express tissue factor (TF) production and procoagulant activity (PCA). In this type of Th cell-monocyte cooperation, both cell-to-cell contact and Th1 cytokines (namely IFN-γ), are required for optimal TF synthesis and PCA, whereas Th2-derived IL-4, IL-10 and IL-13 are strongly inhibitory (Del Prete et al., 1995a).

The factors responsible for the Th cell polarization into a predominant Th profile have extensively been investigated. Current evidence suggests that Th1, Th2 and Th17 cells develop from the same Th-cell precursor under the influence of mechanisms associated with antigen presentation (Kamogawa et al., 1993; Korn et al. 2009). Both environmental and genetic factors influence the Th1 or Th2 differentiation mainly by determining the 'leader cytokine' in the microenvironment of the responding Th-cell. IL-4 is the most powerful stimulus for Th2 differentiation, whereas IL-12, IL-18 and IFNs favor Th1 development (D'Elios et al., 1999). A role has been demonstrated for the site of antigen presentation, the physical form of the immunogen, the type of adjuvant, and the dose of antigen (Constant et al., 1997). Several microbial products (particularly from intracellular bacteria) induce Th1-dominated responses because they stimulate IL-12 production. IFN-γ and IFN-α favor the Th1 development by enhancing IL-12 secretion by macrophages and maintaining the expression of functional IL-12 receptors on Th cells (Szabo *et al.*, 1995). IL-18 sustains the expression of IL-12Rβ, indicating that IL-12 and IL-18 synergize in inducing and maintaining Th1 development (Xu *et al.*, 1998). On the other hand, IL-11 and PGE2 would promote Th2 cell polarization (D'Elios *et al.*, 1999). Other microbial products and stimuli induce a preferential activation of Th17 responses (Codolo et al., 2008a; Korn et al., 2009).

2. Immune responses in *H. pylori* infection

2.1 Innate reponses in *H. pylori* infection

Most of *H. pylori*-infected patients are unable to clear the pathogen, leading to postulate that *H. pylori* might somehow hamper the host immune response. It has been shown that *H. pylori* may interfere with protective immunity by acting on professional APC through the release of its vacuolating cytotoxin (VacA), which impairs antigen processing and the subsequent priming of efficient immune response (Molinari et al., 1998). The failure of clearing *H. pylori* from the gastric environment almost invariably leads to chronic antral gastritis. Colonization of the stomach by *H. pylori* is consistently accompanied by inflammation of the gastric mucosa, which varies according to the host immune reaction against the pathogen. Once in the stomach, *H. pylori* first activates the natural immunity cellular compartment, represented by macrophages and neutrophils. IL-8 expression by gastric epithelium following contact with *H. pylori* plays a major role in the initial host response to the bacterium, since this chemokine acts as a strong chemotactic and activating factor for neutrophils, which in turn contribute to initiate and expand the inflammatory cascade (Crabtree et al., 1993). Furthermore, certain *H. pylori* components, such as HP-NAP, the lipopolysaccharide, VacA or the cytotoxin-associated protein CagA, as well as the urease or the heat shock proteins (HSP) are allowed to cross the damaged layer of gastric cells and to come in contact with macrophages. Activation of macrophages, mainly exerted by HP-NAP, results in the release of several cytokines, including IL-12, IL-1, IL-6, TNF-α, IFN-α and chemokines, such as IL-8. Moreover, also neutrophils are able to produce IL-12 and IL-23 in response to HP-NAP (Amedei et al, 2006). This is an important step of the natural history of *H. pylori* infection, because the local cytokine "milieu", particularly the IL-12 and IL-23 produced by cells of the natural immunity is crucial in driving the subsequent specific T-cell response into a more or less polarized Th1 pattern. Furthermore *H. pylori* may results in activation not only of TLR receptor (e.g. by HP-NAP) but also of the cytoplasmic nucleotide-binding oligomerization domain (NOD)1, member of the NOD-like receptors (NLR) family. In particular, *H. pylori* peptidoglycan, acting in concert with the bacterial type IV ''syringe'', encoded by the cag PAI, following the engagement of NOD1 in gastric epithelial cells, leads to the generation of protective Th1 responses (Kaparakis et al., 2007; Pritz et al., 2007; Watanabe et al., 2010).

2.2 Th response in *H. pylori* infection

The The pattern of cytokines produced by the immunological active cells recruited in the antral mucosa of *H. pylori* -infected patients with peptic ulcer were analyzed by RT-PCR. Antral biopsies from patients with ulcer showed IL-12, IFN-γ, and TNF-α but not IL-4, mRNA expression, whereas virtually no mRNA encoding for cytokines was found in the mucosa of *H. pylori* -negative controls (D'Elios *et al.*, 1997b). In the same biopsies, immunohistochemistry showed remarkable in vivo activation of IFN-γ, but not IL-4, producing T cells (D'Elios *et al.*, 1997c).

Several studies have examined the antigen specificity and the cytokines produced by the *H. pylori*-specific Th cells derived from the antral mucosa of *H. pylori* -infected patients. Gastric biopsies were pre-cultured in IL-2-conditioned medium in order to preferentially expand T cells activated in vivo, and T-cell blasts were cloned according to a high efficiency technique allowing the growth of virtually every single T cell (D'Elios *et al.*, 1997).

In *H. pylori* infected patients the proportion of *H. pylori* -reactive gastric T-cells in each patient was variable, ranging from 2 to 33% of CD4+ clones. The majority of gastric-derived T cells were specific for CagA or for HP-NAP, whereas a minority were specific for VacA, for urease, or for HSP (D'Elios *et al*., 1997b; Amedei *et al*., 2006). Among the *H. pylori* -reactive clones from low grade gastric B-cell lymphoma (MALToma), 25% were specific for urease, 4% for VacA, and 71% proliferated only to *H. pylori* lysate (D'Elios *et al*., 1999). These data suggest that in MALToma urease is an important target of the gastric T-cell response and that some other still undefined antigens of *H. pylori* may be relevant in driving Th and B-cell activation and proliferation.

In peptic ulcer patients, in vitro stimulation with the appropriate *H. pylori* antigens induced the great majority of *H. pylori*-reactive Th clones to produce IFN-γ but not IL-4 (expressing thus a polarized Th1 profile). Under the same experimental conditions, most of *H. pylori*-specific T-cell clones derived from uncomplicated chronic gastritis showed a Th0 phenotype, producing IL-4 and/or IL-5 together with IFN-γ, whereas only one third of *H. pylori*-specific gastric T cells was polarized towards the Th1 effectors (D'Elios et al., 1997d). Also in MALToma patients most of *H. pylori*-specific T cell clones derived from the gastric mucosa were able to produce both Th1 and Th2 cytokines (D'Elios et al., 1999).

Detailed analysis of the antigen-induced B-cell help exerted in vitro by *H. pylori*-reactive gastric T-cell clones provided new information on the mechanisms possibly associated with the onset of low-grade B-cell lymphoma of the gastric MALT, rare complication of chronic *H. pylori* infection. Functional analysis of *H. pylori*-specific Th clones derived from the gastric antrum of infected patients showed that *in vitro* stimulation with the appropriate *H. pylori* antigen resulted in the expression of their helper function for B-cell proliferation and Ig production (D'Elios et al., 1997b). This can provide convincing explanation for the intense B-cell activation in the lymphoid tissue associated with, or newly generated in, the antral mucosa during chronic *H. pylori* infection. Such a sustained *H. pylori* -induced T cell-dependent B-cell activation is responsible for the high levels of specific antibodies found in the serum of *H. pylori* -infected patients (Rathbone et al., 1986; Crabtree et al., 1995). In chronic gastritis patients either with or without ulcer, the helper function to B cells exerted by *H. pylori* antigen-stimulated gastric T-cell clones was negatively regulated by the concomitant cytolytic killing of B cells (D'Elios et al., 1997b). In contrast, gastric T-cell clones from MALToma patients were surprisingly unable to down-modulate their antigen-induced help for B cell proliferation (D'Elios et al., 1999). Indeed, none of the gastric *H. pylori*-specific T-cell clones from MALToma was able to express perforin-mediated cytotoxicity against autologous B cells. Moreover, most Th clones from uncomplicated chronic gastritis induced Fas-Fas ligand-mediated apoptosis in target cells, whereas only a minority of *H. pylori* -specific gastric clones from MALToma patients were able to induce apoptosis in target cells, including autologous B cells (D'Elios et al., 1999).

There are a number of postulated mechanisms whereby *H. pylori* can induce mucosal injury, and some are certainly related to many of the *H. pylori* pathogenic products described (Telford et al., 1994; Tomb et al., 1997). Indeed in many infectious (and non-infectious) diseases, the type of immune response elicited is important for protection, but, under certain circumstances, it may also contribute to the pathogenesis of disease. A number of studies from different research groups seem to agree on that Th1 polarization of immune response to *H. pylori* is associated with more severe disease (D'Elios et al., 1997b; Hauer et al., 1997; Bamford et al., 1998; Sommer et al., 1998). Preferential activation of Th1 cells and the

subsequent production of their cytokines, namely IFN-γ and TNF-α, in the absence of Th2 cytokines can potentiate gastrin secretion and pepsinogen release, as observed in vitro in animal models (Weigert et al., 1996). Data from our laboratory indicate that also in humans TNF-α and IFN-γ are able to dose-dependently stimulate pepsinogen release from isolated gastric chief cells. In this model, simultaneous addition of IL-4 was not synergic, rather inhibitory, on the IFN-γ-induced pepsinogen release by chief cells, whereas it had no effect on the pepsinogen release induced by TNF-α (D'Elios et al., 1998). Moreover Th1 cells are able to induce both tissue factor production by monocytes (Del Prete et al., 1995a) and the activation of coagulation cascade, followed by microvascular thrombosis and consequent alteration of epithelial cell integrity. A number of studies suggest that chronic inflammation (e.g. triggered by infectious agents like *H. pylori*) may be important in the pathogenesis of atherothrombosis (Elizalde et al., 1997; Danesh et al., 1997). Indirect support to the hypothesis that the Th1-type of gastric immune response against *H. pylori* contributes to the pathogenesis of peptic ulcer comes from the observation that in kidney graft recipients (undergoing strong immunosuppression) peptic ulcer and active inflammatory lesions were virtually absent, in spite of a higher prevalence of *H. pylori* colonization (Hruby et al., 1997). The results obtained so far clearly demonstrated that gastric T-cell response to *H. pylori* antigens characterized by a mixed Th1-Th2 cytokine profile is apparently associated with low rate of ulcer complication. The concept that Th2 cytokines, particularly IL-4 and IL-10, are important in balancing and quenching some immunopathological effects of polarized Th1 responses is supported by other clinical and experimental observations. Holding in mind the concept of Th1/Th2 balance, one may reconsider what clinicians know from a long time, that, during pregnancy, patients suffering of peptic ulcer significantly reduce their dyspeptic symptoms and tend to undergo remission for the time of pregnancy (Cappell et al., 2003). This might be an indirect effect of the preferential Th2 "switch" occurring in pregnancy, which makes the mother able to "tolerate" her offspring by inhibiting Th1 responses, which would otherwise promote "graft" (fetus) rejection.

In favor of a role for the immune system in influencing gastric acid secretion and the onset of peptic ulcer disease is the interesting observation in rats that immune cells of gastric mucosa, but not epithelial cells, expressed in vivo detectable mRNA for gastrin, muscarine and histamine receptors. Such information supports the hypothesis that the primary target of antiulcer drugs may primarily be the immune cells in the gastric environment (Mezey et al., 1992). Many studies performed in mice demonstrated that T-cell dependent immune response are needed for protection against *H. pylori* whereas antibody response is not strictly required for protective immunity (Ermak et al., 1998). However if the T-cell response induced against *H. pylori* is not appropriate it may even result in a damage for the host, as demonstrated by several reports also in animal models. Transferring T cells derived from *H. pylori* infected patients into SCID mice has proven to be effective in inducing gastric ulcer in those mice, thus demonstrating that host immunity is involved in the development of peptic ulcers (Yokota et al., 1999). In *H. felis* -infected mice, neutralization of IFN-γ significantly reduced the severity of gastritis, strongly supporting the concept that preferential activation of a Th1-type response, far from being protective, rather contributes to the development and maintenance of gastric immunopathology. The magnitude of *H. felis* -induced inflammation in IL-4-deficient mice was higher than in their wild-type counterparts. Moreover, infection with *H. felis* induced minimal inflammation in BALB/c mice, whose genetic background is prone to high IL-4 production in response to different antigens. The results of these studies

provide further evidence that a polarized Th1 response is associated with gastric inflammation and disease whereas, when a mixed Th1/Th2 response is raised, it is able to reduce the unbalanced proinflammatory Th1 response (Mohamadi et al., 1996). If the hypothesis that some local IL-4 production may result in protection from ulcer is correct, the so-called "African enigma" (i.e. discrepancy between high rate of *H. pylori* infection and low prevalence of peptic ulcer)(Holcombe et al., 1992) may be explained, at least in part, on the basis of the acquired cytokine background of African people living in endemic areas of helminth infection, which is known to elicit strong and persistent Th2-dominated responses. Theoretically, a Th2-oriented host immunological background would be a misfortune for efficient defence against mycobacteria, but would provide at the same time an advantage for developing milder responses to a pathogen like *H. pylori,* so widespread even in infancy. Thus, peptic ulcer may be regarded as the immunopathological outcome of a chronic inflammatory process induced by some *H. pylori* strains in subjects genetically and/or environmentally biased to develop strong Th1-polarized responses.

Although related to *H. pylori* infection, low-grade gastric MALT lymphoma is a very rare complication and represents a model to study the interplay between chronic infection, immune response and lymphomagenesis. This type of lymphoma represents the first described neoplasia susceptible to regression following antibiotic therapy resulting in *H. pylori* eradication (Wotherspoon et al., 1993). A prerequisite for lymphomagenesis is the development of secondary inflammatory MALT induced by chronic *H. pylori* challenge (Isaacson, 1994). The tumor cells of low-grade gastric MALT lymphoma are memory B cells still responsive to differentiation signals, such as CD40 costimulation and cytokines produced by antigen-stimulated T helper cells, and dependent for their growth on the stimulation by *H. pylori* -specific T cells (Hussel et al., 1996; Greiner et al., 1997). In early phases, this tumor is sensitive to withdrawal of *H. pylori* -induced T-cell help, providing an explanation for both the tumor tendency to remain localized to the primary site and its regression after *H. pylori* eradication with antibiotics (Wotherspoon et al., 1993; Bayerdoffer et al., 1995). The growth of neoplastic B cells may depend on evasion from T cell-mediated cytotoxicity. In this regard, gastric T cells from MALT lymphoma showed both defective perforin-mediated cytotoxicity and poor ability to induce Fas-Fas ligand-mediated apoptosis, thus providing a possible explanation for their enhanced helper activity on B-cell proliferation. Both defects were restricted to MALT lymphoma-infiltrating T cells, since specific T helper cells from peripheral blood of the same patients expressed the same degree of either cytolytic potential or pro-apoptotic activity as T cells from chronic gastritis patients (D'Elios et al., 1999). The reason why gastric T cells of MALT lymphoma, while delivering full help to B cells, are apparently deficient in mechanisms involved in the concomitant control of B-cell growth, remains unclear. It has been shown that VacA toxin inhibits antigen processing in APC, but not the exocytosis of perforin-containing granules of NK cells (Molinari et al., 1998). It is possible that, in some *H. pylori* -infected individuals, other bacterial components affect the development or the expression in gastric T cells of regulatory cytotoxic mechanisms on B-cell proliferation, allowing exhaustive and inbalanced B-cell help and lymphomagenesis to occur.

2.3 *H. pylori*, asthma and allergy

The severity and incidence of asthma have increased drastically in the developed nations of the world over the last decades. Although the underlying reason is still unknown, clinical,

epidemiological and experimental evidence indicate that infectious diseases can influence the development of allergic disorders (Strachan et al., 1989; Roumier et al., 2008). Accordingly, an inverse correlation has been demonstrated between the onset of allergic disorders and the incidence of infections. This may be the result of an inhibition of allergic Th2 inflammation exerted by Th1 responses; the latter are elicited by infectious agents and are able to induce the production of IFN-g, IL-12, IL-18 and IL-23 (Herz et al, 2000). This view is supported by studies showing that development of asthma can be prevented in animals by administering live or killed bacteria or their components, which induce Th1 responses (Wohlleben et al., 2006). We demonstrated that *H. pylori* inhibited Th2 responses in asthmatic patients (Amedei et al., 2006). Interestingly, on the basis of large epidemiological studies, a consistent negative association between *H. pylori* infection and the presence of allergic disorders, such as asthma and rhinitis, has recently been proposed (Chen et al., 2007). Although it is an undoubtedly interesting theory, no convincing molecular mechanism has been suggested to support it.

Our studies carried out with *H. pylori* may help in the understanding of this complex issue. We have shown that the addition of the *H. pylori* protein HP-NAP to allergen-induced T-cell lines derived from allergic asthmatic patients led to a drastic increase in IFN-γ-producing T cells and to a decrease in IL-4-secreting cells, thus resulting in a redirection of the immune response from a Th2 to a Th1 phenotype (Amedei et al., 2006). These results suggest that HP-NAP might be the key element responsible for the decrement of allergy frequency in *H. pylori*-infected patients. Several studies were devoted to the definition of new immune-modulating factors able to inhibit Th2 responses and consequently, different compounds have been proposed for the treatment and prevention of asthma, including several TLR ligands mimicking the effects of microbial components, such as dsRNA, CpG-oligodeoxynucleotides and imidazoquinolines (Hirota et al., 2002; Trujillo-Vargas et al., 2005).

We demonstrated that in allergic asthmatic patients, the typical Th2 responses can be redirected toward Th1 by HP-NAP and that the activity of HP-NAP required the engagement of TLR2 (Amedei et al., 2006; Codolo et al., 2008b). To address whether HP-NAP, on the basis of its immune-modulating activity, could be beneficial for the prevention and treatment of bronchial asthma, it was administered via the intraperitoneal or the intranasal route using a mouse model of allergic asthma induced by inhaled ovalbumin (OVA). Groups of nine C57BL/6j, wild-type or *tlr2-/-* mice were treated with OVA alone, or with OVA plus HP-NAP administered intraperitoneally or mucosally. In both systemic and mucosal protocols, mice were treated with OVA according to a standardized procedure consisting of a first phase of sensitization with intraperitoneal OVA and a second phase of induction of the allergic response with aerosolized OVA on day 8, followed by repeated aerosol challenge with the allergen on days 15–18. Control animals were injected with phosphate-buffered saline (PBS) alone and then exposed to aerosolized PBS. In the systemic protocol, mice were treated with intraperitoneal HP-NAP on day 1, whereas in the mucosal protocol mice received intranasal HP-NAP on days 7 and 8 (Codolo et al., 2008b).

After priming and repeated aerosol challenge with OVA, Th2 responses were induced in the mouse lung. Accordingly, following OVA treatment, eosinophils were recruited and activated in bronchial airways, and serum IgE levels increased. Both systemic and mucosal administration of HP-NAP strongly inhibited the development of airway eosinophilia and bronchial inflammation. Likewise, HP-NAP treatment strongly affected the cytokine release

in the lung, reducing the production of IL-4, IL-5 and GM-CSF. Systemic HP-NAP also significantly resulted in both the reduction of total serum IgE and an increase in IL-12 plasma levels. However, no suppression of lung eosinophilia and bronchial Th2 cytokines was observed in *tlr2-/-* mice following HP-NAP treatment (Codolo et al., 2008b). This phenomenon can be explained by the inhibition of the allergic Th2 inflammation seen when Th1 responses are elicited by infectious agents able to induce the production of IFN-γ, IL-12 and IL-23. HP-NAP, by acting on innate immune cells via TLR2 agonistic interaction, induces an IL-12- and IL-23-enriched milieu, and in such a way it represents a key factor able to induce a Th2-Th1 redirection. Furthermore, HP-NAP administration *in vivo* resulted in inhibition of the typical Th2-mediated bronchial inflammation of allergic bronchial asthma. Thus, combined, these results support the view that the increased prevalence and severity of asthma and allergy in Western countries may be related, at least in part, to the decline of *H. pylori* infection, which is able to induce a long-lasting Th1 background, and suggest that the use of a microbial product derived from *H. pylori*, such as as HP-NAP, may help the prevention and treatment of bronchial asthma and allergic diseases. At the same time, we do not suggest infecting people with *H. pylori* or leaving a *H. pylori* infection without antibiotic treatment to treat asthma and allergy.

3. Conclusion

Helicobacter pylori infects almost half of the population worldwide and represents the major cause of gastroduodenal diseases, such as duodenal and gastric ulcer, gastric adenocarcinoma, autoimmune gastritis, and B-cell lymphoma of mucosa-associated lymphoid tissue. Different bacterial and environmental factors, other concomitant infections, and host genetics may influence the balance between mucosal tolerance and inflammation in the course of *H. pylori* infection. *Helicobacter pylori* induces the activation of a complex and fascinating cytokine and chemokine network in the gastric mucosa. The type of innate and acquired immune responses provides an useful model for explaining both different types of protection and the pathogenetic mechanisms of several disorders elicited by *H. pylori*. A predominant *H. pylori*-specific Th1 response, characterized by high IFN-γ, TNF-α, and IL-12 production associates with peptic ulcer, whereas combined secretion of both Th1 and Th2 cytokines are present in uncomplicated gastritis. Gastric T cells from MALT lymphoma exhibit abnormal help for autologous B-cell proliferation and reduced perforin- and Fas-Fas ligand-mediated killing of B cells. In *H. pylori*-infected patients with autoimmune gastritis cytolytic T cells infiltrating the gastric mucosa cross-recognize different epitopes of *H. pylori* proteins and H^+K^+ ATPase autoantigen. An inverse association between *H. pylori* prevalence and the frequencies of asthma and allergies was demonstrated, and the Neutrophil Activating Protein of *H. pylori*, according to its ability in inhibiting allergic inflammation of bronchial asthma, could be the factor responsible for this negative relationship. Given that resistance to antibiotic is increasing and the effectiveness of current therapeutic regimens is decreasing the design of an efficient vaccine for *H. pylori* will represent a novel and very important tool against both infection, peptic ulcer and gastric cancer.

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Part 3

Clinical Management of Peptic Ulcer Patients

14

Helicobacter pylori Infection in Elderly Patients

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1. Introduction

Epidemiological studies report an increased rate of gastrointestinal diseases in subjects older than 65 years, and these diseases constitute one of the most frequent indications for medical consultation in this population (Pilotto, 2004). Oesophageal and gastroduodenal diseases, especially *Helicobacter pylori* (*H. pylori*) infection, are frequent in this population since they account for 40% of the total digestive pathologies in the elderly. Even if data in the literature report an increased prevalence of *H. pylori* infection in the elderly, clinical interest remains low. Only 56% of elderly patients hospitalized for peptic ulcers in the United States were tested for *H. pylori* infection, among whom only 73% were then treated (Ofman et al., 2000). However, studies report an increased rate of peptic ulcer disease (PUD) complications and mortality in older patients (1 per million in young adults aged 20 years to 200 per million after 70 years) (Younger&Duggan, 2002). This alarming finding has stimulated further studies on the pathophysiological and clinical aspects of *H. pylori* infection in the geriatric population. Indeed, recent data reported that *H. pylori* chronic infection plays a role in gastric aging, appetite regulation, and possibly extra-digestive diseases such as Alzheimer disease in the elderly.

2. Epidemiology in the elderly

The principal reservoir for *H. pylori* infection appears to be the human stomach, especially the antrum (Megraud, 2003). In developing countries, the oro-fecal route as well as the oro-oral route coexists because of poor socioeconomic and hygienic conditions (Nurgalieva et al., 2002). In developed countries, the oro-fecal transmission has gradually disappeared, leaving the oro-oral route, which is secondary to vomiting and gastro-oesophageal reflux (Megraud, 2003). Even if socioeconomic progress has led to a decrease in the incidence of the infection in developed countries, the prevalence of *H. pylori* infection still remains high in people born at the beginning of the 20^{e}th century. In fact, Gause-Nilsson *et al.* (Gause-Nilsson et al., 1998) reported that when cohorts of 70 year old subjects born in 1901-1902 and 1922 were compared, the latter cohort showed a significantly lower *H. pylori* positive serology. This difference in *H. pylori* prevalence may reflect changes in socioeconomic conditions. Epidemiologic studies on elderly people, with a mean age of approximately 70 years, reported a prevalence of nearly 60 percent in asymptomatic subjects (Pilotto et al.,

1996; Regev et al., 1999) and more than 70 percent among the most elderly patients with gastrointestinal diseases (Pilotto, 2001; Pilotto&Salles, 2002). Other studies reported a high prevalence of *H. pylori* infection in the most elderly population, especially in institutionalized old people, with a prevalence ranging from 70 to 85 percent (Regev, Fraser, Braun, Maoz, Leibovici&Niv, 1999). These results can be explained by the mode of transmission of *H. pylori* (oro-fecal or oro-oral), taking into account the promiscuity and living conditions in an institution. Regev *et al.* showed that living in an institution increased the risk of *H. pylori* infection, the prevalence of the infection being positively correlated with the duration of stay (Regev et al., 1999). Nevertheless, even if prevalence increases with age, the prevalence curve appears to be flat and tends to decrease after 85 years (Pilotto&Salles, 2002; Salles-Montaudon et al., 2002). Thus, Neri *et al.* showed that *H. pylori* infection passed from 70 to 50 percent after 90 years (Neri et al., 1996). Two hypotheses can explain this trend. The first hypothesis is an underestimation of *H. pylori* infection, because of frequent chronic atrophic gastritis and frequent current or previous use of antisecretory and antibiotic treatments in this frail population. In a previous work, we showed a lower prevalence of *H. pylori* infection (47.7%) than expected in hospitalized elderly people, which can be explained on one hand by the higher polymedication with repetitive antibiotherapies and on the other hand by more frequent gastric atrophic lesions which offers a less favorable ground for *H. pylori* (Salles-Montaudon et al. 2002). The second hypothesis is a premature death of *H. pylori* infected subjects due to various comorbidities, e.g., gastric cancer, peptic ulcer diseases (PUD) and possibly other diseases such as cardiovascular diseases. Based on the data from the PAQUID Cohort study ("Personnes Agées Quid?" which was designed to enrol and follow-up elderly subjects randomly selected in the South-Western France4), we recently evaluated the impact of *H. pylori* infection on the mortality rate in a population of subjects older than 65 years. The follow-up of 605 subjects over 15 years, after adjustment for age, gender, and cardiovascular comorbidity, showed that an *H. pylori* infection was not a risk factor for mortality (HR=1.2, 95% CI [0.94; 1.52]) (Salles et al.).

3. *H. pylori* infection and peptic ulcer disease

The elderly are particularly susceptible to peptic ulcer disease (PUD) and complications due to their higher *H. pylori* prevalence and use of non-steroidal anti-inflammatory drugs (NSAIDs) (Bhala&Newton, 2005; Chow et al., 1998; Jones&Hawkey, 2001). Even if it is well known that the incidence of gastric ulcers increases in elderly people, little is known about gastric mucosal healing alterations during ageing. Newton et al. reported that gastric ageing induces gastric frailty, characterized by a reduction in protective factors, i.e., mucus layer, prostaglandin levels, mucosal growth, and gastric blood flow, and a higher susceptibility to aggressive factors, i.e., NSAID and H. pylori infection (Newton, 2004). Age-related changes could occur as a result of increased exposure to exogenous factors, alterations in the secretion of endogenous aggressive factors, or changes in the production or repair of the mucus bicarbonate layer, the primary barrier against acid and pepsin digestion. The increased risk of severe complications in this population (haemorrhage, gastric perforation) is likely to mask important early symptomatic signs which make PUD treatment difficult in the elderly (Kemppainen et al., 1997). Most of the studies suggested that ulcer pain is less common in this age group. Indeed, the diagnosis of PUD is usually delayed, because symptoms are not easily detected in this population. Typical pain is often absent; only one third of people older than 60 years experience painful symptoms (Seinela&Ahvenainen,

2000). In geriatric practice, anorexia and malnutrition frequently represent alarm symptoms and lead to gastroduodenal endoscopy. Endoscopic exploration, which is most of the time well tolerated in elderly patients, allows a diagnosis in more than 50 percent of the cases (Van Kouwen et al., 2003). Another contributing factor to PUD in the elderly is a high usage of NSAIDs and/or aspirin. Pilotto et al. showed that NSAID use independently increases the risk of peptic ulcer and ulcer bleeding. This risk increases with age in a linear manner, and increases further in the event of comorbidities and polymedication which are common in this population (Pilotto et al., 2003). *H. pylori* infection also independently increases the risk of PUD in the elderly. Clinical studies reported that approximately 50 to 70 percent of elderly peptic ulcer patients are *H. pylori* positive (Pilotto, 2001). The short- and long-term studies performed on elderly patients demonstrated that treatment of *H. pylori* infection in patients with peptic ulcer resulted in ulcer healing in over 95 percent of the patients and significantly improved clinical outcomes, including a decrease in recurrence (Pilotto&Salles, 2002). With regard to the respective responsibility of the two risk factors previously mentioned (AINS, *H. pylori*) in PUD, studies confirmed that both independently and significantly increase the risk of PUD and ulcer bleeding.

4. *H. pylori* infection and gastric aging

The ageing stomach is usually described in terms of the occurrence of gastric atrophic lesions. Thus, a wide range of studies reported an increased prevalence of atrophic gastritis in elderly patients, with rates ranging between 50 to 70% in patients over eighty (Sachs et al., 2003; Younger&Duggan, 2002). Only a few studies provided results on intrinsic gastric ageing and most were animal studies. In contrast, most of the literature reported the strong role played by chronic H. pylori infection in the occurrence of atrophic gastric lesions. The discovery of H. pylori chronic infection has cast a doubt on the reality of the physiological gastric ageing process which is now considered as a process of "pathological gastric ageing". Since the discovery of the strong role of this chronic gastric infection in the development of a gastric atrophy and PUD, we actually consider that most of the observed changes in the stomach that appear during ageing are, in fact, the result of environmental causes, such as chronic infection, nutritional, and pharmacological factors. These environmental factors may participate in inducing gastric frailty with impaired mucosal defence.

4.1 Chronic atrophic gastritis

A series of studies, mainly from Japan, has focused on the long-term effects of *H. pylori* infection and its role in the development of the histological changes that occur with ageing, i.e., atrophic gastritis (Salles, 2007). In a large multicenter trial, authors reported that both atrophic gastritis and intestinal metaplasia were strongly associated with *H. pylori* infection and not with ageing *per se* (Asaka et al., 2001). Interventional prospective studies reported that *H. pylori* eradication induces a significant reduction of inflammatory and atrophic gastric lesions (Ito et al., 2002; Kokkola et al., 2002). Kokkola et al. reported that advanced atrophic gastritis may improve and heal after *H. pylori* eradication in elderly subjects (Kokkola et al., 2002). They followed prospectively 22 elderly men (55–69 years of age) with *H. pylori* infection and atrophic corpus gastritis. During a 7.5-year period prior to eradication therapy, no significant changes were observed in the mean atrophy and IM scores. However, after *H. pylori* eradication, a significant improvement occurred in the mean

histological Sydney system score of inflammation (from 2.2 to 0.5), atrophy (from 2.2 to 1.2) and IM (from 1.6 to 1.1). These findings are in agreement with the results of another study carried out in 132 subjects aged from 34 to 68 years (mean age 50 years) with multifocal (nonautoimmune) atrophic gastritis. Six years after cure of *H. pylori* infection, a significant improvement in antral atrophy was detected in subjects who received anti-*H. pylori* treatment, the effect being greater among those who were free of infection at the end of the trial (Ruiz et al., 2001). Since the response to treatment was similar in patients of different ages, we may assume that the cure of *H. pylori* infection is as recommended in elderly patients with gastric mucosal modifications as it is for young and adult patients. During gastric ageing, histological modifications are frequently observed leading to physiological gastric disturbances. Thus, a high prevalence of chronic atrophic gastritis is frequently observed in the gastric mucosa of elderly people (Ofman et al., 2000; Pilotto, 2004). These histological lesions lead to hypochlorhydria with a risk of bacterial overgrowth in the proximal digestive tract and intestinal malabsorption.

4.2 Gastric acid secretion

Between 1920 and 1980, many studies reported a significant reduction in gastric acid secretion with age. The majority of these studies was retrospective and did not take into account the presence of possible gastric atrophic lesions (Salles, 2007). More recent studies including elderly patients showed that there is no change in acid secretion with age, whereas others even showed an increase in acid secretion. Those studies that demonstrate hyposecretion in elderly patients offer chronic H. pylori infection and atrophic gastritis of the oxyntic mucosa as a reasonable explanation. Shih et al. found that gastric acid secretion does not change with age (Shih et al., 2003). Feldman et al. studied gastric acid secretion in elderly patients and found that basal acid output and peak acid output did not correlate with age (Feldman&Cryer, 1998). Similarly, Lijima et al., reported that gastric acid secretion was well preserved irrespective of ageing, however it seemed to increase with ageing in the H. pylori-negative subjects (Iijima et al., 2004). The decline in gastric acid secretion in H. pylori-positive patients depends on both an increasing prevalence of fundic atrophic gastritis and inflammatory cytokines, i.e., interleukin (IL) IL-1β and TNF-α, which are known to inhibit parietal cells. The mechanism promoting the increase in acid secretion with ageing is unknown. However, since the alteration of acid secretion by the inflammation of oxyntic mucosa can be ignored in the H. pylori-free stomach, two main possibilities could be considered: one is an increase in the total parietal cell mass with age, and the second is an increase in the reactivity of parietal cells. Because the previous studies failed to find any change in parietal cell mass with ageing, the second possibility is more likely.

4.2.1 Gastrointestinal bacterial overgrowth

Only a few clinical studies investigated the prevalence of gastrointestinal bacterial overgrowth in older healthy people, and most of the results showed that bacterial overgrowth occurs rarely during the normal process of gastric ageing, but is rather an iatrogenic process (antisecretory drugs) in the elderly. Mitsui *et al.* performed a study (Mitsui et al., 2006) and included healthy and disabled older people, aged over 70 years. They reported no bacterial overgrowth among healthy patients, but only in disabled or frail older people. Parlesak *et al.* also performed a study in older adults using a hydrogen breath test (Parlesak et al., 2003) and reported a 15.6% prevalence of small bowel bacterial overgrowth. They showed that PPI treatments played a role in increasing the prevalence of

positive breath tests in older adults, which was associated with lower body weight, lower body mass index, lower plasma albumin concentration, and higher prevalence of diarrhea. The pH of gastric acid and bacterial counts in the stomach showed a close correlation. In normal subjects the gastric pH is usually below pH 4, a critical level for protection against enteric pathogens, and the stomach is virtually sterile. At a pH of 4–5 bacteria from the saliva are present in the stomach. A pH greater than 5 allows bacterial, viral and protozoan pathogens to survive and enteric bacteria can be found in the stomach. Other drugs which decrease acid production are anticholinergic drugs and tricyclic antidepressant drugs. In geriatrics, malnutrition is one of the clinical consequences of bacterial overgrowth, and antibiotic treatment may lead to the improvement of the anthropometric parameters of these patients (Lewis et al., 1999).

4.2.2 Vitamin B12 deficiency

Cobalamin or vitamin B12 deficiency is common in elderly patients (Dali-Youcef&Andres, 2009). The Framingham study demonstrated a prevalence of 12% among elderly people living in the community. Other studies focusing on elderly sick and malnourished people living in institutions have suggested a higher prevalence of 30–40%. The main causes of cobalamin deficiency include food-cobalamin malabsorption, pernicious anaemia, and insufficient nutritional vitamin B12. Dietary causes of deficiency are limited to elderly people who are already malnourished with lower albumin level, such as frail elderly patients with severe co morbidities (Salles-Montaudon et al., 2003). First described by Carmel in 1995, food-cobalamin malabsorption is frequent in elderly people, and is characterized by the inability of the body to release cobalamin from food or intestinal transport proteins, particularly in the presence of hypochlorhydria, where the absorption of 'unbound' cobalamin is normal (Carmel et al., 2003). Food-cobalamin malabsorption is caused primarily by chronic atrophic gastritis. In fact, Andres et al, reported in a recent meta-analysis, that cobalamin deficiency, other than those caused by nutritional deficiency, can be treated by oral administration of vitamin B12 in the form of cyanocobalamin (free cobalamin) (Andres et al., 2009). An evidence-based analysis by the *Vitamin B12 Cochrane Group* also supports the efficacy of oral cobalamin therapy. In this analysis, serum vitamin B12 levels increased significantly in patients receiving either oral vitamin B12 alone or patients receiving both oral and intramuscular treatment (Vidal-Alaball et al., 2005). Other factors that contribute to food-cobalamin malabsorption in elderly people include chronic carriage of *H. pylori* and intestinal microbial proliferation, situations in which cobalamin deficiency can be corrected by antibiotic treatment, long-term ingestion of antiacids such as H2-receptor antagonists and PPI, and biguanides (metformin) (Dali-Youcef&Andres, 2009). Sipponen et al, reported normalization of vitamin B12 levels after *H. pylori* eradication (Sipponen et al., 2003).

5. *H. pylori* infection and gastric cancer

Gastric carcinoma and gastric MALT lymphoma have been causally associated with *H. pylori*, and the bacterium has been categorized as a group I carcinogen by the International Agency for Research on Cancer (IARC). The incidence of gastric cancer increases with age worldwide. However, the association between *H. pylori* infection and gastric cancer might have been underestimated due to possible clearance of the infection in the course of disease development, especially among older patients who generally have more severe mucosal

atrophy and intestinal metaplasia in the stomach. Recently, a new analytical investigation to minimize a potential underestimation of the association of *H. pylori* with gastric cancer was performed on Western populations [60]. Applying various more stringent exclusion criteria to minimize a potential bias from this source increased the odds ratio (95% confidence interval) of non-cardia gastric cancer from 3.7 to 18.3 for any *H. pylori* infection, and from 5.7 to 28.4 for cagA-positive *H. pylori* infection (Brenner et al., 2004). A possible mechanism to explain the link between *H. pylori* infection and gastric carcinoma is inflammation which increases the risk of mutations. Many of the mediators and byproducts of inflammation are mitogenic and mutagenic. Release of pro-inflammatory cytokines, reactive oxygen species and upregulation of Cox-2 all contribute to an intragastric environment conducive to neoplastic transformation. The mechanisms involve direct DNA damage, inhibition of apoptosis, subversion of immunity, and stimulation of angiogenesis. In addition, chronic inflammation in the gastrointestinal tract is also known to affect proliferation, adhesion and cellular transformation. There is also evidence of a direct carcinogenic effect of *H. pylori* per se on the gastric mucosa. As mentioned before, the type 4 secretion system encoded by the cag PAI allows the bacterium to inject molecules into the epithelial cells. The CagA protein is one of them. It acts on numerous cell effectors disturbing cell physiology leading to several proneoplastic processes, e.g. activation of growth factor receptors, increased proliferation, evasion of apoptosis, sustained angiogenesis and cell dissociation, and tissue invasion.

6. Impact of *H. pylori* infection on appetite regulation

The results showed that the expression of leptin and ghrelin peptides decreased both in the presence of an *H. pylori* infection and in the presence of atrophic gastritis lesions. The possible role of *H. pylori* infection in the regulation of appetite in the elderly is an interesting new research topic. Studies reported that *H. pylori* eradication appeared to improve certain nutritional parameters, i.e., body mass index (BMI), and albumin (Azuma et al., 2002; Fujiwara et al., 2002). Kamada et al. recently showed a significant increase in the BMI after *H. pylori* eradication among patients suffering from gastric ulcers, with a parallel increase in triglyceride and cholesterol levels (Kamada et al., 2005). In geriatric practice, anorexia and weight loss are often the only symptoms of PUD and are signs warranting endoscopic exploration. It is also crucial to investigate *H. pylori* infection in such cases. Studies performed on adults indicate that inflammatory cytokines may cause anorexia by inducing variations in circulating gastrointestinal hormones, neuropeptides, and NO, all of which can alter food intake (Chapman, 2004; Morley, 2001). Recently, we showed that chronic inflammation in the gastric mucosa affects the expression of gastric satiety inducible peptides such as leptin and ghrelin (Salles et al., 2006). Recent evidence suggests that, in humans and rats, leptin is secreted not only from adipose tissue but also from the gut (Bado et al., 1998). Studies indicate that gastric inflammation induced by *H. pylori* infection raises gastric leptin expression which then induces satiety and lower BMI (Azuma et al., 2001; Konturek et al., 2001). Ghrelin is a newly discovered peptide which is produced mainly in the stomach and is involved in the control of food intake and energy homeostasis in both humans and rodents (Kojima et al., 1999). In a recent study, authors reported that a cure of *H. pylori* infection increased plasma ghrelin, which in turn led to an increased appetite and weight gain (Nwokolo et al., 2003). Consequently, chronic gastric inflammation may induce variations in the expression of both leptin and ghrelin and may play a role in the

pathophysiology of anorexia in elderly patients. In a study on frail elderly patients over 80 years old, we showed that the presence of *H. pylori* chronic gastritis induced a decrease in both leptin and ghrelin gastric production; this finding may, in fact, be due to the high prevalence of atrophic lesions observed in this particular population (Salles et al., 2006). Furthermore, the presence of *H. pylori* chronic gastritis was negatively correlated to the caloric ratio and the body mass index of these aged patients. The decrease in plasmatic and gastric levels of the strong orexigen, ghrelin, could explain the lack of appetite and the malnutrition of aged people who have chronic gastritis lesions due to *H. pylori*

7. *H. pylori* infection and Alzheimer's disease

The risk factors identified for dementia are often inaccessible to intervention (age, gender, genetic). New hypotheses have recently been suggested, such as the possible relationship between *H. pylori* infection and dementia via inflammatory mechanisms, both pro-oxidant and carential. Indeed, in addition to two case-control studies pointing out an association between *H. pylori* infection and Alzheimer disease (Kountouras et al., 2006; Malaguarnera et al., 2004), an interventional study has shown that *H. pylori* eradication positively influences Alzheimer disease manifestations, especially cognitive decline (Kountouras et al., 2009). Preliminary results of a cohort study conducted in our laboratory concluded that *H. pylori* infection was a significant risk factor for developing Alzheimer disease. One of the hypothesis is that *H. pylori* infection could act as a trigger in the genesis or in the accumulation of Alzheimer disease lesions via cerebral hypoperfusion due to atherosclerosis, or via an exacerbation of neuroinflammation.

8. Diagnosis of H. pylori infection in the elderly

Diagnosis of *H. pylori* infection remains difficult in elderly patients because of the characteristics of this population.

8.1 Non-invasive methods

The advantage of these methods is that they are global tests, i.e., *H. pylori* can be detected even if the patchy distribution of the bacteria, when gastric atrophy is present, precludes their histological detection.

8.1.1 Serology

Generally speaking, *H. pylori* immunoglobulin G (IgG) antibodies appear 2 to 3 weeks following infection, and slowly decrease after *H. pylori* eradication. In adulthood, the performance of serology (ELISA) shows 85 to 95 percent sensitivity and 80 to 95 percent specificity (Granberg et al., 1993). Even if most of the epidemiologic studies included serology to detect *H. pylori* infection in the elderly, data concerning the performance of this test remain contradictory for this population. Some authors consider that there is a risk of over-estimating infection in the elderly when using serology, because antibodies remain present for months or even years after *H. pylori* eradication (Kosunen et al., 1992). Indeed, in this population, the prevalence of *H. pylori* infection is significantly higher when detected by serology than by histology. Studies reported that in *H. pylori* positive patients with atrophic body gastritis, after eradication therapy, the time delay concerning the decrease in *H. pylori* IgG did not always correlate with the reduction in gastric inflammation (Kosunen et al.,

1992). This suggests that, in patients with atrophic body gastritis, serology alone may not be valid for assessing the efficacy of eradication treatment. Liston et al. showed that nearly one third of their study patients had positive serology without signs of active *H. pylori* infection (Liston et al., 1996). In addition, other authors reported that serology may not be useful in determining successful eradication post-therapy in the elderly, because of a great heterogeneity in the decrease in IgG antibody titer (6 months or more) (Kosunen et al., 1992). On the contrary, some authors consider that serology may underestimate the infection in the elderly. This could be explained by a possible lack of antibody response due to a frequent immunodeficiency diagnosed in frail elderly people. In fact, immunodeficiency may be the consequence of protein malnutrition which occurs in more than 30 percent of the elderly population (Burns, 2004; Salles-Montaudon et al., 2002). The infection could also be underestimated because of the characteristics of this elderly population, often hospitalized and treated with antibiotics for recurrent urinary tract or pulmonary infections which can induce false negative results.
Immunoblot (Western blot) is another serologic method useful in the elderly, for the detection of antibodies directed against particular antigenic proteins of *H. pylori* (CagA, VacA, urease A and B). Pilotto et al. showed that the presence of a CagA positive *H. pylori* infection was independently correlated with atrophic gastritis and intestinal metaplasia in the elderly (Monteiro et al., 2002; Pilotto et al., 1998).

8.1.2 13Carbon-Urea Breath Test

The ^{13}carbon-urea breath test (^{13}C-UBT) has an excellent diagnostic performance including the post-therapy determination of successful *H. pylori* eradication. The principal disadvantage of this method is the need for specific equipment which associates gas chromatography and mass spectrometry. In the elderly, this test has the advantage of being easily performed with a minimum of cooperation from the patient, and it is very well tolerated. Pilotto et al. demonstrated the excellent performance of this test on elderly patients, with a diagnostic accuracy of 97.9%. They found it useful even for patients with severe cognitive impairment (Pilotto et al., 2000). In a recent study performed on hospitalized patients older than 85 years, we reported that almost one-third of the *H. pylori*-positive patients would have remained undetected without this test, including treated patients (PPIs and antibiotics), or patients with chronic corpus atrophic gastritis [16]. Some studies reported the risk of an overestimation of *H. pylori* infection when using this test on the elderly. This could be explained by a hypochlorhydria due to gastric atrophy, which allows gastric colonization by urease-producing bacteria present in the mouth, oropharynx, and small intestine and decreases the specificity of the test (Chen et al., 2000). Certain situations can, to the contrary, involve an underestimation of *H. pylori* diagnosis in older subjects. Among these various cases, a past gastric resection may decrease the amount of urea present in the stomach, leading to an insufficient *H. pylori* detection.

8.1.3 Stool test

One of the formats of this test is a microwell-based immunoassay (HpSA), which detects *H. pylori* antigens present in human stools (Vaira et al., 1999). This test has a good diagnostic performance and can be easily carried out in routine. The test's lower sensitivity in the elderly can be explained by the higher frequency of chronic constipation in this group. Indeed, the passage of the bacteria into the colon may be prolonged, leading to a degradation of *H. pylori* antigens and jeopardizing their detection. This has been shown

experimentally in stools spiked with *H. pylori*. Moreover, studies have shown that PPI treatment decreases the accuracy of HpSA by increasing the gastric pH and suppressing *H. pylori* colonization (Monteiro et al., 2001). The HpSA test also presents the disadvantage of being more difficult to carry out in this very old population, mainly for practical reasons, in dependent or demented older subjects (Salles-Montaudon et al., 2002).

8.2 Invasive methods

Most of the time, older patients have diagnostic indications for upper gastrointestinal endoscopy, i.e., chronic anemia, dysphagia, epigastralgia, etc. Biopsy sampling per endoscopy permits the detection of *H. pylori* infection by urease test, histological analysis, culture or PCR.

8.2.1 Biopsy urease test

There are several tests available based on the the urease activity of *H. pylori* present in biopsy specimens, among which are the CLO test® and the PyloriTek®. In the elderly, studies reported a lower sensitivity of these tests (57%) compared to histology or serology (Abdalla et al., 1998). As stated previously, the lower sensitivity can be explained by multiple treatments for various infections, and frequent gastric atrophic lesions which may induce a hostile environment for the bacterium.

8.2.2 Histology

In the elderly, the performance of this test increased when biopsy specimens were taken from two areas of the stomach, i.e., the antrum and the body. Indeed, chronic gastric atrophy may induce gastric hypochlorhydria which may in turn reduce *H. pylori* colonization in the antrum. In the elderly, a histological analysis should not be the only diagnostic method for *H. pylori* infection, because of its lower sensitivity as previously stated.

Nevertheless, this method presents the advantage of evaluating the morphological parameters of the gastric mucosa, using the Sydney System classification suggested in 1990 by Price et al. (Price, 1991). The revised classification made in 1994 in Houston is now widely accepted (Genta&Dixon, 1995). These criteria are studied and quantified as either mild, moderate or severe: activity, inflammation, atrophy, intestinal metaplasia, and the presence of *H. pylori* infection. Given the increased incidence of malignancy in the elderly population, histology has become mandatory.

8.2.3 Culture

Microbiological examination of biopsy specimens is considered as the reference technique. Culture provides unique information which is helpful in the management of *H. pylori* infection, in particular the strain's susceptibility to antimicrobial agents (Mégraud, 1996). Concerning all of the "invasive methods", they may underestimate *H. pylori* infection in the elderly because of the frequent antibiotic and PPI treatments, and also the high prevalence of chronic atrophy lesions.

8.2.4 Molecular methods

Molecular diagnosis of *H. pylori* infection presents a real interest. PCR detection of *H. pylori* in gastric biopsies offers very sensitive and accurate results in a short time. Many protocols

have been developed for targeting different genes with specific primers for *H. pylori*. Recently, realtime PCR assays were developed allowing simultaneous detection and quantification as well as the determination of antibiotic susceptibility and genotyping of *H. pylori* (Oleastro et al., 2003).

9. Indications for treatment in geriatrics

9.1 Peptic ulcer disease

The short- and long-term studies performed on elderly patients indicate that treatment of *H. pylori* infection in patients with peptic ulcers results in healed ulcers in over 95% of the patients, and significantly improves the clinical outcome, reducing ulcer recurrence, and histological signs of ulcer-associated chronic gastritis activity [13,86]. It is, thus, strongly recommended to test and treat *H. pylori* infection among the elderly presenting peptic ulcers. Pilotto et al. showed that the rate of peptic ulcer relapse in eradicated older patients was 2 percent versus 42 percent in those non-eradicated (Pilotto, 2001).

9.2 Atrophic chronic gastritis

As stated previously, *H. pylori* eradication induces a decrease in the severity of gastric inflammation, atrophy and intestinal metaplasia (Kokkola et al., 2002). The Maastricht 2-2000 Consensus Report recommends treating patients with atrophic gastritis (Malfertheiner et al., 2002).

9.3 Non-ulcer dyspepsia

The effectiveness of *H. pylori* eradication in patients with non-ulcer dyspepsia is still a matter of debate. A study carried out on a geriatric population showed a significant improvement in symptoms of functional dyspepsia in 70% of the patients two months after eradication (Pilotto et al., 1999). However, the long-term benefit was not studied.

9.4 Gastroesophageal reflux disease

The relationship between *H. pylori* infection and the clinical evolution of gastroesophageal reflux disease has not yet been clarified in elderly subjects (Kountouras et al., 2004; Kountouras et al., 2006). In a study carried out on elderly patients with esophagitis, Pilotto et al. reported that healing of esophagitis after a 2 month treatment with PPIs was similar in *H. pylori*-positive and *H. pylori*-negative patients (Pilotto et al., 2002). Moreover, eradication therapy did not accelerate the clinical response to short-term PPI therapy among these patients. *H. pylori* eradication is thus not recommended for elderly patients with gastroesophageal reflux disease.

9.5 Use of non-steroidal anti-inflammatory drugs

It is now established that most peptic ulcers are caused by *H. pylori* or NSAIDs. Studies showed that *H. pylori* eradication is not sufficient in preventing ulcer bleeding in high-risk NSAID users (Chan et al., 2002; Lai et al., 2003; Pilotto et al., 2000a). It does not enhance the healing of peptic ulcer in patients taking antisecretory therapy who continue to take NSAIDs. However, PPI was superior to the eradication of *H. pylori* in preventing recurring bleeding in patients who are taking NSAIDs.

Among patients with *H. pylori* infection and a history of upper gastrointestinal bleeding who are taking low-dose aspirin, the eradication of *H. pylori* is equivalent to treatment with

omeprazole in preventing recurrent bleeding (Chan et al., 2001). In conclusion, there is no clear proof which supports the systematic eradication of *H. pylori* in elderly patients treated with short- or long-term aspirin and/or NSAID drugs.

10. *H. pylori* eradication in geriatrics

Many clinical trials demonstrated that PPI-based triple therapies for 1 week, as recommended by the Maastricht 2-2000 Consensus Report, were highly effective in the elderly population (Pilotto&Malfertheiner, 2002). *H. pylori* eradication is more effective in elderly people compared to younger individuals. Studies performed on elderly patients also showed that a reduction in the PPI dosage, i.e. omeprazole (20 mg) and pantoprazole (40 mg), from twice daily to once daily did not influence the cure rates of triple therapies consisting of either PPI plus clarithromycin (250 mg b.i.d.) and metronidazole (500 mg b.i.d.), or PPI plus clarithromycin (250 mg b.i.d.) and amoxicillin (1 g b.i.d.) (Pilotto et al., 2001; Pilotto&Malfertheiner, 2002; Pilotto&Salles, 2002).
Bad compliance, which is frequently observed in elderly subjects, and *H. pylori* resistance to antibiotics, are the major reasons for treatment failure in elderly patients (Pilotto&Salles, 2002). Studies reported that metronidazole resistance decreased with age (OR for patients over 60 years = 0.63, 95% CI = 0.48–0.80) and was higher in females than in males (Pilotto&Salles, 2002). The optimal strategy for second-line therapy associates PPI with amoxicillin and metronidazole or PPI with tetracycline and metronidazole, with a recommendation to increase the duration of treatment (10 to 14 days) as well as the metronidazole doses. Concerning the poor compliance in older patients, the use of structured patient counselling and follow-up may have a significant effect on *H. pylori* cure rates and should be part of therapy management.

11. Conclusion

The strongest prevalence of *H. pylori* infection in the elderly as well as the role of *H. pylori* in the occurrence of gastric lesions, in particular ulcer diseases, gastric precancerous lesions, and gastric cancer, render the diagnosis and the eradication of *H. pylori* capital in this population. However, studies evaluating the prevalence, diagnosis and treatment of this infection are still few concerning this population, especially in frail patients older than 80 years.

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Perforated Duodenal Ulcer in High Risk Patients

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1. Introduction

Peptic ulcer disease remains one of the most prevalent disease of the gastrointestinal tract with annual incidence ranging from 0.1% to 0.3% in western countries. There are well-known two major precipitating factors: Helicobacter pylori infection and the use of non-steroidal anti-inflammatory drugs (NSAIDs) and the ulcer incidence increases with age for both duodenal and gastric ulcers.

Peptic ulcer disease (PUD) is considered as a mucosal functional derangements due to intraluminal aggressive factors and defects in endogenous defense mechanisms affecting the mucosa and extend through the muscularis mucosa. Some of these functional defects may be caused by the presence of H pylori colonization of the antral mucosa and antral mucosal metaplasia of the proximal duodenum. In vivo and in vitro data support this concept, particularly with reference to the mechanisms of Helicobacter pylori-induced aberrations in gastric and duodenal mucosal function. Standard medical therapy for peptic ulcer disease includes antisecretory medications as well as antibiotics designed to eradicate H pylori colonization.

Complications of peptic ulcer disease are bleeding, perforation and obstruction. These complications can occur in patients with peptic ulcers of any etiology. Perforation occurs in about 5% to 10% of patients with active ulcer disease. Duodenal, antral and gastric body ulcers account for 60%, 20% and 20% of perforations, respectively, of peptic ulcers. Open and laparoscopic abdominal exploration are always indicated in gastroduodenal perforation. Hemodynamic instability, signs of peritonitis and free extravasation of contrast material on upper gastrointestinal tract contrast studies make the decision for operation more urgent and imperative. But, the advent of proton pump inhibitors and Helicobacter pylori eradication in the management of chronic peptic ulcer disease has reduced the operative treatment of this condition to its complications. Perforated duodenal ulcer remains a major life threatening complication of chronic peptic ulcer disease.

The incidence of peptic ulcer disease in normal populations has declined over the past few years following a more streamlined pharmacological intervention. This can be attributed to the efficiency of histamine 2 (H2) blockers and proton pump inhibitors. Additionally, the diagnosis and eradication of Helicobacter pylori infection, now known to be a major factor in the pathogenesis of peptic ulcer disease, has almost eliminated the role of surgery in the

elective management of peptic ulcer disease. However, the incidence of perforated duodenal ulcers has either remained the same or has been increasing with the resultant increase in the incidence of emergency surgery. Although the use of potent H2 blockers and proton pump inhibitors has caused a marked decline in the incidence of peptic ulcer perforation, no such decline has been seen in the eradication of H. pylori infection.

Patients with perforated duodenal ulcers include those with acute ulcers, such as patients on nonsteroidal anti-inflammatory drugs (NSAIDs) and those with chronic ulcer disease who are refractory to or noncompliant with medical treatment. Another contributing factor to the increased incidence of perforation of duodenal ulcer is the decrease in elective anti-ulcer surgery. Patients presenting with an acute abdomen suggestive of a perforated duodenal ulcer are generally between 40 and 60 years of age although the number of patients over the age of 60 has been gradually increasing. Approximately 50% to 60% of these patients have a history of peptic ulcer disease, while a smaller number have a history of use of NSAIDs. Now, It's settled that H. pylori infection and NSAID use are two independent risk factors associated with perforated duodenal ulcers, and the lack of duodenitis in NSAID users as compared with those with H. pylori infection suggests a differing pathogenesis.

The frequency of perforated peptic ulcer is decreasing among the overall population but it is becoming more frequent among old people. The higher mortality rate in the old population, justifies the search of prognostic factors specific for the elderly.

2. High risk elderly patients

"High risk" surgical patients
Age > 60
Congestive cardiac failure
Ischaemic heart disease
Cardiac arrhythmia
Hypertension
COPD
Pulmonary embolus
Chronic renal insufficiency
Diabetes mellitus with end-organ damage
Long term steroid therapy
Chronic liver disease
Cerebrovascular disease
Peripheral vascular disease

Table 1. Showed patients with high risk of post operative death.

Risk is a term that is understood differently by different individuals depending on expectation and previous experience. The term "high risk surgical patient" is poorly defined. The term should refer to the group of patients, who were considered to be at high risk of post operative death, and were included in studies of pre-operative "optimization" to a pre-determined oxygen delivery [table 1]. From a practical point of view 'high risk' can probably be defined in two different ways: the first is relevant to an individual and suggests that the risk to an individual is higher than for a population; the second compares the risk of the procedure in question with the risk of surgical procedures as a whole. Furthermore, many investigators suggest that surgical patients for whom the probable mortality is greater than 20% should be considered 'extremely high-risk' patients. There are two main components in identification of high risk for surgery. The first relates to the type of surgery and the second to the cardiopulmonary functional capacity of the patient. There are methods that can be used to assess risk in various patient groups and in the author's opinion , the two most useful scoring systems in surgical risk assessment remain the American Society of Anesthesiologists (ASA) score and the patients' clinical criteria. Both of these assessments are simple to use and do not require additional resources. Surgical risk, in turn, has two components: the extent and the duration of the procedure both can cause an increase in postoperative oxygen demand and an increase in cardiac output or an increase in oxygen extraction. The classification of surgical interference is done in accordance with the extension and/or complexity of the procedure, with one or several of the mentioned characteristics:

S1. Minor Surgery: minimal extension, local anesthesia, ambulatory.

S2. Major Simple Surgery: performed on one organ or system, without any other added procedure

S3. Major Complex Surgery: performed on one organ or system, with other procedure or procedures related with the scheduled one, potential important bleeding, perhaps with some surgical problem that can be solved.

S4. Major Multiple Surgery: on several organs or systems, important bleeding, potential perioperative complications, it needs special preparation

S5. “Rescue” surgery, danger of death

The second item is the functional capacity of the patient that determines his ability to support the postoperative demand of increased oxygen consumption and therefore of cardiac output. Myocardial ischemia only becomes part of this equation if the ischemia limits ventricular function and cardiac output.

The definition of “elderly” is controversial and the traditional demographic definitions include those patients exceeding 65 years of age as the functional deterioration is more frequently apparent beyond the age of 70 years. For the elderly, one should categorize age-related pre-existing chronic illness; age related functional physical decline, or preoperative risk status. The most important surgeon responsibility is to decide whether to operate or not when the patient is of high surgical risk. The decision-making process is complex in elderly surgical candidates. Among the currently available risk assessment tools, American Society of Anesthesiologists (ASA) scoring system despite does not measure operative risk, rather it assesses the degree of sickness or physical state prior to anesthesia and surgery. The assessment of cardiac risk is addressed by the Cardiac Risk Index (CRI) in noncardiac surgery and the risks of postoperative respiratory complications are age over 70; perioperative bronchodilator use; abnormal chest x-ray; and high ASA grade.

The Acute Physiological and Chronic Health Evaluation (APACHE) is the best known physiological scoring system. It is based on twelve physiological variables and is currently being used in general and surgical intensive care patients. Age is an independent risk factor built into above mentioned risk prediction tools; ASA, Cardiac Risk Index (CRI) and APACHE. Preoperative Assessment of medication use is highest in elderly persons who require multiple medications to treat their complex set of medical problems. Medications necessary for managing medical conditions can put elderly individuals at risk of medication-induced problems such as adverse drug effects, drug-drug interactions, or drug toxicities. The greater the number of medications taken, the greater the risk of a clinically serious drug-drug interaction and the adverse drug reactions experienced by elderly patients often tend to be more severe than those experienced by younger patients. The reduced organ reserve capacity of elderly persons contributes to this as every organ system loses reserve capacity with age.

3. Epidemiology of perforated PUD

Although there is a decreasing incidence , perforated duodenal ulcer remains a serious condition which generally requires surgical intervention, and is associated with a high mortality rate especially among the elderly. The frequency of perforated peptic ulcer is decreasing among the overall population but it is becoming more frequent among old people. The higher mortality rate in the old population, justifies the search of prognostic factors specific for the elderly. The high mortality from perforated peptic ulcer underlines the importance of risk stratification. Over the past decades, important changes have occurred in the epidemiology of peptic ulcer disease. The discovery of Helicobacter pylori in the early 1980s as a major cause of peptic ulcer disease had a significant impact on the treatment of ulcer disease. The significance of the discovery led to the award of the 2005 Nobel Prize in Medicine to Robin Warren and Barry Marshall [figure 1]. H pylori eradication therapy was proven to cure patients with previous chronic, recurrent ulcer disease.

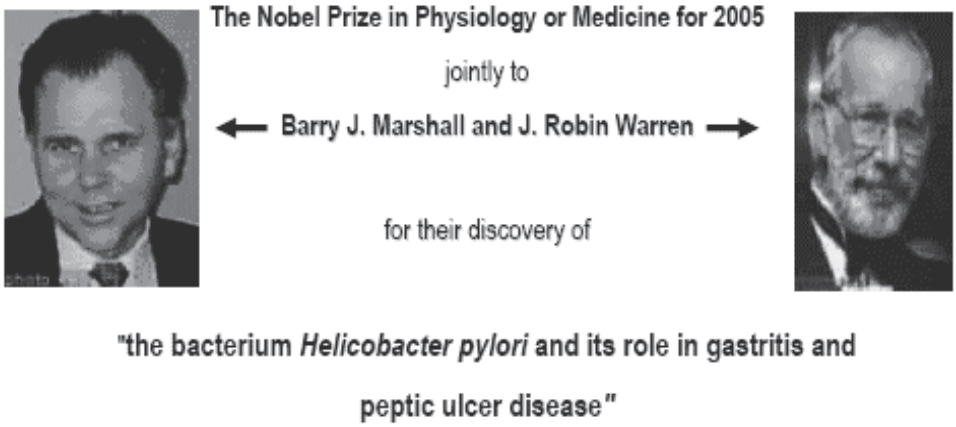

Fig. 1. Nobel Prize winners in Medicine and Physiology at the 2005

Peptic ulcer complications have a high mortality, especially in elderly patients and it is therefore important to understand the epidemiology of this disease in order to investigate if

complications can be prevented. Despite new efficient drugs to treat peptic ulcer disease and increasing knowledge about its aetiology, the incidence of peptic ulcer complications, i.e. perforation and bleeding, have been reported by several groups to be unchanged. Further research into the epidemiology of H pylori infection showed that the prevalence of this bacterium was decreasing over time in recent decades, presumably as a result of improvements in living conditions. The overall decline of peptic ulcer disease is likely to be due to a combination of factors including the introduction of acid suppressive medication, a decreasing prevalence of H pylori in subsequent birth cohorts and the development of eradication treatment for H pylori-positive ulcer patients, which prevents chronic relapsing ulcer disease. The introduction of newer NSAIDs and a tendency for the prescription of lower doses of acetylsalicylic acid for patients with cardiovascular disease may also have contributed to the changing epidemiology of ulcer disease.

With the decreased incidence of ulcer disease, the incidence of ulcer complications may have been affected as well. But most studies showed that the incidence of the most important complication, ulcer bleeding, remained stable notwithstanding the decreasing incidence of peptic ulcers.

The introduction of an endoscopy database allowed for closer investigation of the incidence and epidemiology of gastric and duodenal ulcers, complication rates and classifications. Mortality after perforated and bleeding peptic ulcer increases. An increased burden of comorbidity among elderly patients did not explain the association between advanced age and increased mortality, with the strongest association observed among patients with no history of hospital-diagnosed comorbidity.

Studies on the incidence of perforated duodenal ulcer are limited and data are largely based on findings observed over two decades ago. The epidemiological data on duodenal ulcer perforation was obtained mainly from medical records registry units all over the world for patients admitted with ulcer perforation. The incidence of perforated duodenal ulcer disease increases with advanced age and this increase has been attributed to the high frequency of risk factors for PUD among elderly patients, e.g., Helicobacter pylori colonization or use of non-steroidal anti-inflammatory drugs. Perforated peptic ulcer has an overall reported mortality of 5%–25%, rising to as high as 50% with age. Being closely related to advanced age, increased burden of comorbidity may partially explain the higher mortality among elderly patients.

Several studies support the notion that NSAID is a risk factor not only in uncomplicated peptic ulcer disease, but also in regard to perforated ulcers. The higher risk was maintained during treatment and disappeared after treatment termination. The added risk is dose-dependent and also includes low-dose acetyl salicylic acid. According to a study by Sorensen et al from 2000 the risk increase further when low-dose ASA is combined with NSAID. Proton pump inhibitors (PPI) have become one of the most sold drugs in the world and are nowadays also available over the counter in Sweden. It is a well known fact that PPI protect against peptic ulcer complications in NSAID users. Interestingly the introduction of PPI was almost simultaneous with the beginning of a falling incidence in peptic ulcer complications. H pylori is an important pathogenic factor in peptic ulcer disease, although studies that investigate the connection between H pylori and peptic ulcer complications are somewhat divergent. Helicobacter Pylori is, without a doubt, connected to peptic ulcer disease and its complications , perforation and bleeding, however, other factors such as NSAID and smoking are of great importance as well [figure 2]. Smoking is a another important risk factor for peptic ulcer perforation. smoking more than fifteen cigarettes daily

increased the risk of peptic ulcer perforation 3,5 times. The prevalence of smoking has declined during the last twenty years in most western countries, especially in men, which could perhaps account for fewer ulcer complications.

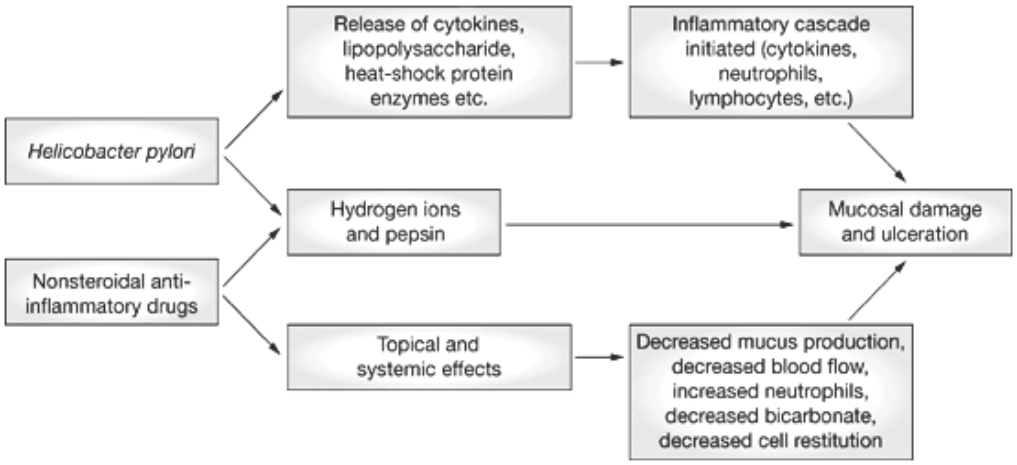

Fig. 2. The synergistic and independent effects of Helicobacter pylori and nonsteroidal anti-inflammatory drugs on gastric mucosal damage.

4. Pathophysiology of perforated peptic ulcer

The duodenal mucosa resists damage from the effect of aggressive factors, such as gastric acid and the proteolytic enzyme pepsin, with the help of several protective factors, such as a mucous layer, bicarbonate secretion, and protective prostaglandins. The epithelial cells of the stomach and duodenum secrete mucus in response to irritation of the epithelial lining and as a result of cholinergic stimulation. A portion of the gastric and duodenal mucus exists in the form of a gel layer, which is impermeable to acid and pepsin. Other gastric and duodenal cells secrete bicarbonate, which aids in buffering acid that lies near the mucosa. Prostaglandins E (PGE) have an important protective role by increasing the production of both bicarbonate and the mucous layer. When an alteration occurs in the aggressive and/or protective factors, a duodenal ulcer occurs such that the balance is in favor of gastric acid and pepsin. Any process that increases gastric acidity (eg, individuals with increased maximal and basal acid output), decreases prostaglandin production (eg, NSAIDs), or interferes with the mucous layer (eg, H pylori infection) can cause such an imbalance and lead to peptic ulcer disease.

Full understanding of the pathophysiology and pathogenesis of duodenal ulcers requires a brief discussion of the two major etiologies: NSAID use and H pylori infection. NSAIDs are pathogenic through their inhibition of the cyclooxygenase-1 (COX-1) pathway, which normally produces protective prostaglandins. These prostaglandins are protective because they augment both bicarbonate and mucous production, as mentioned above. However, perhaps more important, prostaglandins augment mucosal blood flow, and their inhibition leads to impairment of blood flow, leaving the mucosa vulnerable to damage. Infection with H pylori is likely pathogenic by means of a variety of indirect mechanisms as the organism does not generally colonize the duodenum. H pylori infection leads to an inflammatory state

in which high levels of tumor necrosis factor-alpha (TNF-alpha) and other cytokines are produced and in turn stimulate gastric acid production directly by increasing gastrin release from G cells and inhibit somatostatin production by antral D cells. This leads to a net increase in gastric acid secretion, which leads to an increased acid load in the duodenum, overwhelming the mucosal defense. Duodenal acid exposure can lead to gastric metaplasia, whereby the duodenal mucosa can take on characteristics of gastric mucosa. H pylori can then colonize the duodenal mucosa and adhere to cells. This adherence leads to a variety of second-messenger signals, which invoke an immunologic response against those cells causing mucosal damage by host neutrophils and other inflammatory cells. H pylori also affects the gastric and duodenal mucous layer, because this organism produces proteases that degrade the protective mucous layer. Moreover, H pylori infection decreases the production of epidermal growth factor, which normally promotes healing of gastric and duodenal mucosa. H pylori produces proteins that may serve as chemotactic factors for neutrophils and monocytes, which act as proinflammatory cells. H pylori also affects the gastric and duodenal mucous layer, because these organisms produce proteases that degrade the protective mucous layer. H pylori does not lead to the development of gastric and duodenal ulcers through alteration of the bacterial flora. In most cases of perforation, gastric and duodenal content leaks into the peritoneum. This content includes gastric and duodenal secretions, bile, ingested food, and swallowed bacteria. The leakage results in peritonitis, with an increased risk of infection and abscess formation. There are three clinical phases in the process of PPU can be distinguished:

Phase 1: Chemical peritonitis/contamination. The perforation causes a chemical peritonitis. Acid sterilizes the gastroduodenal content; it is only when gastric acid is reduced by treatment or disease (gastric cancer) that bacteria and fungi are present in the stomach and duodenum.

Phase 2: Intermediate stage. After 6–12 h many patients obtain some relief of pain. This is probably due to the dilution of the irritating gastroduodenal contents by ensuing peritoneal exudates.

Phase 3: Intra-abdominal infection. After 12–24 h intra-abdominal infection supervenes

Subsequent third-spacing of fluid in the peritoneal cavity due to perforation and peritonitis leads to inadequate circulatory volume, hypotension, and decreased urine output. In more severe cases, shock may develop. Abdominal distension as a result of peritonitis and subsequent ileus may interfere with diaphragmatic movement, impairing expansion of the lung bases. Eventually, atelectasis develops, which may compromise oxygenation of the blood, particularly in patients with coexisting lung disease.

5. Prognostic factors

The continuing problem with perforated duodenal ulcer stands in contrast to the fall in admissions for uncomplicated duodenal ulcers noted since the 1970's and largely attributed to the introduction of H 2 antagonists. The high incidence of complications necessitates the identification of factors associated with the morbidity and mortality of patients undergoing surgery for perforated peptic ulcer. The patient population with perforation tends to be elderly ; mean age 60–70, chronically ill and those patients often taking ulcerogenic medication. Mortality rate after surgery for perforated duodenal ulcer is much more higher in the elderly that reach up to 50%. This can be explained by the occurrence of concomitant

medical diseases but also by difficulties in making the right diagnosis resulting in a delay of >24 hours. The longstanding perforation more than 24 hours together with major medical illness and preoperative shock collectively predicted the outcome in patients with perforated duodenal ulcer as [table 2].

RISK FACTORS	SCORE
1.Numbers of hrs since ulcer perforation	
24hrs or less	0
More than 24 hrs	1
2.Pre operative systolic BP (mm of Hg)	
100 or more	0
Less than 100	1
3.Any one or more systemic illness	
Absent	0
Present	1

Table 2. Boey score-risk factor to predict mortality

The mortality rate increased progressively with increasing numbers of risk factors: 0%, 10%, 45.5%, and 100% in patients with none, one, two, and all three risk factors of Boey, respectively. Definitive surgery can be done safely in good-risk patients. Simple closure is preferable in those patients with uncomplicated perforations if any risk factor is present. Truncal vagotomy and drainage may be required if there is coexisting bleeding or stenosis. Nonoperative treatment deserves re-evaluation in patients with all three risk factors because of their uniformly dismal outcome after operation.

6. Clinical course of PDU in elderly

Studies have shown that nearly half of patients presenting with complicated peptic ulcer disease (PUD), have no history of the disease. On endoscopy, unsuspected ulcers have been found in people who were taking nonsteroidal anti-inflammatory drugs (NSAIDs). Two courses of the disease were observed: the first is defined by acute disease of less than 24 hours' duration preceding surgery. Classic patients with perforated peptic ulcer disease usually present with a sudden onset of severe sharp abdominal pain that may be generalized pain or epigastric urging these patients assuming a fetal position. Abdominal examination findings are usually consistent with generalized tenderness, rebound tenderness, guarding, and rigidity. However, the degree of peritoneal findings is strongly influenced by a number of factors, including the size of perforation, amount of bacterial and gastric contents contaminating the abdominal cavity, time between perforation and presentation, and spontaneous sealing of perforation. Accordingly, the second course of perforation is of longer duration, starting with various abdominal complaints and presenting more severely only after the first 24 hours. Patients belonging to the second course may also demonstrate signs and symptoms of septic shock, such as tachycardia, hypotension, and anuria, but these indicators of shock may be absent in elderly patients or

in those with other systemic illness. In recent years, patients presenting with perforated duodenal ulcers have tended to be elderly and chronically ill and taking one or more ulcerogenic drugs. Several studies have shown the mean age of such patients to be more than 60 years. In elderly patients, signs and symptoms may be minimal. In patients over age 60 with perforated ulcer, more than 80 % had only mild abdominal pain. Other reported symptoms were dyspepsia, anorexia, nausea, and vomiting. Severe abdominal pain was present in only less than 20 % of patients. Duration of symptoms is usually protracted and delayed. Although minority of those patients had no abdominal findings, most had abdominal tenderness, with up to two thirds having classic signs of peritonitis.. There is a changing scene with perforated peptic ulcer. The older age of presentation, the increased association with non-steroidal anti-inflammatory drugs, associated increased debility, and resulting higher mortality in the elderly, are causing a rethink in management protocols.

7. Management protocol

There are two main accepted regimen of treatment of perforated duodenal ulcer; non-operative and surgical treatment. Non-operative treatment should be rendered in perforated peptic ulcers only when the patient shows definite signs of improvement both symptomatically and clinically, and there is a definite "walling off" of the ulceration, or when the patient's condition is too poor to permit operation. With good operative risk, patients should be prepared for surgery of ulcer-definitive procedure of the surgeon's choice; for example vagotomy and pyloroplasty or antrectomy. Purulent peritonitis would dictate only secure closure of the perforation [table 2].

7.1 Nonoperative treatment

The introduction of novel peptic ulcer drugs, such as H2 receptor blockers and proton pump inhibitors, caused a prompt decline in elective operations for peptic ulcer disease in recent times. On the other hand, surgery for peptic ulcer complications, such as perforations has not changed. Effective medical management of peptic ulcer disease has reduced the incidence of gastric outlet obstruction as a complication, but perforation especially in the elderly remains unchanged and is, in fact, on the increase. There is a changing trend in emergency surgery for perforated duodenal ulcer from definitive anti ulcer surgery to simple closure followed by Helicobacter pylori eradication. Surgical emergency due to a perforated peptic ulcer – whether treated laparoscopically or by open repair – is associated with a significant postoperative morbidity and mortality. Therefore, risk-stratification of these subjects provides surgeons with an important tool to plan the management. The dominant treatment of perforated duodenal ulcer in the first half of the 20th century was surgical closure. In most perforated duodenal ulcers that were successfully surgically closed, the perforation was a harbinger of subsequent major morbidity from peptic ulceration. This was in the form of re-perforation, hemorrhage, obstruction, or intractability. The major concern against simple closure is the possible risk of future serious complications of relapse. Some authors claim that prognosis is not related to the surgical procedure itself and the current policy is not to perform definitive ulcer surgery in cases of PPU. A simple procedure should be the one of choice for an emergency operation and extensive procedures should be reserved only in selected patients despite good results are obtained with simple procedures.

Several facts support an alternative to the currently accepted therapy of the perforated duodenal ulcer, that is, immediate surgical closure of the perforation with or without an ulcer-definitive procedure. The following facts are included:

1. Most ulcers are associated with infection with H pylori, including ulcers that perforate.
2. Almost all ulcers associated with H pylori can be healed with combined medical therapy; ie, antibiotics and proton-pump inhibitors or H2 blockers. The rate of relapse is very low and re-infection is rare.
3. The administration of H2 blockers and proton-pump inhibitors and elimination of NSAIDs are now essential components of medical therapy. Such therapy has favorably affected the natural history of duodenal ulcers, including those that perforate.
4. Approximately half of duodenal ulcers that perforate will have self-sealed when first seen by the physician.
5. The perforation of a duodenal ulcer that has sealed spontaneously can be treated nonoperatively with low morbidity, including releakage and abdominal abscess.
6. Death due to peritonitis reflects protracted leakage and secondary bacterial contamination.
7. Major associated disease is a significant risk factor for death following perforation of a duodenal ulcer.

7.1.1 Principles of conservative treatment

Principles of conservative treatment include nasogastric suction, pain control, antiulcer medication, and antibiotics. Nonsurgical treatment has been recognized for a long time. The first major series was published by Taylor nearly 50 years ago; it reported a mortality rate of 11% in the nonsurgical treatment group, compared to 20% in the surgical group. Since then, because of improvements in operative and postoperative care, the mortality rate with surgical treatment of perforated peptic ulcer has decreased to about 5%. Failure of conservative treatment is generally defined as development of septic shock, multiple organ failure or intra-abdominal abscess. Conservative treatment failure exposes patients to the risk of delayed surgical closure with mortality rates between up to 50% , depending on the timing of secondary surgery. While conservative treatment was first proposed to patients not eligible for surgery, some few investigators have tried this approach in rather fit patients but in fact these studies have reported high mortality compared to the results achieved by surgical repair in elderly or medically frail patients. The systematic introduction of PPI use and HP eradication seems to have favorably influenced the results of conservative therapy through reduction of mortality.

7.1.2 Failure of conservative treatment

Definition of prognostic factors for conservative treatment has been a concern for all investigators. The presence of shock at admission is a major criterion for conservative treatment failure and implies that, even in a moribund patient. The presence of haemodynamic instability militates in favor of prompt surgery. The presence of shock being one of the Boey criteria, has a strong correlation of mortality.

7.2 Surgical Therapy

Surgery is recommended in patients who present with hemodynamic instability, signs of peritonitis and free extravasation of contrast on upper gastrointestinal contrast studies. If

contamination of the upper abdomen is minimal and the patient is stable, a definitive ulcer procedure can be performed. For a perforated duodenal ulcer, this may include a highly selective vagotomy, a truncal vagotomy and pyloroplasty, or vagotomy and antrectomy.

7.2.1 Preoperative preparation

Fluid resuscitation should be initiated as soon as the diagnosis is made. Essential steps include insertion of a nasogastric tube to decompress the stomach and a Foley catheter to monitor urine output. Intravenous infusion of fluids is begun, and broad-spectrum antibiotics are administered. In select cases, insertion of a central venous line for accurate fluid resuscitation and monitoring. As soon as the patient has been adequately resuscitated, emergent exploratory laparotomy should be performed.

7.2.2 Surgical procedures

a. Laparoscopic Surgery

The traditional management of a perforated duodenal ulcer is closure with omental patch and a thorough abdominal lavage. More recently this has been shown to be able to performed using a laparoscope. The only proven advantage of the laparoscopic technique appears to be decreased postoperative pain. Operating times are longer compared to open techniques and hospital time appears to be similar to conventional treatment.

b. Immediate Definitive Surgery

Attempts have been made to improve upon the results of simple closure and lavage in response to the large number of patients more than 25% continue to have symptoms attributable to their ulcer diathesis after surgery. Since the 1940's the concept of immediate definitive ulcer surgery has been raised but debated amongst surgeons. There is good evidence that, in the emergency situation, highly selective vagotomy combined with simple omental patch closure of the perforation, in patients without the risk factors, is just as effective as that performed in the elective setting with less mortality and ulcer recurrence rate. Truncal vagotomy with drainage has its advocates as an expedient operation familiar to most surgeons. Immediate definitive ulcer surgery has not gained widespread popularity as it is associated with a higher mortality than simple closure in patients at risk of suffering from complications of surgery. Many agree that an appropriate approach is to select only those with a chronic history of ulcer disease for more than 3 months and without preoperative risk factors for immediate definitive surgery. A major difficulty is defining preoperatively the patients with chronic ulcer history as many ulcers showed silent history, many patients are too unwell to give a reliable history of their disease and finally, perforations occurs as the first manifestation of the ulcer diathesis.

7.3 Percutaneous peritoneal drainage

The higher mortality rate in the old population, justifies the search of prognostic factors specific for the elderly in whom the difficult management was attributed to their concomitant diseases. The criteria of Taylor's method in selected cases were diagnosis of perforation in less than 12 hours, with stable hemodynamic condition and age not exceeding 70 years. Emergency abdominal operations are commonly performed and carry high morbidity and mortality risk, particularly in elderly patients due to presence of coexisting

cardiopulmonary disease, late admission and presence of peritonitis. So, in high risk elderly patients with perforated duodenal ulcer and established peritonitis, pus should be drained with the least invasive maneuver. Transnasogastric placement of a drainage catheter through the perforated ulcer was said to be as successful as definitive therapy. High-risk peptic ulcer perforation patients can be managed by putting in an intra-abdominal drain supported by conservative treatment with reduced death rate and patients improvements.

7.3.1 Operative technique

In conjunction with conservative measures, percutaneous peritoneal drainage was performed under local anaesthesia through a 3- cm long skin incision at the level of right anterior superior iliac spine and the lateral edge of the rectus muscle. The incision spitted the external oblique aponeurosis, internal oblique and transversus abdominus along the direction of their fibers. Upon interning the peritoneal cavity, the index finger was swiped in all direction to allow protection and good drainage. A wide bored percutaneous intra-abdominal drain.

8. Aims and concerns

Type of surgery	**Morbidity**	**Total no. (%)**
Simple closure (*n* = 29)	a. Pulmonary embolism (1)	8 (27.5)
	b. Septicemia (4)	
	c. Respiratory failure (1)	
	d. Wound infection (1)	
Closure + Acid reduction procedure (*n* = 8)	a. Stomal obstruction (1)	3 (37.5)
	b. Post operative anastamotic leak with septicemia (1)	
	c. Gastric fistula (1)	
Gastric resection (*n* = 17)	a. Wound infection (1)	6 (35.2)
	b. Duodenal blow out (1)	
	c. Respiratory failure (4)	

Table 3. Morbidity related to type of surgery.

Perforated peptic ulcer disease continues to inflict high morbidity and mortality. Although patients can be stratified according to their surgical risk, optimal management has yet to be described. The accepted therapeutic options in patients with perforated peptic ulcer are simple closure or immediate definitive operation. The non-operative management of perforated peptic ulcer has previously been shown to be both safe and effective although it remains controversial. Taylor's conservative treatment, originally proposed for the treatment of choice in perforated acute peptic ulcer in 1951. Today it is reserved for patients considered to be too ill to stand the stress of surgery or in situations where immediate

surgery is unavailable. Minimal surgical intervention (percutaneous peritoneal drainage) can significantly lower the mortality rate among a selected group of critically ill, poor risk patients with perforated peptic ulcer disease.

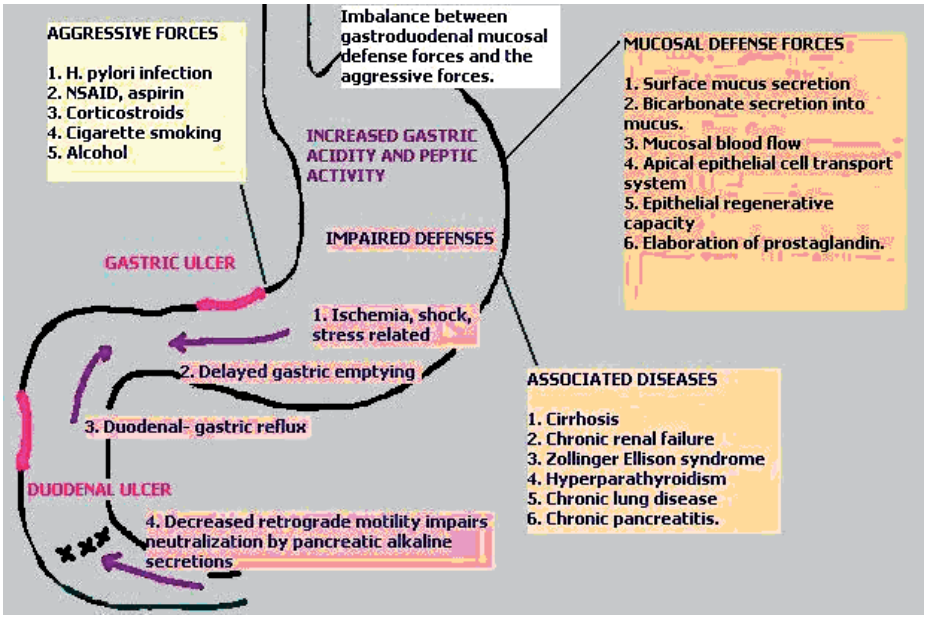

Fig. 3. Shows the imbalance between aggressive factors and protective factors in peptic ulcer disease.

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16

Management of Acute Gastric Ulcer Bleeding

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1. Introduction

Acute gastric ulcer bleeding frequently presents as a gastrointestinal emergency. It has important implications for healthcare costs worldwide. Negative consequences include rebleeding and death usually caused by the functional worsening of concomitant medical conditions, precipitated by the acute bleeding incident. Advances in medical practice in recent decades have influenced the aetiology and management of upper gastrointestinal bleeding (UGIB), but their impact on the incidence and mortality is unclear.

2. Epidemiology

At one time peptic ulcer disease accounted for 50–70% of acute non-variceal UGIB (Barkun et al., 2004). Approximately 80% of these ulcers stop bleeding spontaneously. Gastric ulcer is more frequently the source of UGIB (55% versus 37%); compared to duodenal ulcer (Enestvedt et al., 2008). The current practice to use proton pump inhibitors as ulcer prophylaxis and eradication of *Helicobacter pylori*, has led to a worldwide decrease in the incidence of bleeding from peptic ulcer. However, this seems applicable only to patients younger than 70 years of age (Lanas et al., 2005; Loperfido et al., 2009; Targownik et al., 2006). Recent population-based estimates have suggested that the incidence is about 60 per 100,000 of the population (Lassen et al., 2006), with the incidences related to the use of aspirin and non-steroidal anti-inflammatory drugs on the increase (Ohmann et al., 2005). The mortality associated with peptic ulcer bleeding remains high at 5 to 10% (Lim et al., 2006). The estimated direct medical costs annually incurred in the United States for the in-hospital care of patients with peptic ulcer bleeding amounts to a total of more than $2 billion (Viviane et al., 2008).

3. Pathophysiology

3.1 Risk factors

There are four major risk factors for bleeding peptic ulcers namely *Helicobacter pylori* infection, non-steroidal anti-inflammatory drugs (NSAIDs), stress and gastric acid (Hunt et al., 1995; Hallas et al., 1995). Reduction or elimination of these risk factors lessens ulcer recurrence and rebleeding rates (Graham et al., 1993; Tytgat 1995).

3.1.1 *Helicobacter pylori*

Compared to non-bleeding duodenal ulcers (70–90%), *Helicobacter pylori* plays a lesser role in the aetiology of bleeding and gastric ulcers (Maury et al., 2004). However, it is important to exclude *Helicobacter pylori* as a factor in the aetiology.

Helicobacter pylori eradication should be attempted in all peptic ulcer patients diagnosed with the infection to prevent ulcer recurrence and rebleeding (Hopkins et al., 1996). In Hopkin's report of 19 published studies, the recurrence rates in cured versus uncured *Helicobacter pylori* infection was 6% versus 67% for duodenal ulcer, and 4% versus 59% for gastric ulcer. Various regimens that usually combine one or two antibiotics plus an anti-secretory agent have eradication rates that vary between 80% and 90% (Walsh et al., 1995).

3.1.2 Non-steroidal anti-inflammatory drugs

NSAIDs, including aspirin, frequently cause gastrointestinal ulceration (Lanas et al., 2005; Scheiman 1994). NSAID-induced injury results from both local effects and systemic prostaglandin inhibition effected by blocking cyclooxygenase-1. The majority of these ulcers are asymptomatic and uncomplicated. However, elderly patients with a prior history of bleeding ulcer disease are at increased risk for recurrent ulcer and complications (Hansen et al., 1996; Smalley et al., 1995). NSAIDs are also implicated as critical in the non-healing of ulcers (Lanas et al., 1995). Aspirin in dosages as low as 75 mg daily transfer an increased risk of ulcers and bleeding (Lim et al., 2004).

Combining corticosteroids with NSAIDs doubles the risk of ulcer complications whilst the risk of gastrointestinal bleeding is increased ten fold (Piper et al., 1991). Cyclooxygenase-2 inhibitors reduce the risk of ulcer bleeding only when not combined with aspirin therapy. Of concern, is the increase in incidence of myocardial infarction and cerebrovascular accidents in patients taking selective cyclooxygenase-2 inhibitors. The combination of *Helicobacter pylori* infection and NSAID use may increase the risk of ulcer bleeding; however, the need for eradication of *Helicobacter pylori* in patients who are taking NSAIDs remains controversial. (al-Assi et al., 1996).

3.1.3 Stress-related ulcers

The incidence of stress-related ulcers in intensive care units (ICU) is approximately 0.67. This form of ulceration tends to occur in severely ill patients and is almost certainly triggered by ischaemia due to a combination of decreased mucosal protection and reduced mucosal blood flow (Cooper et al., 1999). It is a frequent cause of acute UGIB in patients who are hospitalized for life-threatening non-bleeding illnesses (Navab et al., 1995). The risk of stress ulcer-related bleeding is increased in patients with respiratory failure and those with a bleeding disorder (Cook et al., 1994). Also, the mortality is higher in patients that present with a UGIB after hospitalization compared to those primarily admitted with UGIB (Zimmerman et al. 1994).

Primary ulcer prophylaxis with anti-secretory agents such as H_2–receptor antagonists or proton pumps inhibitors (PPIs) decreases the risk of stress-related mucosal damage and UGIB in high-risk patients (Cook et al., 1996). Achlorhydria associated with prophylactic acid inhibition effects bacterial growth in the stomach and possible ventilator-associated pneumonia in ICU patients. Furthermore, stress-related ulcers tend to have high rebleeding rates and are not as amenable to endoscopic therapy as patients that present to the hospital with bleeding peptic ulcer (Jensen et al., 1988).

3.1.4 Gastric acid

Gastric acid and pepsin are essential cofactors in the pathogenesis of peptic ulcer (Peterson et al., 1995). Factors such as *Helicobacter pylori*, NSAIDs, or physiologic stress impair the mucosal integrity leading to increased cell membrane permeability and back diffusion of hydrogen ions, resulting in intramural acidosis, cell death, and ulceration. Hyperacidity as is prevalent in patients with Zollinger-Ellison syndrome, is rarely the sole cause of peptic ulceration. However, control of gastric acidity is considered an essential therapeutic manoeuvre in patients with active UGIB.

4. Acute management (Figure 1)

Patients with acute gastric ulcer bleeding frequently present with haematemesis (vomiting of red blood that is suggestive of active bleeding or vomiting of coffee-ground material indicative of older non-active bleeding) and/or melaena (black tarry stools which suggests passage of old blood, usually from an upper gastrointestinal source). Haematochezia (the passage of red blood per rectum) can occasionally be due to massive UGIB as suggested by a hypotensive or shocked patient. Patients who presents with haematemesis and melaena generally have more severe bleeding than those who present with melaena only. Immediate evaluation and appropriate resuscitation are critical as these can reduce mortality in acute UGIB (Baradarian et al., 2004).

4.1 Resuscitation and stabilization

As first priority, the haemodynamic stability (pulse and blood pressure, including orthostatic changes) and the need for fluid replacement must initially be assessed at presentation of a patient with UGIB. A full blood count, urea, electrolytes, creatinine, international normalized ratio (INR), blood type and cross-match should be obtained. If indicated, volume resuscitation should be initiated with crystalloids and blood products in all patients with haemodynamic instability or active bleeding (manifested by haematemesis, bright red blood per nasogastric tube, or haematochezia). Patients with a resting tachycardia ≥ 100 beats per minute, a systolic blood pressure < 100 mmHg, orthostatic hypotension (an increase in the pulse rate ≥ 20 beats per minute or drop in blood pressure of ≥ 20 mmHg on standing), a decrease in haematocrit of ≤ 6%, or transfusion requirement over two units of packed red blood cells) should be admitted to an intensive care unit for resuscitation. The haemoglobin in high-risk patients should be maintained above 10 g/dL, whereas a haemoglobin ≥ 7 g/dL is acceptable in young and otherwise healthy individuals. Patients with active bleeding and a bleeding disorder should be transfused with plasma and platelets if the INR ≥1.5 and the platelets ≤ 50 000/μL respectively.

The vital signs (blood pressure, ECG monitoring, and pulse oximetry), clotting profile and urinary output should be closely monitored. If indicated, elective endotracheal intubation in patients with respiratory failure and decreased consciousness may facilitate endoscopy and decrease the risk of aspiration. Patients older than 60 years, with chest pain or a history of heart disease should also be evaluated for myocardial infarction with electrocardiograms and serial troponin measurements. Nasogastric (NG) tube placement to aspirate and characterize gastric contents can be useful to determine if large amounts of red blood, coffee - grounds, or non-bloody fluid are present. Patients with definite haematemesis do not need an NG tube for diagnostic purposes, but may need one to clear gastric contents before

endoscopy and to minimize the risk of aspiration. Approximately 15% of patients without bloody or coffee-ground material in nasogastric aspirates are found to have high-risk lesions on endoscopy (Aljebreen et al., 2004).

Clinical and laboratory findings are useful to risk-stratify patients (Table 1). The Blatchford score or the clinical Rockall score have been validated as clinical tools in the risk assessment (Blatchford et al., 2000; Rockhall et al., 1996). Poor prognostic factors for bleeding peptic ulcers include the following: age >60 years, comorbid medical illness, orthostatic hypotension, bleeding disorder, bleeding onset in the hospital, multiple blood transfusions and red blood in the NG tube.

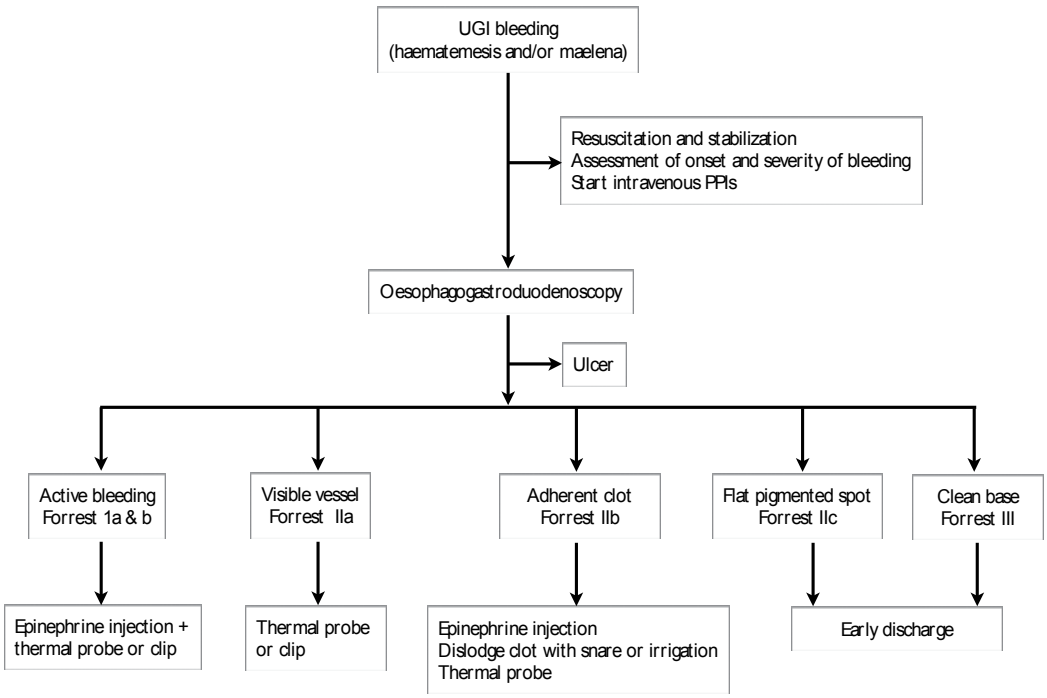

Fig. 1. Approach to upper gastrointestinal (UGI) bleeding. PPIs, proton pump inhibitors.

4.2 Diagnostic endoscopy

Its high sensitivity and specificity in identifying and localizing bleeding lesions, makes upper endoscopy the diagnostic modality of choice for acute UGIB. Early endoscopy within 24 hours of presentation, aids risk stratification of patients and reduces the need for hospitalization. However, it may also expose additional cases of active bleeding and hence increases the use of therapeutic endoscopy. No evidence exists that very early endoscopy (within a few hours of presentation) can reduce risk of rebleeding or improve survival (Tsoi et al., 2009).

A large channel therapeutic upper endoscope should be used to allow for rapid removal of blood from the stomach and to utilize larger endoscopic hemostasis accessories. Well–trained assistants who are familiar with endoscopic hemostasis devices are critical to successful endoscopic hemostasis. At times it may be worth delaying a procedure in order to utilize assistants who are competent at using accessories in emergency situations. Forrest

described an endoscopic classification system that is commonly used (Figure 2). At index endoscopy the prevalence of ulcers with stigmata of recent haemorrhage, defined as Forrest I, IIa and IIb generally accounts for one third and Forrest IIc or III for the remainder (Lau et al., 1998) (Figure 2). An adherent clot is defined as a lesion that is red, maroon, or black and amorphous in texture which cannot be dislodged by suction or forceful water irrigation) (grade IIb). Low-risk lesions include flat, pigmented spots (grade IIc) and clean-base ulcers (grade III) (Figure 3). The inter-observer variation in diagnosing these endoscopic stigmata is low to moderate. At index endoscopy, high-risk lesions with rebleeding rates from 22% to 55% are seen in one–third to one–half of all patients.

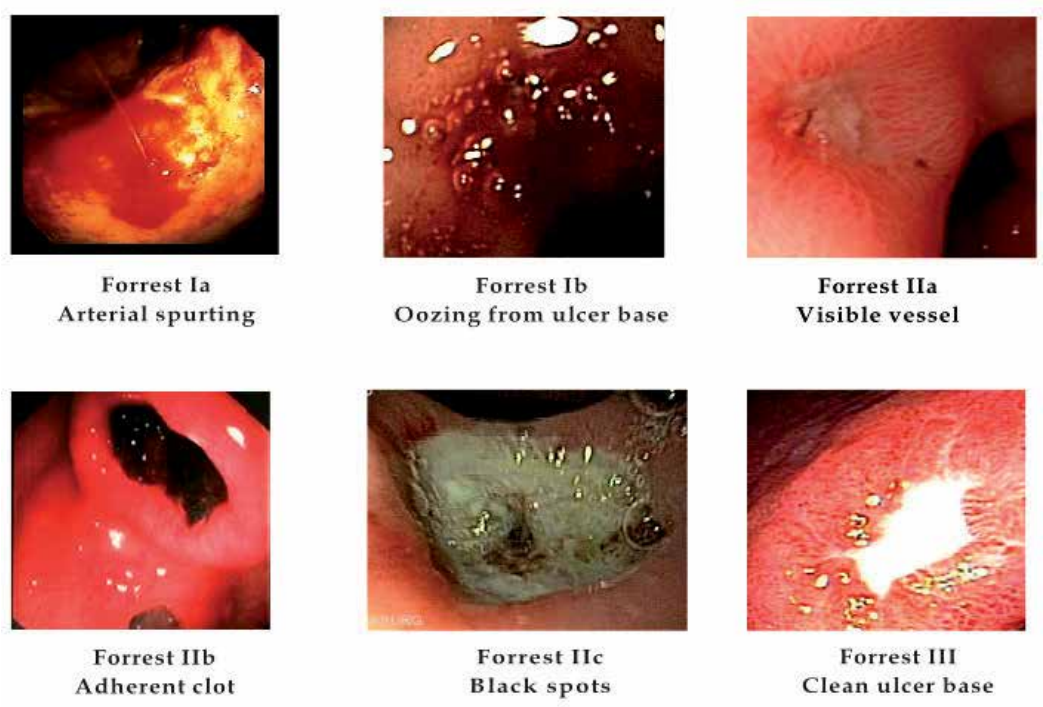

Fig. 2. Endoscopic grading according to Forrest classification

4.2.1 Preparation for emergency esophagogastroduodenoscopy

A large-bore orogastric or NG tube with gastric lavage (use tap water at room temperature) is useful to improve visualization of the gastric fundus on endoscopy; however, this practice has not predictably improved the outcome (Lee et al., 2004). Intravenous erythromycin, as a motilin receptor agonist, promotes gastric motility and substantially improves visualization of the gastric mucosa at index endoscopy. However, erythromycin does not substantially improve the diagnostic yield of endoscopy or the outcome of acute peptic ulcer bleeding. A single 250–mg dose of erythromycin 30–60 minutes before endoscopy should be considered (Carbonell et al., 2006).

Empiric intravenous PPI treatment can be initiated prior to endoscopy in patients that presents with severe UGIB. Several studies and meta-analyses have shown that this practice significantly reduces the proportion of patients with stigmata of recent bleeding at index

endoscopy and therefore the need for endoscopic therapy. However, there is no evidence that PPI treatment affects clinically important consequences, namely mortality, rebleeding or need for surgery (Sreedharan et al., 2010).

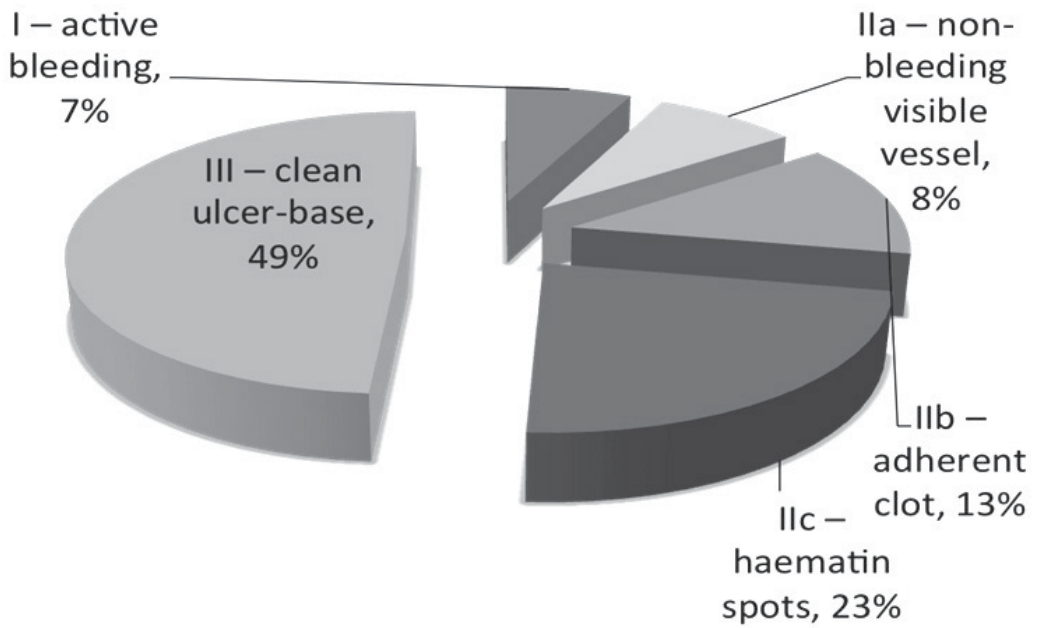

Fig. 2. Stigmata of bleeding prevalence according to the Forrest classification

4.2.2 Stratification of the rebleeding risk

Rebleeding the vital risk factor for mortality increases the rate 5 times compared to patients in whom the bleeding has spontaneously stopped (Church et al., 1999; Forrest et al., 1974). Predictive models evolved to identify high-risk patients for rebleeding and those for early hospital discharge or outpatient care.

The Rockall scoring system is probably the most widely known risk-stratification tool for UGIB. This represents an accurate and validated predictor of rebleeding and death, achieving better results in the prediction of mortality (Rockall et al., 1996). The clinical Rockall score (i.e. the score before endoscopy) is calculated solely on the basis of clinical variables at the time of presentation. For the complete Rockall score the clinical and endoscopic stigmata to predict the risks of rebleeding and death are added; the scale ranges from 0 to 11 points, with higher scores indicating greater risk.

The clinical Rockall and Blatchford scores share mutual features that include the patient's hemodynamic status and comorbid illnesses. These might reduce the need for urgent endoscopic evaluation in patients deemed at low risk. In addition to clinical and laboratory features, endoscopic stigmata can be used to risk-stratify patients that present with acute gastric ulcer bleeding (Table 1).

The endoscopic stigmata of bleeding gastric ulcers provide excellent predictability of the likelihood of rebleeding based on the Forrest classification, which ranges from Ia to III. The risk for rebleeding varies from 55 – 22% in gastric ulcers if left endoscopically untreated

(Laine & Peterson, 1994; Lau et al., 1998). The highest risk is in those with active arterial bleeding (grade I), a non-bleeding visible vessel (grade IIa) and an adherent clot (grade IIb). Additional data are needed to confirm the possible improvement in risk stratification provided by the use of endoscopic Doppler ultrasonography applied directly to the ulcer stigmata.

A. Blatchford Score – At Presentation	Score	B. Rockall Initial Score /7 – Criteria (before gastroscopy)		Score
Systolic Blood Pressure				
• 100 – 109 mmHg	1	Age (years)	• < 60	0
• 90 – 99 mmHg	2		• 60 – 79	1
• <90 mmHg	3		• ≥ 80	2
Blood urea nitrogen				
• 6.5 – 7.9 mmol/L	2	Shock	• "No shock" = (SBP ≥100 mmHg, Pulse <100 /min)	0
• 8.0 – 9.9 mmol/L	3		• "Tachycardia" = (SBP ≥100 mmHg, Pulse ≥100 /min)	1
• 10.0 – 24.9 mmol/L	4		• "Hypotension" = (SBP <100 mmHg)	2
• ≥25 mmol/L	6			
Haemoglobin for men		Co-morbidity	• No major co-morbidity	0
• 12.0 – 12.9 g/dL	1		• Cardiac failure, IHD or any major co-morbidity	2
• 10.0 – 11.9 g/dL	3		• Renal or liver failure, disseminated malignancy	3
• <10.0 g/dL	6	Rockall Full Score /11 – Additional Criteria (after gastroscopy)		
Haemoglobin for women				
• 10.0 – 11.9 g/dL	1	Diagnosis	• Mallory–Weiss tear, no lesion seen, no SRH	0
• <10.0 g/dL	6		• All other diagnosis	1
Other presentation variables			• Malignancy of upper GI tract	2
• Pulse >100/minute	1			
• Maelena	1	Major stigmata of recent haemorrhage	• None – clean ulcer base, flat pigmented spot	0
• Syncope	2		• Blood in upper GI tract, active bleeding, visible vessel, clot	2
• Hepatic disease	2			
• Cardiac failure	2			

Table 1. Risk–stratification tools for UGIB

4.3 Therapeutic endoscopy

Gastric ulcers with a high risk of rebleeding should be treated endoscopically at the initial endoscopy. Since the late 1980s, endoscopic haemostatic therapy has been widely accepted as the first-line therapy for UGIB. Many well–conducted, randomized controlled trials, meta-analyses, and consensus conferences have confirmed the efficacy of endoscopic therapy in this setting (Sacks HS et al. 1990; Cook DJ et al., 1992). These data supported a reduction in recurrent bleeding, the need for urgent surgery, and mortality in patients with high–risk stigmata (Barkun et al., 2003; Adler et al., 2004). However, most of these studies were conducted before the widespread use of PPIs, and predominantly used injection therapy, bipolar-probe coagulation therapy, or a combination of injection and coagulation therapy.

In general, for the highest-risk lesions of active bleeding or non-bleeding visible vessels, endoscopic haemostasis alone will decrease the rebleeding rate to approximately 20–25%. The adjunctive use of PPIs decreases this rate even further. Endoscopic therapy can be broadly categorized into injection therapy, thermal coagulation, and mechanical haemostasis. As no single method of endoscopic thermal coagulation therapy is necessarily superior. Therefore, a familiar haemostatic technique applicable to the identified ulcer stigmata should be used.

4.3.1 Injection therapy

Injection therapy is the most commonly used treatment worldwide, mainly because it is widely available, easy to perform, safe and inexpensive. A disposable needle is used to inject a solution (1:10,000) of diluted adrenaline in normal saline. This mainly has a tamponade-

effect induced by the volume of solution injected (15–25 ml being a standard dose). Although solutions of agents other than adrenaline (such as polidocanol, saline and even dextrose) may have a similar effect, none proofed superior in achieving haemostasis. The injection of sclerosant (including absolute alcohol) should be avoided as extensive and uncontrolled tissue necrosis of the ulcer base can lead to perforation and related complications. Adrenaline injection as definite haemostatic therapy is not recommended for the risk of rebleeding, but it should be followed either by contact thermal therapy or a second injectable agent (e.g. fibrin glue) to avoid further bleeding, the need for surgery and mortality in bleeding peptic ulcer (Vergara et al., 2007). This practice reduces the risk of perforation and subsequent thermal burn damage that might complicate endoscopic therapy.

4.3.2 Thermal devices

Thermal devices are the mainstay of endoscopic haemostasis and can be divided into contact (heater probe, monopolar and bipolar electrocoagulation) and noncontact types (laser treatment, argon plasma coagulation [APC]). Although no single method of endoscopic thermal coagulation therapy is superior, electrocoagulation with bipolar contact probes is more commonly used. Haemostasis of the underlying vessel is achieved when heat is generated during contact of these probes with the bleeding lesion. Thermal contact probes can seal arteries up to 2 mm. The risks of thermal probes include perforation and inducing more bleeding. While the haemostatic effects of contact probes are well established by clinical trials, the use of APC in the treatment of peptic ulcer bleeding has only recently been reported. In a randomised, controlled study comparing APC with heater probe coagulation, the former proofed equally safe and effective (Chau et al., 2003). No significant differences were detected in terms of initial haemostasis at index endoscopy, frequency of recurrent bleeding, requirement for emergency surgery, number of units of blood transfused, length of hospital stay, and mortality rate.

4.3.3 Mechanical devices

Mechanical devices in the form of haemoclips for endoscopic haemostasis in bleeding gastric ulcer disease have gained popularity in recent years. In a landmark study by Cipolleta and colleagues, they compared haemoclips with heater probe thermocoagulation (Cipolletta et al., 2001). The successful application of haemoclips led to a significantly decline in recurrent bleeding (1.8% versus 21%). Deployment of haemoclips on fibrotic ulcer floors may proof problematic, especially when used tangentially, or with the endoscope retroflexed. The difficulty of successful application in these situations may limit the efficacy of haemoclips. These technical problems might be overcome with improvements in future design.

4.4 Control of active bleeding or high-risk lesions

Despite many endoscopists favouring dual endoscopic therapy in patients with severe peptic ulcer bleeding, there is currently no definite recommendation in this regard. In actively bleeding ulcers, an injection can diminish or even stop bleeding; allowing a clear view of the bleeding vessel that in turn facilitates accurate thermal coagulation. Theoretically, the cessation of blood flow prevents dissipation of thermal energy, thereby minimizing tissue injury.

In a systematic review and meta-analysis dual endoscopic therapy proved significantly superior to injection therapy alone. However, it had no advantage over thermal or mechanical monotherapy to improve the outcome of patients with high-risk peptic ulcer bleeding (Marmo et al. 2007). When combining injected substances with thermal coagulation in bleeding peptic ulcer disease, there is a significant risk of complications such as perforation and gastric wall necrosis. Successful application of haemoclips is comparable to thermocoagulation (Sung et al. 2007).

4.5 Managing an ulcer with an adherent clot

In the event of an ulcer with an overlying clot, attempting to remove the clot by targeted washing is critical. Endoscopic removal of the clot by washing or cold snare has been demonstrated to be effective in reducing the recurrence of bleeding (Bini et al., 2003). The findings under the clot (e.g. bleeding vessel, visible vessel, flat spot, clean base) help determine the therapy needed and improve efficacy by allowing treatment to be applied directly to the vessel. A combination of injection with heater probe or bipolar coaptive coagulation is often used and has been shown to be more effective in patients with active bleeding. Vigorous washing of the clot formed after therapy is useful to determine the adequacy of coagulation.

4.6 Treatment of persistent or recurrent bleeding after initial haemostasis

Despite the effectiveness of endoscopic haemostasis, rebleeding occurs in 10–25% of cases, irrespective of the method of treatment. A second attempt at endoscopic control is warranted. Some experts have concerns about the perils of a second endoscopy, which may result in delayed surgery, perforation, and increased morbidity and mortality. Combining techniques is sensible when re-treating the ulcer site as the first attempt at endoscopic therapy might have produced necrosis and weakening of the intestinal wall. By using injection as the first step the thickness of the submucosal layer is increased, thus providing some margin of safety.

4.7 Second-look endoscopy

A planned, second-look endoscopy within 24 hours after initial endoscopic therapy is not recommended on the basis of existing evidence (Barkun et al. 2003; Adler et al. 2004). Even though it proofed to be efficacious in two meta-analyses that second–look endoscopy with heater probe coagulation reduces the risk of recurrent bleeding, it had no overall effect on mortality or the need for surgery (Marmo et al. 2003; Tsoi et al. 2009). Also, this approach may not be cost-effective when profound acid inhibition is achieved by high-dose intravenous PPIs (Spiegel et al. 2003). Second–look endoscopy may be considered in patients who are categorized as high risk for rebleeding (shock at presentation, fresh blood in the stomach, endoscopic stigmata of active bleeding, large ulcers and high lesser curvature gastric ulcers) if adjunctive high–dose intravenous PPI was not commenced. Similarly, if at the time of index endoscopy clots obscured the endoscopic view or the efficacy of the primary endoscopic haemostasis is doubtful, second-look endoscopy may be indicated (Chiu & Sung, 2010).

4.8 *Helicobacter pylori* testing

As one of the main etiological risk factors, all patients with acute bleeding gastric ulcers should be tested for *Helicobacter pylori* infection. Confirmatory testing for *Helicobacter pylori*

in the setting of acute ulcer bleeding may be false negative. Biopsy-based methods, such as rapid urease test, histology, and culture, have a low sensitivity, but a high specificity, in patients with UGIB. The accuracy of ^{13}C-urea breath test remains very high under these circumstances. Stool antigen test is less accurate in UGIB. Although serology seems not to be influenced by UGIB, it cannot be recommended as the initial diagnostic test for *Helicobacter pylori* infection in this setting (Gisbert et al., 2006). Treatment of *Helicobacter pylori* infection is more effective than anti–secretory non-eradicating therapy (with or without long-term maintenance anti-secretory therapy) in preventing recurrent bleeding from peptic ulcer (Gisbert et al., 2004). Therefore, if this infection is not initially detected, it is important to repeat the evaluation subsequently to confirm the initial result. The economic impact of this strategy, especially in young ulcer patients, must be emphasized.

5. Pharmacotherapy

Proton pump inhibitors initiated after endoscopic haemostasis of bleeding peptic ulcer significantly reduced rebleeding compared with placebo or H_2–receptor antagonists (Sung et al., 2009; van Rensburg et al., 2009). The initiation of PPIs before endoscopy significantly decreases the proportion of patients with stigmata of a recent bleed (e.g. visible vessel) and a need for endoscopic haemostasis, but does not reduce mortality, rebleeding, or surgery risks compared with H_2–receptor antagonists or placebo (Dorward et al 2006; Lau et al., 2007). The effects of PPIs are more pronounced in Asian compared with non–Asian populations. There is no role for H_2–receptor antagonist, somatostatin, or octreotide in the treatment of acute bleeding gastric ulcer.

The rationale for using acid inhibition in peptic ulcer disease is based on the observation that the stability of a blood clot is reduced in an acidic environment. Acid impairs platelet aggregation and causes disaggregation. Clot lysis is accelerated predominately by acid-stimulated pepsin. Furthermore, it may impair the integrity of the mucus-bicarbonate-barrier. Thus a pH greater than 6 is necessary for platelet aggregation while clot lysis occurs when the pH drops below 6.

5.1 Role of H_2–receptor antagonists and somatostatin (octreotide)

There are no convincing data to support the use of H_2–receptor antagonists as these drugs do not reliably or consistently increase gastric pH to 6 irrespective of the route of administration (Julapalli & Graham, 2005). These drugs had minimal efficay in clinical trials and the development of tolerance is a problem.

Somatostatin and its analogue, octreotide, inhibit both acid and pepsin secretion and reduce gastroduodenal mucosal blood flow. However, these drugs are not routinely recommended in patients with peptic ulcer bleeding, since contemporary randomized, controlled trials have shown little or no benefit attributable to them, either alone or in combination with an H_2–receptor antagonist. Furthermore, there are no strong data to support the adjunctive use of these drugs after endoscopic therapy for ulcer bleeding. (Arabi et al., 2006).

5.2 Role of proton pump inhibitors

Proton pump inhibitors can increase the intra-gastric pH > 6.0 for 84 – 99% of the day (Lin et al., 1998). Tolerance has not been reported and continuous infusion is superior to intermittent bolus administration (Brunner et al., 1996). Pantoprazole given as an initial 80-

mg bolus injection, followed by 8 mg/h continuous infusion, seems to be the adequate treatment in patients with a high risk of rebleeding. Compared to an initial 80 mg–bolus injection, followed by 6-mg/h continuous insion, it demonstrated a lower inter-individual variability of intra-gastric pH and the pH was ≥ 6 for a greater percentage of time (van Rensburg et al. 2003). About five percent of patients with peptic ulcer bleeding responded poorly to intravenous omeprazole with rebleeding rates higher in patients with a mean intra-gastric pH of less than 6 (Hsieh et al., 2004).
PPIs in bleeding peptic ulcer have shown to reduce the rebleeding rate and the need for surgery, but not mortality whether the patients had an attempt at endoscopic haemostasis or not. PPI therapy for ulcer bleeding proofed more efficacious in Asia than elsewhere. This may be due to an enhanced pharmacodynamic effect of PPIs in Asian patients (Leontiadis et al., 2005). The use of high-dose PPIs (80-mg bolus, followed by 8-mg/h as continuous infusion for 72 hours) has been widely studied and used. However, the most effective schedule of proton pump PPI administration following endoscopic haemostasis of bleeding ulcers remains uncertain. It has been shown in a systemic review and meta-analysis that compared with low-dose PPIs, high-dose PPIs do not further reduce the 30–day rates of rebleeding, surgical intervention, or mortality after endoscopic treatment in patients with bleeding peptic ulcer (Wang et al., 2010).

5.2.1 Proton pump inhibitors – clinical effectiveness and cost-effectiveness

Potent acid-suppressing PPIs do not induce tachyphylaxis and have had favorable clinical results. Recent meta-analyses showed that the use of proton-pump inhibitors significantly decreased the risk of ulcer rebleeding (odds ratio, 0.40; 95% confidence interval [CI], 0.24 to 0.67), the need for urgent surgery (odds ratio, 0.50; 95% CI, 0.33 to 0.76), and the risk of death (odds ratio, 0.53; 95% CI, 0.31 to 0.91), (Bardou et al., 2005; Leontiadis et al., 2006) findings that have also been confirmed in a "real-world" setting (Barkun et al., 2004). In ulcer bleeding, PPIs reduce rebleeding and the need for surgery and repeated endoscopic treatment. PPIs improve mortality among patients at highest risk i.e. patients with active bleeding or a non-bleeding visible vessel (Leontiadis et al., 2007) compared with placebo or H_2–receptor antagonists. PPI treatment initiated prior to endoscopy in UGIB significantly reduces the proportion of patients with stigmata of recent bleeding at index endoscopy but does not reduce mortality, re-bleeding or the need for surgery. The strategy of giving oral PPI before and after endoscopy, with endoscopic haemostatic treatment for those with major stigmata of recent haemorrhage, is likely to be the most cost-effective.
Treatment of *Helicobacter pylori* infection was found to be more effective than anti-secretory therapy in preventing recurrent bleeding from peptic ulcer. *Helicobacter pylori* eradication alone or eradication followed by misoprostol (with switch to PPI, if misoprostol is not tolerated) are the two most cost-effective strategies for preventing bleeding ulcers among *Helicobacter pylori*-infected NSAID users, although the data cannot exclude PPIs also being cost-effective. Further large randomised controlled trials are needed to address areas such as PPI administration prior to endoscopic diagnosis, different doses and administration of PPIs, as well as the primary and secondary prevention of UGIB (Leontiadis et al., 2007).

6. Interventional radiology

Angiography with transcatheter embolization provides a non-operative method to identify and control bleeding when the endoscopic approach fails. Although the technical success

rate can be as high as 90–100%, the clinical success rate varies from 50–83% (Cheung et al., 2009). Embolization might not stop the bleeding permanently.

7. Surgery

The role of surgery in acute peptic ulcer bleeding has markedly changed over the past two decades. The widespread use of endoscopic treatment has reduced the number of patients requiring surgery. Therefore, the need for routine early surgical consultation in all patients presenting with acute UGIB is now obviated (Gralnek et al., 2008).

Emergency surgery should not be delayed, even if the patient is in haemodynamic shock, as this may lead to mortality (Schoenberg, 2001). Failure to stop bleeding with endoscopic haemostasis and/or interventional radiology is the most important and definite indication. The surgical procedures under these circumstances should be limited to achieve haemostasis. The widespread use of PPIs obviated further surgical procedures to reduce acid secretion. Rebleeding tends to necessitate emergency surgery in approximately 60% of cases with an increase in morbidity and mortality (Schoenberg et al.; 2001). The reported mortality rates after emergency surgery range from 2 – 36%.

Whether to consider endoscopic retreatment or surgery for bleeding after initial endoscopic control is controversial (Cheung et al., 2009). A second attempt at endoscopic haemostasis is often effective (Cheung et al., 2009), with fewer complications avoiding some surgery without increasing mortality (Lau et al., 1999). Therefore, most patients with evidence of rebleeding can be offered a second attempt at endoscopic haemostasis. This is often effective, may result in fewer complications than surgery, and is the current recommended management approach.

Available data suggest that early elective surgery for selected high-risk patients with bleeding peptic ulcer might decrease the overall mortality rate. It is a reasonable approach in ulcers measuring ≥2 cm or patients with hypotension at rebleeding that independently predicts endoscopic retreatment failure (Lau et al., 1999). Early elective surgery in patients presenting with arterial bleeding or a visible vessel of ≥2 mm is superior to endoscopic retreatment and has a relatively low overall mortality rate of 5% (Imhof et al., 1998 & 2003). Additional indications for early elective surgery include age >65 years, previous admission for ulcer plication, blood transfusion of more than 6 units in the first 24 hours and rebleeding within 48 hours (Bender et al., 1994; Mueller et al., 1994). This approach is associated with a low 30–day mortality rate as low as 7%.

8. Conclusion and recommendations

Peptic ulcer bleeding, the most common cause for UGIB, is best managed using a multidisciplinary approach. The initial clinical evaluation involves an assessment of haemodynamic stability and the necessity for fluid replacement. Combined with early endoscopic findings (within the first 24 hours), patients can effectively be risk–stratified for recurrent ulcer bleeding and managed accordingly. Those patients with active arterial bleeding or a visible vessel in the ulcer base should receive combined endoscopic therapy (that is, injection and thermal coagulation) as standard of care.

Despite a lack of concrete evidence of high-dose PPIs being more effective than non–high–dose PPIs, an 80–mg bolus followed by 8–mg/h as continuous infusion for 72 hours should be commenced as this is the only method of administration that reliably achieves the desired

high intra-gastric target–pH. Optimal management of bleeding peptic ulcer with an adherent clot should probably include an attempt at endoscopic removal, where after the same treatment to reduce the risk of recurrent bleeding should be affected. In the event of rebleeding after initial successful endoscopic haemostasis repeat–endoscopic therapy should be performed rather than surgery with generally a similar outcome with fewer complications.

For refractory bleeding, transcatheter angiography is equally effective as surgery and should be considered particularly in patients at high surgical risk. Second–look endoscopy should not routinely be performed considering the limited reduction in rebleeding rate and the questionable cost-effectiveness as profound acid inhibition is achieved with current medical treatment. Critical issues are detecting and eradicating *Helicobacter pylori* infection and the resuming NSAIDs or anti-platelet agents when clinically indicated with co-administration of gastro protective agents.

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17

Case Study in Optimal Dosing in Duodenal Ulcer

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1. Introduction

Duodenal ulcers occur in the duodenum – the upper portion of the small intestine as it leaves the stomach. A duodenal ulcer is characterized by the presence of a well-demarcated break in the mucosa that may extend into the muscularis propria [Thompson et al, 2010]. Cimetidine (C) was the first H_2-Receptor Antagonist to receive regulatory approval (in the late 1970s) for the treatment of duodenal ulcers. When it was being developed it was widely held that duodenal ulcers were caused by excessive gastric acid production. In fact the prevailing medical opinion was *no acid, no ulcer*. Sir James Black and colleagues at SmithKline and French Laboratories are credited with the discovery of C. They discovered that histamine released by the H_2-receptor stimulated the production of gastric acid, and that C by blocking the release of this histamine would suppress both normal and food stimulated gastric acid secretion [Nayak & Ketteringham, 1986]. In a reduced acidic environment, ulcers would be able to heal. The first C regimen approved for the treatment of duodenal ulcers in the United Kingdom was 1000 mg per day, given as: 200 mg at breakfast, lunch and dinner, and 400 mg at bed time, for up to 4 weeks. The first regimen approved in the United States for this indication was 1200 mg per day, given as: 300 mg q.i.d. for up to 4 weeks. Subsequently, other indications were obtained, and dosing regimens modified; for example, 800 mg per day, given as 400 mg bid.

In the mid 1980's, based upon data from gastric acid anti-secretory studies at various doses and frequencies of dosing, there was reason to believe that a single night time (hs) dose of 800 mg of C for up to 4 weeks would be the clinically optimal regimen for treating patients with duodenal ulcers. A large, landmark, dose comparison clinical trial [Dickson et al, 1985; Peace et al, 1985; Valenzuela et al, 1985; Young et al, 1989] was undertaken to confirm the effectiveness of 800 C mg hs in the treatment of duodenal ulcers for up to four weeks. When the author was first consulted by the project physician and regulatory affairs expert, the clinical development plan consisted of two, randomized, double-blind, placebo controlled, pivotal proof of efficacy trials with single nighttime dosing for four weeks:

Trial 1: 800 mg C hs vs. Placebo, and Trial 2: 1200 mg C hs vs. Placebo.

Each trial was to enroll 150 patients per treatment group, for a total of 600 patients. One-hundred-fifty patients per group would provide a power of 95% to detect a 20% difference in cumulative four-week ulcer healing rates between the C and Placebo groups with a 1-

sided, Type I error [Peace, 1991a] of 5%. Since conducting these trials would subject ½ the patients to Placebo, the author recommended amalgamating the two trials into a single trial:

Trial 3: 1200 mg C hs vs. 800 mg C hs vs. 0 mg C hs (Placebo)

with 164 patients per treatment group, for a total of 492 patients. One-hundred sixty-four patients per treatment group would provide a power of 95% to detect a difference of 20% in four week ulcer healing rates between any two of the treatment groups with an experiment wise Type I error of 5% (1.67% per each 1-sided, pair-wise comparison). Not only would this trial require fewer patients and be less expensive to conduct, it would also provide a within trial comparison between C doses, for dose discrimination.

Further savings could be realized by incorporating into the Trial #3 protocol, a planned interim analysis after ½ the patients had been entered and completed. At the interim analysis, the efficacy comparisons: 1200 mg C vs. Placebo, and 800 mg C vs. Placebo would be tested. If both were statistically significant, then the entire study could be stopped – if efficacy of the doses were the only objective. If comparing the doses of C was also of clinical importance, then the Placebo arm could be stopped and the two C arms run to full completion to assess dose discrimination. By conducting Trial #3 (instead of the two separate trials) and incorporating the interim analysis, potential savings of up to 190 patients could be realized. Additional savings would be expected due to less time required to conduct the trial [Peace, 1990, 1991b}.

The primary objective in conducting a clinical trial of C in the treatment of duodenal ulcers with a single nighttime dose was to demonstrate that 800 mg C was clinically optimal. We therefore added a 400 mg dose and replaced the 1200 mg dose with a 1600 mg dose (a two-fold increase among consecutive doses) in the final trial protocol, which was IRB approved.

2. Materials and methods

2.1 Objective

Both primary and secondary efficacy objectives were identified in the final protocol. The primary objective addressed ulcer healing. The secondary objective addressed upper gastrointestinal (UGI) pain relief.

The primary objective was to confirm that C given as a single nighttime dose of 800 mg for up to 4 weeks was clinically optimal in healing duodenal ulcers. Clinically optimal meant that 800 mg C was effective (significantly superior to placebo), that 800 mg C was superior to 400 mg C, and that 1600 mg C was not significantly superior to 800 mg C. Symbolically the primary (note p subscript of H) objective derives from three null and alternative hypotheses:

$$H_{p01}: P_{uh800} = P_{uh0}, \quad H_{p02}: P_{uh800} = P_{uh400}, \quad H_{p03}: P_{uh1600} = P_{uh800}$$

$$H_{pa1}: P_{uh800} > P_{uh0}, \quad H_{pa2}: P_{uh800} > P_{uh400}, \quad H_{pa3}: P_{uh1600} \neq P_{uh800}.$$

where P_{uh0}, $P_{uh400,}$ P_{uh800} and P_{uh1600} represent the cumulative ulcer healing (uh) rates by week 4 in the Placebo, 400 mg C, 800 mg C and 1600 mg C treatment groups, respectively, under single nighttime (hs) dosing. Specifically, H_{pa1}, H_{pa2} and H_{p03} comprised the primary study objective.

Symbolically, the secondary (note s subscript of H) objective derives from the three null and alternative hypotheses:

$$H_{s01}: P_{pr800} = P_{pr0}, \quad H_{s02}: P_{pr800} = P_{pr400}, \quad H_{s03}: P_{pr1600} = P_{pr800}$$

$$H_{sa1}: P_{pr800} > P_{pr0}, \quad H_{sa2}: P_{pr800} > P_{pr400}, \quad H_{sa3}: P_{pr1600} \neq P_{pr800}.$$

where P_{pr0}, P_{pr400}, P_{pr800} and P_{pr1600} represent the UGI pain relief (pr) rates in the Placebo, 400 mg C, 800 mg C and 1600 mg C treatment groups, respectively, under single nighttime (hs) dosing. Specifically, H_{sa1}, H_{sa2} and H_{s03} comprised the secondary study objective.

Of the six possible pairwise comparisons among the 4 dose groups, only three comprised the study objective. The other three: 1600 mg C versus 0 mg C, 1600 mg C versus 400 mg C, and 400 mg C versus 0 mg C were not part of the study objective and thus did not exact a Type I error penalty (i.e. the overall Type I error of 5% was 'Bonferonnied' across the three pairwise comparisons comprising the study objective, and not across the 6 possible pairwise comparisons).

2.2 Designing and planning the investigation

The trial was multicenter, stratified, randomized, double-blind and Placebo (0 mg C) controlled. Neither patients, investigators nor their staff knew the identity of the C regimens. As there had been reports [Korman et al, 1981; Korman et al, 1983; Lam & Koo, *1983;* Barakat et al, 1984] of the influence of smoking on the healing of duodenal ulcers at the time of protocol development, patients were stratified by smoking status within each center prior to randomization to the treatment groups. Smoking strata were Light Smokers and Heavy Smokers. Patients who smoked at most 9 cigarettes per day comprised the Light Smoker stratum. Patients who smoked at least 10 cigarettes per day comprised the Heavy Smoker stratum.

2.3 Blinded treatment groups

Blinded treatment group medication was packaged using the existing regulatory approved 400 mg C tablet. A 400 mg Placebo tablet was formulated identical to the 400 mg C tablet except that it contained 0 mg C. Blinded trial medication for the four treatment groups was packaged in blister packs for 4 weeks of nightly treatment as identified below:

0 mg C Group: Four 400 mg Placebo tablets
400 mg C Group: One C 400 mg tablet + three 400 mg Placebo tablets
800 mg C Group: Two C 400 mg tablets + two 400 mg Placebo tablets
1600 mg C Group: Four C 400 mg tablets.

2.4 Sample size determination

The trial was designed to recruit and enter enough patients to complete one-hundred sixty-four (164) per treatment group, for a total of 656 patients. One-hundred sixty-four patients per treatment group would provide a power of 95% to detect a difference of 20% in cumulative four week ulcer healing rates between any two of the treatment groups with an experiment wise Type I error rate of 5% (1.67% per each 1-sided, pair-wise comparison). This number was inflated to account for a 15% drop out rate. A cumulative four week healing rate of 45% among Placebo treated patients [de Craen et al, 1999] in previous trials was used in the sample size determination.

2.5 Entry requirements and assessment schedule

Patients were required at entry to have an endoscopically confirmed duodenal ulcer of size at least 0.3 cm, and either daytime or nighttime UGI pain. After providing informed consent, at the preliminary examination or baseline visit, patients provided a history (including prior use of medications, particularly anti-ulcer ones or antacids), underwent a physical examination, had vital signs measured, provided blood and urine samples for clinical laboratory assessments, in addition to having UGI pain assessed and undergoing endoscopy. Patients were also instructed how to use a daily diary to record the severity of daytime or nighttime UGI pain, as well as to record any adverse experience or concomitant medication use. Diaries and trial medication were dispensed and the patients instructed to return at weeks 1, 2 and 4 of the treatment period for follow-up endoscopy, UGI pain assessment and assessment of other clinical parameters. Antacids were provided to patients for relief of severe pain during the first six days/ nights of therapy only, and were limited to 4 tablets per day of low acid-neutralizing capacity. Table 1 summarizes clinical assessments made throughout the trial.

Follow-up endoscopic evaluation was carried out following strict time windows (Table 1) at week 1 (Days 7-8), week 2 (Days 13-15) and week 4 (Days 26-30). Patients whose ulcers were healed at any follow-up endoscopy were considered trial completers and received no further treatment or endoscopic assessment.

Clinical Parameter	Preliminary Examination[1]	Week 1 (Days 7-8)	Week 2 (Days 13-15)	Week 4 (Days 26-30)
History	Y			
Physical Exam	Y			
Vital Signs	Y	Y	Y	Y
Adv. Events		Y	Y	Y
Con. Meds	Y	Y	Y	Y
Endoscopy	Y	Y	Y	Y
Pain Assessment	Y	Y	Y	Y
Clin. Labs.	Y	Y		Y

[1] After providing Informed Consent

Table 1. Clinical Evaluation Schedule

2.6 Primary and secondary endpoints

The **primary efficacy data** was ulcer healing at week 1, 2 or 4. Ulcer healing was defined as complete reepithelization of the ulcer crater (normal or hyperemic mucosa), documented by endoscopy. The **primary efficacy endpoint** was cumulative ulcer healing at week 4 (healed at week 1 or week 2 or week 4).

Secondary efficacy data were the severity ratings of daytime and nighttime UGI pain recorded by the patient on the daily diary card. The severity of daytime pain was recorded just prior to going to sleep at night. The severity of nighttime pain was recorded upon arising in the morning. At each follow-up visit, the physician would review the diary card

and record the most severe rating of daytime and nighttime pain since the previous clinic visit. Daytime and nighttime UGI pain were rated separately according to the following scale:

0 = None = I had no pain
1 = Mild = I had some pain, but it didn't bother me much
2 = Moderate = I had pain that was annoying, but it didn't interrupt my activities
3 = Severe = I had pain which was so bad I couldn't do my usual activities

For nighttime pain, activities reflected sleep. The **secondary efficacy endpoint** was whether the patient was free of daytime or nighttime pain at weeks 1, 2 or 4.

2.7 Conducting the investigation

When the trial was conducted, there was great pressure to complete it as quickly as possible. This was due in part to Ranitidine's rapid gains into the antiulcer market, of which C had exclusivity for several years. Approximately 60 centers were recruited. The centers were rigorously and frequently monitored for conformity to protocol and federal regulations, in an attempt to minimize violations to protocol and collection of questionable if not unusable data. Roughly half of the sites were monitored by in-house Clinical Monitoring Personnel (CRA = Clinical Research Associates). The remaining sites were monitored by an outside Contract Research Organization (CRO).

A fairly heavy advertisement campaign was initiated to recruit possible trial participants. Ads ran on television and radio and appeared in the print media. In addition circulars were posted in public areas such as supermarket and laundromat bulletin boards. The ads were targeted to adults who had been having UGI or ulcer like pain, but who were otherwise healthy.

Weekly meetings were held during the conduct of the trial to monitor progress and deal with any issues. A proactive approach to clinical data management was taken. Data collection forms (DCFs) were expressed by each clinic to the data management group (or picked up by the CRA) where they were rapidly reviewed for completeness, legibility, entered into the computerized trial database, verified and quality assured. The goal was to provide a quality assured database for statistical analysis in as short a time as possible after each patient completed the protocol.

At the time the duodenal ulcer trial was conducted, there was no commercially available 800 mg C tablet. The commercially available 400 mg C tablet was used. Therefore a blood level trial that demonstrated bioequivalence [Randolph et al, 1986a] between a new 800 mg C tablet formulation (to be marketed) and two-400 mg C commercially available tablets had to be conducted with results available by the completion of the duodenal ulcer trial. Results from these two trials as well as that from specified drug interaction studies provided the primary data to support filing a supplemental new drug application (SNDA) to the FDA for the approval of C as a single 800 mg tablet taken at bedtime for the treatment of duodenal ulcers.

2.8 Statistical analysis methods

2.8.1 Methods

Descriptive and inferential methods were used in presentations and analysis of the trial data using procedures (PROCS) in the Statistical Analysis System (SAS). Both tables and graphs reflecting the number of patients, the mean (percent for dichotomous data) and standard deviation by treatment group and time of assessment were developed.

Inferential analyses, significance tests and confidence intervals, derived from an analysis of variance model containing fixed effects of center, strata and treatment group, with contrasts specified for the pairwise comparisons of interest. P-values for the pairwise comparisons comprising the primary trial objective were used for statistical inference. Confidence intervals were used as the basis of inference for secondary trial objectives and for the three pairwise comparisons not a part of the trial objective.

Since there were many centers and relatively few patients per treatment group per strata per center were expected, 12 blocks reflecting smoking status (2 levels)-by-baseline ulcer size (6 levels) were defined *a priori* (Table 2). An analysis of variance model containing the fixed effects of blocks and treatment was also used to assess the effect of treatment adjusted for blocks.

Gereralizability (poolability) of treatment effects was assessed by running an analysis of variance model with block, treatment group and block-by-treatment interaction. In these analyses the sole interest was the P-value for the interaction term. The blocking factor was smoking status-by-baseline ulcer size as defined in Table 2. A separate analysis that included the factors: smoking status, baseline ulcer size, their interaction, and the interaction of each of these with treatment was also performed.

Light	[0.3]
Light	(0.3; 0.4]
Light	(0.4; 0.5]
Light	(0.5; 1.0)
Light	[1.0]
Light	(1.0; 3.0]
Heavy	[0.3]
Heavy	(0.3; 0.4]
Heavy	(0.4; 0.5]
Heavy	(0.5; 1.0)
Heavy	[1.0]
Heavy	(1.0; 3.0]

Table 2. Smoking Status by Ulcer Size (cm) Blocks

Bivariate plots of the proportion of patients with ulcers remaining unhealed and the proportion of patients with UGI pain (daytime or nighttime) by time of endoscopic evaluation and treatment group were developed. These plots illustrate the rate of ulcer healing and pain relief across the times of endoscopic evaluation.

2.8.2 Interim analysis

Prior to finalizing the protocol, we considered including an interim analysis plan. Incorporating such a plan could result in completing approximately ½ the planned number of patients. More importantly, it could reduce the time from starting the trial to filing the SNDA. The idea was accepted initially, but later rejected by upper management; so the final protocol did not include an interim analysis plan.

However after the trial started, there was a push to conduct an interim analysis. A plan was developed to conduct an interim (mid-study) analysis after ½ the patients had entered. The

plan was filed by in-house regulatory affairs personnel with the FDA. Essential features of the plan ensured preservation of the Type I error and safe guarded blindedness among investigators, patients, and in-house personnel. We hired an outside consulting group that generated dummy investigator, patient and treatment group identification. The group also computed the P-values associated with the 3 pairwise comparisons comprising the study objectives and reported them to FDA Biometrics and in-house statistical personnel. The trial was not stopped and ran to completion, eventually enrolling 768 patients. The final results, based upon more than twice the number of patients in the interim analysis, were similar to those of the interim analysis in terms of estimates of treatment effects.

3. Results and discussion

3.1 Interim or mid study analysis results

3.1.1 Numbers of patients and baseline characteristics

Table 3 summarizes the number of patients available for the mid-study, interim analysis. Three hundred and thirty-seven (337) were randomized of which 315 [Peace et al, 1985; Valenzuela et al, 1985] were considered evaluatable [Peace, 1984] for efficacy for at least one follow-up visit. The fact that 17 more patients were assigned to the 1600 mg C group illustrates that slight imbalance across treatment groups can occur in randomized trials consisting of many centers.

Table 4 contains descriptive results of data available at baseline for mid-study analysis patients by C treatment group. The treatment groups appear balanced in terms of demographic characteristics, UGI pain and ulcer size, although the 800 mg C group had patients with the largest ulcers.

	Total	0 mg	400 mg	800 mg	1600 mg
# Randomized	337	76	83	85	93
# Evaluatable					
Week 1	304	67	80	73	84
Week 2	235	46	63	60	66
Week 4	174	41	47	47	39
≥ 1 week	315	71	82	75	87

Table 3. Number of Patients by Treatment Group (Mid Study Analysis)

3.1.2 Distribution of patients according to ulcer size

Table 5 provides the distribution of patients at baseline according to ulcer size. Ten percent (10%) of patients had ulcers of size 0.30 cm; 12.5% had ulcers of size greater than 0.30 but at most 0.40 cm; 17.8% had ulcers of size greater than 0.40 cm but at most 0.50 cm; 27.2% had ulcers of size between 0.50 cm and 1.00 cm; 17.8% had ulcers 1.00 cm in size; and 14.7% had ulcers of size greater than 1.00 cm but at most 3.00 cm.

Table 6 provides the distribution of patients in the Placebo group by baseline ulcer size whose ulcers had healed by 4 weeks. Seventy-one percent (71%) of Placebo patients with ulcers of size 0.30 cm healed; 78% of Placebo patients with ulcers of size greater than 0.30 but at most 0.40 cm healed; 45% of Placebo patients with ulcers of size greater than 0.40 cm but at most 0.50 cm healed; 41% of Placebo patients with ulcers between 0.50 cm and 1.00 cm in size healed; 30% of Placebo patients with ulcers 1.00 cm in size healed; and 25% of Placebo patients with ulcers of size greater than 1.00 cm but at most 3.00 cm healed. Table 6

reflects a strong negative correlation (or trend) between baseline ulcer size and ulcer healing by 4 weeks; i.e. the smaller the ulcer, the greater is ulcer healing by 4 weeks.

Characteristic	Statistic	0 mg	400 mg	800 mg	1600 mg
Age (yr)	Mean	42	40	44	42
Height (in)	Mean	67	67	67	67
Weight (lb)	Mean	169	160	163	160
Sex	Male (N)	50	62	51	55
	Female (N)	26	21	34	38
Race	Caucasian(N)	44	50	58	61
	Black (N)	24	21	18	24
	Other (N)	8	12	9	8
Day Pain	Mean	2.89	3.13	2.91	2.92
Night Pain	Mean	2.68	2.84	2.80	3.05
Ulcer Size(cm)	Mean	0.76	0.71	0.85	0.75
Smoking	Heavy (N)	40	45	45	48
	Light (N)	36	38	40	45

Table 4. Baseline Characteristics (Mid Study Analysis)

Ulcer Size (cm)	% Patients
[0.30]	10.0%
(0.30; 0.40]	12.5%
(0.40; 0.50]	17.8%
(0.50; 1.00)	27.2%
[1.00]	17.8%
(1.00; 3.00]	14.7%

Table 5. Distribution by Ulcer Size - Mid Study Analysis

Ulcer Size (cm)	Healed
[0.30]	71%
(0.30; 0.40]	78%
(0.40; 0.50]	45%
(0.50; 1.00)	41%
[1.00]	30%
(1.00; 3.00]	25%

Table 6. Cumulative 4-week Ulcer Healing Rates: Mid Study Analysis Placebo Patients

3.1.3 Influence of smoking and ulcer size on ulcer healing

Figure 1 provides a summary of the cumulative proportion of patients across all treatment groups with healed duodenal ulcers by week of endoscopy and smoking status. Figure 1 reflects a strong negative correlation between smoking status and ulcer healing; i.e. light smokers have a higher percentage of healed ulcers than do heavy smokers at all weeks of endoscopy.

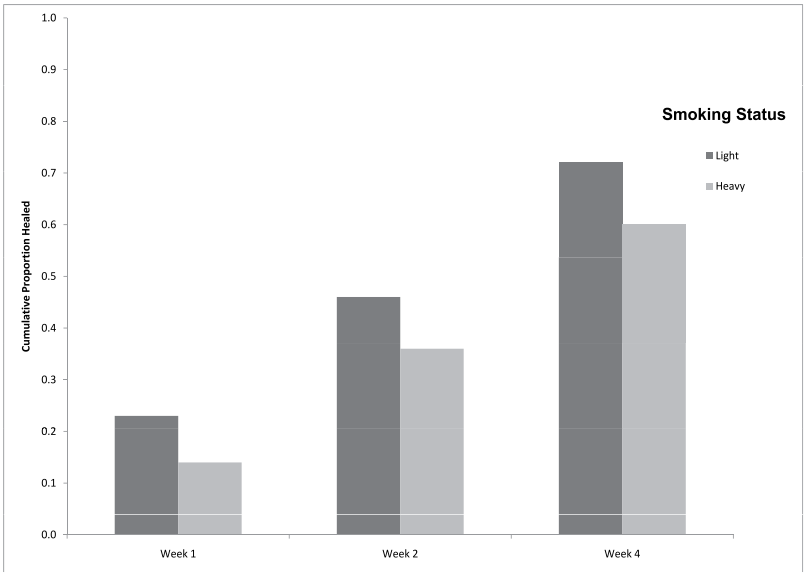

Fig. 1. Cumulative Proportion Healed: Light vs Heavy Smokers, Combined Treatment Groups

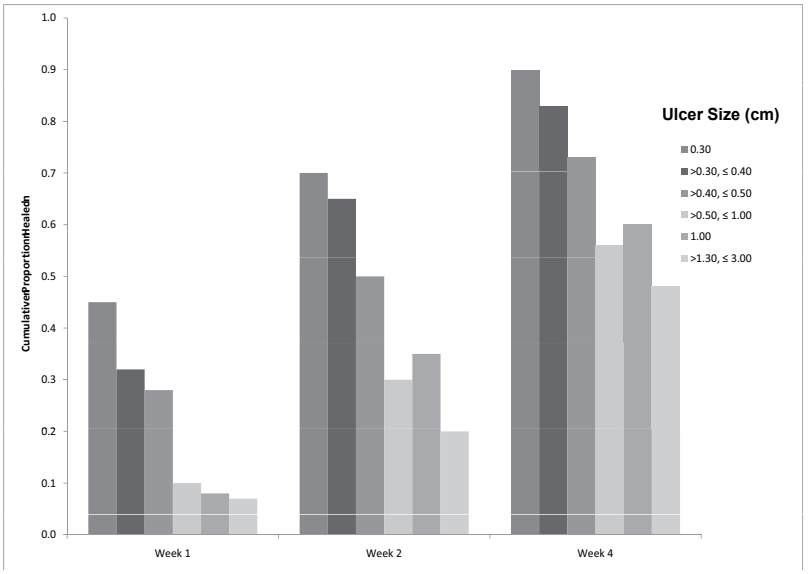

Fig. 2. Cumulative Proportion Healed by Ulcer Size, Combined Treatment Groups

Figure 2 provides a summary of the cumulative proportion of patients across all treatment groups with healed duodenal ulcers by week of endoscopy and baseline ulcer size. Figure 2 reflects a strong negative correlation between ulcer size and ulcer healing; i.e. patients with smaller ulcers have a higher percentage of healed ulcers than do patients with larger ulcers at all weeks of endoscopy. Note that the categories of ulcer size in Figure 2 are those that were defined *a priori*.

The negative correlation between ulcer size and healing is sharpened when collapsing the six ulcer size categories into three (Figure 3).

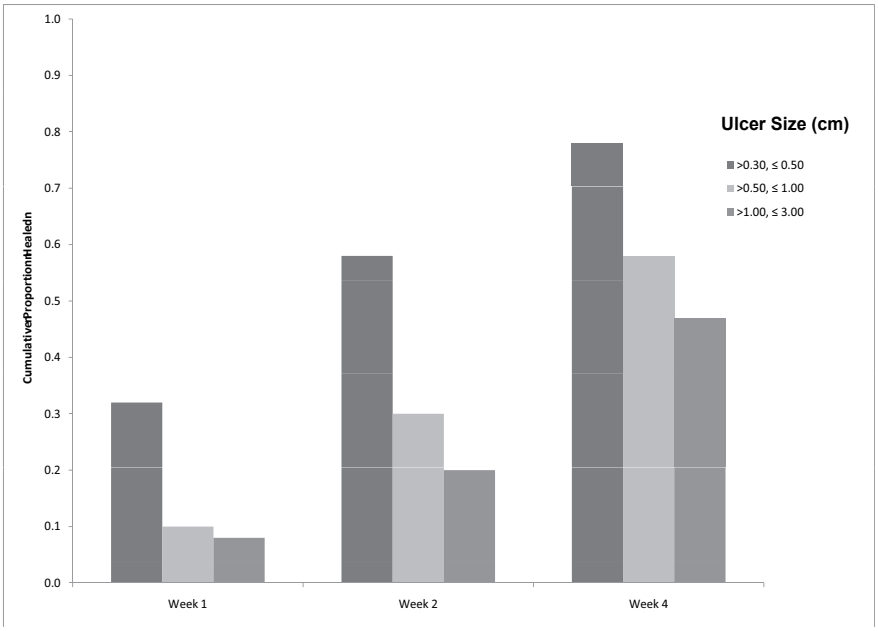

Fig. 3. Cumulative Proportion Healed by Ulcer Size, Combined Treatment Groups

3.1.4 Cumulative ulcer healing

The cumulative duodenal ulcer healing rates are summarized [Peace et al, 1985; Valenzuela et al, 1985] in Figure 4 by week of endoscopy and treatment group. The healing rates were: 19%, 18%, 16% and 21% at week 1; 29%, 37%, 38% and 49% at week 2; and 41%, 62%, 72% and 74%; for the Placebo, 400 mg C, 800 mg C and 1600 mg C groups respectively. At week 4: 800 mg C was effective (P = 0.0002) as compared to Placebo; 800 mg C was marginally superior to 400 mg C (P = 0.1283); and 1600 mg C provided no clinically significant greater benefit {δ = 0.0156: 90% CI on ratio of 1600 mg C/ 800 mg C = (0.86; 1.18)} than did 800 mg C. Even though 800 mg C healed 10% more ulcers than did 400 mg C, the P-value for this comparison did not achieve statistical significance. Therefore, the mid-study analysis did not demonstrate that 800 mg C was clinically optimal as formulated in the trial objective.

3.1.5 Generalizability assessment

Table 7 provides a summary of the assessment of generalizability (poolability) of treatment effect across smoking status, baseline ulcer size and smoking status-by-baseline ulcer size.

All of the P-values are large and therefore provide no evidence of lack of generalizability of treatment effects across these subpopulations.

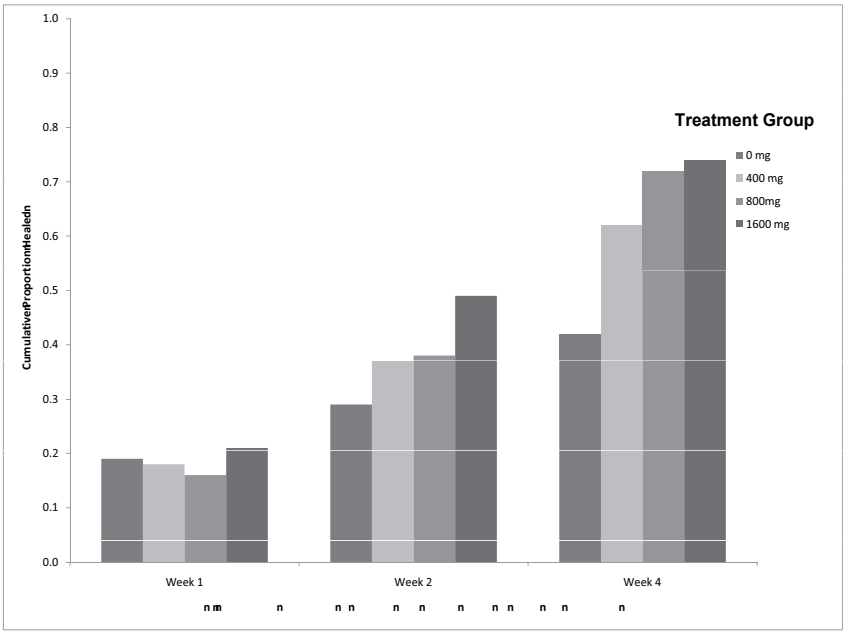

Fig. 4. Cumulative Proportion of Patients with Healed Ulcers by Week and Treatment Group

Source	F-value	P-value
Smoke x Size	1.11	0.3559
Smoke x Dose	0.40	0.7518
Size x Dose	1.12	0.3359
Smoke x Size x Dose	0.78	0.7038

Table 7. Assessment of generalizability: Smoking Status by Ulcer Size Subpopulations, Mid Study Analysis

3.1.6 Complete UGI pain relief and ulcer healing

To illustrate changes in duodenal ulcer healing and complete relief of UGI pain jointly, bivariate plots (Figure 5 and Figure 6) were generated. To develop these plots, the means (proportions) of each endpoint were computed by treatment or dose group and each endoscopy evaluation. The means, corresponding to each endoscopy evaluation and dose group identification, along with the ranges (0; 1) of each endpoint, were output to a data file. The data file was accessed by a graphical software package and a plot generated of the mean pairs by dose group. In generating the plots, the horizontal axis reflects the range of one endpoint and the vertical axis reflects the range of the other endpoint. In plotting the pairs of means for each dose group, the endoscopy evaluation corresponding to each pair appears as a floating index on the graph of each dose group.

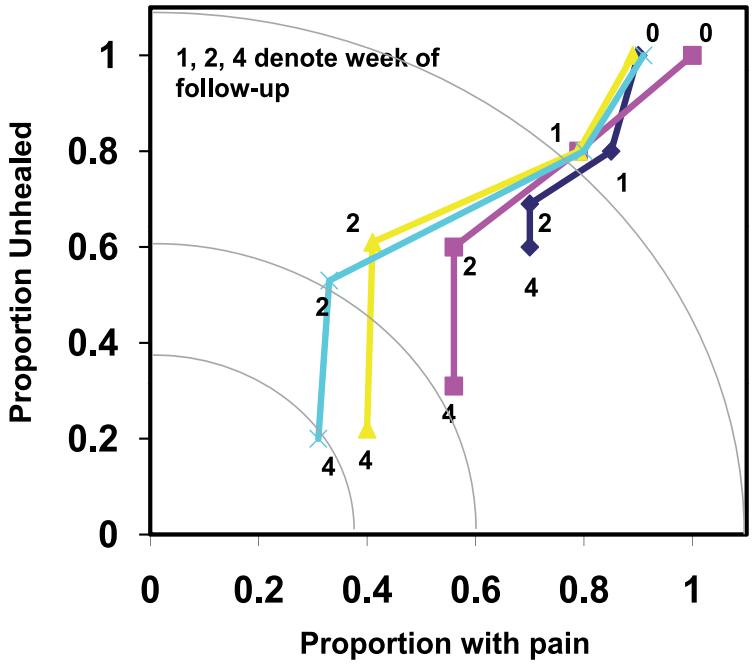

Fig. 5. Proportions of patients with Daytime Pain and Unhealed Ulcers, by Treatment Group (Mid-Study Analysis)

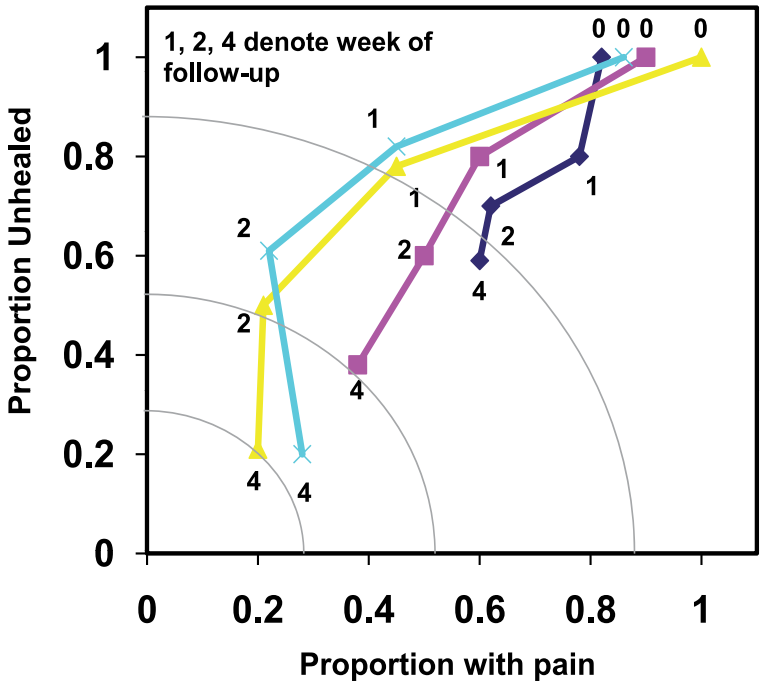

Fig. 6. Proportions of Patients with and Unhealed Nighttime Pain Ulcers, by Treatment Group (Mid-Study Analysis)

In Figures 5 and 6, the horizontal axis reflects the proportion of patients with UGI pain, and the vertical axis reflects the proportion of patients with unhealed ulcers; rather than proportions of patients without UGI pain and with healed ulcers. The (1,1) point therefore reflects where the patients are at baseline, and the (0,0) point reflects the ideal therapeutic goal of a treatment or dose by the final visit. For a broader discussion of bivariate plots, references [Peace & Tsai, 2009and Peace & Chen, 2010] may be seen.
Figure 5 is the bivariate plot of daytime UGI pain and lack of ulcer healing. Figure 6 is the bivariate plot of nighttime UGI pain and lack of ulcer healing. The fact that all dose groups do not begin at the (1,1) point is due to the fact that some patients had daytime UGI pain but not nighttime UGI pain and vice versa. Focusing on week 4 results, Figures 5 and 6 reflect a beautiful picture of dose response, both univariately and bivariately.

3.2 Final study analysis results

At the final study analysis, 168, 182, 165 and 188 patients [Young et al, 1989] were efficacy evaluatable, in the Placebo, 400 mg C, 800 mg C and 1600 mg C groups, respectively. The cumulative duodenal ulcer healing rates are summarized in Figure 7 by week of endoscopy and treatment group. The healing rates were: 17%, 16%, 15% and 21% at week 1; 30%, 40%, 42% and 48% at week 2; and 41%, 62%, 73% and 77%; for the Placebo, 400 mg C, 800 mg C and 1600 mg C groups respectively. At week 4: 800 mg C was effective ($P < 10^{-8}$) as compared to Placebo; 800 mg C was superior to 400 mg C ($P = 0.023$); and 1600 mg C provided no clinically significant greater benefit {$\delta = 0.04$: 90% CI on ratio of 1600 mg C/ 800 mg C = (0.96; 1.17)} than did 800 mg C. Therefore, the study demonstrated that 800 mg C was clinically optimal.

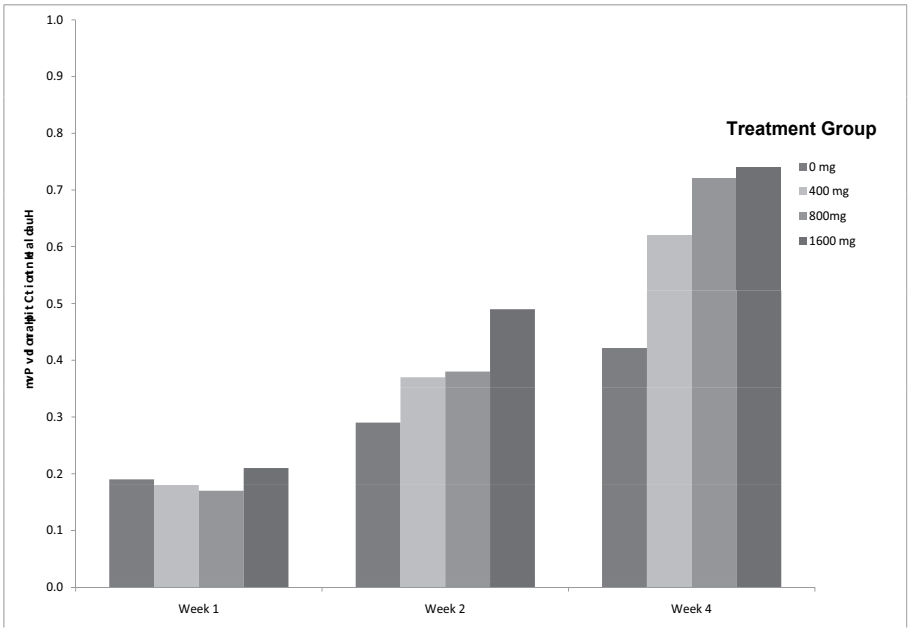

Fig. 7. Cumulative Proportion of Patients with Healed Ulcers by Week and Treatment Group (Final Study Results)

3.3 Other considerations

3.3.1 Bioequivalence trial of two-400 mg tablets and One-800 mg tablet

At the time the duodenal ulcer trial was conducted, there was no commercially available 800 mg C tablet. The commercially available 400 mg C tablet was used. Therefore a blood level trial that demonstrated bioequivalence [Randolph et al, 1986a] between a new 800 mg C tablet formulation (to be marketed) and two-400 mg C commercially available tablets had to be conducted with results available by the completion of the duodenal ulcer trial.

3.3.2 Cimetidine-by-drug interaction trials

Since C was widely prescribed (the prescription leader at the time), a change in dosage regimen, particularly a larger dose, required other trials involving the new 800 mg C regimen. We conducted specific Cimetidine-by-drug interaction trials exploring whether 800 mg C altered the circulating levels of other widely prescribed drugs. The drugs selected were Theophylline [Seaman et al, 1985; Randolph et al, 1986b; Randolph et al, 1986c] and Lidocaine [Frank et al, 1983] and Warfarin [Sax, et al, 1987].

3.3.3 Study in the elderly

At the time the duodenal ulcer trial was conducted, the FDA IND/ NDA rewrite was in progress, which among other specifics, stipulated that pharmaceutical companies should conduct studies in the elderly to explore whether doses of drugs posed a drug dose-by-age interaction. In addition, conducting clinical efficacy trials in the elderly was gaining sway. We actually developed a protocol for a small clinical trial comparing the 800 mg C to Placebo in elderly (age ≥ 65 years) patients with duodenal ulcers. The trial was to enroll 100 patients balanced across the 800 mg C and Placebo groups.

However, prior to starting the trial the author subset the final database for the trial described in this chapter and found it contained 101 elderly patients of which 19 were in the Placebo group and 23 in the 800 mg C group. Randomization in the large trial did not guarantee balance across treatment groups in this subset of elderly patients. Therefore the treatment groups were compared statistically in terms of baseline characteristics, and found to be comparable. Sixteen (16) of 23 (75.6%) elderly patients treated with 800 mg C experienced ulcer healing, as compared to 6 of 19 (32%) in the Placebo group {δ = 38%; 95% CI = (10.3%;75.6%)}. Since there was evidence in the original trial database that 800 mg C was effective in the elderly, there was no need to conduct a separate clinical efficacy trial in the elderly.

Results from the duodenal ulcer trial, the bioequivalence trial and the Cimetidine-by-drug interaction trials provided the primary data to support filing a supplemental new drug application (SNDA) to the FDA for the approval of C as a single 800 mg tablet taken at bedtime for the treatment of duodenal ulcers.

3.4 Innovative aspects of the clinical trial program

There are several aspects of this program that were rather innovative.

3.4.1 Interim analyses to drop placebo arms

Interim analyses plans that would allow dropping of the placebo arm after establishing efficacy of the doses, while allowing the dose arms to run to completion for dose discrimination, were developed.

3.4.2 Third party blinding during interim analyses

Interim analysis plans that safeguarded company personnel from knowing the identity of investigators, of patients and treatment groups were developed. These included: a. using an outside data management group who generated an analysis data set in which dummy treatment group labels, investigator id and patient id, while preserving the original randomization appeared; and b. having the outside data management group provide the blinded data set to the company statistician and to the FDA plus the file containing the IDs directly to the FDA.

3.4.3 Trial objectives as only three of six pairwise comparisons

The study objective was formulated as only 3 of six pairwise comparisons among the four dose groups while preserving the overall experiment wise Type I error across these three comparisons. The other 3 comparisons could be investigated, preferably using confidence intervals, but they should not invoke a Type I error penalty on the study objective.

3.4.4 Giving up information on center differences

Instead of using centers as a blocking factor in the primary analyses, the 12 classifications of smoking status-by-baseline ulcer size was used as the blocking factor due to small numbers of patients per treatment group per center and due to the prognostic importance of smoking status and baseline ulcer size.

3.4.5 Assessment of type of monitoring by treatment group

An assessment of differences in treatment effect between sites monitored by in-house personnel and those monitored by the CRO was conducted. There was no treatment-by-type of monitoring interaction, although the healing rates were generally lower among CRO monitored sites.

3.4.6 Association between ulcer healing and smoking status and ulcer size

The duodenal ulcer trial definitively established for the first time negative correlations between ulcer healing and smoking and ulcer healing and baseline ulcer size. Effectiveness estimates of ulcer healing were adjusted for smoking status and baseline ulcer size.

3.4.7 Utilization of bivariate graphical methods

The duodenal ulcer trial was the first to utilize bivariate plots to profile ulcer healing and UGI pain relief jointly. The plots illustrated strong dose response in terms of ulcer healing and UGI pain relief separately and jointly.

3.4.8 Establishing effectiveness based on a subset analysis

Efficacy of the 800 mg C dose was established in the elderly based on a subset analysis. The trial entered a large enough elderly population to demonstrate that 800mg C was effective in elderly. That's a plus for conducting a trial larger than necessary to establish the effectiveness of each dose.

3.4.9 Maximum use of patients screened with UGI Pain

The focus of this manuscript has been to review features of the land mark, dose comparison trial of once nightly C in the treatment of duodenal ulcer. This trial was one of three clinical

trials comprising a major clinical trial program. Each center conducted three protocols: the one discussed in duodenal ulcer, but also one in gastric ulcer and one in dyspepsia.
Patients were recruited on the basis of having experienced ulcer like symptoms including epigastric UGI pain. Those who satisfied general entry criteria and who gave consent were endoscoped. If duodenal ulcer (DU) was confirmed, they entered the DU trial. If gastric ulcer (GU) was confirmed, they entered a GU trial, and if there was no DU or GU, they entered a dyspepsia trial. This latter protocol provided a rather stringent definition of dyspepsia: Ulcer like symptoms including epigastric UGI pain not explained by the presence of DU or GU. This concurrent protocol method maximized the utility of the advertisement effort to get patients to the clinic who were experiencing ulcer like symptoms.

4. Conclusions

The SNDA clinical trial program that led to approval of clinically optimal dosing of the first H2-receptor antagonist: Cimetidine, in the treatment of duodenal ulcers has been reviewed in detail as a case study. The program included a landmark clinical trial that not only definitively established 800 mg C hs for 4 weeks as the clinically optimal dosing regimen, but also was the first to definitively establish negative associations between ulcer healing and smoking status and ulcer size, as well as the first trial to establish bivariate dose response in terms of ulcer healing and relief of UGI pain. Clinical optimality of 800 mg C hs was defined as 800 mg C being effective as compared to placebo; 800 mg C being more effective than 400 mg C; and 1600 mg C not being more effective than 800 mg C.
In addition, to make maximal use of patients screened, the program included clinical trials of the 800 mg C regimen in dyspepsia and in gastric ulcers. Further, the program also included drug interaction trials of the 800 mg C dose with widely used drugs and a bioequivalence trial of a new 800 mg C tablet compared to two, 400 mg tablets of the commercially available formulation. The bioequivalence trial was required as the clinical trial in DU was conducted using the commercially available 400 mg tablet at the time of study conduct.
Since the development of Cimetidine and other H_2-receptor antagonists: Ranitidine (Glaxo), Famotidine (Merck) and Zinatidine (Lilly), and the proton pump inhibitors (e.g. Prilosec and Prevacid) more is known about the causes of ulcers in the duodenum and stomach. It is now widely held that duodenal and gastric ulcers are caused by chronic use of NSAIDS: non-steroidal, anti-inflammatory medications (that decrease endogenous prostaglandin production), and by interference with the protective gastric mucosal layer from *Helicobacter pylori* infection [Thompson et al, 2010]. Current treatment consists of a combination of two antibiotics (clarithromycin and either amoxicillin or a nitroimidazole), and a proton pump inhibitor with the primary aim of eradicating H. Pylori infection [Gisbert et al, 2003]. Bismuth-based regimens are also used for second-line rescue therapy.

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Part 4

Treatment and Prevention Strategies of Peptic Ulcer

18

Conventional and Novel Pharmaceutical Dosage Forms on Prevention of Gastric Ulcers

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1. Introduction

Peptic ulcer formation in either the stomach or duedonum is due to an imbalance between erosive factors such as hydrochloric acid and pepsin and the ability of the gastroduodenal mucosal to protect and heal itself (1). Unlike duedonal ulcers, in which the importance of acid secretion is indisputable, gastric ulcers can develop despite only minimal amounts of acid.
On the other hand, it has become apparent that consumption of nonsteroidal anti-inflammatory drugs (NSAIDs) and stomach colonization by Helicobacter pylori (H.pylori) are the two most common causes of peptic ulcer disease. The prevention and management of NSAID related gastrointestinal (GI) complications are well recognized and in many cases successfully treated. However, the understanding and treatment of H. pylori-induced ulcers are still in progress (2).

2. NSAIDs induced gastric ulcers

NSAIDs are mainly indicated for mild to moderate pain of somatic origin. Due to their anti-inflammatory effect, they are among the agents most frequently used against musculoskeletal and rheumatic disorders throughout the world (3). Other indications include osteoarthritis, soft-tissue injury, renal colic, postoperative pain, and dental procedures. The efficacy of NSAIDs may vary by patient and by indication. In case of inefficacy, substitution by a NSAID from a different chemical class is a reasonable therapeutic option.
In 1899, acetylsalicylic acid was released to the pharmaceutical market (4). Almost 40 years passed before it was realized that aspirin may damage the gastric mucosa (5). Later, drugs having similar effects were recognized and thus termed `aspirin-like drugs' or NSAIDs. The main therapeutic actions proved to be analgesic, antipyretic and anti-inflammatory through inhibition of the cyclooxygenase (COX) enzyme system (6,7). During the past 15 years the number of NSAIDs has doubled. Along with the discovery in 1990 of the inducible form of the cyclooxygenase system, i.e. COX-2 (8), and development of COX-2-specific inhibitors, NSAIDs may now be classified as either (i) non-selective NSAIDs, i.e. aspirin and non-

aspirin NSAIDs; (ii) COX-2 preferential inhibitors; and (iii) COX-2-specific inhibitors (coxibs) or COX-1-sparing NSAIDs (9).

NSAIDs may be grouped as salicylates (with as prominent member aspirin itself), arylalkanoic acids (diclofenac, indomethacin, nabumetone, sulindac), 2-arylproprionic acids or profens (ibuprofen, flurbiprofen, ketoprofen, naproxen), *N*-arylanthranilic acids or fenamic acids (mefenamic acid, meclofenamic acid), pyrazolidine derivates (phenylbutazone), oxicams (piroxicam, meloxicam), sulfonanilides (nimesulide), and others (10). As a group, NSAIDs are structurally diverse and differ in pharmacokinetic and pharmacodynamic properties, but ultimately they share the same mode of action. Like aspirin, nonaspirin NSAIDs inhibit the production of prostaglandins by blocking the COX enzyme, causing analgesic, antipyretic, and anti-inflammatory benefits, but at a risk for increased gastric bleeding (11). Two COX isoforms have been identified and referred as COX-1 and COX-2. The inducible COX-2 is an important regulator to generate prostaglandins that mediate inflammation and pain, whereas the constitutive COX-1 is responsible for maintenance of the integrity of gastric mucosa and platelet aggregation (1).

However, aspirin and nonaspirin NSAIDs differ fundamentally in the way the COX enzyme is inhibited. Aspirin inhibits COX by noncompetitive and irreversible acetylation, where an acetyl group is covalently attached to a serine residue in the active site of the COX enzyme, rendering the COX enzyme permanently inaccessible for the biotransformation of arachidonic acid into PG H_2.

Conversely, nonaspirin NSAIDs competitively and reversibly inhibit the COX enzyme during only part of their dosage interval. This distinction is exemplified by their differential effects on platelet aggregation (10).

The gastroduodenal adverse effects include dyspepsia without endoscopically proven damage, asymptomatic endoscopic lesions of submucosal haemorrhage, erosions and ulcers, and-most important-ulcer complications (3). It is highly likely that the ulcerogenic effects of NSAIDs are directly related to their ability to suppress prostaglandin synthesis in the stomach. Prostaglandins play an important role in the GI tract: they mediate several components of mucosal defence (blood flow, mucus and bicarbonate secretion and mucosal immunocyte function). There is a good correlation between the ability of an NSAID to suppress gastric prostaglandin synthesis and its ulcerogenic action. NSAIDs, including acetyl salicylic acid, also have topical irritant properties that may contribute to their ability to damage the gastric mucosa. The majority of NSAIDs are weak acids with an ionisation constant in the range of 3.5. In the strongly acid environment of gastric juice, drugs are non-ionized and freely cross the cell membrane into the mucosal cells. The elevated intracellular pH promotes dissociation to its ionized form with subsequent intra-epithelial accumulation. The phenomenon of ion trapping and/or ability of these drugs to uncouple oxidative phosphorylation represent two important steps in the topical irritancy of NSAIDs. Thus, intragastric acidic pH plays an important role in the topical or systemic adverse effects of NSAIDs on the gastroduodenal mucosa (12).

Established risk factors for NSAID-associated ulcer complications include patient-specific factors such as advanced age, female gender, a history of peptic ulcer, and drug-specific factors such as the use of non-selective NSAIDs (type, dose, duration, multiple use) and concomitant anticoagulant drugs or corticosteroids. Probable risk factors comprise H.pylori infection and heavy consumption of alcohol, whereas use of selective serotonin re-uptake inhibitors, smoking and a number of other factors have also been proposed to contribute. Knowledge of absolute risk estimates is important for clinical decision making (3).

There is consistently clear evidence that advanced age is a major risk factor for complicated ulcer disease. The risk increases at least linearly with age in both NSAID-unexposed and NSAID-exposed individuals (13,14).

There is good evidence from meta-analysis that males have a two-fold higher risk of ulcer complications compared to females (15). However, among NSAID users, women have both a greater relative risk (RR) than men (RR 5.0 versus. 3.5) (15) and a higher absolute risk, with number needed to harm (NNH) among women being about 50 versus 75 in men (13).

Patients with a history of peptic ulcer have an overall almost six-fold increased risk of ulcer complications (15, 16). Even though the relative risk of NSAID use is lower in patients without a history of ulcers than in patients with a prior ulcer (odds ratio (OR) 5.0 versus 2.5), NSAIDs are still more dangerous (17) due to the higher base-line risk of ulcer complications among the latter.

Heavy alcohol use was found to be associated with an increased risk of bleeding peptic ulcer (18,19). Previous dyspepsia may be associated with an increased risk of ulcer complications (20). NSAID-related dyspepsia is often treated with a proton pump inhibitor to heal a possible underlying ulcer. Some data suggest that the use of H_2-receptor antagonists can mask dyspepsia that may herald an ulcer bleeding. In clinical practice, therefore, proton pump inhibitors are often preferred (3).

The interaction between *H. pylori* and the use of NSAIDs in the development of gastroduodenal ulcers is less clear. *H. pylori* infection and NSAID use may represent independent but synergistic risk factors (21,22). A recent meta-analysis of 21 studies that evaluated the relationship between *H. pylori* and NSAIDs in the development of gastroduodenal ulcers found that the risk for uncomplicated ulcers was 4 times as high in *H. Pylori* positive compared with *H. pylori*-negative patients, irrespective of NSAID use (OR, 4.03), and 3 times as high in NSAID users compared with nonusers, irrespective of *H. pylori* status (OR, 3.10) (22). Furthermore, the risk of uncomplicated ulcers was almost twice as high among *H. pylori*-positive compared with *H. Pylori*-negative NSAID users (OR, 1.81), and 17.5 times higher among *H. pylori*-positive NSAID users compared with *H. pylori*-negative nonusers. Possible explanations for the increased risk of ulcers in *H. pylori*-positive NSAID users are deterioration of the mucosal barrier caused by inflammation, increased acid secretion, a higher level of apoptosis in the infected mucosa, and decreased gastric adaptation to NSAIDs (23).

Patients with rheumatoid arthritis seem to be at increased risk of having ulcer complications compared with patients with osteoarthritis (24). This difference may, however, be explained at least partly by use of higher doses of NSAIDs in patients with rheumatoid arthritis. Some studies have indicated that patients with a history of heart failure are at increased risk of ulcer complications (25). Moreover, recent data suggest that diabetes mellitus may increase the risk as well (20).

Solid evidence from landmark studies (26, 14), and good meta-analyses (15, 16) indicate that the use of ibuprofen and diclofenac is associated with a lower risk of gastroduodenal adverse effects. The use of naproxen, indomethacin and aspirin constitutes an intermediate position, while the use of piroxicam and ketoprofen is associated with a higher risk. Moreover, clear evidence indicates that a high dose of an NSAID is associated with an enhanced risk of ulcer complications in a dose-dependent fashion. (13, 16, 27, 28, 29). Moreover, users of multiple NSAIDs are at the highest risk (OR 9.0; 95% confidence interval (CI), 5.9±13.6) followed by switchers (OR 6.2; 95% CI, 4.7±8.1) compared with single-NSAID users (OR 4.6; 95% CI, 3.9-5.4) (13).

Initially, it was suggested that short duration of NSAID therapy may be associated with a higher risk of ulcer complications (26,30) perhaps explained by gastric adaptation. However, recent cohort studies and meta-analyses indicate that the risk of ulcer complications remains constant during NSAID exposure (15,31,32). After discontinuation of NSAIDs the risk of ulcer complications declines rapidly, however, being increased during 2 months before returning to the base-line level.

Whether patients are exposed to NSAIDs or not, anticoagulants increase the risk of bleeding from pre-existing ulcers because of their antihaemostatic properties. NSAIDs are prescribed to anticoagulant users in about 13% of elderly subjects, and the risk of ulcer complications is heavily increased (Relative risk (RR)12.7, 95% CI, 6.-25.7; excess risk 2.4%; and NNH_{1yr}~40) (33). Anticoagulants alone also increased both the relative and the absolute risk (RR 4.3, 95% CI, 2.6-7.2; excess risk 0.68%; NNH_{1yr} ~147).

One out of seven elderly subjects may use both NSAIDs and corticosteroids (34). Other studies (13) have confirrmed the relationship and estimated that the excess relative risk due to the interaction between NSAIDs and steroids accounts for almost 60% of all cases using both NSAIDs and steroids.

The use of selective serotonin re-uptake inhibitors (SSRI) seemed to increase the risk of upper GI bleeding threefold (OR 3.0; 95% CI, 2.1-4.4) (35). Concomitant use of NSAIDs, however, increased the risk substantially, with an OR of 15.6 (95% CI, 6.6±36.6), suggesting an important interaction between NSAIDs and SSRI.

With the discovery of the 2 COX isoenzymes, COX-1and COX-2, it was hypothesized that the continuous production of local gastroprotective prostaglandins is mainly COX-1 dependent, while the inducible production of inflammatory prostaglandins is mainly COX-2 dependent. Most traditional NSAIDs were found to be nonselective inhibitors of both COX isoforms (36). An ideal NSAID would selectively inhibit the inducible COX-2 isoform, thereby reducing inflammation and pain, without acting on the constitutive COX-1 isoform, thereby minimizing toxicity. On the basis of this hypothesis, several COX-2-selective NSAIDs were developed in the 1990s. Celecoxib (Celebrex®), rofecoxib (Vioxx®), and valdecoxib (Bextra®) received FDA approval for use in rheumatoid arthritis and osteoarthritis, while celecoxib and rofecoxib were also approved for use in acute pain. Two other COX-2 selective NSAIDs, etoricoxib (Arcoxia®) and lumircoxib (Prexige®), received European approval for use in rheumatoid arthritis, osteoarthritis, and acute gout or osteoarthritis, respectively. COX-2-selective NSAIDs demonstrate comparable analgesia and anti-inflammatory effects to nonselective NSAIDs in patients with rheumatoid arthritis and osteoarthritis (36-40). At their defined therapeutic doses, COX-2-selective NSAIDs show at least a 200- to 300-fold selectivity for inhibition of COX-2 over COX-1 (36). Many studies have evaluated the efficacy of COX-2-selective NSAIDs on reducing the risk of NSAID ulcers. In 2000, 2 pivotal outcome studies, the Celecoxib Long-term Arthritis Safety Study (CLASS) and Vioxx Gastrointestinal Outcome Research study (VIGOR), demonstrated that COX-2-selective NSAIDs decrease the risk for both endoscopic NSAID ulcers and serious NSAID ulcer complications when compared with nonselective NSAIDs (41,42).

The Multinational Etoricoxib and Diclofenac Arthritis Long-term program was a pooled intent-to-treat analysis of 3 randomized comparisons of etoricoxib (60 or 90 mg daily) and diclofenac (150 mg daily) in 34,701 rheumatoid arthritis or osteoarthritis patients (43). Overall, GI events were significantly less common with etoricoxib than with diclofenac.

In the Therapeutic Arthritis Research and GI Event Trial, 18,325 osteoarthritis patients were randomized to lumiracoxib 400 mg once daily, naproxen 500 mg twice daily, or ibuprofen

800 mg 3 times daily for 52 weeks (44). In the patients not taking aspirin, the cumulative incidence of serious NSAID ulcer complications (bleeding, perforation, or obstruction) was significantly lower with lumiracoxib than with naproxen or ibuprofen (hazard ratio, 0.21; 95% CI, 0.12 to 0.37). However, there was no significant difference in the patients concurrently taking aspirin. Furthermore, there were more myocardial infarctions with lumiracoxib, especially as compared with naproxen (0.38% versus 0.21%), although the differences were not statistically significant.

Several tentative conclusions may be drawn from these and other studies. First, the use of COX-2-selective NSAIDs significantly reduces the risk of NSAID ulcers and of serious NSAID ulcer complications. However, long-term efficacy remains debatable. Second, concurrent use of low-dose aspirin for primary or secondary prevention of cardiovascular or cerebrovascular disease negates the gastroprotective effect of COX-2-selective NSAIDs. This observation may be directly related to effect of aspirin, which irreversibly blocks COX-1 in the GI tract (45). Third, the use of COX-2-selective NSAIDs increases the risk of myocardial infarction, as compared with the nonselective NSAID naproxen (10). The highly selective COX-2 inhibitors such as rofecoxib showed reduced GI side effects but their possible role in increasing cardiac adverse effects has resulted in the withdrawal of rofecoxib and valdecoxib from the market (1).

3. Strategies to enhance the safety profile of NSAIDs

Two strategies have been employed to enhance the safety profile of NSAIDs: the use of concomitant medication to protect the gastroduodenal mucosa and the development of safer anti-inflammatory drugs: COX-2 selective inhibitors, nitric oxide-donors NSAIDs, phospholipid-coupled NSAIDs, N-enatiomers of NSAIDs (12). The other way to enhance the safety profile of NSAIDs is to use rectal drug delivery systems or modified release formulations. These are less ulcerogenic included methods to reduce topical effects such as enteric coating, rectal administration, or sustained relese oral formulations. It is now well established that the point prevalence of peptic ulcer disease in patients receiving conventional NSAID therapy ranges between 10 and 30%, which is a 10- to 30-fold increase over that found in the general population (46). In a study that examined the prevention of NSAID-related ulcer complications in 8843 arthritis patients, it was reported that, over a 6-month trial period, 0.76% of patients (or 1.5% annually) experienced upper GI complications (25). The US Food and Drug Administration (FDA) similarly estimates that 2-4% of patients taking conventional NSAID for one year experience symptomatic ulcer or potentially life-threatening ulcer complications (47). The Arthritis, Rheumatism and Aging Medical Information Systems (ARAMIS) reported that the overall annual incidence of hospitalization for GI events was 1.3%, the rate was 6 times higher in patients with RA who were taking NSAID than in those who were not (24). Despite a reduction in the rate of hospitalisation (24,48), it has been established that 1 out of 175 users of conventional NSAIDs in the USA will be hospitalised each year for NSAID-induced GI damage (49). The mortality of hospitalised patients remains about 5-10%, with an expected annual death rate of 0.08% (24).

4. Suppository formulations

The advantages of suppositories as conventional formulations compared to other dosage forms are reduction of side effects, such as GI irritation and avoidance of disagreable taste,

first pass effect, and undesirable effects of meals on drug absorbtion (50-54). There are indications for using this route of administration such as when the oral administration of medication is difficult due to non-compliance of patient or when GI motility is severely impaired. In addition the oral route can not be used in some patients due to oral or oesophageal injuries or ulceration and in convulsing neonates rectal administration is easier than parenteral or oral administration (55,56).

After dissolving a suppository containing NSAID in the rectal fluids and absorption by the rectal mucosa, the NSAID will be distributed to the various body compartments. The upper haemorrhoidal vein will drain the drug into portal system while the middle and lower haemorrhoidal veins drain it directly into the inferior vena cava which explains why the drug bioavailability may be modified according to the position of the suppository into the rectum. At least a part of the drug absorbed will bypass the liver and its first pass metabolism (which is of great importance for high clearance drugs but not for low clearance drugs such as most of the NSAIDs) will be decreased. It was known that, NSAIDs are variably, but usually well absorbed rectally, thereby reducing the risk of GI ulceration and NSAID suppositories are one approach, besides many others, that is proposed to limit NSAID-induced gastropathy. This proved to be true at least in one study conducted on 45 normal volunteers who received either indomethacin or placebo suppositories, or oral indomethacin. Both suppositories seemed to be better tolerated than oral formulation (57).

Ersmark H. et al. (58) have used in their study piroxicam and indomethacin suppositories for painful coxarthrosis. Six orthopaedic clinics in Sweden made a comparison of the effects and side effects of Piroxicam (20 mg) and Indomethacin (100 mg) suppositories in 261 patients with painful coxarthrosis on the waiting list for total hip replacement. The study was designed as a single blind study over 4 weeks. Amount of pain and range of motion was registered before the trial and compared with findings after 4 weeks, including reported side effects. Both drugs gave satisfactory pain relief without any appreciable variation on weightbearing or at rest. On the other hand, the trial showed a significant difference ($p = 0.0033$, Student's-test) between the two drugs as regards the frequency of side effects from the lower gastrointest GIinal tract, where piroxicam had a lower rate compared with indomethacin. No serious complications occurred; 16 patients dropped out, 8 in each group.

Carrabba M. et al. (59) compared the local tolerability, safety and efficacy of meloxicam 15 mg suppositories with piroxicam 20 mg suppositories over a 3-week period in a single-blind, randomized study in patients with osteoarthritis. They found that local adverse events occurred in 11.9% of patients receiving piroxicam and 6.9% of those receiving meloxicam. Overall, GI adverse events were the most frequent of all 11.9% of piroxicam-treated patients. In both groups, about 90% of global tolerability assessments were classified, by the investigator and the patient, as either very good or good. They concluded that meloxicam 15 mg suppositories showed excellent local tolerability accompanied by good safety and efficacy in osteoarthritis, which was comparable to that of an established NSAID administered by the rectal route, and to that previously observed with oral formulations of meloxicam 15 mg.

Hatori M. et. al (60) used in their study 231 patients aged 16 to 75 years with osteoarthritis of the knee joint. Each patient received 20 mg of piroxicam daily as a suppository administered before sleep; 75% of the patients were treated for 14 days or longer. Overall treatment outcome was excellent in 34% according to physicians' ratings and in 36% according to the patients' self-ratings, good in 39% and 41%, fair in 22% and 17%, and unimproved in 5% and

7%, respectively. Side effects were reported by 3% of the patients. They concluded that treatment of osteoarthritis with piroxicam suppositories is safe and effective.

Aärynen M. and Palho J. (61) studied with 15 patients having rheumatoid arthritis or osteoarthritis. They received a single dose (20 mg) piroxicam (Felden) as suppository. Serum piroxicam concentrations were assayed by fluorometry 1, 2, 4, and 8 h after the installation of the suppository, the mean values being 1.3, 1.9, 1.8, and 1.8 mg/l, respectively. Then the patients continued on oral piroxicam 20 mg daily for maximum 3 weeks, and serum piroxicam levels (mean 6.3 mg/l) were checked at the end of this period. Nine patients then continued on piroxicam suppositories 20 mg daily for one week, and serum piroxicam levels (mean 4.5 mg/l) were again assayed at the end of this maintenance. Pain at rest, pain on motion, and joint movement restriction were scored on day 1, after oral maintenance, and after rectal maintenance. Reduced scores were found with time, but the only statistically significant effect was in the overall subjective pain relief measured after oral maintenance. Rectal irritation was recorded in one patient. They concluded that a) absorption of piroxicam from suppository was adequate, b) it was possible to maintain adequate serum piroxicam levels by repeated administration of suppository for one week, and c) the GI toleration was acceptable in these patients selected for showing poor tolerance towards other nonsteroidal antiinflammatories.

In a placebo-controlled double-blind trial analgesic effectiveness and tolerability of alpha-methyl-4-(2-thienyl-carbonyl)phenylacetic acid (suprofen, Suprol) 300 mg suppositories were evaluated for 45 informed patients suffering from chronic pain due to osteoarthritis; the subjects were treated rectally, t.i.d., for 10 days. Suprofen proved to be statistically significantly superior to placebo in all the variables considered for evaluation of the analgesic effect, i.e., pain intensity and relief scores, sum of pain intensity differences, total pain relief , global assessments by investigator and patient. In particular, the efficacy of suprofen was judged by the physician good or very good in 86.3% of the patients. Similar frequencies of rectal side-effects were observed in both treatment groups, with slightly but not significantly higher incidence in the group treated with suprofen. Haematologic and clinical chemistry laboratory tests showed no statistically significant alterations due to the treatment (62).

Efficacy and toleration of piroxicam suppositories 20 mg, given once daily for 4 weeks were assessed in 96 patients suffering from degenerative joint disease and 20 patients suffering from rheumatoid arthritis. The mean scores of objective parameters measured (tenderness, swelling, limitation of movement) decreased significantly 2 and 4 weeks after initiation of therapy. Patients' self-evaluation of pain and stiffness also significantly improved during the trial. Overall evaluation of efficacy and toleration were excellent or good in more than 80% of patients. Local toleration was excellent in all but two patients (63).

In a 15 day double-blind clinical trial 39 patients affected with rheumatic disease have been enrolled to evaluate the therapeutic effect of rectal administration of Piroxicam, in comparison with Indomethacin. At the end of the study, 20 patients had been treated with Piroxicam and 19 with Indomethacin. Nine patients in the Indomethacin group and one in the Piroxicam group dropped-out. Both drugs safety resulted good in the patients who completed the study, whereas 5 out of 10 dropped-out patients stopped the trial in consequence of severe side-effects of Indomethacin. Piroxicam induced a very good improvement in 76% of the patients, moderate in 19% and no improvement in 5%; Indomethacin induced a very good improvement in 75% of the patients, moderate in 15% and no improvement in 10%. No significative modifications resulted from the control of the

laboratory blood tests. Piroxicam (30 mg/die) showed a therapeutic activity similar to Indomethacin (100 mg/die). The rectal administration of Piroxicam can be then considered a very good alternative to the oral one, particularly in the patients in which oral use of NSAID is counter-indicated (64).

Ketoprofen administered via the rectal route seemed to be valuable when given at night to patients with various rheumatic syndromes and may be particularly useful for patients who show gastric intolerance of the capsules. Anal intolerance was noted in 12% of the patients (65).

The relative risks associated with anti-inflammatory drug prescription for patients with an earlier history of drug-associated gastro-intestinal disturbance have been investigated by Bunton RW. et. al (66) in a retrospective study. Under these circumstances ibuprofen was well tolerated. The risks associated with modified salicylates (principally aspirin in enteric-coated form) and indomethacin suppositories also appeared to be relatively slight.

Together with NSAIDs a lot of other drugs such as alendronate sodium (ALD) have GI side effects. ALD is a bisphosphonate medication used in the treatment and prevention of osteoporosis. Absorption of ALD as oral formulation is very poor (0.5-1%). Its bioavailability can decrease with food effect. It has some GI adverse effects such as gastritis, gastric ulcer, and esophagitis. Asikoglo et al. (67) developed in their study a rectal formulation of ALD as an alternative to oral route and investigated the absorption of it by using gamma scintigraphy. For this reason, ALD was labelled with Technetium-99m (^{99m}Tc) by direct method. They found that the rectal absorption of ^{99m}Tc-ALD from suppository formulation was possible. According to their results, this formulation of ALD can be suggested for the therapy of osteoporosis as an alternative route. Asikoglu et al. (68) developed in another study a vaginal suppository formulation of ALD and they showed that the vaginal absorption of ^{99m}Tc-ALD from suppository formulation was also possible.

It was known that sustained release (SR) suppositories together with suppositories are other important formulations to reduce the GI side effects. In the case of drugs that are rapidly eliminated from the systemic circulation, frequent administration would be needed to maintain the therapeutic plasma concentration. To reduce the frequency of dosing, several approaches have been performed to prepare SR suppositories by using various polymer such as chitosan (69), Eudragit (70-72), cellulose acetate phthalate (73), carboxyvinyl polymer (74), and various hydrogel formulation (75,76), were also investigated. Özgüney et. al. (77) prepared SR suppositories of ketoprofen (KP). Since KP produces gastro-intestinal side effects and its administration rectally is considered as a serious alternative to the oral route (75). KP is an appropriate model drug for formulation of controlled release dosage forms due to its short plasma elimination half-life and poor solubility in unionized water, which affects its bioavailability (78). They designed KP SR suppositories according to the 3^2x2^1 factorial design as three different KP:Eudragit RL 100 ratios, three particle sizes of prepared granules and two different PEG 400:PEG 6000 ratios. The conventional KP suppositories also prepared with Witepsol H 15, Massa Estarinum B, Cremao and the mixture of PEG 400:PEG 6000. The dissolution studies of suppositories prepared was carried out according to the USP XXIII basket method and it was shown that the dissolution time was sustained to 8 hours. In addition, they determined antiinflammatory activity of SR suppository as significantly extended according to the conventional suppositories.

Güneri et. al. (79) reported that formulation of sustained-release suppositories using ibuprofen-ethylcellulose microspheres was attempted. Ibuprofen was an appropriate candidate for sustained-release formulation because of its short half-life (1.8-2 hrs) and

undesired GI effects when it is administrated through oral route, such as peptic ulceration and GI bleeding.

There are a lot of studies on SR suppositories prepared with NSAIDs to reduce their GI side effects (80,81).

Liquid suppository formulations are newer SR suppository formulations according to the conventional suppository formulations. Conventional suppositories are solid forms which often cause discomfort during insertion. The leakage of suppositories from the rectum also gives uncomfortable feelings to the patients. In addition, when the solid suppositories without mucoadhesivity reach the end of the colon, the drugs can undergo the first-pass effect. To solve these problems, Choi et.al. (82) developed a novel in situ-gelling and mucoadhesive acetaminophen liquid suppository with gelation temperature at 30–36°C and suitable gel strength and bioadhesive force. Poloxamer 407 (P407) or/and poloxamer 188 (P188) were used to confer the temperature-sensitive gelation property.The mixtures of P407 (15%) and P188 (15–20%) existed as a liquid at room temperature, but gelled at 30–36°C. They studied bioadhesive polymers such as polyvinylpyrrolidone, hydroxypropylmethylcellulose, hydroxypropylcellulose, carbopol and polycarbophil to modulate the gel strength and the bioadhesive force of acetaminophen liquid suppositories. Choi et. al. (83) showed in their another study that liquid suppository A [P 407:P 188:polycarbophil:acetaminophen (15:19:0.8:2.5%)], which was strongly gelled and mucoadhesive in the rectum, showed more sustained acetaminophen release profile than did other suppositories and gave the most prolonged plasma levels of acetaminophen in vivo. Liquid suppository A also showed higher bioavailibility of acetaminophen than did the conventional formulation and it did not cause any morphological damage to the rectal tissues.

Özguney et. al. (84, 85) prepared a liquid suppository formulation using P407, P188, ketoprofen and various amounts of different bioadhesive polymers (PVP, CMC, HPMC and Carbopol 934 P). Because of the gastro-intestinal side effects ketoprofen was choosen as active ingredient. They investigated the release and mechanical characteristics of the formulations . As to the obtained results of in vitro drug release studies, Carbopol has the bigest effect on release rate among the bioadhesive polymers. It was seen that the release rate decreased with increasing of Carbopol cocentration. The release rate decreased between the formulations having highest or lowest concentrations of Carbopol in percent of 20 at 8. hour.

5. Enteric-coated formulations

Enteric-coated (EC) products are designed to minimize exposure of a drug to the acidic pH in the stomach, which could result in its degradation, or to decrease gastric side effects such as ulcers, perforations and bleeding due to the local effects of the drug on the gastric mucosa. Cellulose acetate phthalate is the polymer most commonly use for enteric coating. The core in such a formulation is coated with this polymer, which does not dissolve at a gastric pH. The dissolution of coating begins at a higher intestinal pH (generally at a pH higher than 5) as the tablet transist out of stomach into the intestine. Generally, the enteric-coated products are tablets. However, small beads or spheroids can be covered with an enteric coating and than these beads can be placed in a hard gelatin capsule (86). Ethylcellulose and cellulose acetate phthalate capsules in the form of Snap-Fit type hard gelatin capsules were developed for controlled release and enteric-coated dosage forms

respectively. The capsules were drilled in different diameters by using laser and filled with concentrated drug solution. In vitro and in vivo drug releases were investigated (87). In an another study, the enteric-coated capsules were prepared using hydroxypropylmethyl cellulose phthalate and examined in vitro and in vivo drug releases (88,89).

Although diclofenac sodium (DFNa), is a conventional NSAID, it could be fully utilized without harmful side effects if it was properly formulated (90). When it comes to oral administration of DFNa, at least two requirements should be considered: (a) perfect drug retention under gastric conditions, and (b) sufficient drug release during intestinal residence time. To achieve these requirements, a variety of controlled release formulations for DFNa have already been reported. In terms of pH-responsive matrices, water-soluble matrix tablets containing DFNa coated with hydroxypropyl methylcellulose phthalate (HPMCP) for delayed release of DFNa (91) and DFNa-loaded pH-sensitive microspheres comprising of poly(vinyl alcohol) and poly(acrylic acid) interpenetrating network for the delivery of DFNa to intestines were prepared and evaluated in vitro (92). Novel enteric microcapsules were reported, and in vivo evaluation of dosage forms showed successful pharmacodynamic activities (93).

Due to the necessity to pass intact through the stomach for reaching the duodenum for absorption, the pantoprazole is formulated as solution for intravenous administration (lyophilized powder for reconstitution) or as gastric-resistant tablets (oral delayed-release dosage form). In the case of oral administration, the enteric coating prevents pantoprazole from degradation in the gastric juice (at pH 1–2, pantoprazole degrades in few minutes) (94). As a general rule, the multiple-unit products show large and uniform distribution; they are less affected by pH and there is a minor risk of dose dumping (95). Besides, these new drug delivery systems, as the polymeric microparticles, are also proposed to improve absorption, distribution, and bioavailability of acid labile drugs (96,97). As they rapidly disperse in the GI tract, they can maximize drug absorption, minimize side effects, and reduce variations in gastric emptying rates and intersubject variability (98,99).

Caldwell J.R. et al. (100) compared in their study efficacy and GI tolerability of a new enteric coated formulation of naproxen (NAP-EC) with standard immediate release naproxen (NAP-STD). For this reason one hundred seventy-nine patients with osteoarthritis and one hundred seventy-six patients with rheumatoid arthritis at high risk for developing GI side effects to NSAID therapy were enrolled in a double blind, parallel, multicenter study. All patients had either discontinued as NSAID during the previous one year or required cotreatment with antiulcer drugs for control of GI complaints related to NSAID use. The treatments were evenly divided in both diagnostic cohorts. As to the obtained results of their study, except for minor differences in alcohol consumption, baseline characteristics of patients in both treatment groups were statistically similar. Both naproxen formulations were highly efficacious by all variables of disease activity when changes were measured from baseline. No statistically significant between formulation difference was found in the primary efficacy variable, overall disease activity. Overall, between formulation differences in efficacy measures were few, though most favored NAP-STD. GI complaints were reduced by 15% (51% NAP-EC vs 60% NAP-STD, $p = 0.077$) and GI complaints thought to be drug related were reduced by 36% (16% NAP-EC vs 25% NAP-STD, $p = 0.024$). Withdrawals due to GI complaints were reduced by 37% in the NAP-EC group (12% NAP-EC vs 19% NAP-STD, $p = 0.054$), and withdrawals due to GI complaints judged to be drug related were reduced by 55% in the NAP-EC group (6% NAP-EC vs 12% NAP-STD, $p = 0.025$). They concluded that enteric coated naproxen is an effective treatment for osteoarthritis and

rheumatoid arthritis. All observed differences in GI tolerability favor NAP-EC over NAP-STD.

The damaging effect of enteric-coated and plain naproxen tablets on the gastric mucosa was studied in 12 healthy subjects before and after 7 days' treatment in a randomized, double-blind, double-dummy, cross-over trial. Both formulations of the drug caused mucosal lesions, but the extent of the damage was significantly decreased after enteric-coated naproxen as compared with plain tablets. The subjects' preference was significantly in favour of the enteric-coated naproxen tablets. The plasma naproxen concentration was significantly higher after treatment with enteric-coated naproxen than after treatment with plain tablets. In conclusion, the results of the study indicate that naproxen might damage the gastric mucosa by local and systemic effects and that the local effect might be prevented by enteric coating of the tablets (101).

Aabakken L. et al. (102) studied the GI side effects of three formulations of naproxen in 18 healthy male volunteers. In a Latin-square design crossover study, the subjects received 500 mg naproxen twice daily for 7 days as plain tablets, enteric-coated tablets, or enteric-coated granules in capsules. The 51Cr-EDTA absorption test was performed before and at the end of each drug period, to evaluate changes in the distal gut. The test dose was instilled distally in the duodenum to prevent lesions in the stomach from interfering with the evaluation. Upper endoscopy was performed at the same intervals, scoring changes in the middle and distal duodenum separately from findings in the stomach and duodenal bulb. The nature and severity of adverse effects were recorded for each treatment period. Non-parametric methods were used for statistical evaluation. All drugs induced a significant increase in 51Cr-EDTA absorption, but they did not detect any difference between the three formulations. All formulations were associated with a significant increase in all the endoscopic findings monitored. Enteric-coated tablets induced significantly less lesions than enteric-coated granules in the stomach and duodenal bulb, and an advantage over plain tablets was indicated. No difference was seen in the middle and distal duodenum. The proximal endoscopic scores were not correlated to those found in the middle and distal duodenum. Evaluation of the small and large bowel should probably be included in clinical studies of NSAIDs, but their findings suggest that the importance of transfer of mucosal lesions to the distal gut by enteric coating may have been overemphasized.

The effects of plain and enteric-coated fenoprofen calcium (Nalfon, Dista, Indianapolis, Ind.) on GI microbleeding were studied in 32 normal male volunteers in a randomized, open-label, parallel trial at two inpatient research facilities. A 1-week placebo (baseline) period preceded 2 weeks of fenoprofen therapy (enteric coated or plain, 600 mg q.i.d.). Fecal blood loss was measured by 51Cr-tagged erythrocyte assay and averaged over days 4 to 7 (baseline) and 11 to 14 and 18 to 21 (active therapy). At one center GI irritation was evaluated endoscopically before and after active therapy. Endoscopy showed both formulations to cause mucosal damage not evident by subject-reported symptoms. Four of the 16 subjects developed asymptomatic duodenal ulcers. Mean daily fecal blood loss was significantly lower ($P = 0.03$) with enteric-coated (mean +/- SD, 1.104 +/- 0.961 ml/day) than with plain fenoprofen calcium (mean +/- SD, 1.686 +/- 0.858 ml/day), suggesting that tolerance of fenoprofen can be improved with administration in an enteric-coated form (103).

When administered on a chronic high-dosage regimen, enteric-coated aspirin granules produced significantly less gastric damage than plain aspirin or aspirin-antacid combinations. Clinically meaningful damage occurred in all subjects receiving plain aspirin, 93% of those receiving aspirin-antacid combination and only 27% and 20% of those

receiving enteric-coated aspirin granules qid and bid, respectively. All three aspirin formulations were taken as 1 g qid (4 g/day) and an additional group received enteric granules administered as 2 g bid (4 g/day). Gastric damage was assessed by means of endoscopy carried out after seven days of treatment. Enteric granules are equally safe when administered on a bid or qid regimen (at same total daily dosage) and, in a bid regimen, should provide a compliance advantage for patients on high-dose therapy for diseases such as rheumatoid arthritis (104).

6. Sustained and controlled release formulations

The development of oral sustained and controlled release formulations offers some benefits: controlled administration of a therapeutic dose at the desired delivery rate, constant blood levels of the drug, reduction of side effects, minimization of dosing frequency and enhancement of patient compliance (105).

The basic rationale for the development of controlled drug delivery is to modulate the magnitude and duration of drug action(s), and to dissociate it from the inherent properties of the drug molecule. To enable optimal design of controlled release systems, a thorough understanding of the pharmacokinetics and pharmacodynamics of the drug is necessary.

In many cases, the development of SR dosage forms is somewhat empirical. It is often based on the sole objective of reducing the dosing frequency or fluctuation between peak and trough plasma concentrations (C and Cmax respectively) associated with conventional tablet or capsule formulations. The development process tends to be based on an intuitive pharmacodynamic rationale assuming that the magnitude of response elicited by the drug is closely related to changes in its plasma concentration (106).

In general, almost all drugs cause side effects or have extraneous activity in addition to their primary therapeutic function. An important principle in the design of a proper delivery system for a drug is the conconsideration that each of the pharmacologic effects of the drug has its own pharmacodynamic profile. Furthermore, while a certain pharmacological effect is considered as a therapeutic response,

larger intensities of the same effect are regarded as undesired (and possibly toxic). Thus, an important advantage of SR formulations is that by narrowing the range of drug concentrations (especially, by reducing Cmax levels) the delivery system enables the minimization of the adverse effects associated with elevated drug concentrations. This pharmacodynamic principle has been widely applied as a means to improve drug therapy (107). There are numerous examples to demonstrate modulation of adverse effect of NSAIDs by SR formulations (108-115).

Lipid-based formulations have attracted increasing attention for improvement of bioavailability of hydrophobic drugs in comparison with solid dosage forms (116). In fact, lipid microspheres composed of lecithin and soybean oil were tested as carriers for hydrophobic NSAIDs (117). Unlike many of NSAIDs, DFNa is basically watersoluble at neutral pH, making it difficult to exist in an oil-based formulation. Although self-emulsifying drug delivery system (SEDDS) composed of goat fat and Tween 65 was also applied to diclofenac (90).

Twenty-five inpatients with chronic inflammatory rheumatic disease were entered into a double blind crossover trial. Consecutive treatment regimens consisted of a single daily dose of Bi-Profenid 150 mg at 8 pm for 3 days and a single placebo tablet at 8 pm for 3 days. Order of treatment regimens was randomly assigned. Bi-Profenid proved highly superior to

placebo with a very significant (p less than 0.01) difference in effectiveness on nocturnal pain, morning stiffness and pain evaluated on the pain scale. During the short treatment period no significant clinical side-effects were recorded. The authors conclude that Bi-Profenid is effective at a daily dosage of 150 mg, thus enabling to adjust prescriptions to actual needs when pain is not continuous throughout the 24 hours (118).

Schumacher HR. et al. (119) described a new extended-release formulation that maintains therapeutic plasma ketoprofen concentrations for up to 24 hours. A single 200-mg capsule thus provides daytime and nighttime symptom control. Small pellets, enclosed in a gelatin capsule, are released in the stomach but release their contained ketoprofen only after reaching the nonacidic environment of the small intestine. Diurnal fluctuations in plasma concentrations of ketoprofen are reduced, and the drug does not accumulate in plasma with extended use. The half-life of the drug from this dosage form is not significantly affected by the increasing age of the patients. The efficacy of extended-release ketoprofen in British clinical trials has been comparable to that of conventional ketoprofen or naproxen. Safety profiles have been comparable to profiles of other NSAIDs; adverse effects have usually been mild and transient, although, as with other NSAIDs, ulcers and bleeding can occur. Extended-release ketoprofen appears to be a good choice for the symptomatic treatment of rheumatoid arthritis and osteoarthritis. Convenient once-daily administration may help improve patients' compliance.

An open study was carried out in 46 patients with osteoarthritis of the hip to compare the efficacy and tolerance of treatment with ketoprofen given either as 100 mg capsules twice daily or as 2 capsules of 100 mg ketoprofen in a controlled-release formulation given once daily. The results of subjective and objective assessments before and during 3-months' treatment in the 48 patients who completed the trial showed both treatments produced improvement in all parameters, except for the time taken for inactivity stiffness to develop, and there was no significant difference between treatments in terms of efficacy. The controlled-release preparation, however, was significantly better tolerated than the ordinary capsule form. Minor haematological and biochemical changes during treatment were noted but these were not of clinical importance. Six patients, 2 receiving the controlled-release and 4 receiving the ordinary formulation of ketoprofen, were withdrawn because of lack of efficacy or unacceptable side-effects (120).

A multi-centre, double-blind, crossover study was carried out in 80 patients with rheumatoid arthritis to compare the efficacy and side-effect profiles of two formulations of indomethacin. Patients were allocated at random to receive 75 mg indomethacin per day either as 1 controlled-release tablet at night or as 1 immediate-release capsule given 3-times a day for a period of 4 weeks before being crossed over to receive the alternative treatment for a further 4 weeks. Pain scores, daily symptomatology and the requirement for escape analgesia recorded by both investigator and patient indicated that controlled-release indomethacin tablets, 75 mg given at night, was as efficacous as immediate-release indomethacin capsules given 3-times daily. However, the controlled-release formulation had a superior side-effect profile with a reduced incidence of abdominal/epigastric pain compared to the immediate-release preparation (121).

Prichard PJ. et al. (122) have compared acute gastric bleeding caused by a new slow release preparation of indomethacin (indomethacin Continus) with that caused by aspirin and other indomethacin preparations. In a randomized crossover study, blood loss into timed gastric aspirates was determined in 20 healthy volunteers after receiving, over 96 h, either placebo, aspirin (600 mg four times daily; 17 doses) indomethacin BP (50 mg three times daily; 13

doses), Indocid-R (75 mg twice daily; 9 doses) or indomethacin Continus (75 mg twice daily; 9 doses). A venous blood sample was also taken during each treatment period for subsequent determination of alpha 1-glycoprotein, and for drug assay. Gastric bleeding on placebo was 1.4 (0.7-2.8) microliters 10 min-1 (mean, 95% CI). Both aspirin and the indomethacin preparations caused significantly more bleeding (P less than 0.05). Rates of bleeding after aspirin, indomethacin BP, Indocid-R, and indomethacin Continus were respectively 22.0 (10.7-47.2) microliters 10 min-1, 4.4 (2.2-9.1) microliters 10 min-1, 10.8 (5.3-22.3) microliters 10 min-1, and 5.1 (3.0-10.6) microliters 10 min-1. 4. Rates of bleeding after indomethacin BP and indomethacin Continus, but not Indocid-R, were significantly less than after aspirin (P less than 0.01). Salicylate or indomethacin was detectable in the plasma of all subjects after the active treatment periods, except for one instance involving a subject allocated indomethacin BP. Indomethacin levels were significantly higher 2 h after Indocid-R than with indomethacin BP or indomethacin Continus. 6. alpha 1-acid glycoprotein levels were not significantly affected by prior treatment with aspirin or indomethacin.

GI blood loss was measured in 30 healthy male volunteers before and during 4 weeks of oral treatment with either tiaprofenic acid tablets 300 mg twice daily, tiaprofenic acid sustained action (SA) capsules 600 mg once daily, or indomethacin SR capsules 75 mg once daily, in an open parallel-group study of 38 days' duration. Autologous erythrocytes labelled with 51Cr were given intravenously on the first study day. GI blood loss was measured by comparing faecal and red blood cell 51Cr activity during the second and fourth weeks of drug treatment. Blood loss was significantly greater during treatment with all 3 active preparations than during the pretreatment period, but this comparison is of limited value because placebo was not given in parallel and because in 4 subjects, who had to have their erythrocytes relabelled, there was no pretreatment data. The tiaprofenic acid SA group had consistently lower blood loss than the tiaprofenic acid tablet group. Both these groups also had consistently lower blood loss than the indomethacin SR group, although the difference between the treatment groups was not significant. Blood loss during the fourth week of treatment was less than during the second week of treatment for both the tiaprofenic acid SA and indomethacin SR capsule groups. With tiaprofenic acid tablets, blood loss was very similar at weeks 2 and 4 but this result should be viewed with caution because data at week 2 were missing for 3 subjects. Thus, formulation of tiaprofenic acid as a sustained action capsule does not appear to increase gastric irritancy as measured by faecal blood loss (123).

Fourty adult patients with coxarthrosis were treated for 30 days with oral diclofenac sodium at the daily dose of 150 mg: 20 of these were administered one 150 mg prolonged-release capsule per day, the other 20 received one 50 mg enteric-coated tablet every 8 hours. The presence and severity of several symptoms and signs (various pain types, cramps, morning stiffness, impaired function capacity), the intensity of pain through the Visual Analogical Scale and some laboratory tests (Erythrocyte Sedimentation Rate, C-reactive protein, Rheuma test) were controlled to monitor drug efficacy. The routine laboratory tests of blood, liver and kidney function, the GI tolerance of the two administered formulations and the appearance of any adverse event were controlled to monitor drug tolerability. Both administration schemes yielded very positive results as to treatment efficacy, although the prolonged-release capsule often induced a somewhat quicker response. At the end of the one-month treatment more than half of patients in both groups registered disappearance of several symptoms and a noticeable reduction of the remainder ones. Systemic tolerability was also good, with superimposable results in the two groups; GI tolerance on the contrary was better in the recipients of the prolonged-release capsules (2 cases of dyspepsia) with

respect to those treated with the enteric-coated tablets (2 cases of gastric pyrosis and 2 cases of gastralgia). No adverse events were registered (124).
A double-blind, double-dummy, crossover study was carried out in 8 centres to compare the efficacy and tolerability of 'controlled-release' ketoprofen tablets (200 mg) with that of indomethacin suppositories (100 mg) in out-patients with definite or classical rheumatoid arthritis. Patients were allocated at random to receive a daily bedtime dose of either 1 ketoprofen tablet or 1 indomethacin suppository plus the dummy of the other formulation for a period of 3 weeks. They were then crossed over to the alternative treatment for a further 3 weeks. Daily diary records were kept by patients of the number of night-time awakenings due to pain, pain severity at awakening in the morning and the duration of early morning stiffness. Treatment efficacy was also assessed at the end of each trial period by means of an articular index and by physician's and patient's overall evaluation of response. Adverse effects spontaneously mentioned by the patients or elicited by direct questioning using a symptom check-list were recorded. Statistical analysis of the results from 83 evaluable patients showed that the 'controlled-release' tablet formulation of 200 mg ketoprofen was equally as effective as the 100 mg indomethacin suppository in the treatment of rheumatoid arthritis, especially with regard to pain at awakening and morning stiffness. Side-effects in both groups were those commonly seen with non-steroidal anti-inflammatory drugs and, as expected, GI and CNS disturbances predominated. Overall, side-effects were fewer with ketoprofen than with indomethacin (125).
There are several histological studies which showes that controlled release formulations of NSAIDs are alternatives for preventing of gastric lesions.
Nishihata T. et al. (126) showed in their study that the increased solubility of sodium diclofenac in a suppository base in the presence of lecithin resulted in a slow release of sodium diclofenac from the base. Rat rectal mucosal damage caused by sodium diclofenac was moderated by the administration of the lecithin suppository, probably due to the low concentration of sodium diclofenac in the rectal fluid due to a slow release of sodium diclofenac from the lecithin suppository.
A mefenamic acid-alginate bead formulation (127, 128) and mefenamic acid spherical agglomerates (129) prepared with various polymethacrylates were developed in different studies and evaluated histologically. Histological studies showed that the administration of mefenamic acid in alginate beads or spherical agglomerates prevented the gastric lesions.
Another work reports on a new pharmaceutical formulation for oral delivery of diclofenac sodium (DFNa). Although DFNa itself is water-soluble at neutral pH, it was readily suspended in soybean oil via complex formation with an edible lipophilic surfactant and a matrix protein. The resulting solid-in-oil (S/O) suspension containing stably encapsulated DFNa in an oil phase markedly reduced the risks for GI ulcers upon oral administration even at the LD50 level in rats (ca. 50 mg/kg DFNa) (90).

7. H. pylori induced gastric ulcers

H. pylori is a gram-negative microaerophilic non-invasive spiral bacillus which has the ability to colonize the gastric mucosa (130). It causes indolent but chronic inflammation in the gastric mucosa and its clinical course is highly variable (131). It has a powerful urease enzyme which catalyses hydrolysis of urea to ammonia, enabling the bacteria to survive in the acid milieu. Although it induces a strong host local and systemic immune response (which is important in pathogenesis) it has also developed mechanisms to evade host

immunity. This means that following initial infection, which usually occurs in childhood, it is able to persist lifelong in the absence of effective treatment. This persistent infection and inflammation underlies disease, which usually occurs in adults. Worldwide, *H. pylori* colonizes >50% of the population and is by far the most important cause of peptic ulcers and gastric adeno-carcinoma. Its prevalence varies from more than 80% in developing countries to less than 20% in some developed countries, where it is steadily falling due to improved hygiene and sanitation, and possibly increased antibiotic use.

Only about 15% of individuals infected with *H. pylori* develop a peptic ulcer: who develops disease depends on bacterial, host and environmental factors (130). The infection is usually limited to the antrum, resulting in hypersecretion of acid and the development of duodenal ulcers, which is basically an acid injury. However, the infection sometimes spreads proximally, causing diffuse inflammatory damage to the gastric mucosa in the body of the stomach and resulting in a gastric ulcer. The inflammation induced by H. Pylori damages the natural defence of the gastric mucosa (131).

The risk of ulceration is higher with more virulent strains. The best-described virulence determinants are expression of active forms of a vacuolating cytotoxin (VacA) (132) and possession of a protein secretory apparatus called Cag (cytoxin-associated gene products) that stimulates the host inflammatory response (133). Cag+ strains interact more closely with epithelial cells and induce release of pro-inflammatory cytokines, thereby increasing inflammation. Host genetic susceptibility and environmental factors may affect the risk; for example, smoking is strongly associated with peptic ulceration in *H. pylori*-infected individuals (134). *H. pylori*-induced duodenal ulceration arises in people with antral-predominant gastritis (135). Gastric ulceration occurs on a background of pangastritis, often arising at the highly inflamed transitional zone between antrum and pylorus, particularly on the lesser curve. Identical hormonal changes occur, but acid production from the inflamed corpus is reduced or normal.

H. pylori appears to be responsible for 95% of the cases of gastritis and 65% of gastric ulcers (136). Although most individuals with H. pylori are asymptomatic, there is now convincing evidence that this bacterium is the major etiologic factor in chronic dyspepsia, H. pylori-positive duodenal and gastric ulcers and gastric malignancy (137, 138). Consequently, H. pylori eradication is now recognized to be the correct approach along with conventional therapies in the treatment of the disease. Options that have been considered to treat peptic ulcer disease include taking drugs such as antacids, H -blockers, antimuscarinics, proton pump inhibitors and combination therapy for gastritis associated with H. pylori. The eradication of H. pylori is limited by its principle unique characteristics. Once acquired, it penetrates the gastric mucus layer and fixes itself to various phospholipids and glycolipids on the epithelial surface, including phosphatidylethanolamine (139), GM3 ganglioside (140) and Lews antigen (141). For effective H. pylori eradication, therapeutic agents have to penetrate the gastric mucus layer to disrupt and inhibit the mechanism of colonization. This requires targeted drug delivery within the stomach environment. Although most antibiotics have very low in-vitro minimum inhibitory concentrations against H. Pylori, no single antibiotics has been able to eradicate this organism effectively. Currently, a drug combination namely ''triple therapy'' with bismuth salt, metronidazole and either tetracycline or amoxycillin with healing rates of up to 94% has been successfully used (142-144). The principle of triple therapy is to attack H. pylori luminally as well as systemically. The current treatment is based on frequent administration (4 times daily) of individual dosage forms of bismuth, tetracycline and metronidazole (Helidac Therapy, consisting of

262.4 mg bismuth subsalicylate, 500 mg tetracycline and 250 mg metronidazole). The associated limitations are the complex dosing regimen/frequency, large amount of dosage forms and reduced patient compliance. Therefore, a successful therapy not only includes the selection of the right drugs but also the timing and frequency as well as the formulation of the delivery system (2).

8. Dosage forms with prolonged gastric residence time

More than 50% of the pharmaceutical preparations on the market are for oral administration. The advantages of this route include the ease of administration, and avoidance of the pain and discomfort associated with injections. However, for drugs whose target is the stomach, such as antibiotics against H.pylori for local treatment of gastric ulcer, the development of oral drug delivery systems meets with physiological obstacles such as limited residence time and inefficient drug uptake by the gastric mucosa (145). Long-term monotherapy of gastric ulcer patients with amoxycillin is ineffective even at high daily doses, apparently due to limited contact time with the target site when administered in a conventional oral dosage form (138, 146-148).

The degredation of antibiotics in gastric acid may be the other reason of ineffectiveness (149). Local diffusion of the drug in the mucosa appears to be essential for achieving bactericidal levels in both healthy subjects (138) and patients: for example, more complete eradication of H. pylori was achieved by applying a new method of topical therapy in which an amoxycillin solution was kept in contact with the stomach for 1 h (150). The development of oral amoxycillin dosage forms with prolonged gastric residence time is therefore an attractive goal. Several strategies have been developed in order to prolong the gastric residence time of dosage forms and target the gastric mucosa, including the use of floating, floating in situ gelling, swelling, expanding and bioadhesive forms (151-156). A new strategy is proposed for the triple drug treatment (tetracycline, metronidazole and bismuth salt) of Helicobacter pylori associated peptic ulcers. The design of the delivery system was based on the swellable asymmetric triple layer tablet approach, with floating feature in order to prolong the gastric retention time of the delivery system. Tetracycline and metronidazole were incorporated into the core layer of the triple-layer matrix for controlled delivery, while bismuth salt could be included in one of the outer layers for instant release. Results demonstrated that sustained delivery of tetracycline and metronidazole over 6–8 h can be easily achieved while the tablet remained afloat. The floating aspect was envisaged to extend the gastric retention time of the designed system to maintain effective localized concentration of tetracycline and metronidazole. The developed delivery system has potential to increase the efficacy of the therapy and improve patient compliance (2).

Floating in situ gelling system of clarithromycin (FIGC) was prepared using gellan as gelling polymer and calcium carbonate as floating agent for potentially treating gastric ulcers, associated with H.pylori. The in vivo H. pylori clearance efficacy of prepared FIGC and clarithromycin suspension following oral administration, to H. pylori infected Mongolian gerbils was examined by polymerase chain reaction (PCR) technique and by a microbial culture method. FIGC showed a significant anti-H. pylori effect than that of clarithromycin suspension. It was concluded that prolonged GI residence time and enhanced clarithromycin stability resulting from the floating in situ gel of clarithromycin might contribute better for complete clearance of H. Pylori (157). Rajinikanth P.S et al. (149) developed in their another study a intra-gastric floating in situ gelling system for controlled delivery of amoxicillin for the treatment of peptic ulcer disease caused by H.pylori. They

prepared gellan based amoxicillin floating in situ gelling systems (AFIG). The in vivo H. pylori clearance efficacy of the formulation was examined by the same technique . It showed a significant anti-H. pylori effect in the in vivo gerbil model. It was noted that the required amount of amoxicillin for eradication of H. pylori was 10 times less in AFIG than from the corresponding amoxicillin suspension. The results further substantiated that the prepared AFIG has feasibility of forming rigid gels in the gastric environment and eradicated H. pylori from the GI tract more effectively than amoxicillin suspension because of the prolonged GI residence time of the formulation.

A gastroretentive drug delivery system of DA-6034, a new synthetic flavonoid derivative, for the treatment of gastritis was developed by using effervescent floating matrix system (EFMS). The therapeutic limitations ofDA-6034 caused by its low solubility in acidic conditions were overcome by using the EFMS, which was designed to cause tablets to float in gastric fluid and release the drug continuously. The release of DA-6034 from tablets in acidic media was significantly improved by using EFMS, which is attributed to the effect of the solubilizers and the alkalizing agent such as sodium bicarbonate used as gas generating agent. DA-6034 EFMS tablets showed enhanced gastroprotective effects in gastric ulcer-induced beagle dogs, indicating the therapeutic potential of EFMS tablets for the treatment of gastritis (158).

In another example, it was found that in normal volunteers ionexchange resins achieved excellent distribution in the gastric cavity and had a prolonged gastric residence time, 20-25% remaining for 5.5 h. (155). More recent results by the same group indicate that the mechanism by which resin particles adhere to the mucosa is unlikely to be chargebased, since they persist in the stomach regardless of whether they bear a non-adhesive polymer coating and regardless of whether the stomach contains food (156). Other authors have recently shown that ion-exchange resins also interact with other mucosal surfaces, such as the nasal mucosa (159). Because of this reason, microparticles consisting of amoxycillin-loaded ion-exchange resin encapsulated in mucoadhesive polymers (polycarbophil and Carbopol 934) were prepared.

As reported in this review, the drug delivery systems have an important role on prevention of NSAID related or H.pylori induced gastric ulcers.

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Spices as Alternative Agents for Gastric Ulcer Prevention and Treatment

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1. Introduction

1.1 Important aspects on ulcer pathogenesis

World-wide, peptic Ulcer disease (PUD) is considered as a common gastrointestinal disorder. It develops as a result of altered balance between offensive and defensive factors. Offensive (aggressive) factors disrupt normal mucosal integrity and allow H+ back diffusion with a subsequent cellular injury. Helicobacter pylori (H. pylori) and nonsteroidal anti-inflammatory drugs (NSAID) represent the major aggressive factors associated with PUD. Experimentally induced gastric ulcer has expanded our knowledge on ulcer pathogenesis. Indomethacine, 80% ethanol and pyloric ligation are the methods commonly applied in experimental ulcer models. Other universally accepted experimental ulcer models include 0.2 mol/L NaOH, 25% NaCl, stress induced by swimming (1), acetylsalicylic acid (2), cold-restraint (3) and hypothermic restraint (4).

A major event in the pathogenesis of NSAID induced gastric ulcer is represented by inhibition of prostaglandin (PG) synthesis, enhancement of gastric acid secretion, suppression of bicarbonate secretion, glutathione (GSH) levels, mucosal circulation, cell proliferation and growth as well as alteration of gastric mucosal barrier integrity. Inhibition of PG biosynthesis enhances generation of leukotrienes and other products of the 5-lipoxygenase pathway (5). These products disrupt the mucosal barrier with subsequent enhancement of gastric mucosal permeability for H + ions and Na+ ions and reduction of transmucosal potential difference (6, 7). Furthermore, NSAID uncouple mitochondrial oxidative phosphorylation, affect mitochondrial morphology, reduce the intracellular ATP levels and alter the normal regulatory cellular function (8). These processes promote erosions and ulcer formation. In addition, generation of reactive oxygen species (ROS) is also considered as a major factor contributing to ulcer pathogenesis. Another, prostaglandin-independent pathway of gastric ulcer pathogenesis is induced by enhanced endothelial adhesion, activation of polymorphonuclear cells (PMN) with subsequent release of oxidative byproducts (9, 10). PMN activation induces depletion of GSH and sulfhydryl compounds (SH) in tissue with enhanced mucosal myeloperoxidase (MPO) and malondialdehyde (MDA) concentration (11). Myeloperoxidase is considered as a marker of oxidative process induced by PMN tissue infiltration.

Similarly, in ethanol-induced gastric mucosal injury there is enough evidence to suggest the role of oxidative burst. Ethanol-induced oxidative damage is commonly associated with

generation of ROS, leading to oxidative stress. Acute ethanol treatment induced oxidative damage is associated with a decreased GSH content in gastric tissue along with an increased MDA and xanthine oxidase activity (12). The oxygen free radicals-induced lipid peroxidation affects mitochondrial energy metabolism and plays a critical role in the pathogenesis of acute ethanol-induced gastric mucosal injuries (13). On the mitochondrial level, ethanol-induced intracellular oxidative stress causes also mitochondrial permeability transition with mitochondrial depolarization that precedes gastric mucosal cells necrosis. This process can be prevented by intracellular antioxidants, such as GSH (14). Acute ethanol administration is also associated with inhibition of catalase and, glutathione peroxidase (GPx) activities with significant increase of MDA contents, MPO activity, and cellular apoptosis (15). Furthermore, ethanol induces inhibition of SH with enhancement of superoxide dismutase (SOD) and glutathione reductase (GR) activities (16). The extent of oxidative damage in stomach as indicated by the ulcer index, gastric mucosal MDA content and alteration of mitochondrial ultrastructure is correlated with ethanol exposure and concentration (13).

2. Introduction in spices

Spices are used in several parts in the world as food additives and carminatives. Since ancient times they are also applied in the traditional management of a variety of disorders. Currently their therapeutic value has gained a considerable interest and several investigators have reported their effects in laboratory animals and in man. It has been experimentally demonstrated that spices, herbs, and their extracts possess antibacterial (17) antifungal (18,19), vermicidal, nematocidal, molluscicidal properties (20-22), anti-inflammatory and antirheumatic activity (23-25), hepatoprotective (26,27), nephro-protective (28), antimutagenic, anticancer potentials (28-30) and antihypercholesterimc potentials (31-34).

A tremendous number of studies have evaluated the antiulcer effect of spices. Although some investigators have reported deleterious effect of certain spices such as red and black pepper, the majority have demonstrated rather a cytoprotective activity in animal (35-37) as well as in human (38).

3. Factors involved in ulcer healing

In the presence of mucosal barrier disruption, several factors including acid, bile acids, NSAID and ethanol promote H+ back diffusion and enhance the susceptibility to develop ulcer. On the other hand, optimal mucosal microcirculation and bicarbonate secretion with formation of an alkaline buffer layer at the epithelial surface is considered as a first line of mucosal defense and gastroprotection. Other factors including prostaglandins (PG), growth factors (GF), nitric oxide (NO) or calcitonin gene-related peptide (CGRP), as well as some gut hormones such as gastrin, cholecystokinin (CCK), leptin, ghrelin, gastrin-releasing peptide (GRP) and melatonin are involved in mucosal defense system and the ulcer healing process. The protective action of gut hormones is attributed to the release of cyclooxygenase-2 (COX-2) and PGE2 at the ulcer margins (39, 40) or activation of sensory nerves (41). In addition, Tumor necrosis factor – α (TNF-α), released during gastric mucosal injury, activates PG pathway and promotes epithelial cell repair and healing (42). EGF and other growth factors are also pivotal for the process of mucosal healing. Furthermore, in response to gastric injury and inflammation, gastrin and parietal cells contribute to the regulation of mucosal proliferation (43).

4. Why spices, herbs and plant extracts are considered as an alternative ulcer therapy?

Ulcer healing and prevention of recurrence represent the central goals of treatment. The treatment is targeted at either counteracting aggressive factors like acid, pepsin, active oxidants, platelet aggravating factor (PAF), leukotrienes, endothelins, bile or exogenous factors including (NSAID) or enhancing mucosal defense such as mucus, bicarbonate, blood flow, PG and NO (44). Antiscretory drugs including H_2-receptor antagonists (H_2-RA) and proton pump inhibitors (PPI) alone or combined with antibiotics in the presence of H. pylori infection are currently considered as the most acceptable drugs for ulcer treatment. The main action of antisecretory drugs is acid suppression. These agents lack effect on other factors involved in ulcer pathogenesis and therefore, do not meet all treatment goals. In addition, acid suppressors are expensive and associated with adverse effects and ulcer recurrence. Hence, efforts are on to search for suitable alternative treatment from medicinal plants resources. Already a large percentage of world population relies on medicinal plants to treat a variety of disorders including PUD. In addition to their ability to act on various pathogenetic factors, they are cheap and easily accessible. Furthermore, a large number of spices and plant extracts evaluated by various researchers for their anti-ulcer effects have a favorable outcome. (29, 45-48)

The antiulcer effect of spices/herbs is based on the activities of their chemical constituents, which attenuate the gastric secretion, enhance mucosal integrity, interfere with oxidative burst, NO, SH compounds and inhibit H. pylori growth. Due to their variable phytochemical constituens they may exhibit antisecretory, cytoprotective, antoxidant or combined activities.

5. Herbs and gastric secretion

A variety of spices and their extracts possess a potent antiscretory activity. Pylorus-ligation in rats represents the model of antisecretory studies. A number of spices, herbs and plants methanolic or aqueous extracts possess an antisecrertoy activity. For instance, Cissus quadrangularis , Maytenus ilicifolia, phytosphingosine, Cecropia glaziovii Sneth (Cecropiaceae), alkaloid extract and 2-phenylquinoline obtained from the bark of Galipea longiflora (Rutaceae) and Landolphia owariensis induce significant inhibition of acidity, pepsin content and ulcer index (49-54), respectively. This potent antisecretory action of spices and plant extracts is likely related to their flavonoid content. Maytenus ilcifolia is considered among flavenoid-rich plant extracts (55).

In rats with pylorus ligation, antisecretory effect of methanolic extract of Momordica charantia L. is demonstrated by decrease in acidity, pepsin content and ulcer index with an increase in gastric mucosal content (56).

Likewise, the protective effect of Cissus quadrangularis extract is mediated by inhibition of gastric secretion with decrease in ulcer index as well as enhancement of mucosal defense (49).

6. Herbs and cytoprotection

Several spices and plants extracts promotes ulcer healing via enhancing gastric mucosal content beside their antisecretory activity in pylorus-ligated rats. Ginger rhizome extract-induced cytoprotective activity is based on its antioxidative and gastric mucosal protective

activities (1). Total carotenoid and astaxanthin esters protect mucin, enhance antioxidants enzymes level and H+, K+-ATPase inhibitory activity (57). Furthermore, boswellic acid-induced gastroprotection depends on generation of cytoprotective PG, enhanced gastric mucosal resistance, and inhibition of leukotriene synthesis (3). Similarly, Galipea longiflora (Rutaceae) protect gastric mucosa by enhancement of mucus content and antisecretory activity (53). In addition to its antioxidant activity, Cissus sicyoides , induce increase of NO and SH compounds and enhances defense mechanism (58). In pylorus-ligated rats, beside its antiscretory action, Momordica charantia L. extract also significantly increases gastric mucosal content (56). Increase in Glycoprotein level, gastric mucin content and SH concentration are essential for the gastroprotection. Their levels are raised by treatment with Cissus quadrangularis extract (49)

7. Herbs and antioxidants

Recently, oxidants are found to play a critical role in PUD pathogenesis. Experimental NSAID and ethanol induced microvascular and gastric mucosal injuries are at least partially caused by ORS release (59). Therefore, implementations of agents with antioxidative properties are useful for the prevention of injuries and promotion of gastric ulcer healing. Many spices have phytochemicals with antioxidative activities. For instance, Coriander contains many antioxidant constituents including d-linalool, borneol, geraniol, geranyl acetate, camphor, carvone, which are responsible for its antioxidative property (60). Black cumin (Nigella sativa), peperine and thymoquinone, the active constituents of pepper and Nigella sativa, respectively have also the ability to inhibit ROS in experimentally induced gastric lesions in rats (29, 60, 61).

Many other spices/herbs and plant extracts protect against experimentally-induced gastric mucosal injuries through their potential antioxidative effect. These include ginger rhizome, carotenoid and astaxanthin esters, Cissus sicyoides extract, isopulegol and the herb collection Korniozil (1, 57, 58, 62, 63), respectively. Through the interaction with endogenous PG and antioxidative properties, isopulegol, monoterpene a constituent of essential oils of several aromatic plants, induce significant gastroprotection. Total carotenoid and astaxanthin esters increase the levels of the antioxidant enzymes catalase, SOD, and GPx in gastric homogenate and protect gastric mucin (57). Korniozil also protects against experimentally induced stress ulcers, with restoration of lipid peroxidation and antioxidative system function along with enhancement of gastric mucous coat regeneration (63). Ginger rhizome extract gastroprotective activity is also based on restoration of antioxidant enzymes and gastric mucin generation in addition to inhibitory effect on H. pylori growth (1). Due to its antioxidative properties, Cissus sicyoides oral extract increase also NO and SH and induces also protection (58]

The alkaloid indigo, obtained from the leaves of Indigofera truxillensis Kunth (Fabaceae), prevents ethanol induced depletion of SH and GPx activity, inhibits GR and MPO activities and partially inhibits gastric mucosa DNA damage caused by ethanol (64).

8. Herbs combined activities

Due the presence of several active constituents, some spices/herbs and plant extracts protect the gastric mucosa via different mechanisms. For instance, Weikang decoction acts as antisecretory, cytoprotective and antioxidative agent. It enhances mucosal thickness, NO in

gastric tissue, PGE2 in plasma, (EGF) content in gastric juice and SOD in plasma. In addition it inhibits also MDA and endothelin in plasma (65). Many other spices like Rocket Eruca sativa, black cumin, black pepper, clove, cardamom, caraway , peppermint, saffron, coriander and anise possess also antisecretory, cytoprotctive and antioxidative activities (4, 29, 66-73). They also replenish gastric wall mucus concentration and SH levels and significantly reduce MDA level.

Besides its H. pylori bactericidal effect, Davilla elliptica also enhances NO, H_2O_2, TNF - α production and GSH bioavailability. These activities are related to its phytochemical constituents acylglycoflavonoids, phenolic acid derivatives and tannins (74). Also Brazilian medicinal plant methanolic extracts have an anti-H. pylori effect and protect the gastric mucosa by increasing PGE2, antisecretory and gastroprotective properties (75) The gastroprotection of Vochysia tucanorum Mart. methanolic extract and buthanolic fraction provided by the antioxidant activity and maintenance of gastric mucosa NO levels is interrelated to its phytochemical constituent Triterpenoid (76)

9. Herbs- PG interaction

Gastric protection is maintained in a state of equilibrium between aggressive and protective factors. In experimental ulcer model, indomethacin increases acid secretion, activates oxidative stress and inhibits the release of cyclooxygenase-1 (COX-1), PGE2, bicarbonate, and mucus (77). Similar to conventional NSAID, COX-2 inhibitors also delay the healing of chronic gastric ulcer and suppress the epithelial cell proliferation, angiogenesis and maturation of the granulation tissue in experimental animals. COX-2 is important for gastric mucosal defense (78). Indomethacin- induced gastric damage is associated with an increase of acid and oxidative parameters and inhibition of protective factors such as COX- 1, PGE2, bicarbonate, and mucus release (77).

Generally, PG are products of arachidonic acid and their biosynthesis is influenced by local, hormonal and neural factors. They stimulate gastric and duodenal bicarbonate secretion and the production of mucus glycoproteins. PG are also able to protect the gastric mucosa against experimentally induced gastric injuries in an acid independent manner known as “cytoprotection" (79). PG play a pivotal role in the gastric mucosal defensive system and contribute to the overall protective process against gastric mucosal injuries. They exhibit a variety of defensive mechanisms including mucus-alkaline secretion, mucosal hydrophobicity, mucosal microcirculation, tissue lysosomes stabilization, SH preservation, rapid proliferation and mucosal cells renewal. Mucosal integrity protection can also be accomplished even by small quantities of PG. Gastroprotection is attained by stimulation of mucosal protective PG biosynthesis or by the inhibition of preulcerogenic arachidonic acid metabolites (80). In addition, PG antiulcer activity is determined mainly by their antioxidant property with inhibition of lipid peroxidation as well as SOD and catalase activities (81).

Other mediators involved in gastric mucosal protection besides PG include growth factors, NO, CGRP and some gut hormones such as gastrin and CCK. In addition, leptin, ghrelin and gastrin-releasing peptide (GRP) have also the ability to protect gastric mucosa against corrosive agents-induced mucosal damage. Gut hormones protective activity is attributed to PG release (41). Ulcer healing is controlled by contribution of growth factors and gut hormones, increase of COX-2 induction and local PGE release in the ulcer area. Endogenous PG generated at ulcer margin play a key role in ulcer cure (82)

In acute injury, in the presence of PG-mediated paracellular space closure, mucosal permeability, PG helps the mucosal permeability to recover with epithelial restitution (83,

84). The ulcer healing by endogenous PG is mediated by PGEP4 receptors, as well as involvement of COX-2 in the early stage and COX-1 in the late stage of healing. Bacterial lipopolysaccharide contributes also through COX and endogenous PG genes activation to gastric mucosal protection in rats (85).

Mainly through their effect on PG, several plant extracts promote ulcer healing. For instance, Hyptis spicigera essential oil major constituents, monoterpenes, enhances PGE2-induced gastric mucus and reduces ulcer size in addition to increasing COX-2 and EGF expression in gastric mucosa and acceleration of ulcer healing (86).

Other spices and plant extracts involved in activation of PG synthesis and healing of gastric mucosal injuries include Boswellic acid, isopulegol, Teucrium polium (3, 62, 87). Endogenous PG and PGEP1 receptors play a key role in the adaptive protection (88). Chilli is believed to be detrimental to the gastric mucosa, however, its active ingredient capsaicin, decreases acid secretion and activates the defensive system by enhancing mucus, alkali secretions as well as mucosal microcirculation and hence, it prevents ulcer formation. Furthermore, capsaicin stimulates afferent neurons in the stomach and transmits signals to the central nervous system, which trigger an anti-inflammatory response and gastroprotection (89). Furthermore, Citrus lemon, Alchornea triplinervia and Myristica malabarica have demonstrated gastroprotective effect. Citrus lemon belongs to Rutaceae family and contains two main components, limonene and β-pinene. In ethanol and indomethacin gastric ulcer models, while Citrus lemon and limonene induce complete gastroprotection, β-pinene is not effective. Citrus lemon and limonene protective effect is linked with PGE2 and mediated by enhancing mucus secretion, HSP-70 and VIP (90). The antiulcer effect of ethyl acetate fraction of Alchornea triplinervia, a medicinal plant used in Brazil to treat gastrointestinal ulcers, is mainly related to its flavonoids content and mediated by increasing gastric mucosal prostaglandin PGE2 levels (75).

While PGE2, and vascular endothelial Growth Factor (VEGF) levels decrease, EGF and endostatin levels increase in indomethacin- induced ulceration in mice. Through modulation of PG synthesis and angiogenesis, Myristica malabarica plant extract restores these parameters. In comparison omeprazole, which offered similar healing, did not alter these parameters (91).

Coenzymes Q10, an essential cofactor in the mitochondrial electron transport pathway possess a potent antioxidant action. Pretreatment of indomethacin induced gastropathy with CoQ10 prevents ROS generation, mitochondrial dysfunction, vascular permeability erosions, ulcers and helps to restore PGE_2, NO and GSH levels (92).

10. Herbs and EGF

Growth factors and their receptors are also important for maintaining physiological function of gastric mucosa. They maintain and enhance defensive and inhibit aggressive factors. Following acute mucosal injury and during the initial stages of experimental gastric ulcer healing, R-associated tyrosine kinase is essential for regulation of cell proliferation, EGFR gene activation, EGFR phosphorylation, and increased mitogen-activated protein (MAP) kinase activity. H. pylori is a major cause of PUD and contributes also to inhibition of healing. In experimental gastric ulcer model, H. pylori vacuolating cytotoxin interfers with ulcer healing and inhibits cell proliferation, binding of EGF to its receptor, EGF-induced EGFR phosphorylation, and MAP and extracellular signal-related kinase (ERK-2) activation (93,94).. Growth factors and their receptors are pivotal for the process of gastroprotection

and ulcer healing. EGF and transforming growth factor (TGF)-α and their common receptor (EGFR) inhibit gastric secretion, boost overexpression of growth factors, blood flow at ulcer margin and promote cell proliferation with ulcer healing (95). The process of gastric mucosal tissue repair and healing is controlled by EGFR activation (96). EGF-induced gastric epithelial cells proliferation is likely intervened by ERK /COX-2 pathway (97). Various GF exhibit different functions of the mucosal repair. They are implicated in the process of tissue healing with cell migration, proliferation, differentiation, secretion, and degradation of extracellular matrix. While EGF , TGF- α , and trefoil factors (TFFs), usually present in the gastric juice or mucosa, as well as hepatocyte growth factor (HGF) are responsible for epithelial structure reconstitution, basic fibroblast growth factor (bFGF), (VEGF), transforming growth factor-β (TGF-β) and platelet derived growth factor (PDGF), are essential for connective tissue reconstitution(98,99).

In gastric mucosal injury, EGF released from salivary glands and TGF-α from gastric mucosa are of particular value in mucosal integrity maintenance and repair. EGF and TGF-α have similar spectra of biological activity in the repair mechanism. Accumulation of EGF and EGFR overexpression in the ulcer area contributes together to repair process. During the ulcer healing process they activate cells migration from the ulcer margin and cell proliferation along with formation of granulation tissue and microvessels, angiogenesis (100). During initial stage of experimental ulcer healing, EGFR-associated tyrosine kinase plays an essential role in the regulation of cell proliferation by activation of the EGFR gene, EGFR phosphorylation, and enhancement of MAP kinase activitay. The presence of H. pylori vacuolating cytotoxin counteracts this process (93).

Numerous growth factors accelerate gastric epithelial and mesenchymal injury healing in vitro with acceleration of cell migration and proliferation. Gastric epithelial healing is mainly accelerated by a group of growth factors including EGF, TGF-α and HGF, while mesenchymal healing is predominantly accelerated by TGF-β and bFGF. Both, gastric epithelial and mesenchymal injury healing are significantly accelerated by PDGF, factor-betabeta and insulin-like growth factor-1 (IGF-1). During the healing process, IGF-1 regulates the gastric epithelial-mesenchymal interaction (96,101).

In injured gastric mucosa, growth factors TGF-α, HGF and IGF accelerate epithelial restitution and variably regulate the regeneration of human gastric epithelial cells through modulation of cell shape adaptation, migration and proliferation (102). Growth factors endorse EGFR-dependent PI3K activation, which promotes cell migration and restitution in injured human gastric epithelial monolayers (103).

Smoking is known as a risk factor for PUD. The detrimental effect of smoking is exhibited by inhibition of cell proliferation, mucus secretion and angiogenesis due to deficiency in EGF biosynthesis and its mRNA expression. The shortage of these factors is responsible for the delay in ulcer healing (104).

Several spices and plant extracts such as Mexican tea herb and pilular adina herb, Chuanxiong spices, Capsaicin, Kuiyangping, Weitongning and Angelica sinensis interact with EGF synthesis and hence contribute to the gastroprotection. For instance, Mexican tea herb and pilular adina herb stimulate NO, EGF secretion and EGFR expression and herewith protect the gastric mucosa integrity (105). Capsaicin-sensitive nerves induced ulcer healing is mediated by stimulation of EGF expression in salivary glands, serum and gastric mucosa (106). Also Kuiyangping, promotes ulcer healing in rats and decreases recurrence via increased expression of EGF and EGFR mRNA (107). Furthermore, Weitongning herb increases EGF and NO content in ulcer scars, and hence improves ulcer healing and reduces recurrence (108). In experimental myocardial infarction, Angelica and Chuanxiong spices

promote endothelial cell proliferation and VEGF expression(109) and likewise may also promote angiogenesis and tissue repair in experimental ulcer. In indomethacin-induced gastric mucosal injury, crude extract from Angelica sinensis promotes EGF-mediated gastric mucosal healing via DNA synthesis, stimulation and augmentation of EGF mRNA expression (110). Picrorhiza kurroa (Scrofulariaceae) rhizomes possess an antioxidative property indicated by reduction of thiobarbituric acid reactive substances (TBARS) and protein carbonyl in addition to enhancing expression of EGF, VEGF, COX-1 and 2 enzymes associated with an increase of mucin and mucosal PGE2, which explain its ability to heal indomethacin-induced acute gastric injury in mice (111). Similarly, Myristica malabarica spice constituting two major antioxidants, malabaricone B and malabaricone C suppressed thiobarbituric acid reactive substances and protein carbonyls levels. Malabaricone C is more potent in modulating expression of EGF receptor and COX isoforms, mucin secretion, PGE2 synthesis and in controlling all these factors (112). Furthermore, ulcer cure by malabaricone B and malabaricone C is related to their ability to modulate angionetic factors. They significantly increase the mucosal EGF level serum VEGF level and microvessels formation. In contrary, the healing effect of misopristol and omeprazole is not correlated with angiogenesis enhancement (113).

11. Herbs and nitric oxide

In combination with other factors, NO significantly add to mucosal protection. The inflammatory process is mediated by inducible nitric oxide synthase (iNOS) and interleukin-8 (IL-8). Nitric oxide donors (SIN-1 and NOC-18) augment IL-8 and nitrite in mRNA, expression of IL-8. Production of large amounts of NO by iNOS may activate NF-kappaB and AP-1 and the expression of IL-8 in gastric epithelial cells (114). While iNOS is found in inflammatory cells in ulcer bed, NOS is located at the vascular endothelium and mucosal cells in normal and ulcerated gastric tissues. Endothelial NOS and NO significantly contribute to ulcer healing (106,115). Maintenance of NO synthesis is essential for an adequate mucosal defense. Conversely, Inhibition of NO synthesis in mucosal injury models is associated with an increase in ulcer index and asymmetric dimethylarginine (ADMA) levels along with a significantly decreased dimethylarginine dimethylaminohydrolase (DDAH) activity. ADMA Administration is associated with an inflammatory process with inhibition of NO synthesis and elevation of TNF-α levels and indicates the importance of ADMA in precipitating gastric mucosal injury (116). Such a process can be prevented by the use of extracts obtained from herbs and plants rich in phenolic compounds. Methanolic extract and buthanolic fraction of Vochysia tucanorum Mart., possess an antioxidant activity and protect NO levels in gastric mucosa. This protective effect is probably mediated by its phenolic compounds containing various active phytochemical constituents, triterpenoids (76). Triterpenoids are also active constituents of Croton reflexifolius and may explain its gastroprotective effect. Pretreatment with NOS inhibitor attenuates the gastroprotective effect induced by polyalthic acid (117). Furthermore, plant-extract-induced gastroprotective activity is likely related to the enhancing effect on release of NO in addition to NOS inhibitor expression and gastric microcirculation (118).

12. Herbs and SH compounds

The pathogenesis of gastric ulcer is complex. Several endogenous substances including SH compounds are important for the cytoprotection. They are involved in motivation of PG

synthesis, protection of gastric mucosal integrity as well as in the antioxidative process. SH mucosal concentration is suppressed, especially in ethanol-induced gastric mucosal injuries. Preservation of mucosal microcirculation for rapid restitution and cell proliferation is considered as a key target of gastroprotection by either PG or SH compounds (119).
Several spices and plant extracts have protective effect against ethanol-induced SH depletion. Among these spices, Black seed, coriander, peppermint, black pepper, clove, anise aqueous suspension, and rocket replenishe ethanol-induced gastric wall mucus and SH depletion in experimental studies (4,66,67,71-73). Similarly, Ginkgo biloba extract preserves mucosal function via inhibition of ethanol-induced SH and gastric wall mucus depletion and lipid peroxidation (120). Methanolic extracts of C. sicyoides and Commiphora opobalsamum (L.) Engl. (Balessan) also enhances the defense system in rodents and inhibits gastric injuries through SH and NO involvement (58).

13. Herbs and cytokines

Altered immune system function significantly contributes to the pathogenesis of ulcer disease, particularly T-helper lymphocytes and released cytokines. The gastroprotection induced by Phyllanthus emblica L. also upregulates anti-inflammatory cytokine IL-10 concentration through its antioxidative activity, modulates anti-inflammatory cytokines and inhibits pro-inflammatory cytokines TNF-α and IL-1β (121).
The process of ulcer formation is considerably induced and regulated by IL-1β, TNF-α, IL-4, -6, -8, -12 cytokines. Cytokines, IL-1β and IL-1RN genes modulate the inflammatory response and therefore play an important role in the course of the disease (122).
In many gastric injuries, TNF-α is involved in the induction of chemokine expression. It increases the number of macrophages and monocyte chemotactic protein-1 (MCP-1) mRNA expression in mucosal scar. Increased MCP-1 may play a key role in regulating leukocyte recruitment and chemokine expression in gastric ulcer. TNF-α increases also macrophage inflammatory protein (MIP)-2 and cytokine-induced neutrophil chemoattractant (CINC-2α) mRNA expression and MPO activity (123). Cytokine gene polymorphisms influence mucosal cytokine expression and the degree of inflammation in H. pylori infection (124).
Furthermore, IL-1β enhances adhesion molecules expression, intercellular adhesion molecule 1 and leucocytic β2 integrins as well as the concentrations of TNF-α in ulcer scar and contributes to the recurrence of gastric ulcers in rats. The presence of gastric acid is important for the recurrence process of IL-1β-induced gastric ulcer. Gastric acid activates the inflammatory process in scarred mucosa during ulcer recurrence (125).
The outcome of H. pylori infection is influenced by the host response, which in susceptible individuals determines the development of ulcer. In H. pylori infected antral mucosa response is associated with an increase of proinflammatory IL-1β , IL-6, TNF α cytokines, and IL-8; the immunoregulatory gamma interferon (IFN-γ); and the anti-inflammatory TGF-β (126). A correlation between genetic polymorphisms and H. pylori-related diseases is well-established. While IFN-γ +874 AA genotype is associated with cagA positive infections, IL-10 −819 TT and TNF-A −857 TT are associated with intestinal metaplasia and duodenal ulcer, respectively (127).
Among various gastropathies gastritis is the only gastric disorder associated with significant oxidative stress marker expression of TNF-α, IL-8 and H. pylori cagA+/vacAs1 genotype. These probably represent the main oxidative markers responsible for ROS level increase with a decrease of the expression of the Manganese superoxide dismutase (MnSOD) and GPx (128).

In Western countries, polymorphism of pro-inflammatory cytokine genes is associated with the development of duodenal ulcer and gastric cancer. Similarly, polymorphisms in TNF-α rather than IL-1β are associated with an increased risk for gastric ulcers and gastric cancer in Japan. Increased risk of gastric ulcer development is associated with carriage of the alleles TNF-α-857 T, TNF-α-863 A and TNF-α-1031 C. Simultaneous carriage of more than one high-producer allele of TNF-α further increase the risks for gastric ulcer and cancer (129). In chronic H. pylori infection Pro-inflammatory cytokines are produced in the gastric mucosa by inflammatory cells. In contrast to Asians, in western population the inflammatory cytokine gene polymorphisms IL-4-590, IL-6-572 and IL-8-251 are more associated with development of PUD. Polymorphisms of these and other cytokines such as IL-1β, IL-1RN and TNF-α, may help to predict those at higher risk to develop peptic ulcer and those , who require H. pylori eradication(130).

Spices and other plant extracts may interfere with cytokines function, regulate the inflammatory process and help in ulcer healing. In gastric ulcer model, both curcumin and bisdemethoxycurcumin, a yellow pigment in rhizomes of Curcuma longa, promote gastric ulcer healing. While curcumin suppress iNOS and TNF-α protein production, bisdemethoxycurcumin lowers the increased iNOS protein expression level without any effect on TNF-α. The gastroprotective property of bisdemethoxycurcumin is related to its capability to decrease gastric acid secretion and suppress iNOS-mediated inflammation (131). Medicinal plants may also modulate lipopolysaccharide-induced proinflammatory cytokine production in murine macrophage cells and in mice treated with the stimulant lipopolysaccaharide. This has been demonstrated by the use of three herbal constituents, apigenin (chamomile), ginsenoside Rb1 (ginseng) and parthenolide (feverfew). All of these herbal constituents have inhibited lipopolysaccaharide-induced IL-6 and/or TNF-α production in culture(132).

14. Herbs and H. pylori

H, pylori represents the main cause for PUD and its eradication is imperative for ulcer healing and reduction of ulcer recurrence rate. The current eradication rate is below 90% and the resistance rate is growing up. Therefore, the search for potent H. pylori bactericidal agents from plants resources is emerging. Several spices and plant extracts possess H. pylori growth inhibitory activities. Curcumin (133), black cumin (134), eugenol, cinemaldeyde (135), turmeric, cumin, ginger, chilli, borage, black caraway, oregano and parsley (136) have an anti-H. pylori activity. Oil extract of Chamomilla recutita affects H. pylori morphological and fermentative properties and inhibits urease production (137). H. pylori adhesion to the gastric mucosa, an important stage of infection is inhibited by extracts of turmeric, borage and parsley (136), curcumin and its methanolic extract restrain the growth of all strains of H. pylori in vitro (133)]. Moreover, eugenol and cinnamaldehyde have prevented growth of H. pylori obtained from human gastric tissue, and inhibited the growth of all 30 tested H. pylori strains, with a lack of resistance (135). Besides, phenolic compounds of Oregano (Origanum vulgare L.), a Mediterranean herb, possess an inhibitory effect on H. pylori growth (138).

Also, aqueous-ethanol extracts of over 25 of Pakistani medicinal plants including Mal. philippines (Lam) Muell. Mallotus philippines (Lam) Muell., Curcuma amada Roxb., Myrisctica fragrans Houtt., and Psoralea corylifolia L have potent anti-H. pylori activity (139). In addition, methanolic extract of 25 0f 50 Taiwanese folk medicinal plants have also

demonstrated compelling anti-H. pylori action (140). Furthermore, of 53 Mexican traditional medicinal plants especially extracts of Artemisia ludoviciana subsp. mexicana, Cuphea aequipetala, Ludwigia repens,and Mentha x piperita and methanolic extracts of Persea americana, Annona cherimola, Guaiacum coulteri, and Moussonia deppeana have verified a persuasive H. pylori inhibitory effect (141).
At last, the anti- H. pylori effect of 70 Greek plant extracts and a variety of commercially available herbs used in traditional medicine such as extracts of Chamomilla recutita, Conyza albida, Origanum vulgare Anthemis melanolepis, Cerastium candidissimum, Dittrichia viscosa, and Stachys alopecuros have inhibited a standard strain and 15 H. pylori clinical isolates (142).

15. Adverse events

Spices, herbs and other plant extracts have been used in traditional medicine for thousands of years. Recently, in several parts of the world there is a growing acceptance for using these agents to treat various conditions including PUD. Most of these extracts have been effective; however their safety and toxicity have not been well-evaluated. The increasing use of herbal medicine is expected to be more frequently associated with adverse reactions. Clinical evaluation of these adverse effects is not easy due lack of standardization, randomization, adequate number of patients and difficulty in using an appropriate placebo. Herbs are believed to be safe and have no adverse effect. However similar to other drugs they may induce intrinsic or extrinsic adverse effects. Some of their multiple constituents, such as anti-cancer plant-derived drugs, digitalis and the pyrrolizidine alkaloids are cytotoxic. Nevertheless, their adverse effects are less frequent than those of synthetic drugs (143).
Hepatotoxicity induced by curcumin and its derivatives (144) as well as by turmeric and its ethanolic extract in vulnerable mice has been reported (145). Also animals treated with Cinnamon zeylanicum, Piper longum and R. chalepensis have developed abnormalities in liver, spleen, lung or reproductive organs, in addition to an increase in count and motility of sperm and decrease in hemoglobin level (146,147). Kava (Piper methysticum), used as anxiolytic herb in Western countries has been potentially found to be hepatotoxic. Its hepatotoxicity is correlated with overdose, prolonged treatment, concurrent medication, and the quality of raw material (148). Suspected herb-induced liver injury (HILI) is evaluated by the causality score using this multidisciplinary approach and Roussel Uclaf Causality Assessment Method (RUCAM) (149).
In addition to hepatic toxicity, alteration of body weight has been described in rodents treated with Foeniculum vulgare ethanolic extracts and Ruta chalepensis (150). Furthermore, in experimental model, piperine has decreased mating performance and fertility and intrauterine injection has caused loss of implants without histological abnormalities (151). Herbal-induced toxicity is influenced by herbs related factors (quality, dose and nature of constituents) and individual risk factors (genetics, age, concomitant drugs, and concomitant diseases) (152). Therefore, simultaneous administration of herbs with conventional medications should generally be discouraged (153).
Herbal medicine-associated adverse reactions are expected to occur more frequently as a result of the fast mounting use of these agents in treatment. Some commonly used herbs like St John's wort (Hypericum perforatum), a popular herbal anti-depressant, lead to a decrease of the activity of immunosuppressive agents i.e.cyclosporine and subsequent tissue rejection in transplanted patients. Like other medicinal plants it also interferes with cytochrome P450 activity and metabolism of other drugs (154).

Examples of Drugs known to interact with St John's wort include besides cyclosporine tacrolimus as well as HIV non-nucleoside and protease inhibitors (155). Other drugs interfering with St. John's wort CYP 3A4 induction include , oral contraceptives.and indinavir(156).

Literature review of 128 case reports or case series, and 80 clinical trials have revealed that St John's wort-induced cytochrome P450 and P-glycoprotein induction, decreases plasma levels of a large variety and frequently used medications. Clearance of caffeine and midazolam may be influenced by Echinacea (157).

Herbal agents such as St. John's wort, interact differently with various drugs. It may increase the clearance of some medications via cytochrome P-450 mixed-function oxidase or through P-glycoprotein efflux pump modulation. On the other hand, it may decrease digoxin, theophylline, warfarin, protease inhibitors, cyclosporine, tacrolimus, and tricyclic antidepressants concentration with subsequent reduction of their therapeutic effect. A third category of drugs such as procainamide carbamazepine and mycophenolic acid are not affected by St. John's wort. herb (158).

Therapeutic drug monitoring is usually estimated by immunoassay technique. The potential interference St. John's wort, with commonly by this method monitored drugs has been evaluated. A significant interference with digoxin, quinidine, procainamide, N-acetyl procainamide theophylline, tricyclic antidepressants, phenytoin, carbamazepine, valproic acid and phenobarbital serum levels is lacking (159). Due to unwanted effects, ginseng and ginkgo should not be combined with anticoagulants and valerian with barbiturates (160). Elderly patients are more likely to develop diseases and ingest more medications. They are also prone to develop suppression of cytochrome P450 (CYP) activity. Taking herbal agents make them more vulnerable to herb-drug interactions (161). Herbal toxicity may also affect other central organs like the kidney. Case reports of interstitial fibrosis progressing to chronic renal failure and termed as aristolochic acid nephropathy may complicate treatment with slimming herbs belonging to Aristolochia family (162). Despite all of these reports of adverse events, spices are generally safe when used in standared doses. Popular traditional Chinese medicine has relatively less adverse effects and appears safer than other drugs (163).

The safety of herbal agents during pregnancy has been evaluated in 392 pregnant women 8% have reported taking chamomile, licorice, fennel, aloe, valerian, Echinacea oil 27, propolis and cranberry. Only four out 109 have reported insignificant adverse events in form of constipation after tisane, rash and itching after local application of aloe or almond oil. A higher incidence of threatening miscarriage and preterm labors was observed among regular users of chamomile and licorice (164).

In disparity, many spices and plant extracts, in commonly used dose, up to 500mg/kg body weight have not exhibited adverse effect. These include cardamom (62, 68), black pepper (66), clove (67), caraway (69), saffron (70),coriander(71), peppermint (72), anise (73), davilla elliptica and nitida (74) ,Brazilian medical plants (75) and Alchornea triplinervia (76) and Hyptis spicigera Lam (86). Even in pregnancy, ginger, peppermint, and Cannabis have been used to treat nausea were effective and lack clinical evidence of harm (165). Clinically, spices like turmeric and curcumin have been well-tolerated even with high doses and lack any toxicity (166).

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Ethnopharmacology as Current Strategy in the Search of Novel Anti-Ulcerogenic Drugs: Case of a Brazilian Medicinal Plant (*Maytenus ilicifolia* Mart. ex. Reissek)

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1. Introduction

Several medical products of natural origin were conceived in traditional systems of knowledge and practice that has been transmitted over centuries and which continuously change. In actual scenario, researchers of many countries involved in the modern drug discovery processes are becoming increasingly aware of the value of their traditional knowledge, while global pharmaceutical industry is looking for alternative solutions to reduce the crescent innovation deficit and enhance the development of new products.

Has been systematically showed that aleatory screening of plants used traditionally by pharmaceutical industries in the search for new leads or drugs is vastly expensive and requires much time. On the other hand, ethnodirected approach to traditional knowledge has been extremely useful in screening and identification of plants with bioactive compounds with potential application in drug development. This approach consists in selecting species according to the indication of specific population groups in certain contexts of use. The ethnodirected approach has significantly increased the chances of discovery of new biomolecules with potential therapeutic application while reduce the cost and time involved in this process. Beyond this approach provide a shortcut to the discovery of active compounds that could serve as a basis for rational drug development, it also provides a mechanism for pre-screening on the therapeutic properties of the species collected. Most of these compounds are part of routinely used traditional medicines and hence their tolerance and safety are relatively better known than any other chemical entities that are new for human use. Thus, traditional medicine based on ethnodirected bioprospecting offers an unmatched structural variety as promising new leads.

In the context of ethnodirected studies, the ethnopharmacological research has shown a great contribution in selecting plants and discovery of compounds with pharmacological potential. Ethnopharmacology is a strategy used in the investigation of plants with medicinal properties, combining information acquired from users of medicinal plants (traditional communities and experts), with chemical and pharmacological studies. While in the past the typical industrial drug discovery process made the use of aleatory selection and systematic bioassays to find promising compounds for a particular target,

ethnopharmacology goes the opposite way, tries to understand the pharmacological basis of culturally important medicinal plants, testing their efficacy in the laboratory.
Currently, research centers and pharmaceutical industries have driven the search for new drugs of plant origin with effective activity to fight several diseases that today present a limited treatment, including gastrointestinal ailments. In the case of gastric ulcers, several plants extracts described in the specific cultural context are being investigated in the search for sources of effective biomolecules in reducing the damage to gastric mucosa.
Gastric hyperacidity and ulceration of the stomach mucosa due to various factors are serious health problems of global concern. Peptic ulcer disease (encompassing gastric ulcer and duodenal ulcer) affect a large portion of the world population and are triggered by several factors, including stress, smoking, nutritional deficiencies, and ingestion of non-steroidal anti inflammatory drugs. Today, there are two main approaches for treating peptic ulcer. The first deals with reducing the production of gastric acid and the second with re-enforcing gastric mucosal protection. Although a number of anti-ulcer drugs such as H_2 receptor antagonists, proton pump inhibitors and cytoprotective agents are available, all these modern pharmacological approach have side effects and limitations. Moreover, development of drug tolerance and incidence of recurrences make the efficacy of allopathic drugs arguable. Therefore, there is urgent need to find alternatives that have antiulcerogenic properties. This has been the basis for the development of new anti-ulcer agents, which include herbal substances that could serve as leads for the development of new drugs.
In view of the importance of finding new plant compounds for the management of gastric ulcers in the context of current health, this chapter aims to show plant species and crud drug preparations with antiulcer activity identified within the ethnopharmacological approach. Furthermore, will be described the main phytochemicals responsible for the antiulcer therapeutic properties of plant extracts. Finally, by analyzing the case of *Maytenus ilicifolia*, will address aspects from preliminary phytochemical analysis and experimental tests with different preparations of this plant, and the process of isolation of fractions of the ethanol extract of *Maytenus ilicifolia* for identification of bioactive constituents related to its gastroprotective action.

2. Contribution of ethnopharmacology in the selection of natural products with potential application in health care

Chemical substances derived from plants have been used to treat human diseases since the dawn of medicine. Currently, the health care based in natural products is still the mainstay of about 75 - 80% of the whole world population, and the major part of traditional therapy involves the use of plant extracts (Gilani & Atta-ur-Rahman, 2005). Recognizably, natural products remain an important source for the discovery of new drugs and is estimated that about 13000 plant species worldwide are known to have been used in drugs formulation. About 60% of anticancer and 75% of anti-infective drugs approved from 1981-2002 could be traced to natural origins (Patwardhan & Vaidyab, 2010). Studies on sources of new drugs from 1981 to 2007 reveal that almost half of the drugs approved since 1994 are based on natural products (Harvey, 2008). Currently, it is estimated that about 80% of molecules used in drugs sold worldwide are derived from natural products and that over hundred new natural product-based leads are in clinical development (Butler, 2008; Bhutani & Gohil, 2010). Moreover, despite the tremendous development of chemical synthesis today, 25% of prescribed drugs in the world are of vegetable origin (Balunas & Kinghorn, 2005; Bhutani &

Gohil, 2010). Aspirin, atropine, artimesinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, quinidine, reserpine, taxol, tubocurarine, vincristine and vinblastine are a few important examples of what medicinal plants have given us in the past. Most of these plant-derived drugs were originally discovered through the study of traditional cures and folk knowledge of indigenous people and some of these could not be substituted despite the enormous advancement in synthetic chemistry (Sekar et al., 2010).

Due to growing drug discovery from natural products, researchers and pharmaceutical industries has been increasing interest in traditional health practices used around the world (Patwardhan, 2005). This interest has been renovated for decades due to systematic demonstrations that plants are the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer et al., 1999). Since the reported data so far available on plants are comparatively meager before the number of plant population, ethnopharmacologists, botanists, microbiologists and natural-product chemists world over today, is constantly still in search of medicinal efficacy of plants and their phytochemicals. Furthermore, the wide spectrum of therapeutic activity makes the natural products attractive candidates for further research (Vlietinck & Van Den Berghe, 1991). In this context, a new recognition has been given to ethnopharmacology, traditional, complementary and alternative medicines, which re-emerging as new strategic options in health attention, has provided valuable clues of plants with bioactive compounds potentially usable in the production of new drugs (Harvey et al., 2010; Patwardhan & Vaidyab, 2010). The World Health Organization's Commission on Intellectual Property and Innovation in Public Health also has duly recognized the promise and role of traditional medicine in developing affordable drugs for the treatment of health problems (Patwardhan, 2005a; Patwardhan & Vaidyab, 2010).

The ethnopharmacological approach is currently employed to study numerous medicinal plants and vegetable preparations from traditional ethnic groups (Elisabetsky & Nunes, 1990). Although the clinical efficacy of these preparations is reported by traditional practices, they have not been scientifically validated. Thus, ethnopharmacologists typically develop working hypotheses derived from field observations having as one of its main goals to enhance the knowledge of local communities incorporating scientific findings to traditional accounts. In this context, the central questions that direct the ethnopharmacological research is if a specific plant extract used in the cultural context to cure some diseases present a pharmacological basis that explains the effects traditionally indicated. In this process, ethnopharmacological discoveries started with field observations and ended in new pharmacological insights (Gertsch, 2009). Therefore, ethnopharmacology research is transdisciplinary, touching on areas like anthropology, ethnobiology, and as the name implies, pharmacology (Raza, 2006; Gertsch, 2009).

The systematic screening ethnopharmacology-based of plant species with the purpose of discovering new potential bioactive compounds is a routine activity in many laboratories (Elisabetsky, 2002). Traditionally, the etnopharmacological research on the medicinal plants should be extended with the identification and isolation of specific phytochemicals. After these processes, only from the careful scientific examination of these isolated compounds could lead to standardization and quality control of the products to ensure their safety. It is after such evaluation that vegetable derivatives can be approved for the development of new products used in health care (Vlietinck & Van Den Berghe, 1991, Elisabetsky, 2002; Patwardhan, 2005b).

Several advantages can be achieved with the adoption of ethnodirected method for screening bioactive components. Due to traditional use of vegetable products in specific communities for the prevention or treatment of various health conditions, it is possible to meet preliminary criteria for safety for human consumption and any adverse effects of such use (Elisabetsky & Nunes, 1990; Vlietinck & Van Den Berghe, 1991). Coupled with better cultural acceptability of natural products and reduced cost is encouraging for both the consuming public and national health care institutions to consider plant medicines as a complementary practice to synthetic drugs (Elisabetsky & Nunes, 1990; Elisabetsky, 2002; Patwardhan, 2005a).

3. Peptic ulcers and herbal medicine

Peptic ulcer disease (PUD) is one of the most common, chronic gastrointestinal disorder in modern era. Now it has become a common global health problem affecting a large number of people worldwide and also still a major cause of morbidity and mortality (Sen et al., 2009). An estimated 15,000 deaths occur each year as a consequence of PUD (Dharmani & Palit, 2006).

Ulcer is an open sore that develops on the inside lining of the stomach (a gastric ulcer) or the small intestine (a duodenal ulcer). Both types of ulcers are also referred to as PUD and can be characterized by inflamed lesions or excavations of the mucosa and tissue that protect the gastrointestinal tract. The most common symptom of a peptic ulcer is a burning or gnawing pain in the center of the abdomen (stomach) (Tarnawski, 2005, Vyawahare et al., 2009).

In the past, it was mistakenly thought that the main causes of peptic ulcers were lifestyle factors, such as diet, smoking, alcohol and stress. While these factors may play a limited role, it is known that the leading cause of peptic ulcers is a type of bacteria called *Helicobacter pylori* (*H. pylori)* can infect the stomach and small intestine; and in some people, the bacteria can irritate the inner layer of the stomach and small intestine, leading to the formation of an ulcer (Dulcie et al., 1997). Peptic ulcer occurs due to an imbalance between the aggressive (acid, pepsin and *H. pylori*) and the defensive (gastric mucus, bicarbonate secretion, prostaglandins, nitric oxide, growth factors and innate resistance of the mucosal cells) factors (Falcão et al., 2008b). Painkillers known as nonsteroidal anti-inflammatory drugs (NSAIDs), which include aspirin and ibuprofen, are the second most common cause of peptic ulcers that can irritate the lining of the stomach and small intestine in some people, particularly if they are taken on a long-term basis (Tarnawski, 2005; Vyawahare et al., 2009).

Traditionally, there are two main approaches for treating peptic ulcer. The first deals with reducing the production of gastric acid and the second with re-enforcing gastric mucosal protection (Sen et al., 2009). According to the old hypothesis, acid secretion was thought to be the sole cause of ulcer formation and reduction in acid secretion was thought to be the major approach towards therapy. However, in the light of recent evidences this concept has changed. The modern approach for the ulcer treatment mainly targets the potentiation of the gastrointestinal defensive system preventing ulceration by inhibiting acid secretion, increase gastroprotection, increase epithelial cell proliferation and stop apoptosis for effective ulcer healing process (Bandhopadhyay et al., 2002).

Recently, there has been a rapid progress in the understanding of the pathogenesis of peptic ulcer. Most of the studies focus on newer and better drug therapy for the prevention and treatment of peptic ulcer. These have been made possible largely by the availability of the proton pump inhibitors, histamine receptor antagonists, drugs affecting the mucosal barrier

and prostaglandin analogues (primarily misoprostol) (Hoogerwerf & Pasricha, 2006). However, the clinical evaluation of these drugs showed development of tolerance and incidence of relapses and side effects that make their efficacy arguable. Furthermore, most of these drugs produce several serious adverse reactions including toxicities, arrhythmias, impotence, gynaecomastia, arthralgia, hypergastrinemia, haemopoeitic changes and even may alter biochemical mechanisms of the body upon chronic usage (Vyawahare et al., 2009). This has been the rationale for the development of alternative approach in recent days for the research of new antiulcer drugs medicaments from traditional medicinal system, which includes herbal drugs.

For many years, herbal medicines were generally indicated only as coadjutant gastrointestinal therapy to conventional drugs and when these drugs presented adverse effects and are used during a long-term. Due to several plants encountered in many countries have been reported to poses marked antiulcerogenic activity, the role of natural medicine in management of gastrointestinal diseases has been rethought (Schmeda-Hirschmann & Yesilada, 2005). Thus, the investigation of traditional knowledge, popular medicine and the development of new medicaments based in natural products for the treatment of diseases like peptic ulcer have been indicated as a absolute requirement of our time (Sen et al., 2009).

Medicinal plants and their derivatives have been an invaluable source of therapeutic agents to treat various disorders including PUD (Borrelli & Izzo, 2000). The use of vegetable extracts used in popular medicine and their phyto-constituents as drug therapy to treat major ailments has proved to be clinically effective for the treatment of PUD (Dharmani and Palit, 2006). Furthermore, the use of plants and their phytoconstituents in the treatment of gastrointestinal diseases is promising due to the broad spectrum of action on various defensive mechanisms like antioxidant, antinflamatory, imunomodulatory, cytoprotective and antisecretory (Newall et al., 1996).

Although several plants have showed beneficial gastroprotective effects, earlier publications, and researchers from around the world, have pointed out that relatively little of the world's plant biodiversity has been extensively screened for bioactivity (Harvey et al., 2010), and this scenario extends to most plants that have traditional indication for the management of gastric ulcers.

4. Ethnopharmacological discovery of antiulcer crude drugs

Treatment of gastrointestinal ailments with natural products is quite common in traditional medicine worldwide. The importance of ethnopharmacological studies in the search for plants with gastroprotective activity is emphasized by the observation that the first drug effective against gastric ulcer was carbenoxolone, discovered as a result of research on a commonly used indigenous plant, *Glycyrrhiza glabra*. Also, studies on cabbage, previously employed as an anti-ulcer agent in folk medicine, has led to the development of gefarnate, a drug used for the treatment of gastric ulcers (Akhtar & Munir, 1989). Thus, a search among medicinal plants is still important, despite the progress in conventional chemistry and pharmacology in producing effective drugs (Harvey et al., 2010).

Currently, new therapeutic approaches with medicinal plants based in traditional knowledge and in ethnopharmacological screening have received particular attention in the prevention and/or treatment of gastric diseases such as PUD. In this context, there are reports of a large variety of plants species with antiulcerogenic potential in several countries. Examples of plants and their phytochemicals with antiulcer activity investigated in ethnopharmacological studies are sowed in table 1.

Botanical name	Parts used*	Actives phytochemicals	Ulcer model
Aclepiadaceae			
Hemidesmus indicus	root	alkaloids, tannins, phenols, saponins	aspirin, pylorus ligation, cyteamine
Anacardiaceae			
Anacardium occidentale	leaves	glycosylated quercetin, glycosylated myricitin, catechin, proanthocyanidin, biflavonoid amentoflavone	ethanol + HCl
Asteraceae			
Centaurea solstitialis	spiny flowers	sesquiterpene lactones, chlorojanerin 13-acetylsolstitialin A, solstitialin A	ethanol, HCl, indometacin cold stress, serotonin
Clusiaceae			
Calophyllum brasiliense	stem bark	flavones, flavonols, triterpenoids, xanthones, steroids	ethanol, indomethacin, cold stress, pyloric ligation
Combretaceae			
Anogeissus latifolia	bark	glycosides, leucocyanidin, ellagic and flavellagic acid, galic acid	ethanol, aspirin, cold stress, pylorus ligation
Euphorbiaceae			
Alchornea castanaefolia	leaves, bark	quercetin-3-O-β-D-galactopyranoside, quercetin-3-O-α-L-arabinopyranoside, myrecetin-3-O-α-L-arabinopyranoside, quercetin, galic acid, amentoflavone, glycolipids, free sugars	ethanol + HCl, acetic acid cold stress, pylorus ligation, indomethacin
Emblica officinalis	fruits	flavonoids, phenols, curcuminoides, phyllembelic acid, tannins,	ethanol, aspirin, cold stress, pylorus ligation,

Fabaceae			
Desmodium gangeticum	root	alkaloids, steroids, pterocarpanoids flavone, isoflavonoid glycosides, N-oxides, b-amyrone, tryptamines, phospholipids	ethanol, aspirin, cold stress, pylorus ligation
Spartium junceum	flowers	alkaloids, saponins, spartitrioside	ethanol, pylorus ligation, cold stress
Labiatae			
Ocimum sanctum	leaves	eugenol, carvacrol, caryophyllene, apigenin, luteolin, orientin, molludistin, ursolic acid	ethanol, acetic acid, aspirin, reserpine, pylorus ligation
Liliaceae			
Aloe vera	leaves	alkaloids, sterols, gelonins, saponins, fatty acid, glycoproteins	HCl, pylorus ligation
Asparagus racemosus	root	alkaloids, steroids, saponins, flavonoids, phenols, tannins, terpenes	ethanol, aspirin, cold stress, pylorus ligation,
Malphiaceae			
Byrsonima crassa	leaves	quercetin-3-o-b-D-galactopyranoside quercetin-3-o-a-L-arabinopyranoside amentoflavone, catechin, epicatechin	ethanol + HCl
Meliaceae			
Azadirachta indica	bark	phenols, phenolic diterpenoids, glycosides, isoprenoids, essential oils flavonoids, tannins	ethanol, aspirin, indomethacin, histamin
Oleaceae			
Jasminum grandiflorum	leaves	alkaloids, saponins, phenois flavonoids, carotenoids, glycosides	pylorus ligation + aspirin ethanol, acetic acid

Sapindaceae			
Allophylus serratus	leaves	ß-sitosterol, phenacetamide, flavonoids, glycosides	ethanol, aspirin, acetic acid, cold stress
Rhizophoraceae			
Rhizophora mangle	bark	polyphenols, catechin, epicatechin, chlorogenic, gallic and ellagic acids, gallotannins, elagitannins	diclofenac
Rubiaceae			
Rubia cordifolia	root	anthraquinones, iridoid glycoside, bicyclic hexapeptides, triterpenes	pylorus ligation
Scrophulariaceae			
Scoparia dulcis	aerial parts	cirsitakaoside and quercetin	pylorus ligation, histamine, bethanechol
Simaroubaceae			
Quassia amara	bark	alkaloids, b-carbonile, cantin-6, steroids, quassinoids, terpenes	ethanol, HCl
Solanaceae			
Solanum nigrum	Fruits	tannins, alkaloids, carbohydrates, anthocyanins	ethanol, indomethacin, pylorus ligation, cold stress
Utleria salicifolia	rhizome	steroids, alkaloids, terpenoids, saponins, tannins	ethanol, acetic acid cold stress, pylorus ligation
Zingiberaceae			
Zingiber officinalis	Root	alkaloids, flavonoids, phenols, monoterpenoids, sesquiterpenoids	methanol, acetone, HCl
Amomum subulatum	fruits	anthocyanins, aurone, flavone, essestial oils	ethanol, aspirin, pylorus ligation

Table 1. Plants with antiulcerogenic activity from ethnopharmacological studies.
*Gastroprotective effects were obtained using crude preparations of all the plants described

Important questions related with crude drugs are the necessary amount of plant part to provide a healing response, traditional way of preparation (infusion, decoction and maceration), concentration (plant/solvent ratio), frequency and duration of treatment. Unfortunately, this basic information is not always present in ethnopharmacological studies and this fact is surprising as there should be a realistic approach to the doses to confirm the reputed effectiveness of the crude drugs. Extraction yields and doses recommended in traditional medicine were not taken into account in most cases. This fact clearly suggested the need for guidelines when looking for gastroprotective crude drugs or gastroprotective compounds from medicinal plants (Schmeda-Hirschmann & Yesilada, 2005).

In anthropological and ethnobiological investigations of popular herbal therapies practices has been indicated that as a common way of preparation, plants are used in traditional medicine as infusions or decoctions, but in some localities also as macerates, either in water or in alcoholic beverages (Schmeda-Hirschmann & Rojas de Arias, 1990). In particular cases, the treatment may also be applied by direct ingestion of the material.

As a common popular practice, the plant material (in the range of 5–50 g of dry plant material per liter) is placed in a pot of solvent (most commonly hot or coldwater), while resinous materials which would not dissolve in polar solvents are directly swallowed. As the percent (w/w) extraction yields of plant material presents a great variability depending on the extraction solvent, processing temperature and time, doses corresponding to 5–50 g of dry plant material are about 0.5–10 g extract for an adult user (60–70 kg) corresponding to ca. 100–150 mg of crude extract per kg of body weight. In the current scenario, it has been observed that most studies have investigated the antiulcerogenic properties using different animal models at doses of plant extracts ranging from 5 to about 2000 mg/kg. However, the doses between 25 and 800 mg/kg have indicated great gastroprotective effect of crude plant extracts, range that is more realistic considering the tolerable human consumption. Higher doses may not be realistic since they will not be used or recommended in traditional medicine (Schmeda-Hirschmann & Yesilada, 2005).

Although crude drugs obtained through the use of herbal preparations with fresh or dried plants and concentrated plant extracts have broad applicability in folk medicine, the viability of this practice in the pharmaceutical industry is arguable. Is widely recognized that crude drugs exhibit a wide spectrum of phytochemicals with different biological activities. However, few of these compounds have pharmacological activities of interest for the treatment of specific health conditions, and although these compounds are present in crude drugs, can also be phytochemicals with antagonistic activity or even harmful to human health. Furthermore, another important aspect is the difficulty of standardization in the phytochemical composition of these crude drugs since there is a wide variation in levels of the chemical components related to where the raw material was obtained (Leite, 2009).

In this context, following the identification of the efficacy of crude herbal preparations in different health conditions, the method ethnopharmacological predicts the development of several research stages aiming to identify, concentrate and isolate phytochemical components to test the biological activities of each compound identified in the crude drug. In this process, it is possible to focus the investigations on the phytochemicals responsible for the desired biological effects (Fabricant & Farnsworth, 2001; Bhutani & Gohil, 2010). Thus, through this approach it is possible to achieve the level of control required in pharmacological studies in order to determine the therapeutic dose, toxicity and mode of use of specific phytochemicals, criteria that must be strictly defined where the intention is to discover and develop new pharmaceuticals derived from plants for commercial purposes

(Koehn & Carter, 2005). A basic scheme of the ethnopharmacological method is represented in the figure 1. Based in this method several phytochemicals with antiulcer activity were discovered, and was clearly demonstrated that the main benefits of these crude drugs are linked to alkaloids, flavonoids, saponins and tannins.

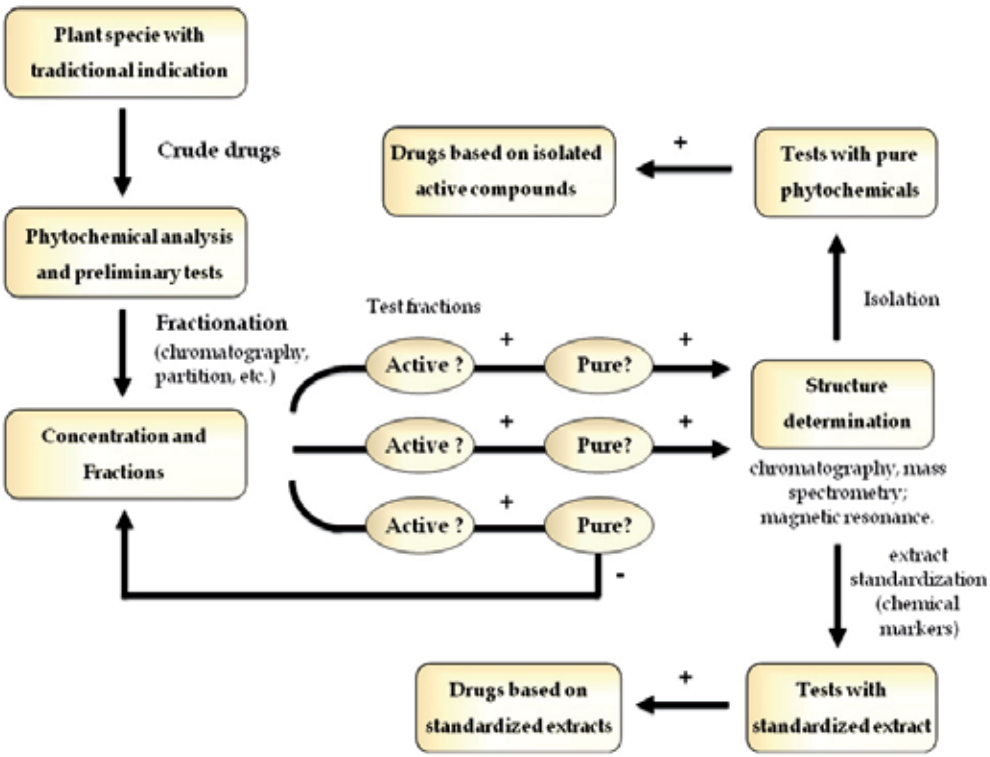


Fig. 1. Generic representation of ethnopharmacological method in a bioassay-guided fractionation for the investigation of phytochemicals with therapeutic properties used in drugs development (adapted from Koehn and Carter, 2005).

5. Antiulcer properties of specific phytochemicals

5.1 Alkaloids

The alkaloids are a diverse group of low molecular weight nitrogen-containing compounds derived mostly from amino acids. These secondary metabolites are found in about 20 % of plant species (Ziegler & Facchini, 2008). Plants containing alkaloids with antiulcer properties are showed in table 2. Furthermore, these phytochemicals represent a group of natural products that has had a recognized impact in medicine and are being used in management of gastrointestinal ailments. Clinically, alkaloids they are used to block the muscarinic activity of acetylcholine showing antispasmodic and antisecretory effects in the treatment of spastic colitis, gastroenteritis and peptic ulcer. In a previous literature review were identified fifty-five naturally derived alkaloids with antiulcer activity such as imidazole, indole, isoquinoline, non-nitrogen heterocycle alkaloid, phenylalkylamide, piperidine, pyrazine, pyridine, pyrrolidine, pyrrolizidine, quinolizidine and tropane alkaloids (Falcão et al., 2008a).

Botanical name	Parts used	Ulcer model
Apocynaceae		
Himatanthus lancifolius	bark	ethanol, pylorus ligation
Annonaceae		
Enantia chlorantha	bark	ethanol, HCl pylorus ligation
Apocynaceae		
Voacanga africana	fruits	ethanol, HCl, pylorus ligation, indomethacin
Asteraceae		
Mikania cordata	leaves	diclofenac
Senecio brasiliensis	flowers	ethanol, HCl, pylorus ligation, indomethacin
Buxaceae		
Pachysandra terminalis	leaves	cols stress
Flabaceae		
Sophora flavescens	leaves	acetic acid, cold stress
Ranunculaceae		
Coptis chinensis	rhizoma	ethanol, acetic acid pylorus ligation
Coptis japonica	rhizoma	ethanol
Rubiaceae		
Pausinystalia yohimbe	bark	cold stress
Rutaceae		
Galipea longiflora	bark	ethanol, HCl, bethanecol

Table 2. Plants containing alkaloids with anti-ulcer activity.

Among the different alkaloids showing potent pharmacological properties are the narcotic analgesic morphine, the antimicrobial berberine and the sympathomimetic ephedrine. These isoquinoline alkaloids occur mainly in plants belonging to families Papaveraceae, Berberidaceae and Ephedraceae (Ziegler & Facchini, 2008). In murine model of gastric damage induced by reserpine, aspirin or indomethacin, morphine and ephedrine presented

significant antiulcer activity (Al-Shabanah et al., 1993; Sandor & Cuparencu, 1977). In addition, the alkaloid 7,8-dihydro-8-hydroxypalmatine obtained from the bark of *Enantia chlorantha* was effective to increase gastric mucus and accelerated ulcer-healing production after gastric lesions caused by acetic acid. Positive effect was also evidenced when ulceration of gastric mucosa was induced using HCl/ethanol and pylorus ligature (Tan et al., 2000). Other alkaloids isolated from *Coptidis* rhizome, coptisine and 8-oxocoptisine, showed protection of gastric mucosa similar to that offered by gastroprotective conventional drugs such as cimetidine and sucralfate (Hirano et al., 2000, 2001).

Alkaloids derived from *Voacanga africana* was assayed for cytoprotective, anti-secretory and ulcer healing actions. Through enteral administration, alkaloid fraction inhibited ulcer formation in a dose-dependent way in several models of gastric damage (HCl/ethanol, absolute ethanol, HCl/ethanol/ indomethacin, pylorus ligation, cold restraint stress, and histamine). These alkaloids have gastric anti-secretory effects similar to histamine receptor blockers and decreased the gastric acid secretion. Moreover, its cytoprotective and ulcer healing effects are associated to its property to strengthen gastric mucosal defenses by stimulating mucus synthesis (Tan & Nyasse, 2000). When combined with ranitidine, a synergistic anti-secretory effect was observed (Tan et al., 2002). In addition, alkaloids such as matrine, 13-alpha-hydroxymatrine and oxy-matrine isolated from *Sophora flavescens* were able to decrease the acid secretion and inhibited the gastric motility in experimental model of gastric ulcers induced by pylorus ligature (Zhu et al., 1993; Yamazaki, 2000).

The pyrrolizidine alkaloids integerrimine, retrorsine, senecionine, usaramine and seneciphylline were extracted from *Senecio brasiliensis*. These alkaloids demonstrate significant activity in acute and chronic gastric ulcers. In this investigation, gastroprotective effects of alkaloids were associated to the stimulation prostaglandin synthesis in gastric mucosal and free mucus, reduction of exfoliation of superficial cells, hemorrhages and blood cell infiltration, events that can be mediated by increased expression of epidermal growth factors (Toma et al., 2004).

5.2 Flavonoids

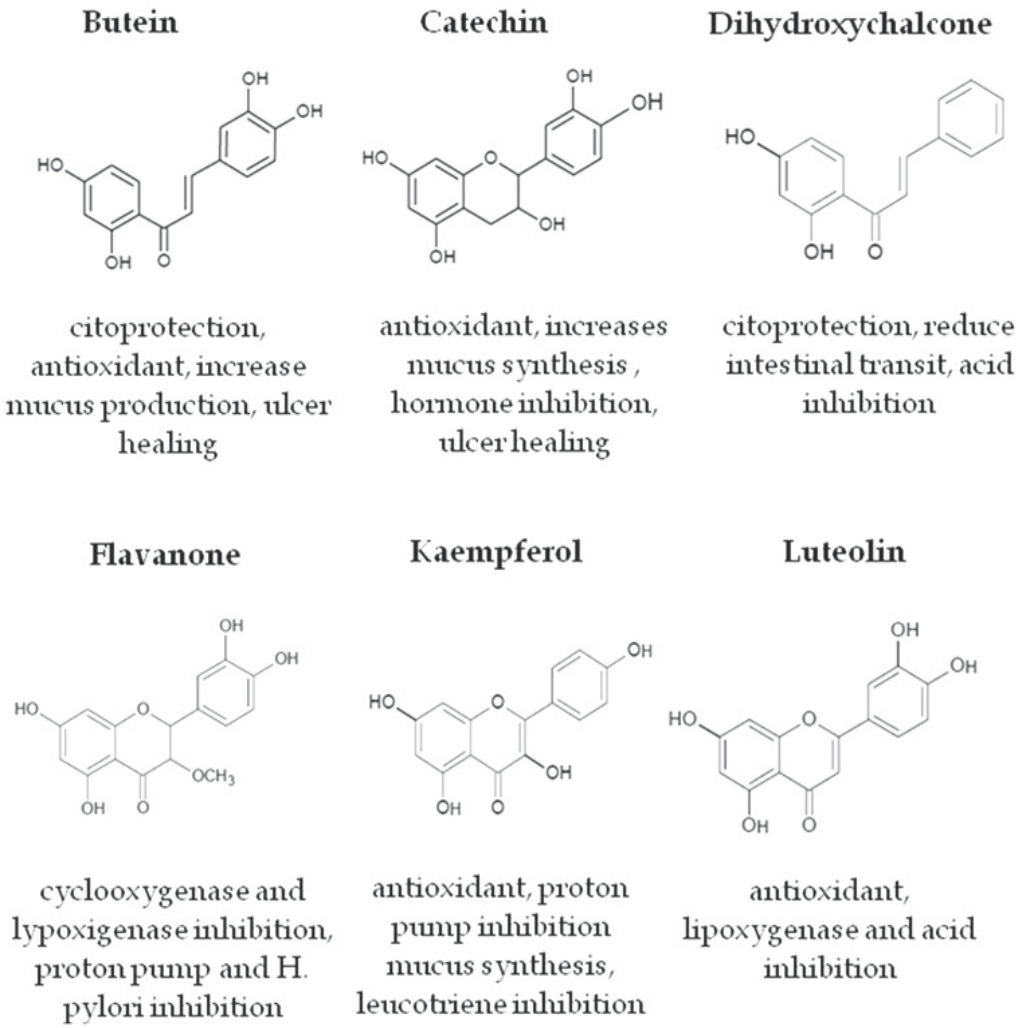
Flavonoids are important constituents in human diet that are also found in several medicinal plants used in popular medicine around the world (Di Carlo et al., 1999). These molecules represent a highly diverse class of secondary metabolites derived from vegetable material comprising about 9,000 structures with a wide range of biological effects, including antiulcer activity (Mota et al., 2009).

There are several studies with flavonoids naturally derived were found to be able to protect the gastric mucosa reducing the number and intensity of the lesions induced by a variety of ulcerogenic agents such as ethanol, HCl, acetic acid, aspirin, diclofenac, indomethacin, reserpine, cold stress and pylorus ligation (Borrelli & Izzo, 2000). Currently, the flavonoids catechin, flavanone, flavone, kaempferol, naringin, naringenin, quercetin, and rutin have been most commonly cited as having important gastroprotective effect in experimental models of duodenal and gastric ulcers.

Several mechanisms have been proposed to explain the gastroprotective effects of flavonoids; these include increase of mucosal prostaglandin content, decrease of histamine secretion from mast cells and inhibition of *H. pylori* growth (Beil et al., 1995). Furthemore, flavonoids have been found to be free radical scavengers with an important role in

protection against ulcerative and erosive lesions of the gastrointestinal tract. Due to low toxicity, flavonoids could have a therapeutic potential ideal for treatment of gastrointestinal diseases associated with *H. pylori* infection, i.e. type B gastritis and duodenal ulcer (Di Carlo et al., 1999, Martín et al., 2000). Common flavonoids with antiulcer activity are shown in Figure 2.

In experiments with murine model of ethanol-induced gastric ulcers, the flavonoids naringin and quercetin displayed marked antiulcerogenic effects. In particular, naringin at a dose of 400 mg/kg had a significant gastroprotective effect, reducing the number and severity of ulcerative lesions. It is suggested that the gastroprotective property of naringin occurs through a complex non-prostaglandin dependent mechanism that involved an increase in the mucus synthesis and their viscosity. Free-radical scavenging also seems to be implicated in this protective activity (Martín et al., 2000).



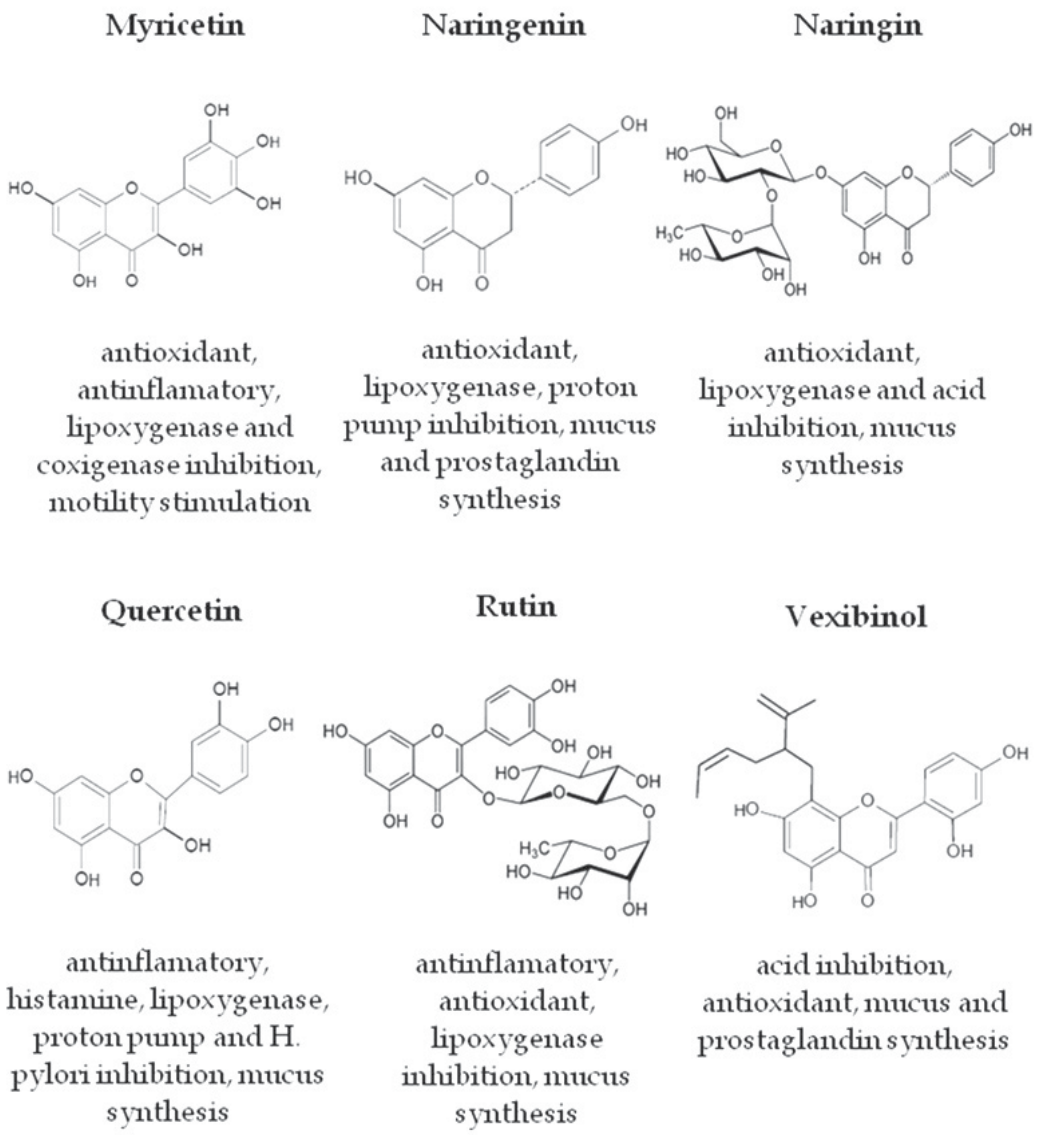


Fig. 2. Flavonoids with antiulcerogenic properties and their mechanisms of action.

Administration of Quercetin at a dose of 200 mg/kg also showed beneficial effects by reducing the occurrence of ulcers and increased the amount of glycoprotein content of gastric mucus. A proposed mechanism of action for the effects of Quercetin is a cytoprotective effect mediated by antioxidant properties, stimulation of prostaglandin and inhibition of leukotriene production, events that reinforce the defensive compounds of the gastrointestinal wall (Alarcon de la Lastra et al., 1994; Di Carlo et al., 1999). In a rat model of gastric damage induced by acidified ethanol, the antiulcer effects of flavone, quercetin, naringin, rutin and kaempferol were previously related to the synthesis of platelet activating factor (PAF), a recognized ulcerogenic agent (Izzo, 1996). In this study,

intraperitoneal administration of quercetin, rutin and kaempferol reduced tissue erosion in a dose-dependent manner (25-50 mg/kg), while naringin reduced gastric damage only at high dose levels (200-400 mg/kg) and flavone was inactive. Gastric mucosa of rats exposed to acidified ethanol presented large amounts of PAF and the treatment with flavonoids proved to have protective effects against the gastric damage when the doses utilized were able to reduce PAF synthesis. Although other protective mechanisms cannot be excluded, evidences indicate that the degree of PAF inhibition produced by flavonoids was an important factor associated with the reduction of gastric injuries (Izzo et al., 1994; Di Carlo et al., 1999).

The influence of flavonoids on gastric acid secretion, mucosal prostaglandin production and *H. pylori* growth were also previously investigated (Beil et al., 1995). In this study, the flavonoids Flavone, Flavanone and Quercetin reduced *H. pylori* growth and the acid production in response to histamine and dibutyryl cyclic AMP stimulation. All flavonoids tested also inhibited the gastric proton pump (H^+/K^+), however, no inhibitory action was observed on the formation of prostaglandin E. In this context, flavone and flavanone increased PGE release, and quercetin was inactive in this process. Thus, was possible concluded that due to low toxicity of flavonoids and the effective gastroprotective properties described (antisecretory action, stimulation of prostaglandins, inhibition of *H. pylori*), these compounds presented a promissory therapeutic potential for the direct treatment of ulcerative diseases or for the development of drugs with this purpose (Di Carlo et al., 1999).

5.3 Saponins

Saponins are largely distributed in plants and are characterized as a specific kind of glycosides. They exhibit haemolytic properties and are highly toxic in direct contact with the blood stream. According to the structure of the aglycone or sapogenin two forms of saponin are recognized, the steroidal and triterpenoid type, the latter form being found in high concentrations in many plant species (Samuelsson, 1992; Borrelli & Izzo, 2000).

Antiulcer activity of several plant species containing high amounts of Saponins has been continuously indicated in different experimental ulcer models. The main species investigated in ethnopharmacological studies are shown in Table 3. Was previously demonstrated that liquorice root contains about 2%-12% of glycyrrhizic acid and the seeds of the horse-chestnut up to 13% of aescin (Newall et al., 1996; Borrelli & Izzo, 2000). Among these, saponins isolated from the rhizome of *Panax japonicus* and the fruit of *Kochia scoparia* (with about 20% of saponins) showed significant gastro-protective properties by inhibiting the amount and severity of ulcerative lesions (Matsuda et al., 1998). Furthemore, oleanolic acid oligoglycosides extracted from the same plants showed antiulcer effects on ethanol- and indomethacin-induced gastric damage. In another study, methanol extract of *Panax japonicus* rhizome also was able to protect gastric mucosa against stress- or HCl-induced ulcers (Yamahara et al., 1987; Borrelli & Izzo, 2000).

Aescin, a mixture of saponins encoutered in the seeds of *Aesculus hippocastanum*, has been shown to possess a marked antiulcer property (Marhuenda et al., 1993). For this compound, the gastroprotective effect has been associated with an inhibition of gastric acid and pepsinogen secretion. However, in a model of gastric ulceration ethanol-inducedm aescin was also effective in preventing gastric lesions (Marhuenda et al., 1994; Borrelli & Izzo, 2000). As in this model acid and pepsin do not play a significant role, the evidences indicates that other protective mechanisms are involved in the antiulcer action of aescin Furthermore,

Botanical name	Parts used	Materials tested	Ulcer model
Araliaceae			
Polyscias balfouriana	leaves, roots	ethanolic extract	aspirin
Asteraceae			
Aster squamatus	aerial parts	hydroalcoholic extract	ethanol, pylorus ligation
Chenopodiaceae			
Kochia scoparia	fruits	isolated saponin	ethanol, indomethacin
Combretaceae			
Pteleopsis suberosa	bark	aqueous extract	indomethacin
Compositae			
Calendula officinalis	rhizome	isolated saponin	arsenic, butadione, pylorus ligattion
Dilleniaceae			
Davilla rugosa	stem	hydroalcoholic extract	acetic acid, ethanol, HCl, cold stress
Fabaceae			
Spartium junceum	Flowers	ethanolic extract	ethanol
Icacinaceae			
Pyrenacantha staudtii	Leaves	aqueous extract	indomethacin, serotonin, cold stress
Menispermaceae			
Rhigiocarya racemifera	Leaves	aqueous extract	indomethacin, serotonin, reserpine
Mimosaceae			
Calliandra portoticensis	Leaves	ethanolic, aqueous extract	cold stress, pylorus ligation
Sapindaceae			
Aesculus hippocastanum	seed	mix of saponins	ethanol, pylorus ligation cold stress
Sapindus saponaria	fruits, leaves	hydroalcoholic extract	cold stress
Sapotaceae			
Mimusops elengi	bark	hydroalcoholic extract	ethanol, pylorus ligation

Scarabaeoidea			
Panax binnatifidus	rhizome	isolated saponin	psychological stress
Theaceae			
Camellia sinensis	seed	methanolic extract	ethanol

Table 3. Plants containing saponins with anti-ulcer activity.

mucus synthesis mediated by prostaglandin seems not to be an able mechanism to explain the role of aescin-induced gastro-protection due to inability of saponin to stimulate the prostaglandin production in model of ethanol-induced gastric ulceration (Marhuenda et al., 1994; Borrelli & Izzo, 2000).
In a general context, the current information indicate that the antiulcer protective activities of the saponins are not due to inhibition of gastric acid secretion but probably due to activation of mucous membrane protective factors (Borrelli & Izzo, 2000).

5.4 Tannins

Plants produce tannins as protective substances, found in the outer and inner tissues. Tannins are by definition phenol compound with sufficiently high molecular weight and different chemical structures occurring in medicinal and food plants that are utilized world-wide. This phytochemical presents several remarkable biological and pharmacological activities and an important meaning for human health. Tannins are used in medicine primarily because of their astringent properties, which are due to the fact that they react with the proteins of the layers of tissue with which they come into contact (Samuelsson, 1992; Borrelli & Izzo, 2000). Moreover, the used of tannins against peptic ulcer, diarrhea and as an antidote in poisoning by heavy metals are described in medical literature.
Several plants with anti-ulcer activity containing high levels of tannins are showed in Table 4. In a previous investigation, a crude extract of *Linderae umbellatae* exhibited a marked anti-peptic and antiulcerogenic activity (Ezaki et al., 1985; Borrelli & Izzo, 2000). In this study, condensed tannins such as (+)-catechin, (-)-epicatechin, proanthocyanidin, cinnamtannin B1 and D1 (monomers, dimers, trimers and tetramers) have been isolated and their anti-peptic and anti-ulcer activity confirmed in experimental models of gastric lesions induced by pylorus-ligation in rats and stress in mice. Significant biological differences were observed between the chemicals structures of tannins. Monomers and dimers, did not presented inhibitory activity on peptic activity in vitro, while trimers exhibited higher inhibition of peptic activity compared to tetramers. In mice with pylorus ligation, trimers and tetramers markedly reduced the peptic activity of gastric juice. Furthermore, monomers and dimers slightly suppressed the peptic activity in this experimental model (Ezaki et al., 1985; Borrelli & Izzo, 2000). As monomers and dimers proved to be inactive in vitro, it is possible that their activity is not related to the direct inhibition of pepsin in vivo, but mainly related to influence on the secretion mechanism of pepsin.
Additional mechanisms have been related to the antiulcer action of tannins. This phytochemicals are known to coat the outermost layer of the mucosa and to render it less permeable and more resistant to chemical and mechanical injury or irritation (Asuzu & Onu, 1990; Borrelli & Izzo, 2000). When a low concentration of tannins is applied to the mucosa,

Botanical name	Parts used	Extract	Ulcer model
Anacardiaceae			
Myracrodruon urundeuva	bark	ethyl-acetate tannin fraction	ethanol, indomethacin
Schinus terebinthifolius	bark	aqueous	cold stress
Celastraceae			
Maytenus ilicifolia	leaves	aqueous	indomethacin
Combretaceae			
Combretum dolichopetalum	roots	ethanolic	indomethacin, cold
Pteleopsis suberosa	bark	chloroform, aqueous	Indomethacin, cold stress
Euphorbiaceae			
Excoecaria agallocha	bark	aqueous	diclofenac
Fabaceae			
Sesbania grandiflora	bark	ethanolic	indomethacin
Fagaceae			
Quercus suber *Quercus coccifera*	leaves	isolated tannins	ethanol
Lauraceae			
Linderae umbellatae	steam	isolated tannins	cold stress
Meliaceae			
Entandrophragma utile	bark	aqueous	ethanol
Menispermaceae			
Rhigiocarya racemifera	leaves	aqueous	reserpine, serotonin,
Myrtaceae			
Syzygium cumini	bark	isolated tannin	ethanol + HCl
Scrophulariaceae			
Veronica officinalis	aerial parts	hydroalcoholic	indomethacin, reserpine

Table 4. Plants containing tannins with anti-ulcer activity.

only the outermost layer is tanned, becoming less permeable and affording an increased protection to the subjacent layers against the action of bacteria, chemical irritation, and, to a

certain extent, against mechanical irritation. In another hand, high concentrations of tannins often cause coagulation of the proteins of the deeper layer of the mucosa, resulting in inflammation, diarrhea and vomiting. The discovery of the inhibitory effects of tannins on lipid peroxidation in rat liver mitochondria and microsomes was followed by the uncovering of several effects related to improving the several gastrointestinal symptoms, activity that may be related to inhibition of lipoxygenase products related to metabolism of arachidonic acid (Okuda, 2005; Borrelli & Izzo, 2000). In addition, gastroprotective protective effects are related to antioxidant, vasoconstricting and antihemorrhagic properties of tannins (Borrelli & Izzo, 2000), which has also been linked to inhibition of *H. pylori* growth by several hydrolysable tannins (Funatogawa et al., 2004).

6. Screening of the constituents from *Maytenus ilicifolia* with anti-ulcerogenic activity

The beneficial medicinal effects of plant materials typically result from the secondary products present in the plant, and usually are not attributed to a single compound but a combination of the metabolites. In an extensive literature review, was identified, in quantitative terms, that the gastroprotective properties of crude drugs plant-based are attributed mainly to the presence of flavonoids, being found about 53 flavonoid compounds with antiulcer activity (Mota et al., 2009). However, currently there are sufficient evidence that in crude vegetables preparations the gastroprotective effects are also deeply influenced by other phytochemicals such as alkaloids, saponins and tannins. Therefore, efforts should be directed towards isolation and characterization of the active principles and elucidation of the relationship between structure and activity, followed by attempts for modulation of its activity potential by chemical modification. Furthermore, detailed analysis of the active constituents of natural drugs should be directed towards clinical relevance and to maintain indispensable reproducible quality in biological evaluation.

The ethnopharmacology constitutes an important and reliable method in bioprospecting of phytochemicals with anti-ulcer activity. The case of the discovery of the gastroprotective activity of *Maytenus ilicifolia* constitutes an appropriate example that clearly demonstrates the important role of ethnopharmacology in elucidating the pharmacological basis that is associated with a large part of traditional knowledge about medicinal plants and that waiting to be discovered.

Maytenus ilicifolia Mart. ex. Reissek belongs to the Celastraceae, a pantropical family native to southern Brazil, Paraguay, Uruguay, and northern Argentina. The plant is a small medicinal evergreen shrub that grows to a height of five meters bearing leaves and berries that resemble holly and is popularly known as “espinheira santa” (holy spine), “cancerosa”, “cangorosa”, “maiteno” and “espinheira divina” (divine spine) (Cordeiro et al., 2006). *Maytenus ilicifolia* is widely used as a traditional medicine in many countries of South America and its leaves are traditionally used as a remedy for gastrointestinal diseases, including dyspepsia and gastric ulcers (Leite et al., 2001, 2010). They are found in the local commerce as capsules, powders, dried leaves, or as aqueous or aqueous-alcoholic preparations.

The antiulcerogenic activity of *Maytenus ilicifolia* leaves is well documented. Has been showed that its aqueous extract causes significant reduction in the number of gastric ulcers induced by both indomethacin and cold-restraint stress in rats. This protection was similar

to that observed with cimetidine, a well known histamine H2 receptor antagonist. Chemical constituents obtained from this plant extracts with solvents of different polarities are terpenoids, flavonoids, tannins and polysaccharides (Leite et al., 2001).

Souza-Formigoni et al., (1991) used boiling water extract of *Maytenus ilicifolia* leaves against ulcer lesions induced by indomethacin and cold-restraint stress in rats and found that both the oral and intraperitoneal administration of the extract had a potent antiulcerogenic effect against both types of ulcers. In this study, several phytochemicals were identified in crud extract of *Maytenus ilicifolia* and apparently polyphenols and flavonoids were the main compounds linked to gastroprotective effects evidenced. However, in a study conducted by Martins et al., (2003), antiulcer effects of *Maytenus ilicifolia* spray-dried powders obtained of an ethanol extract was mainly related to the tannins content in the crude extract. Thus, results of these studies suggested that a number of active constituents might be present in crude extract to control ulcerative lesions. However, of the major bioactive component of *Maytenus ilicifolia* that offers antiulcer effects remained still not well understood.

In a previous study, Baggio and collaborators reported the potent *in vivo* gastroprotective properties of a flavonoid-rich fraction separated from the leaves of *Maytenus ilicifolia*, *Maytenus ilicifolia*, containing epicatechin (3.1%) and catechin (2%) as major constituents, which was correlated with the in vitro inhibition of rabbit gastric H+,K+-ATPase activity (Baggio et al., 2007). Aiming to further the investigation on the bioactive constituents from *Maytenus ilicifolia* leaves, Leite et al., (2010) carried out a phytochemical investigation of an ethanol extract of *Maytenus ilicifolia* leaves for the isolation of compounds which were further used as chemical markers to monitorize an activity-guided fractionation of a lyophilized aqueous extract of *Maytenus ilicifolia* leaves. Finally, high performance liquid chromatography analyses of aqueous extract and its chromatographic fractions were carried out, aiming at establishing a correlation between gastroprotective effect and chemical composition. In this study, fractionation of aqueous extract led to 5 fractions containing different flavonoids such as the tri-flavonoid glycosides mauritianin, trifolin, hyperin, epi-catechin, a tetra-glycoside kaempferol derivate and the monosaccharide galactitol. Chemical structures of the phytochemicals identified are showed in Figure 3. These fractions were evaluated in rats for their effects on gastric secretion volume and pH in a model of pylorus ligation. Considering the results of the study it was possible to conclude that only fractions containing mauritianin and tetra-glycoside kaempferol derivate caused significant increase of gastric volume and pH, thus indicating that these glycosides play an important role on the gastroprotective effect of *Maytenus ilicifolia* leaves. Compounds identified in the other fractions had a less important contribution to gastroprotective effect since they have not disclosed significant activity on gastric volume and pH of rats. Gastric mucus is believed to play an important role in the defensive mechanism against gastric ulceration. The protective effect of mucus as an active barrier may be attributed to the glycoproteins, which have the property of holding water in the interstices, thus obstructing the diffusion of hydrogen ions. Stress has been shown to decrease the amount of mucus adhering to the gastric mucosa (Jorge et al., 2004). Hence, increase in synthesis of mucus, according to results obtained in this study with *Maytenus ilicifolia*, is consistent to those found by some authors (Bravo et al., 1990; Sairam et al., 2002), suggesting that such increase is an important factor for antiulcer protection.

Because of mucosa protection, extracts of *Maytenus ilicifolia* may represent an important clinical alternative in antiulcerogenic therapeutic, though, further studies are needed to

evaluate the real usefulness of this extract in the prevention and treatment of peptic ulcers. Thus, the screening of the constituents from *Maytenus ilicifolia* with antiulcer activity used in this study, clearly illustrate as a phytochemical investigation directed by an ethnopharmacologycal approach can be of great value in the search for compounds with potential use for the development of new and more efficient drugs used in management of ulcerative diseases and others health disorders. The occurrence of tetra-glycosylated flavonoids in this specie afford a valuable chemical marker for the quality control of the Brazilian *Maytenus* marketed as phytomedicines.

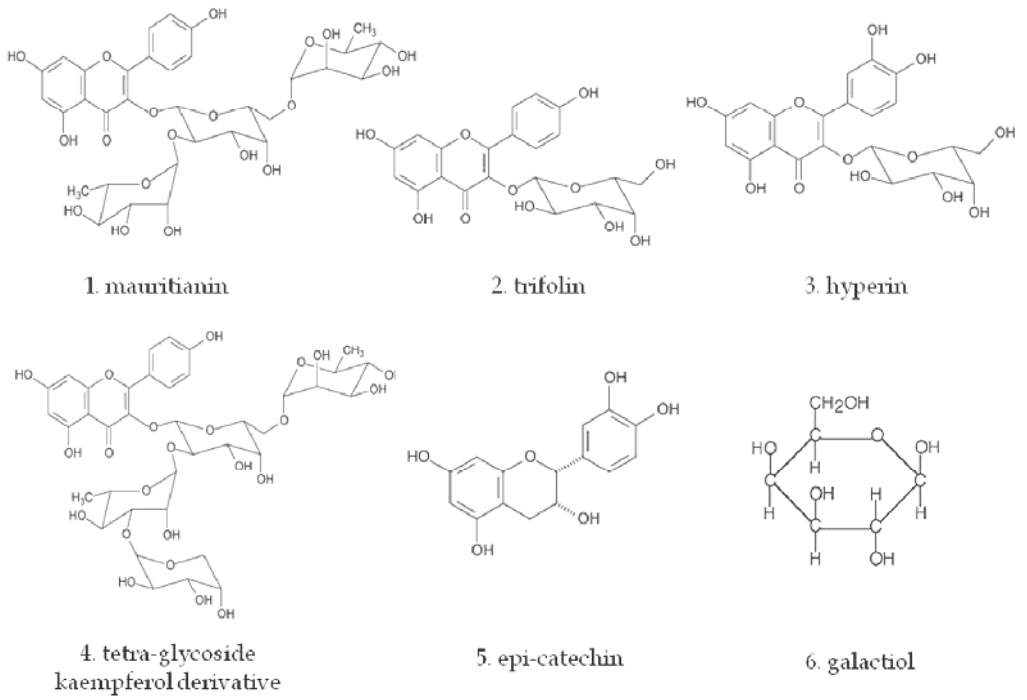


Fig. 3. Phytochemicals identified in a lyophilized aqueous extract of *Maytenus ilicifolia* leaves.

7. Conclusion

Currently, there is a positive trend in favor of traditional, complementar and integrative therapies both in scientific research and health care. Furthermore, in recent decades has been observed strengthening of approaches related to health care such as ethnopharmacology, reverse pharmacology, phytotherapy, systems biology and personalized medicine. Ethnopharmacology has already played recognized importance in the discovery of plants with medicinal potential and in the development of natural health care practices, and is likely to play more significant role in the years to come. It would not be surprising to see that the use of herbal medicines will be gradually accepted in the main stream of conventional medicine. Due to acceptance that the diversity of chemical substances found in vegetable materials may have different biological effects of interest with potential

applications in many different health conditions, it is believe that there will be a growing trend in the use of novel natural products and development of chemical libraries based on these products in drug discovery campaigns.

Plant resources have proved to be an important source for the discovery of new substances with antiulcer potential. Researches in this area have been targeted for both the isolation of active principles, such as to obtain standardized extracts. Polyphenolic compounds, including flavonoids, have been the subject of increasing interest since *in vitro* and *in vivo* biological assays indicated that flavonoids can mediate a range of mechanisms related to anticancer, antitumor, and anti-oxidant activities, among other. The contribution of the flavonoids to the dietary intake of polyphenolics compounds is considerable. In fact, cereals, legume seeds, fruits, wine, and tea contain significant amounts of flavonoids and their derivatives.

In Brazil, studies of the species *Maytenus ilicifolia* have advanced considerably, reinforcing the use in folk medicine, where preparations from the leaves of this species are used as an antiulcer treatment. This species is part of the cast of the Brazilian Pharmacopoeia, being the phenolic constituents used as chemical markers. After several chemical investigations, preclinical and clinical (Phase I, II and III) studies, herbal preparations that include this species obtained registration with the Health Surveillance Agency (ANVISA), the Brazilian agency that regulates the registration of these products. The species is also included in the list of herbal medicines that the Brazilian government provides in the pharmaceutical assistance of the Unified Health System (SUS), and their herbal medicine, therefore, proven by the government as to its effectiveness and safety. In *Maytenus ilicifolia* the 3-*O*-glycosides of quercetin and kaempferol are the most common group of flavonoids. It is known that the sugar moiety is an important factor for the bioavailability of the flavonoid derivatives.

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21

Anti-Ulcerative Potential of Some Fruits and the Extracts

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1. Introduction

Concern about the effects of various foods on human health has risen significantly in recent years. Plant-based foods, including fruit and vegetables, are regarded as important for human health. It is believed that plant-based diets have positive effects on health due to their phytochemical components. Plant extracts that contain various phytochemicals have been used in a numbers of studies to assess their biological effect on health, but the precise roles of phytochemicals in human health are still unclear in many cases.

There are many reports indicating that various plant extracts and related phytochemicals can act as ulcer preventing agents. Studies have shown that extracts of plants used in ayurvedic medicine (traditional medicine native to India) display a certain level of efficiency on gastric ulcer prevention in animals (Ajaikumar et al., 2005; Bhatnagar et al., 2005; Mishra et al., 2009). Moreover, extracts from vegetables, such as artichoke (*Cynara Scolymus*) leaf (Ishida et al., 2010), rocket or arugula (*Eruca sativa*) (Alqasoumi et al., 2009), Indian cluster bean 'Guar' (*Cyamopsis tetragonoloba*) (Rafatullah et al., 1994), cabbage (*Brassica oleracea*) (Akhtar & Munir, 1989) and basil (*Ocimum basilicum*) (Akhtar & Munir, 1989), also have been reported to have certain effect on gastric ulcer prevention in rats.

Studies on fruit extracts using experimental gastric ulcer in rodents, have revealed antiulcerative activity, for banana (*Musa* species) (Pannangpetch et al., 2001), pomegranate (*Punica granatum*) (Ajaikumar et al., 2005), dates (*Phoenix dactylifera*) (Al-Qarawi et al., 2005), cluster fig (*Ficus glomerata*) (Rao et al., 2008), prickly pear (*Opuntia ficus indica*) (Galati et al., 2003), Indian cherry (*Cordia dichotoma*) (Kuppast t al., 2009), dried papaya (*Carica Papaya*) (Rajkapoor et al., 2003) etc.

There are various experimental models for gastric ulcers such as ethanol-, aspirin-, indomethacin- or stress-induced gastric ulcers. We have used ethanol-induced gastric ulcer (or gastric mucosal injury) in rats to find effective extracts from underutilized fruits, including immature fruits. Among these underutilized fruits, we have found that Chinese quince (*Pseudocydonia sinensis* (Thouin) C. K. Schneider) extracts were the most effective against gastric mucosal injury. Extracts from European (normal) quince (*Cydonia oblonga* Miller) fruit also showed activity, but in our experiments, Chinese quince extracts were superior to the quince (cv. Smyrna) extracts on a same weight basis.

Chinese quince and quince fruits have been used in traditional medicines in Asian and western countries, respectively. Chinese quince is believed to be native of China (Zhejiang province) and is now widely planted in Japan, China, and Korea. As for quince, the primary

area of natural growth seems to be the eastern Caucasus and Transcaucasus, and it is cultivated in all countries with worm-temperate to temperate climates (Khoshbakht and Hammer, 2006).

Fig. 1. Chinese quince (*Pseudocydonia sinensis* Schneid.) fruits on tree.

The Chinese quince fruit is inedible when raw because of its hard flesh, strong astringency, and high acidity. This characteristics are similar to those of the quince fruit, but Chinese quince has numerous stone cells that are larger than those in the quince fruit, making the flesh more unpleasant to eat. Therefore, these fruits, especially the Chinese quince fruit, are usually consumed as processed food products such as concentrated juice extracts, liquor, jam, jelly, glutinous starch syrup, crystallized fruit, and throat lozenges. The dried fruit has also been used in traditional medicine in the form of hot water extracts, for its antitussive and/or expectorant properties. Thus homemade medicines from Chinese quince fruit (including fruit liquor, decoction, syrup, and paste) have been said to have antitussive, expectorant, antispasmodic, and antidiuretic actions, and have been used for combating respiratory infections, intestinal dysregulation, and diuresis, and for treating people with a weak constitution. On the other hand, European quince fruits have been used as traditional medicines to treat cough (Kültür, 2007), constipation (Khoshbakht and Hammer, 2005) and also as stomach's comforter (Wilson, 1999).
In this chapter, studies on extracts of Chinese quince are presented and the efficacy of other fruit extracts and some of their chemical components on ulcer is also discussed.

2. Efficacy of fruit extracts and fruit products against HCl/ethanol induced mucosal injury in rats

2.1 Hot-water extracts from underutilized fruits and byproducts

2.1.1 Introduction

Attempts to find a use for underutilized fruits, including immature fruits picked through fruit thinning during fruit growth, have been made because those fruits have been known to

be rich in various phytochemicals. Moreover, there is an interest in residue of fruits (or pomace), by-products in the food industry, because it has become a big problem of waste disposal. For these reasons, a research project to discover a use for under-utilized fruits and industrial by-products of fruit processing has been carried out. In this research project, over 30 plant materials including underutilized fruits and horticultural by-products were collected and hot-water extracts were made to assess their biological activities. We investigated the antiulcerative potential of hot-water extracts from selected plant materials using an HCl/ethanol-induced ulcer model in rats for screening purpose. In addition, some chemical components and free radical scavenging activity were also measured.

2.1.2 Materials and methods

Plant materials were collected at various places in the Nagano prefecture, Japan. Hot-water extracts were obtained by boiling each plant material in four times its volumes of water for 1 hour. The suspended solution was filtered using two layers of cheesecloth and a filter paper, concentrated, then lyophilized. For determination of antiulcerative activity, male Wistar rats were orally administered 2.5 ml of water (control) or sample suspention containing 200 mg of extracts 30 min before gastric ulcer induction. The gastric mucosal lesions that lead to acute gastric ulcer were induced by oral administration of 1.5 ml of 150 mM HCl/ethanol (40:60, v/v) solution (Mizui & Doteuchi, 1983). Animals were sacrificed under anesthesia 60 min after the HCl/ethanol administration. Stomachs were removed, opened along the greater curvature, rinsed with physiological saline solution and stretched on balsa boards. The degree of gastric mucosal damage was evaluated from digital pictures using a computerized image analysis system. Percentage of the total lesion area (hemorrhagic sites) to the total surface area of the stomach except the forestomach was defined as the ulcer index. For chemical component analysis in the hot-water extracts, total polyphenol and polyuronide contents were determined using Folin-Ciocalteu method (Singleton & Rossi, 1965) and 3,5-dimethylphenol assay (Schott, 1979), respectively. Proanthocyanidin content was determined using butanol-HCl assay. Free radical scavenging activity (RSA) was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Brand-Williams et al., 1995).

2.1.3 Results and discussion

In our experiment, many fruit extracts tested displayed some level of efficacy against gastric mucosal injury (antiulcerative activity) induced by HCl/ethanol in rats (Fig. 2). Among the fruit extracts, Chinese quince fruit extracts showed the strongest activity on the same weight basis although quince fruit, immature apple and apple pomace extracts also had a significant activity. Lack of clear activity in liquor residues of Chinese quince fruit suggests that the active ingredients were eliminated by dissolution in the alcoholic solution. Because boiling-water can extract polyphenolic compounds and cell wall polysaccharides effectively, the fruit extracts contained these components at various concentrations (4.4–106 mg/gDW for total polyphenols; 7.9–46 mg/gDW for total polyuronides). Study of the relationship between ulcer index and the presence of chemical components or radical scavenging activity indicated that total polyphenol and proanthocyanidin content tended to have a negative relation to the ulcer intensity induced by HCl/ethanol. Meanwhile, polyuronide content and radical scavenging activity do not seem to bear any relation with the ulcer index. However, it has been reported that antioxidant capacity is related to prevention of gastric ulcer because oxygen radicals generated from neutrophils have an important role in formation of

gastric lesions (Matsumoto et al., 1993). Our results indicate that not only radical scavenging activity, but also composition of antioxidants or other components were strongly related to the antiulcerative property of the fruit extracts. Hot-water extracts from Chinese quince fruit that were rich in procyanidins seemed to have a significant potential as an antiulcer agent. The effect of polyphenols and polysaccharides on experimental gastric ulcer is described later (in section 3). Additionally, it is not negligible that hot-water extracts from quince fruit or apple by-products also had moderate activity of ulcer prevention.

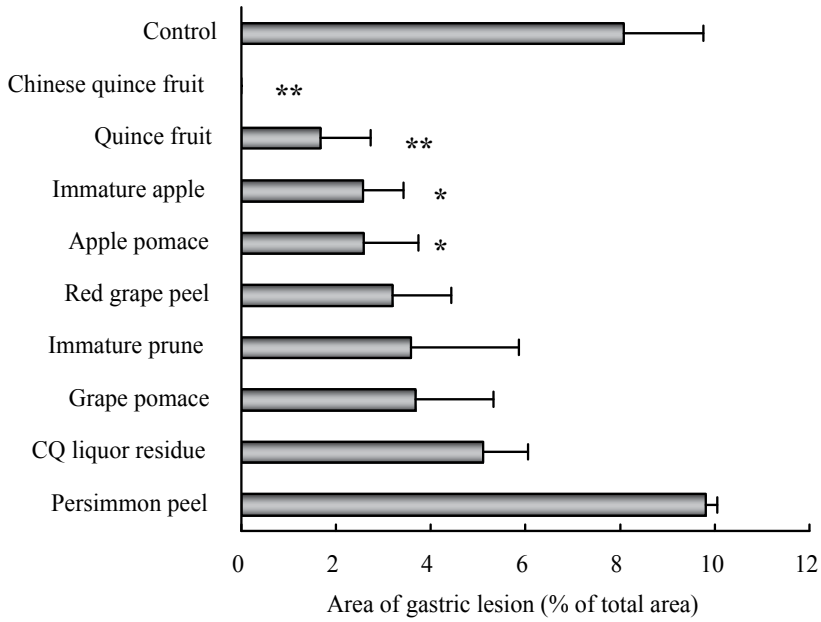


Fig. 2. Antiulcerative activity of hot (boiling)-water extracts from underutilized fruits or by-products. Rats were orally administered 2.5 ml of water (control) or a solution containing 200 mg of fruit extract 30 min before ulcer induction by 150 mM HCl/ethanol (40:60, v/v). Data are mean ± SE (n=7 for control; n=5 for Chinese quince and quince fruits group; n=3 for other study group). * $P< 0.05$; ** $P< 0.01$ vs control (Student-t test). CQ, Chinese quince.

2.2 Boiling-water extracts and jelly of Chinese quince fruits

2.2.1 Introduction

Chinese quince and quince fruit are normally consumed after being processed into jam, jelly, fruit paste (quince cheese), or fruit liquor. During processing, the fruits are often heated or boiled for extended periods of time. Moreover, quince juice and jelly have been traditionally used as folk medicine for treating stomach illness (Kloss, 1999; Wilson, 1999). Quince marmalade has been believed to help digestion, to comfort or strengthen the stomach (Wilson, 1999). Although it is unclear whether the antiulcerative properties of the fruits was part of the folk medicine knowledge, study of food function including the antiulcerative properties of boiling-water extracts of these fruits is interesting and meaningful. Therefore, we investigated the chemical characteristics and preventive efficacy of boiling-water extracts and jelly made from the Chinese quince and quince fruits on HCl/ethanol induced gastric lesions in rats.

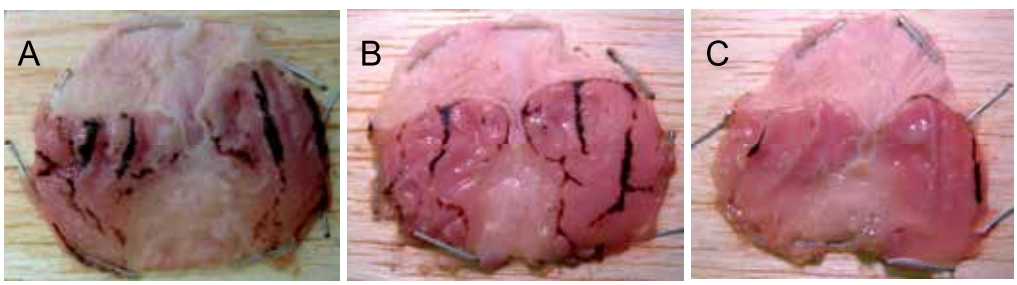
2.2.2 Materials and methods

Commercial ripe fruits of the Chinese quince 'Kegai' and quince 'Smyrna' were obtained at a local orchard in the Nagano prefecture, Japan. For boiling water extraction, fruit were cut into small pieces, put into 3 times their volumes of boiling water, and boiled for up to 4 hr. An small volume of boiling water was added every hour to make up for evaporation. The boiled fruit extract was filtered using 2 layers of cheesecloth and gently squeezed, brought to a volume of 800 ml (from 200 g fruit) and stored in a freezer until use. Fruit jelly was made using the boiled fruit extract as follows: 200 mL of extract (from 50 g of fruit) was mixed with 50 g of superfine sugar and reduced by boiling for 50 min to make 70 g of jelly. The procedure to determine antiulcerative activity and chemical components was as described above.

2.2.3 Results and discussion

In the experiment with Chinese quince extracts, administration of 2.5 ml of fruit extracts obtained by boiling for 2 hr significantly prevented the gastric mucosal lesions induced by HCl/ethanol but extracts obtained by boiling for 1 hr did not show significant activity (Fig. 3). Because boiling for extended periods of time has the advantage of breaking cell wall polysaccharides and to extract chemical components from the fruit tissue, the extracts obtained by boiling for 2 hr had more phytochemicals such as antioxidants than that obtained by boiling for 1 hr. In fact, amount of polyphenols extracted from 100 g of the fruit tissue after 1 hr and 2 hr of boiling was 791 mg (62.3%) and 985 mg (77.6%), respectively. Likewise, the amount of pectic polysaccharides extracted from 100 g of tissue after boiling for 1 hr and 2 hr was 291 mg (34.6%) and 365 mg (43.5%), respectively. Therefore, prolonged heating (boiling) in processing of Chinese quince fruit may be beneficial from a viewpoint of antiulcerative activity in HCl/ethanol induced ulcer.

Because the boiling water extracts of Chinese quince and quince fruits are rich in pectic polysaccharides and organic acids, they can easily form gels by addition of sugar and brief heating. To determine whether the gelling products (fruit jelly) retain the antiulcerative activity, Chinese quince and quince jellies made from the extract obtained by boiling for 2 hr were used for the study. The administration of jelly made from extracts of either fruits strongly prevented the development of gastric lesions (Table 1). This indicates that the preventive effect was retained even after jelly manufacturing. The antiulcerative activity of Chinese quince jelly was stronger than that of quince jelly. This may be due to the difference of polyphenolic content and radical scavenging activity in the jellies. The actual polyphenolic composition is currently being analyzed, but procyanidins (the major component in Chinese quince and quince fruit) in the jellies may be an important factor.



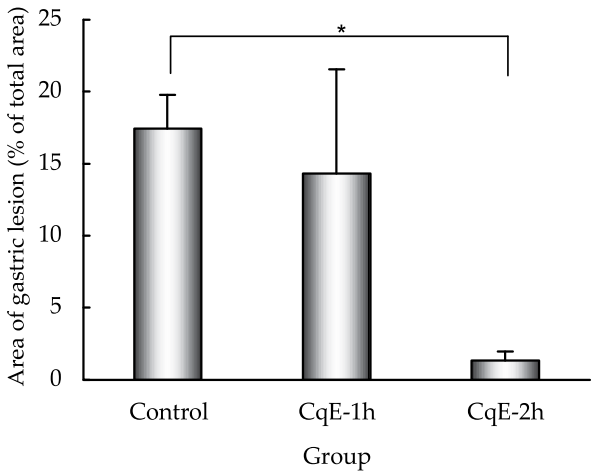


Fig. 3. Antiulcerative properties of boiling-water extracts of Chinese quince fruits on gastric legions induced by HCl/ethanol in rats. Photographs shows mucosal surface of rat stomach. Rats were administered 2.5 ml of water (A; control) or fruit extracts obtained by boiling for 1 h (B) and 2 h (C) then gastric ulcer was induced by administration of 1.5 ml of HCl/ethanol. Histogram shows percentage of area of gastric lesion to total surface area of stomach. Data are mean ± SE (n=19 for the controls; n=3 for each extract). * $P < 0.05$. CqE, Chinese quince extracts.

	Chinese quince	Quince
Reddish color (*A*458)	0.61 ± 0.02	0.56 ± 0.12
pH	2.9	3.2
Brix (%)	83 ± 3.3	79 ± 2.4
Viscosity (Pa·s)	66.2 ± 24	33.1 ± 11
Polyphenol content [a] (mg/100 g)	704 ± 18	166 ± 2.5
Polyuronide content [b] (mg/100 g)	33.3 ± 1.6	41.0 ± 0.8
Radical scavenging activity (EC_{50} [c])	132 ± 3.9	23.6 ± 9.1
Antiulcerative activity [d]		
Area of gastric legion [e]	0.04 ± 0.01	0.47 ± 0.22
Inhibition ratio (%)	99.5 ± 0.16	93.5 ± 3.1

[a] (–)-epicatechin equivalent (Folin-Ciocalteu assay).
[b] α-D-galacturonic acid equivalent (Dimethylphenolic assay).
[c] Expressed as the dilution factor needed to decrease the initial DPPH concentration by 50%.
[d] Rats (n = 5) were administered 2 ml of a diluted jelly solution (1 g jelly + 1 ml of water). Control rats (n = 19) were administered with water.
[e] Percentage of legion area in total surface area of stomach. The value for the control rats was 17.5 ± 2.3 (%).

Table 1. Characteristics of Chinese quince and quince fruit jellies made from the extracts obtained by boiling for 2 hours.

2.3 Juice extracts of Chinese quince and apple fruits

2.3.1 Introduction

There are some commercial juice extracts of Chinese quince fruit available, but the production is very limited in Japan. Unlike other fruits such as apple, it is difficult to separate the juice of Chinese quince fruit from the pulp after homogenization using a blender. This is because their large amounts of fiber absorb the juice such that almost no liquid remains. Therefore, merely squeezing the mealy homogenate is not an effective means of juice extraction; hence, large quantities of sugar are often added to create a sucrose osmotic gradient, and then the juice is extracted. Practically, the crushed fruit obtained using a hammer crusher is added to a quantity of sugar approximately equal to 80% of the fruit weight and macerated for about three months. The mushy pulp is then squeezed in a pressing machine to obtain the juice extracts that contain approximately 60% sugar. This is the simplest method to obtain juice extracts from Chinese quince fruit. Because boiling-water extracts of Chinese quince fruit have a strong antiulcerative potential, we tried to see the effect of the juice extracts of Chinese quince fruit on prevention of the gastric mucosal lesions. In addition, the effect of apple juice was also investigated a comparison.

2.3.2 Materials and methods

Chinese quince fruit extract (juice extracted by using osmotic pressure as described above) and apple juice (cloudy type) were purchased from a local market affiliated to a juice factory in Nagano prefecture, Japan. The Chinese quince extract contained 60% (w/w) of sugar and had a pH of 3.4. The apple juice was made from 'Fuji' apples and contained >12% Brix and 0.25% organic acid. Treatment of rats including the induction of gastric mucosal injury was as described above except that the volume of sample solution administered was 3 ml. In addition to measurement of lesion area, myeloperoxidase (MPO) activity of mucosa was also measured because this enzyme indicates amount of infiltrating leukocytes. For this experiment, a crude enzyme solution was prepared from homogenized mucosa randomly collected with a razor blade from the inner surface of the frozen stomach. MPO activity was measured spectrophotometrically using 3,5,3',5'-tetramethylbenzidine (TMB) and 0.3% H_2O_2 in acetate buffer (pH 5). Free radical scavenging activity of the extract and juice was measured using DPPH radical. Polyphenolic composition was analyzed using PDA-HPLC.

2.3.3 Results and discussion

The HCl/ethanol-induced gastric lesions were strongly suppressed in rats that were given Chinese quince extracts and apple juice but the effect was stronger in those given Chinese quince extract (Fig. 4). The intensity of the gastric lesions, as quantified by the percentage of the injury surface area, was 20% in control rats versus 0.002% and 2.1% in rats given Chinese quince extract and apple juice, respectively. MPO activity in gastric mucosa (22.3 U/mg protein in controls) also was suppressed significantly ($P < 0.05$) in rats given Chinese quince extract (10.5 U/mg protein), and the activity tended to be suppressed in rats given apple juice (11.6 U/mg protein) as well.

The free radical scavenging activity of Chinese quince extract, expressed as the volume (ml) that can scavenge 50% of DPPH, was 4 times stronger than that of apple juice (Table 2). From these results, it appeared that the preventative effect of Chinese quince extract or apple juice might be due to the radical scavenging capacity and the suppression of leukocyte

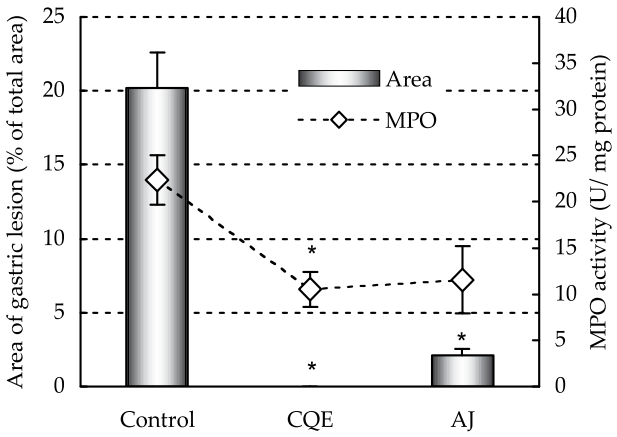


Fig. 4. Antiulcerative property of commercial Chinese quince extract and apple juice in rats.

Rats were administered 3 mL of water (control) or test solution (extract or juice) 30 min before gastric ulcer induction by HCl/ethanol. Vertical bars indicate SE (n=5). * $P < 0.05$ vs control. (from Hamauzu et al., 2008)

	Chinese quince extract	Apple juice
Free radical scavenging activity (EC_{50}) [a]	0.03 ± 0.001	0.12 ± 0.01
Soluble pectin (mg/100 mL) [b]	1.3 ± 0.07	4.9 ± 0.2
Total phenolics (mg/100 mL) [c]	342.2 ± 21.5	85.0 ± 6.4
Phenolic composition [d]		
(+)-Catechin	nd	0.57 ± 0.07
(–)-Epicatechin	3.7 ± 0.6	3.1 ± 0.09
Procyanidin B1 [e]	2.3 ± 0.2	1.3 ± 0.03
Procyanidin B2 [e]	7.3 ± 1.9	4.1 ± 0.07
Oligomeric procyanidins [e]	11.9 ± 3.2	tr
Polymeric procyanidins [e]	106.1 ± 38.8	nd
3-Caffeoylquinic acid [f]	4.9 ± 0.7	nd
5-Caffeoylquinic acid	5.5 ± 0.5	17.0 ± 0.2
Phloretin derivative [g]	nd	0.86 ± 0.01
Phlorizin	nd	0.70 ± 0.01

Data are mean ± SE. Abbreviations: nd, not detected; tr, trace.
[a] Values are volume (mL) of sample that can scavenge 50% of DPPH.
[b] Values are expressed as α-galacturonic acid equivalent.
[c] Values are expressed as (–)-epicatechin equivalent in Folin–Ciocalteu method.
[d] Values are results of HPLC analysis and expressed as mg/100 mL.
[e] Values were calculated using standard curve for (–)-epicatechin.
[f] Values were calculated using standard curve for 5-caffeoylquinic acid.
[g] Values were calculated using standard curve for phlorizin.

Table 2. Free radical scavenging activity, soluble pectin content, total phenolic content and phenolic composition of Chinese quince extract and apple juice (from Hamauzu et al., 2008)

migration to the gastric mucosa, which could be indicated by lowered activity of MPO, a marker enzyme of leukocytes. It has been thought that leukocytes migrate to the site of inflamed mucosa after injury by HCl/ethanol and subsequently expand the lesion area by producing reactive oxygen species, including free radicals (Osakabe et al., 1998). Therefore, suppression of leukocyte migration may be an important mechanism of action in the antiulcerative activity as well as radical scavenging capacity of the fruit extract and juice. There was a remarkable difference not only in polyphenolic content but also in the chemical composition of Chinese quince extract and apple juice (Table 2). The major polyphenols in Chinese quince extract were polymeric procyanidins, whereas predominant component in apple juice was 5-caffeoylquinic acid (chlorogenic acid). This difference might be the cause of the different strength of the two fruit extracts in terms of antiulcerative activity. Although apple juice had relatively weaker activity than Chinese quince extracts, the preventive effect of apple juice against HCl/ethanol-induced gastric lesions is also worth noting.

3. Effect of fruit components on the experimental gastric ulcer in rats

3.1 Polyphenolic compounds

Some polyphenolic compounds have been reported to have antiulcerative activity and are believed to be the main factor of the beneficial effects of medicinal plants in some cases. Extracted polyphenols or particular polyphenols belonging to the flavonoids family of compounds (such as quercetin, rutin, naringenin) (de Lira Mota et al., 2009), catechin and proanthocyanidins (Saito et al., 1998; Iwasaki et al., 2004) and phenolic acids (such as caffeic, ferulic, *p*-coumaric acids) (Barros et al., 2008) were reported to have certain efficacy in animal models. For example, Alarcón de la Lastra et al. (1994) reported that oral pretreatment with the highest dose of quercetin (200 mg/kg), 120 min before absolute ethanol administration, was most effective in necrosis prevention. Moreover, flavonoids such as quercetin, flavone and flavanone have been shown to inhibit growth of *Helicobacter pylori* in a dose-dependent manner *in vitro* (Beil et al., 1995). (+)-Catechin has been reported to protect gastric mucosa against ischaemia-reperfusion-induced gastric ulcers by its antioxidant activity and mucus protection (Rao et al., 2008). Proanthocyanidins (condenced tannins) are polymers of a variable number of flavan-3-ol (catechins) units. The most abundant of proanthocyanidins are procyanidins which are widely distributed in the plant kingdom. Saito et al. (1998) studied the antiulcer capacity of pure procyanidin oligomers and showed that the antiulcer activity of a series of procyanidins increased as the degree of polymerization of the catechin unit increased. Oligomers longer than three catechin units showed a strong protective effect against stomach mucosal injury. In our research, we have shown that administration of highly polymerized procyanidins isolated from pear fruit (cv. Winter Nélis) with 60%(v/v) acetone after washing with 80%(v/v) methanol strongly suppressed the induction of gastric mucosal lesion (Hamauzu et al., 2007). The preventative effect of these molecules was clearly in histological sections (Fig. 5).

Moreover, semi-purified Chinese quince polyphenols that mainly consist of polymeric procyanidins also showed strong antiulcerative activity in a dose-dependent manner (Fig. 6). The effect was observed in the ulcer index (area of gastric lesion) and myeloperoxidase activity. Apple polyphenols also showed antiulcerative activity but it was not dose-dependent. This may be due to the presence of chlorogenic acid, the predominant component in apple polyphenols (see below).

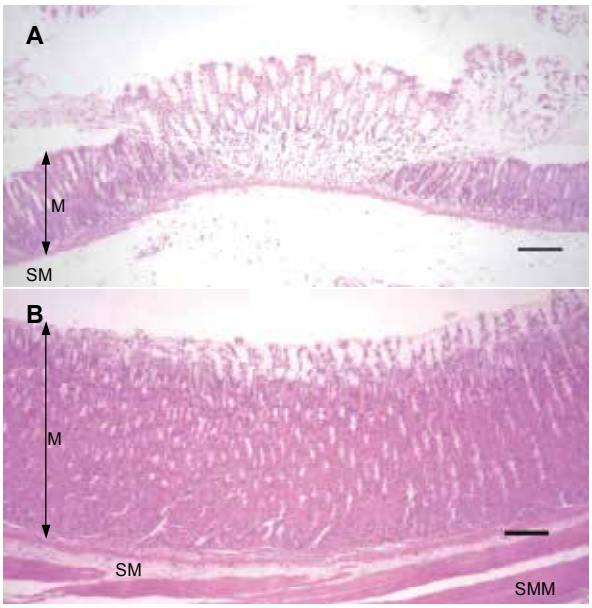


Fig. 5. Histological section analysis of rat gastric mucosa after treatment of HCl/ethanol. (A) Water was administered before induction of gastric mucosal lesions by HCl/ethanol. (B) Pear procyanidins, in an aqueous solution, were administered before induction of the lesion. M, mucosal layer; SM, submucosal layer; SMM, smooth muscle layer. Bar: 100 µm.

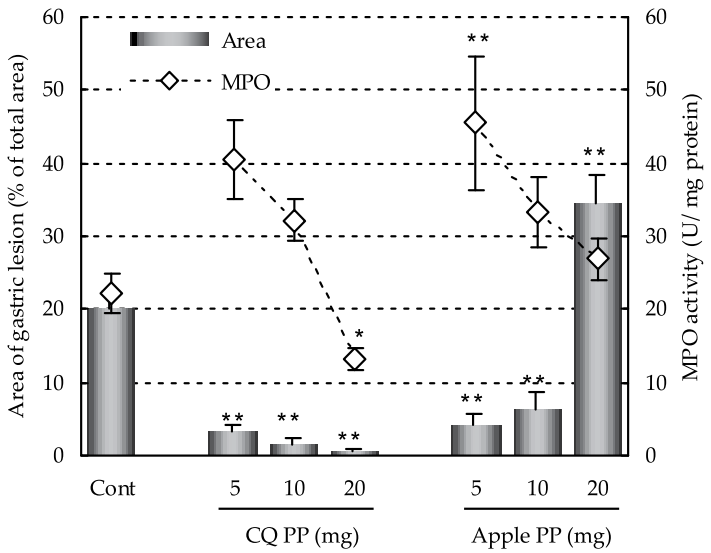


Fig. 6. Intensity of gastric lesions and mucosa myeloperoxidase activity (MPO) in rats that were administered 1.5 ml of water (control) or a solution of semi-purified Chinese quince polyphenols (CQ PP) or apple polyphenols (Apple PP) before treatment with HCl/ethanol. Bars indicate SE (n=15 for control group; n=5 for CQ PP and Apple PP group). * $P < 0.05$; ** $P < 0.01$ vs control.

The efficacy of procyanidins that have a high mDP may be due to both radical scavenging activity and affinity to the gastric mucosa. Procyanidins have affinity to protein (because they are a kind of tannin) and the affinity is known to depend in their degree of polymerization. Saito et al. (1998) reported that procyanidins such as pentamers and hexamers strongly bound to BSA. The highly polymerized procyanidins isolated from 'Winter Nélis' pear had a very high value of mean degree of polymerization (mDP = 89). Moreover, the mDP of procyanidins contained in semi-purified polyphenols from Chinese quince fruit was approximately 19, whereas that in apple polyphenols was 3–4. Their affinity to protein was actually affected by the mDP (Fig. 7 upper panel). Because of their high affinity to protein, fruit procyanidins having high mDP may have potential to bind to the mucosa. Additionally, radical scavenging activity of semi-purified Chinese quince polyphenols was stronger than that of apple polyphenols (Fig. 7 lower panel). Therefore, Chinese quince polyphenols may be superior to apple polyphenols in gastric protection because of the radical scavenging activity and its continuance on the gastric mucosa.

Thus, the mechanism of protection of the mucosa by fruit procyanidins may be both physical and chemical. By binding strongly to the mucosa, procyanidins build a protective layer against ethanol, reducing leukocyte migration, and then deploying a local antioxidant protection against free radicals. The real chemical pathway for activation and migration of leukocytes is not well understood, and it is difficult to say at which level procyanidins prevent this migration.

In our research, we have observed that chlorogenic acid-rich phenolic extract or chlorogenic acid standard showed a negative effect on prevention of gastric lesions when it was administered in excess dose.

Chlorogenic acid (5-caffeoylquinic acid) is a phenolic compound and is widely distributed in plant kingdom. It is observed in coffee beverage, blueberries, apples and ciders (Clifford, 1999). Coffee beans are one of the richest dietary sources of chlorogenic acid and for many consumers must be the major dietary source for this molecule. Chlorogenic acid has been reported to have a series of biological effects in vitro and in vivo, such as antioxidant capacity, radical scavenging activity, antimutagenic/anticarcinogenic effect, inflammation inhibiting and endothelial protective properties, etc. (Chang & Li, 2005), and thought that the compound might contribute to body health promotion to some extent. Zhao et al. (2008) has reported that chlorogenic acid has the down-regulative effects on the H_2O_2- or TNF-α-induced secretion of interleukin (IL)-8, a central pro-inflammatory chemokine involved in the pathogenesis of inflammatory bowel diseases, in human intestinal Caco-2 cells. In relation to the gastric ulcer prevention, Graziani et al. (2005) reported that chlorogenic acid was equally effective as apple extracts in preventing oxidative injury to gastric cells.

However, in some cases, chlorogenic acid seems to be ineffective in preventing gastric ulcers in animal models. Ishida et al. (2010) reported that oral administration of chlorogenic acid (4 mg/kg or 16 mg/kg, respectively) was ineffective to prevent absolute ethanol-induced or restraint plus water immersion stress-induced gastric ulcer in male Sprague-Dawley strain rats.

In our experiment, administration of a high dose (20 mg/rat; approx. 80 mg/kg b.w.) of chlorogenic acid tended to enhance the gastric lesion induced by HCl/EtOH in male Wistar rats. We also observed that a high dose (20 mg/rat) of semi-purified apple polyphenols (rich in chlorogenic acid) enhanced the ethanol-induced gastric lesions in rats. These findings suggest that chlorogenic acid has potential to increase some factors that progress gastric

lesions in ethanol-induced ulcer model when it administered at high dose. The actual mechanism is unclear, but chlorogenic acid seems to stimulate gastric acid secretion. It has been reported that chlorogenic acid affects the expression of gastric acid secretion-related proteins in human gastric cancer cell (Rubach et al., 2008). The excessive secretion of hydrochloric acid, the main constituent of gastric acid, in the stomach is considered an important factor in the formation of peptic ulcer (Welgan, 1974).

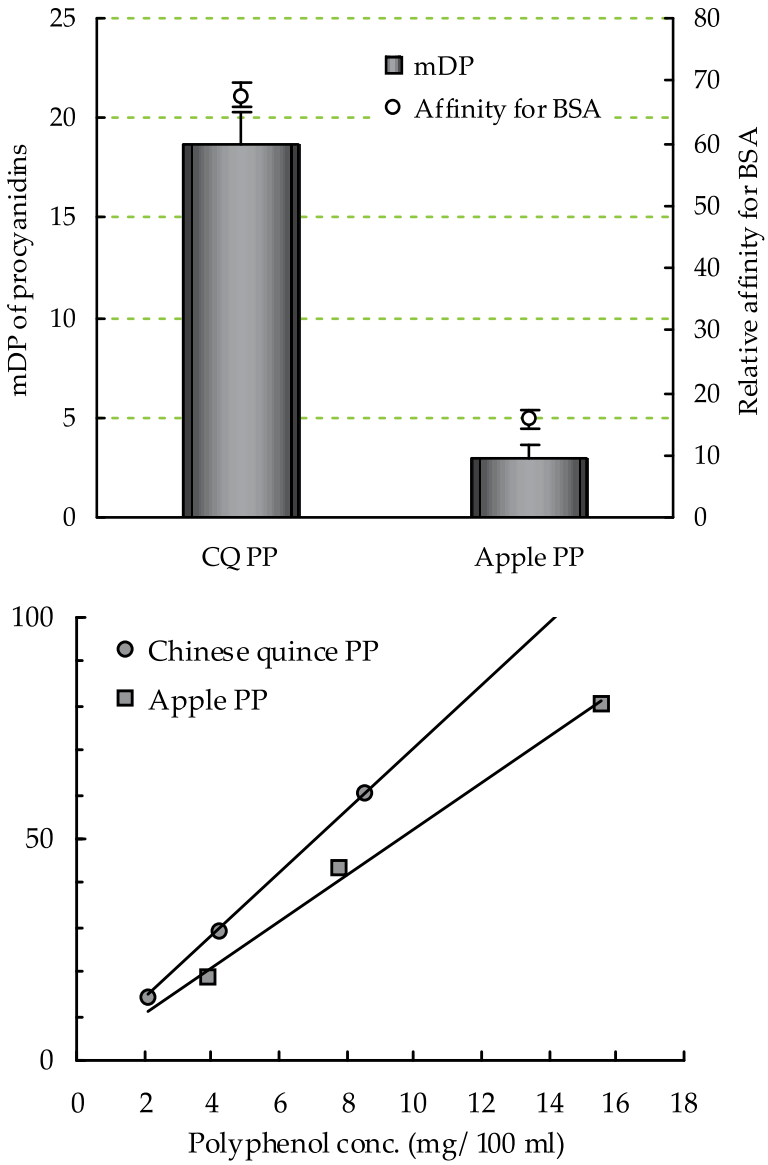


Fig. 7. Mean degree of polymerization (mDP) of procyanidins, relative affinity for bovine serum albumin and free radical scavenging activity of semi-purified Chinese quince polyphenols (CQ PP) and apple polyphenols (Apple PP).

Although high dose of chlorogenic acid or apple polyphenols have the potential to promote gastric mucosal lesions, normal consumption of apple polyphenol has been shown to prevent gastric ulcer in rats (Graziani et al., 2005). We also have confirmed that administration of cloudy apple juice suppressed gastric mucosal lesions induced by HCl/ethanol (section 2.3). Therefore, it should be emphasized that the natural concentration of phenolics in both apple fruit and juice may not cause any deteriorating effect on HCl/ethanol-induced gastric lesions and, in fact, may have some health benefit. This may indicate that excessively purified compounds may have adverse effects on health under particular conditions, even though they are known as health-promoting components.

3.2 Dietary fiber

Fruits contain high amount of soluble- and insoluble-fiber components. Soluble-fiber, such as pectic polysaccharides (pectin), might be an effective ingredient in gastric ulcer prevention because some soluble polysaccharides or mucilage were reported to have antiulcerative activity. For example, a galactomannoglucan with an estimated weight–average molar mass of 415,000 g/mol, obtained from an aqueous extract of the mesocarp of fruits of catolé palm (*Syagrus oleracea*), significantly inhibited gastric lesions induced by ethanol in mice, showing a gastroprotective property (da Silva & Parente, 2010). Lemnan, a pectic polysaccharide of duckweed *Lemna minor*, was also reported to be a potent gastroprotective agent for chemical and emotional stress models in animals (Khasina et al., 2003); it enhanced resistance of the stomach tissue to various ulcerogenic factors (emotional stress, indomethacin, pesticide 2,4-D).

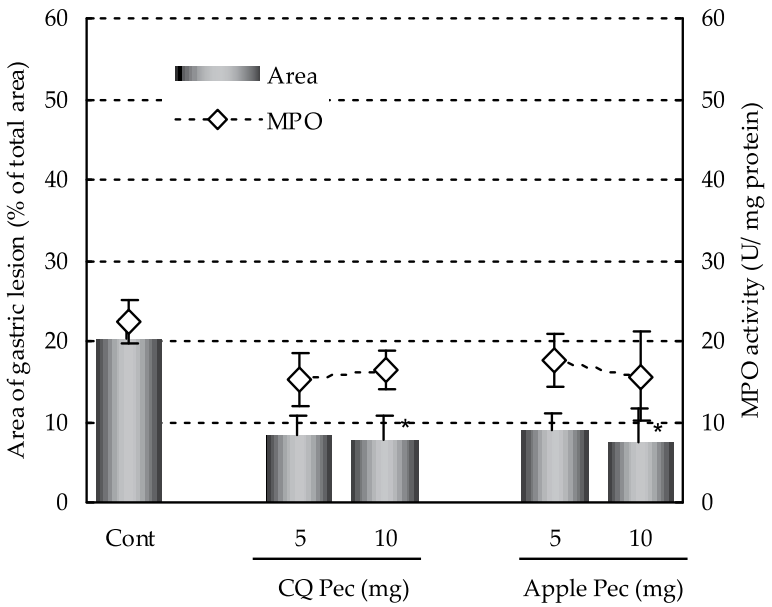


Fig. 8. Intensity of gastric lesions and myeloperoxidase (MPO) activity of mucosa of rats that were administered water (control), soluble pectin from Chinese quince fruit (CQ Pec) or commercial apple pectin (Apple Pec) before treatment with 150 mM HCl/ethanol (40:60, v/v). * $P < 0.05$ vs control.

In our research, soluble pectin extracted from Chinese quince fruit and commercial apple pectin both showed antiulcerative activity (Fig. 8). However, the effect seemed weaker than that of extracted polyphenols, especially in case of Chinese quince fruit. Therefore, pectic polysaccharides may partly contribute to antiulcerogenic activity together with polyphenols.

4. Conclusions

Many fruits, especially medicinal fruits, have been reported to have antiulcerative activity in animal experiment. Chinese quince fruit extract show strong activity for the prevention of gastric mucosal lesions induced by HCl/ethanol in rats. The effect is probably due to a high content of procyanidins that exhibit antioxidant activity and affinity to proteins. The preventative effect of fruit extracts on gastric mucosal lesions is retained even after prolonged heating (as observed in the effect of fruit jelly). Moreover, pectin, a cell wall polysaccharide, may enhance the effect of polyphenols on the prevention of gastric lesions. Meanwhile, some other fruit products such as apple juice and hot-water extract of quince also have a significant effect. However, a high dose of chlorogenic acid may promote the ethanol-induced gastric lesions. This indicates that excess intake of purified compounds should be avoided even if it is a natural antioxidant. Future research to elucidate the mechanisms of action of fruit polyphenols that prevent or increase the gastric lesions that lead to ulcer will be needed.

5. Acknowledgment

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22

Herbal Treatment of Peptic Ulcer: Guilty or Innocent

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1. Introduction

Normally there is a balance between the protective factors (e.g. mucus, bicarbonate, prostaglandins, nitric oxide and normal blood flow) and aggressive factors (e.g. acid plus pepsin, active oxidants, leukotrienes, endothelins, bile or exogenous factors including nonsteroidal anti-inflammatory drugs). Peptic ulcer develops when aggressive factors overcome the protective mechanisms (Borrelli & Izzo, 2000). *Helicobacter pylori*, nonsteroidal anti-inflammatory drugs and acid-pepsin hypersecretion are the major factors that disrupt this equilibrium. There is other type classified as idiopathic and may be related to defective mucosal defence mechanisms due to tobacco use, psychological stress (stress gastritis), rapid gastric emptying or genetics (Calam & Baron, 2001).
Drug treatment of peptic ulcers is targeted at either counteracting aggressive factors or stimulating the mucosal defences (Tepperman & Jacobson, 1994). The ideal aims of treatment of peptic ulcer disease are to relieve pain, heal the ulcer and delay ulcer recurrence (Borrelli & Izzo, 2000).

2. Aim of the work

The aims of this chapter are to review data about their herbs current usage by patients with peptic ulcer, evidence for their efficacy, the mechanisms by which they might act, and, lastly, their adverse effects on the body.

3. Herbal treatment of peptic ulcer

Tyler defines herbal medicines as "crude drugs of vegetable origin utilized for the treatment of disease states, often of a chronic nature, or to attain or maintain a condition of improved health (Tyler, 1994).
In spite of the progress in conventional chemistry and pharmacology in producing effective drugs, the herbal medicine might provide a source of treatment by many people in the world. In many cultures herbal knowledge was said to have been handed down from the gods. Herbs had been used by all cultures throughout history because patients are often

unaware of the potential problems caused by herbal medicines. In addition, their physicians commonly lack knowledge about these compounds. This factor results in the perception by physicians that herbal drugs are ineffective placebos that can simply be ignored. Some physicians view use of these products as a threat to their paternalistic role and sternly admonish their patients or angrily label them as being crazy (Crone & Wise, 1998).

3.1 Examples of herbs used in treatment of peptic ulcer

Solanum nigrum (family: Solanaceae) commonly known as black nightshade, deadly nightshade, sunberry, makoy, fragrant tomato, duscle, Hound's berry , petty Morel , wonder berry, popolo or wonder cherry. It is effective in treatment of peptic ulcers. The raw juice of its leaves is given either separately or in conjunction with other beneficial juices (Akhtar & Munir, 1989).

A condensed tannin, polyflavonoid tannin, catechol-type tannin non-hydrolyzable tannin or flavolan has been isolated and their anti-peptic and anti-ulcer activity confirmed experimentally (Vasconcelos et al., 2010). When a low concentration of tannin is applied to the mucosa, only the outermost layer is tanned, becoming less permeable and affording an increased protection to the subjacent layers against the action of bacteria, chemical irritation, and, to a certain extent, against mechanical irritation. Tannins may promote a mechanic barrier that protects the stomach from ulcer formation and facilitates ulcer healing (Borrelli & Izzo, 2000).

Saponins (family: Sapindaceae) are so-called because of their soap-like effect, which is due to their surfactant properties. Saponins isolated from the rhizome of panax japonicas, the fruit of kochia scoparia (which contain approximately 20% of saponins) some oleanolic acid oligoglycosides extracted from P. japonicas, K. scoparia and a methanol extract of P. japonicus rhizome have been demonstrated to possess gastro-protective properties (Matsuda et al., 1998).

Licorice or glycyrrhiza glabra (family: Leguminosae) also known as lacrisse (German), licorice root, liquorice, reglisse (French), regolizia (Italian), suessholz, sweet licorice, sweet wood. It is one of the most widely used medicinal plants in the world, commonly used in European, Arabian and Asian traditional medicine systems. Licorice is very effective in the treatment of stomach ulcers. It soothes the irritation of the inner lining of the stomach caused due to excessive acids. Its root is taken, dried and then soaked overnight in water. This is taken in an infusion with rice gruel. This is such an effective treatment that it is used in conventional allopathic medicine also (Hayashi & Sudo, 2009).

Plants containing mucilages traditionally used in several countries in the treatment of gastric ulcer include althaea officinalis (marshmallow), cetraria islandica (Iceland moss), malva sylvestris (common mallow), matricaria chamomilla (chamomile) and aloe species (Capasso & Grandolini, 1999). Myrrh (meaning bitter), an oleo-gum-resin obtained from commiphora molmol, contains up to 60% gum and up to 40% resin (Newall et al., 1996). Myrrh pre-treatment produced a dose-dependent protection against the ulcerogenic effects of different necrotizing agents (Al-Harbi et al., 1997). The protective effect of myrrh is attributed to its effect on mucus production or increase in nucleic acid and non-protein sulphydryl concentration, which appears to be mediated through its free-radical scavenging, thyroid-stimulating and prostaglandin- inducing properties. Also aloe seems to be able to speed wound healing by improving blood circulation through the area and preventing cell death around a wound (Borrelli & Izzo, 2000).

3.2 Potential benefits and mechanism of action

Experimental studies have demonstrated that the herbs have gastroprotective activity against gastric mucosal injury induced by ethanol (Souza et al., 2007), ischemia reperfusion (El-Abhar et al., 2002), indomethacin (Souza et al., 2007), alcohol toxicity (Kanter et al., 2005) or stress (Khaled, 2009) in rat.

The mechanism of herb-induced gastroprotection varies according to the nature and chemical constituents of the herbs. The main functions including; inhibition of acid plus pepsin secretion (Baggio et al ., 2007), cytoprotective (by enhancement of epidermal growth factor content in gastric juice, nitric oxide and H+, K+-ATPase inhibitory activity in gastric tissue, PGE2 in plasma, inhibition of endothelin in plasma, an increase in mucosal thickness (Fan et al., 2007) and mucus content in the gastric mucosa) (Kamath et al., 2008), bactericidal activity, inhibition of the growth and activity of *helicobacter pylori* (Mahady et al., 2002) and antioxidant activities (and the ability to scavenge reactive oxygen species) (Souza et al., 2007), isolated or in combination, are responsible for gastric mucosal protection (Zaidi, et al., 2009). Moreover, plantextract- induced gastroprotection is probably related to the enhancing effect on NOS inhibitor expression, gastric microcirculation (Al Mofleh, 2010). Herbs could protect the gastric mucosa by increasing the bioavailability of arachidonic acid, resulting in biosynthesis of the cytoprotective prostaglandins in the stomach (Tsuji et al., 1990). Moreover, herbs have also been reported to produce a marked inhibition on the release of leukotrienes, which cause mucosal tissue injury and hypoxemia (Mansour, 1990).

3.3 Risks of herbal treatment of peptic ulcer

It is important to acknowledge that all conventional drugs have potential toxicities. However, in contrast to herbal products, conventional drugs undergo trials and postapproval surveillance that define these toxicities, giving practitioners data on that to weigh risks and benefits of treatment. The therapeutic window and dosage are also defined, as are the constituents of the medicine. Because of rigorous quality control, each pill has the same ingredients as another. Adverse reactions to herbal medicines are probably underrecognized and underreported (D'Arcy et al., 1991). Herbal medicines can produce unwanted side effects, toxicity and herbal drug interaction caused by their pharmacologic properties.

A-Side-effects and toxicity of herbal therapy

i. Direct side-effects and toxicity of herbal therapy

Nausea, diarrhea, and skin reactions are common side effects of a wide variety of herbal medicines (tannins, mucilages, saponins and solanum nigrum). Also there is a serious side effects of herbal remedies on the liver (tannins and Licorice) include liver injury, acute and chronic hepatitis, hepatic failure and possibly hepatic tumours (Chandler, 1987). While most of the adverse effects on the digestive tube are self-limiting and relatively trivial, the same is not true of herb-induced hepatotoxicity, in which fatalities have been reported with alarming frequency (Chitturi & Farrell, 2000). More serious side effects of herbal medicines may include hypertension, heart failure (licorice), anaphylaxis (matricaria chamomilla), and lupus-like symptoms (D'Arcy et al., 1991). Ventricular arrhythmias, intravascular hemolysis, hemorrhage, renal failure, and pulmonary hypertension have all been linked to the active chemical components found in herbal remedies (Larrey et al., 1992). Psychoactive effects in several herbal medicines have produced behavioural, cognitive, mania and emotional

disturbances (Capwell, 1995). Most of these herbs are not recommended for woman with pregnancy or breast feeding (Roulet et al., 1988).

Black nightshade is UNSAFE. It contains a toxic chemical called solanin. At higher doses, it can cause severe poisoning. Signs of poisoning include irregular heartbeat, trouble breathing, dizziness, drowsiness, twitching of the arms and legs, cramps, diarrhea, paralysis, trembling, paralysis, coma, and death (Duke, 1985).

In sensitive individuals, a large intake of tannins may cause bowel irritation, kidney irritation, liver damage, irritation of the stomach and gastrointestinal pain. A correlation has been made between esophogeal or nasal cancer in humans and regular consumption of certain herbs with high tannin concentrations (Lewis, 1977). Tannins interfere with iron absorption through a complex formation with iron when it is in the gastrointestinal lumen which decreases the bioavailability of iron. There is an important difference in the way in which the phenolic compounds interact with different hydroxylation patterns (gallic acid, catechin, chlorogenic acid) and the effect on iron absorption. The content of the iron-binding galloyl groups may be the major determinant of the inhibitory effect of phenolic compounds. However, condensed tannins do not interfere with iron absorption (Brune et al., 1989).

Saponins are harmful if swallowed or inhaled. They cause irritation to skin, eyes and respiratory tract. Symptoms include redness, itching, and pain. Saponin inhalation causes sneezing and may irritate the respiratory tract. They cause haemolysis of RBC's if reach the blood. Frequent ingestion of small amounts of saponin results in chronic githagism (a disease, similar to lathyrism, that results in pain, burning and prickling sensations in lower extremities, and increasing paralysis) (Hostettmann and Marston, 2005).

Excessive consumption of licorice is known to be toxic to the cardiovascular system and may produce oedema (van Uum, 2005). Comparative studies of pregnant women suggest that licorice can also adversely affect both IQ and behaviour traits of offspring (De Smet, 2002). In large amounts, licorice containing glycyrrhizin can cause high blood pressure, salt and water retention, and low potassium levels, which could lead to heart failure (Blumenthal et al., 2000).

Mucilage side effects include bloating, abdominal pain, flatulence and oesophageal obstruction. Matricaria chamomilla (chamomile) causes symptoms of an allergic reaction such as rash, itching, swelling, dizziness and trouble breathing (Andres et al., 2009). Althaea officinalis is generally regarded as safe. However, the potential for marshmallow to cause allergic reactions or low blood sugar, genotoxicity, carcinogenicity and/or reproductive and developmental toxicity has been noted anecdotally (Büechi et al., 2005). Taking aloe by mouth is unsafe, especially at high doses. There is some concern that some of the chemicals found in aloe latex might cause cancer. Additionally, aloe latex is hard on the kidneys and could lead to serious kidney disease and even death (Poppenga, 2002).

ii. Indirect Side-Effects and Toxicity of Herbal Therapy

The use of herbal therapy may be complicated by several indirect adverse effects. People initially consulting herbal practitioners may suffer from misdiagnosis and consequent delay in obtaining effective conventional treatment (Angell & Kassirer, 1998). Others may delay or forego appropriate conventional options in favour of ineffective unconventional ones. When expectations of alternative therapy are high, failure to obtain relief from symptoms, particularly if treatment has been expensive, could also be construed as an adverse effect (Langmead & Rampton, 2001).

B-Drug–herb Interactions

A pharmacodynamic interaction occurs when substances act at the same receptor, site of action or physiologic system. Pharmacodynamic interactions result in an antagonistic or additive drug effect (Anastasio et al., 2000). A drug or substance that accentuates or interferes with the absorption, distribution and elimination of a second drug or substance produces a pharmacokinetic interaction. This mechanism is the most frequent cause of adverse interactions, commonly caused by altered drug elimination. Induction of elimination can result in a decreased therapeutic benefit whereas inhibition of drug elimination can produce excessively increased dose related toxicity (Nicole & Mitchell, 2003).

Saponins and mucilage can interfere with the absorption of other medicines within the gut if they are taken at the same time (Mohammed, 2009).

Several medications may cause potentially negative drug interactions with licorice. Some of these medications include blood pressure medications (beta blockers, calcium channel blockers, and nervous system inhibitors), certain diuretics (such as bumetanide, chlorothiazide, chlorthalidone, ethacrynic acid, furosemide, hydrochlorothiazide, metolazone and torsemide), hypoglycemics and corticosteroids (D'Arcy et al., 1991). These licorice drug interactions can result in serious problems, such as low blood potassium and low blood calcium (Blumenthal et al., 2000). Licorice should not be taken concurrently with corticosteroid treatment (Poppenga, 2002). Concurrent use of furosemide may potentiate development of acute renal failure. Potassium loss due to other drugs, e.g. thiazide diuretics, can be increased. With potassium loss, sensitivity to digitalis glycosides increases (D'Arcy et al., 1991). Licorice should not be administered in conjunction with spironolactone or amiloride (Poppenga, 2002).

It is mentioned in some literature sources (Barnes et al. 2002, Poppenga, 2002) that absorption of concomitantly administered medicines can be delayed due to mucilage protecting layer. Potential risks of chamomile include interference with warfarin and infant botulism in very young children (Biancoa et al., 2008). Aloe may increase K + loss and potentiate cardiac glycosides and antiarrhythmic agents such as quinidine. Increased K + loss when used with other drugs, such as diuretics, with similar effect on K +. Laxative effect may reduce absorption of other drugs (Poppenga, 2002).

4. Conclusion

Herbal medicine is prescribed by the herbalists symptomatically—based on signs and symptoms alone—rather than as a result of a full understanding of the underlying disease. Proper diagnosis is totally absent. As any plant, medicinal herbs contain many chemicals that are subjected to change with changing conditions of the environment, especially storage. The discriminate and proper use of some herbal products is safe and may provide some therapeutic benefits, but the indiscriminate or excessive use of herbs can be unsafe and even dangerous (Borrelli & Izzo, 2000).

There is an urgent need for further scientific assessment of the potential benefits and dangers of the huge range of herbal medications available. Herbal preparations used for medicinal purposes should require licensing by an independent national body in order to improve their quality and safety, and to ensure that claims of efficacy are validated by randomized controlled trials.

The general public, as well as pharmacists, general practitioners and hospital doctors, should be aware, particularly, of the risks associated with the use of herbal remedies,

whether on their own or in combination with other herbal or conventional medicines. The incorporation of a short course on alternative and complementary therapy in medical school curricula would help achieve this end.

Lastly, because of the potential for side effects, toxic reactions, and unwanted drug-drug interactions, it is essential for physicians to ascertain if their patients are taking herbal medications. So if you are thinking about using herbal medicine it would be a good idea to check with your physician about possible adverse reactions and interactions with medications you may be taking before starting (D'Arcy et al., 1991).

IF YOU NEED ONE WORD "Do not take herbs internally except under the supervision of a qualified professional".

Herbs you're guilty until proven innocent by researchers!

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23

In Vitro and *In Vivo* Anti-*Helicobacter pylori* Activity of Natural Products

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1. Introduction

Since old times plants have been a resource used by human beings an important sources of biologically active products. Recently, efforts have been made in order to identify new antiulcerogenic drugs from natural sources, having as the main target the *Helicobacter pylori*, a bacterium considered as the most important etiological agent of the human peptic ulcer. It has been shown to be a rich source of bioactive substances, having antifungal, gastroprotective, analgesic, anti-HIV, antibacterial, antitumoral properties and inhibitor of gastric H+,K+-ATPase and angeotensin-converting enzyme. This chapter aims to demonstrate methods anti-*Helicobacter pylori in vitro* and *in vivo* for screening of plant extracts.

Helicobacter pylori was identified in 1982 by Marshall and Warren (1984) and quickly became the subject of countless microbiological, histological, epidemiological, immunological, ecological and clinical studies (Vaz, 2005). This organism has its nomenclature revised, starting with Campylobacter pyloridis, and a correction of the name was originally Greek to Latin, Campylobacter pylori (Marshall and Goodwin, 1987) and organisms like Campylobacter. Taxonomic studies have led to reclassification, resulting in the name Helicobacter pylori (Goodwin and Armstrong, 1990).

H. pylori is a bacillus, Gram negative, microaerobic, spiral and curved (Dunn et al. 1997). It has two to six flagella that provide motility to it to resist the rhythmic contractions of stomach and penetrate the gastric mucosa. It has 2.4 to 4.0 mm in length and 0.5 to 1.0 mm in width (Brown, 2000).

The identification and isolation of *Helicobacter pylori* allowed for a considerable development of knowledge about peptic ulcer (Marshall and Warren, 1984). This pathogen is considered the main etiological agent of human peptic ulcer, with a worldwide prevalence rate of about 40% in developed countries and over 80% in developing countries (Shi et al., 2008).

H. pylori produces a number of virulence factors that may have different associations with the disease. The establishment of chronic infection may be influenced by host genetic factors as well as the blood group ABO and Lewis-blood group antigen and differences in susceptibility to particular strains of *H. pylori* (Brown, 2000).

To establish and maintain the infection, H. pylori expresses a variety of different types of maintenance factors that allow bacteria to colonize and remain within the host and virulence factors, which contributes to the pathogenic effects of bacteria, with emphasis on gastric inflammation, mucosal barrier disruption gastric and changes in gastric physiology (Dunn

et al. , 1997). *H. pylori* is a genetically highly diverse bacterium, featuring several genotypes which have been associated with virulence factors and risk of gastric disease and other outcomes of infection. Among these, the vacA gene, which encodes a cytotoxin of vacuolization, is present in all types of *H. pylori*. This gene is also strongly associated with high levels of inflammation and epithelial damage in the gastric mucosa, caused by cagA gene is a marker for the presence of PAI pathogenicity (cag pathogenicity island) (Ladeira et al., 2003).

In previous studies, infection with different types of Helicobacter, as H. mustela, H. felis, H. heilmanii, and H. pylori in mice, cats, pigs, monkeys and gerbils have been described suggesting its relevance to human infection, but these animal models do not mimic the infection of H. pylori in humans because of the lack of virulence factors of infecting organisms, such as vacA and cagA encoded cytotoxins, required for the mucosal damage, inflammation and ulcer formation (Konturek et al., 2000). Moreover, some of these animals are large and unwieldy, there is a need to test commonly used in animal models such as mice, which could be used to study various aspects of infection by H. pylori, ulcer healing and therapy of infection (Ross et al., 1992).

Results in experimental studies in animal models using rodents, concluded that H. pylori alone causes little or no effect on the gastric mucosa of intact rats. However, this organism can cause persistence of pre-existing ulcers and chronic active inflammation. Presence of predisposing factors leading to disruption of the integrity of the gastric mucosa may be necessary for the H. pylori enhancement inflammation and tissue damage to the stomach of these animals (Konturek et al 2000). Whereas peptic ulcer is generally a disease which results from the circumscriptive loss of tissue in regions of the digestive tract that may come into contact with the stomach's chloride peptic secretion (Coelho, 2003). In general, it is caused by an imbalance between aggressive and defensive factors of the gastric mucosa (Rao et al., 2000). It seems that the Helicobacter pylori takes advantage of this situation to colonize and settle in the gastric mucosa.

2. Experimental protocols

2.1 Animals

Male Wistar albino rats (160-210 g) and male Swiss-Webster mice (25-30 g), can be used. The animals should be kept in propylene cages at 26±2^{o}C under 12h light-dark cycle, with free access to water and restricted access to food, 2 hours/day (9-10a.m. and 6-7p.m.).

2.2 Microorganism

The strain of *Helicobacter pylori* ATCC 43504 (vacA and cagA positives) can be used to express the factors that determine their virulence. Stock cultures can be maintained in Mueller-Hinton broth at -20^{o}C.

2.3 Botanical material

Plants should be carefully collected and treated to prevent fungal contamination. The plants should be deposited, registered and taxonomically verified.

2.4 Extract preparation and phytochemical analysis

Plants should be cleaned, dried at room temperature and shredded in an electric mill with a sieve with a mesh size of 40μm, until powder be obtained. The dried powder should be

successively macerated (1:5, w/v), with hexane, dichloromethane, ethyl acetate, methanol and water-ethanol 75%, for 7 days each. Every extract should be separated by filtration and concentrated under reduced pressure at, approximately, 40ºC, with the residual solvent being eliminated in an incubator at 40ºC. To prepare the dichloromethanic fraction (Fig. 1), the crude dichloromethanic extract should be submitted to silica filtration using dichloromethane. The preliminary phytochemical analyses of the hydroethanolic extract and the dichloromethanic fraction may follow the methodology described by Matos (1998).

Fig. 1. Scheme of preparation of extracts and fractions

2.5 In-vitro assays

2.5.1 Disk diffusion

For the disk diffusion assay, serial dilutions of the hexanic, ethyl acetate, dichloromethanic, methanolic and hydroethanolic extracts from plants, should be prepared, in order to obtain the following doses: 0.0625; 0.125; 0.25; 0.5 and 1 mg/disk. The sterile disks utilized (6 mm - CECON®) should be imbibed in 25µL of each dose of extract and fraction. The extract- or fraction-imbibed disks should be deposited on the surface of the plate inoculated with *H. pylori*, in a suspension of $6x10^8$ CFU/mL (McFarland turbidity standard 2), using clarithromycin (15µg - CECON®) as the standard drug, incubated at 37°C under microaerophilic conditions in an atmosphere of 5 to 15% O_2 and 5 to 10% CO_2 for 3-5 days. After this period, the growth inhibition halos should be quantified with a digital pachymeter. The diameters of inhibitory zones should be measured in duplicate and mean values ≥10 mm are considered active (Fig. 2).

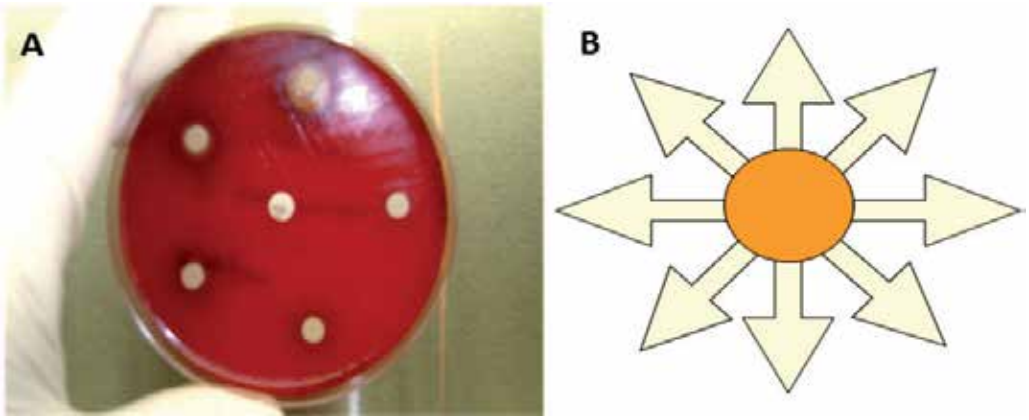

Fig. 2. Photograph (A) and scheme (B) of a disk diffusion

2.5.2 Broth microdilution

The broth microdilution assay allows the determination of the Minimum Inhibitory Concentration (MIC). To each well in the microplate should be added 100µL of Mueller-Hinton broth, supplemented with 10% foetal calf serum inoculated with $6x10^8$ *H. pylori* (McFarland turbidity standard 2), 100µL of the hexanic, ethyl acetate, dichloromethanic, methanolic and hydroethanolic extracts from plants, should be also added to reach the final concentrations of 0.0625; 0.125; 0.25; 0.5 and 1 mg/mL. Clarithromycin (5 mg/mL) is used as the standard drug for growth inhibition. Next, the microplate (Fig. 3), should be incubated at 37°C under microaerophilia in an atmosphere of 5 to 15% O_2 and 5 to 10% CO_2, for 3-5 days. After incubation, the plates should be visually examined and each well should be replicated in blood agar (Mueller-Hinton agar with 5% sheep blood), to determine whether growth had occurred, with the MIC defined as the lowest concentration to cause complete bacterial growth inhibition (bactericidal activity).

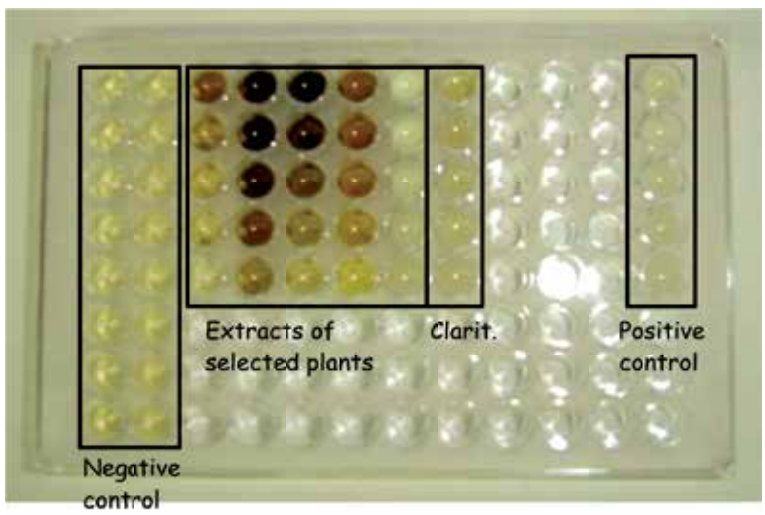

Fig. 3. Photograph of a microdilution plate

2.6 In-vivo assays

2.6.1 Acute toxicity evaluation

The acute toxicity evaluation of each extract of plant should be performed in mice (n = 4). The animals should be treated orally (p.o.) with extract at 250, 500, 1000, 3000 and 5000 mg/kg doses. A control animal should be used for each dose, having received the vehicle (distilled water, 10 mL/kg). After the administration of the extract or vehicle, the animals should be observed individually in appropriate cages (open field) at 0, 15 and 30 minutes; 1, 2, 4 and 8 hours and, once every day, for 14 days. The results for the general behavioural observations should be recorded in a table adapted from Malone (1977).

2.6.2 Ulcer induction and colonization by *H. pylori*

Rats should be ulcerated by acetic acid according to method described by Takagi et al. (1969), with modifications. After ulcer induction, the animals should be kept in propylene cages, with daily access to commercial food restricted to the time periods of 9-10 a.m. and 5-6 p.m., allowing for adequate fasting for administration of *H. pylori*, and of extract at 50, 100 and 200mg/kg doses of preparations, as well as of the standard drugs (amoxicillin 50mg/kg + clarithromycin 25mg/kg + omeprazole 20mg/kg).

According to the method described by Konturek et al. (1999), with modifications, 24h after ulcer induction by acetic acid, the animals should be inoculated intragastrically with 1 mL of *H. pylori* ATCC 43504 ($9x10^8$) suspended in Mueller-Hinton broth, by using a cannula appropriate for orogastric gavage. For the animals in the control, Sham and acetic acid-induced ulcer groups without *H. pylori* infection, only Mueller-Hinton broth should be orally administered. The orogastric inoculation of *H. pylori* should be maintained twice a day for 7 days, whereas the administration of extracts from plants and of the standard drugs, twice a day, for 14 consecutive days, starting from the third day after ulcer induction by acetic acid. After treatment, the animals should be sacrificed by cervical dislocation; blood can be collected from the inferior vena cava. The stomachs should be removed for evaluation of gastric lesions, the ulcerated area (mm^2) should be measured and the healing rate (%) and then determined according to method described by Takagi et al. (1969). Prostaglandin E2 (PGE2) levels should be measured from gastric mucosal scrapings and a fragment from each stomach can be used for the histopathological exam and for the urease determination.

2.6.3 Determination of PGE_2 concentration

The concentration of PGE_2 in scrapings of gastric mucosa should be quantified by ELISA using a commercial kit (Parameter®, R&D Systems). The mucosal scrapings (100 mg) should be homogenized with 1 mL phosphate buffer and centrifuged at 3,000 RPM at 4°C for 10 min. The PGE_2 levels should be determined according to the manufacturer's instructions.

It has been demonstrated that PGE_2, derived from COX-1 and COX-2, is involved in the regulation of gastric mucosa inflammation and also contributes to maintaining its integrity during infection by *H. pylori* through several mechanisms, including augmentation of the gastric mucosal flow, synthesis of mucus and bicarbonate, inhibition of gastric motility, and the release of enzymes, free radicals from neutrophils and gastric secretion (Chao et al., 2004).

2.6.4 Urease production determination

With the aid of tweezers, a fragment of gastric tissue should be inserted in the centre of a minitube containing urease gel (NEWPROV®). Inoculation times should be recorded, the minitubes should be kept at room temperature and the change in colour should be evaluated after 1 hour, and whenever it is negative, a final reading should be taken after 24 hours. A urease test should be considered positive if an alkaline reaction has developed (red or dark pink colour), and negative when there are no changes in the medium's colour (yellow or light orange).

Urease, an enzyme produced by *H. pylori*, acts by promoting the hydrolysis of urea, a substrate that is present in gastric juice under physiological conditions, leading to the production of ammonium, that behaves as a receptor for H^+ ions and generates a neutral pH inside the bacteria, thus contributing to the survival of these organisms in the highly acidic environment of the stomach (Ladeira et al., 2003). The rapid urease test is considered one of the most useful and cheapest tests among the invasive assays, with a 100% sensitivity and 89.5% specificity (Ogata et al., 2002), although false positive results may occur given the other bacterial species that might be isolated from the oral and/or gastric cavities (*Proteus mirabilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Staphylococcus aureus*) and that also produce urease (Osaki et al., 2008).

2.6.5 Determination of cytokines IL-1β, TNF-α, IL-10 and VEGF

Total blood should be collected from the inferior vena cava, in tubes containing 5% EDTA, centrifuged at 3000 RPM for 10 min., and the plasma should be separated and frozen at -20ºC until the assay. For measuring plasma levels of IL-1β, TNF-α, IL-10 and VEGF, a plex kit for rat cytokines and chemokines (RCYTO-80K) should be used according to the manufacturer's instructions, and the fluorescence should be determined through a Luminex ® device.

The literature refers to IL-1β as being the most potent among the known gastroproctective agents (Kondo et al., 1994; El-Omar, 2004). TNF-α also inhibits gastric secretion, although to a lesser extent than IL-1β and is found, together with IL-10, elevated in patients with chronic gastritis associated with *H. pylori* infection (El-Omar, 2004). VEGF has been mentioned as a cicatrisation-promoting factor in gastric ulcer (Okabe and Amagase, 2005).

2.6.6 Histopathological analysis

After their removal, half of each stomach should be fixated in 10% buffered formalin and embedded in paraffin. From each block, two 5µm sections should be made, one being stained by hematoxilin and eosin (HE) and the other by a modified Giemsa stain for H. pylori detection.

All tissues should be examined by a pathologist, according to criteria established by Dixon et al. (1996), and the following parameters should be analyzed:

- Inflammation - presence of lymphocytes and plasmocytes in the lamina propria;
- Activity – characterized by the presence of neutrophils inside the superficial and glandular epithelial layers;
- Regeneration - characterized by a proliferative response to epithelial lesion, in which epithelial cells presenting larger hyper-stained / excessively stained nuclei, with an increase in the nucleus-cytoplasm ratio, and observation of occasional mitotic figures;
- Atrophy – reduction of glandular structures;

- Metaplasia - presence of caliciform cells with an intestinal morphology.

2.6.7 Statistical analyses

Results for the parametric tests should be expressed as mean ± standard error of the mean (S.E.M.). Statistical significance should be determined by one-way analysis of variance (ANOVA), followed by Tukey-Kramer or Dunnett's post-test. For frequency comparisons, Fisher's exact test should be used, and p values <0.05 should be considered significant.

3. Some considerations

Gastrointestinal diseases are one of the most important causes of the previously high morbidity rates in non-industrialized countries, which have been lowered, in part, by the many drugs employed for treatment of peptic ulcers. However, such drugs may have several side effects, on top of their high financial cost for underprivileged populations (Borrelli and Izzo, 2000). Therefore, the use of medicinal plants and the development of phytotherapies, at a low cost, would represent an alternative for treatment of gastrointestinal problems for a large population segment that does not have access to medication (Sartori 1997). In Brazil, numerous plant extracts are used in conventional medicine to treat many digestive disorders (Falcão et al., 2008).

With respect to the *in vitro* assays, it is important to emphasize that the disk diffusion method is recommended for studying polar substances, given that it allows the evaluation of different compounds against a microorganism and, therefore, establishes its antibacterial spectrum. For non-polar extracts the employment of diffusion techniques seems inadequate, since they do not readily diffuse in agar. In the broth microdilution method, the compound to be tested is mixed into the proper liquid medium that had been previously inoculated with the microorganism, allowing determination as to whether the compound is bacteriostatic (minimum bacteriostatic concentration) or bactericidal (minimum bactericidal concentration – MBC). It presents a higher sensitivity to drugs than the disk diffusion method because it permits direct contact between the drug and the microorganism and is, therefore, appropriate for assays assessing either polar or nonpolar substances (Rios and Recio, 2005).

With respect to the *in vivo* assays, the establishment of a persistent infection by *H. pylori* in laboratory animals that completely reproduces the basic characteristics of human infections (an intense active chronic gastritis, either antral or diffuse), their complications (mucosa atrophy and intestinal metaplasia) and their associated pathologies (peptic ulcer, gastric adenocarcinoma and lymphoma) is not easy to accomplish. Chronic ulcer by acetic acid injection into the gastric subserosa area, differently from acute ulcers, penetrates the muscle layer of the glandular area and, occasionally, relapses after wound healing, and is highly similar to the human gastric ulcer in light of its pathological characteristics and healing process (Okabe and Pfeiffer, 1972).

Previous results indicate that Wistar rats with pre-existing gastric ulcers, experimentally produced by acetic acid injection, developed active ulcers when exposed to *H. pylori*, similar to the results of Konturek et al. (1999), which were obtained with a different species (Souza *et al*, 2009).

In order to evaluate the *in vivo* anti-*H. pylori* activity and to verify the presence of this bacteria in the gastric mucosa of ulcerated infected rats, the urease test and histopathological

analysis should be carried out and monitored by the degree of cicatrisation of the gastric lesion.

The histopathological exam is considered one of the most specific tests for diagnosing *H. pylori* infections, presenting 98% sensitivity and 97% specificity (Lin et al., 1996; Ogata et al., 2002). The histopathological findings confirm the results found in the urease test, in which treatment of animals, ulcerated and inoculated with *H. pylori*. Moreover, parameters such as inflammation, ulcer persistency and neutrophilic activity, which are characteristics of *H. pylori* infection.

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24

Prevention of Gastric Ulcers

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1. Introduction

Upper gastrointestinal tract integrity is dependent upon the delicate balance between naturally occurring protective factors as mucus or prostaglandins and damaging factors as hydrochloric acid present normally in the digestive juices. An imbalance causes peptic ulcer formation and destruction of gastrointestinal tract mucosal lining. Ulcer may develop in the esophagus, stomach, duodenum or other areas of elementary canal. In women, gastric ulcers are more common than duodenal ulcers, while in men the opposite is true.

The ulcer irritates surrounding nerves and causes a considerable amount of pain. Obstruction of the gastrointestinal tract may occur as a result of spasm or edema in the affected area. The ulcer may also cause the erosion of major blood vessels leading to hemorrhage, hematemesis and/or melena. Deep erosion of the wall of the stomach or the intestine may cause perforation and peritonitis, which is a life-threatening condition needing emergency intervention. Duodenal ulcers are almost always benign but stomach ulcers may turn malignant. Although mortality rates of peptic ulcer are low, the high prevalence of the disease, the accompanying pain and its complications are very costly.

The ongoing rapidly expanding research in this field provides evidence suggesting that, with therapeutic and dietetic advances, gastric ulcer may become preventable within the next decade. This could be achieved by strengthening the defense mechanisms of the gastric mucosa and, in parallel, limiting the aggression of predisposing factors causing gastric ulceration. The defenses of the gastric mucosa are incredibly efficient under normal mechanical, thermal or chemical conditions. These defenses can endure insults from food, gastric enzymes and acid secretion. Even trauma caused by a biopsy wound is dealt with and can heal relatively fast, within hours.

However, under certain condition, some risk factors may contribute to mucosal injury and initiation of gastric ulcer, as psychological stress, increased hydrochloric acid secretion, Zollinger Ellison syndrome and family history of gastric ulcer. Conditions associated with increased risk of gastric ulcer include also chronic disorders as liver cirrhosis, chronic obstructive pulmonary disease, renal failure, organ transplantation and rheumatoid arthritis. In addition, severe physical stress as in case of burns, major surgery or head trauma may also contribute as risk factors.

Avoidable risk factors that may predispose to gastric ulcer include smoking, high consumption of alcohol and intake of some medications as non-steroidal anti-inflammatory drugs. Some factors are thought to aggravate already established gastric ulcer, but are no longer considered risk factors predisposing to it, as ingestion of too hot or cold foods or drinks, eating spicy food and intake of caffeine. The key cause of gastric ulcer is now known

to be the infection by a certain gram negative bacterium called Helicobacter pylori. Although the mechanism by which the infection by this bacterium leads to ulcer formation is not yet fully understood, it is believed that infection decreases the normal immunity of the gastrointestinal tract wall, which in turn weakens the mucosa and makes it vulnerable to ulceration under the acidic effect of gastric secretions.

Avoiding risk factors is the first line in prevention of gastric ulcer. Smoking cessation and alcoholic consumption minimization may help in reducing the risk of ulcer formation. In addition, sanitary food and drinking habits to avoid infection with Helicobacter pylori may help in ameliorating the initiation of gastric ulcer and its recurrence. Therapeutic interventions to eradicate Helicobacter pylori can also prevent ulcer formation and its transformation into gastric cancer, one of the major complications of chronic gastric ulcer. Avoiding unnecessary intake of ulcer-inducing over-the-counter medications may help in reducing the prevalence of gastric ulcers.

Active therapeutic measures can aid in preventing gastric ulcers in predisposed groups and in patients with healed gastric ulcer to avoid its recurrence. Such therapeutic interventions may be of natural herbal sources or medicinal drugs. A number of traditional anti-ulcer drugs may be used in prevention as well as in treatment of gastric ulcer. Proton pump inhibitors, histamine H_2 receptor antagonists and mucosal protective agents can thus all be used as protective drugs against initiation of gastric ulcer in predisposed groups as well as prevention of remittent attacks. Recent investigations showed that a number of drugs, other than traditional anti-ulcer medications, can help in prevention of gastric ulcer formation. Herbal compounds can also protect against gastric ulcer and they have the advantage of being safer, cheaper and usually having limited, if any, side effects.

In this chapter, a collection of updated recent information published about gastric ulcer protection is gathered. Information in this chapter can be considered as guidelines for clinical practice to direct medical personnel perception to preferred approaches to prevent gastric ulcer as established by scientifically valid research. Making such information available may also increase public awareness of preventive means of gastric ulcer, which may aid in decreasing the suffering of a large number of populations exposed to the disease worldwide.

2. Avoidance of gastric ulcer risk factors

The best and cheapest method to prevent gastric ulceration is the avoidance of risk factors resulting in the occurrence of the disease. Avoiding Helicobacter pylori infection, alternation of life style and substitution of ulcer-inducing medications with less harmful drugs can thus contribute largely to prevent gastric ulcer disease (Fig. 1). Unfortunately, some risk factors are unavoidable. One of the strongest risk factors for initiation of a gastric ulcer is the presence of prior ulcer disease with history of ulcer complications as previous perforation or hemorrhage.

Zollinger-Ellison Syndrome is another unavoidable cause of gastric ulceration. In this syndrome, tumors producing gastrin hormone (gastrinomas) in the pancreas and duodenum stimulate gastric acid secretion. The large amounts of excess acid produced cause gastro-intestinal ulceration. Ulcers may form in the stomach, duodenum, jejunum or other atypical sites in the elementary tract. The incidence of this disease is less than 1% and men are more affected than women. The syndrome is suspected in patients with ulcers who are not infected with Helicobacter pylori and who have no history of non-steroidal anti-inflammatory drugs use. Diagnosis is confirmed by measurement of serum gastrin hormone

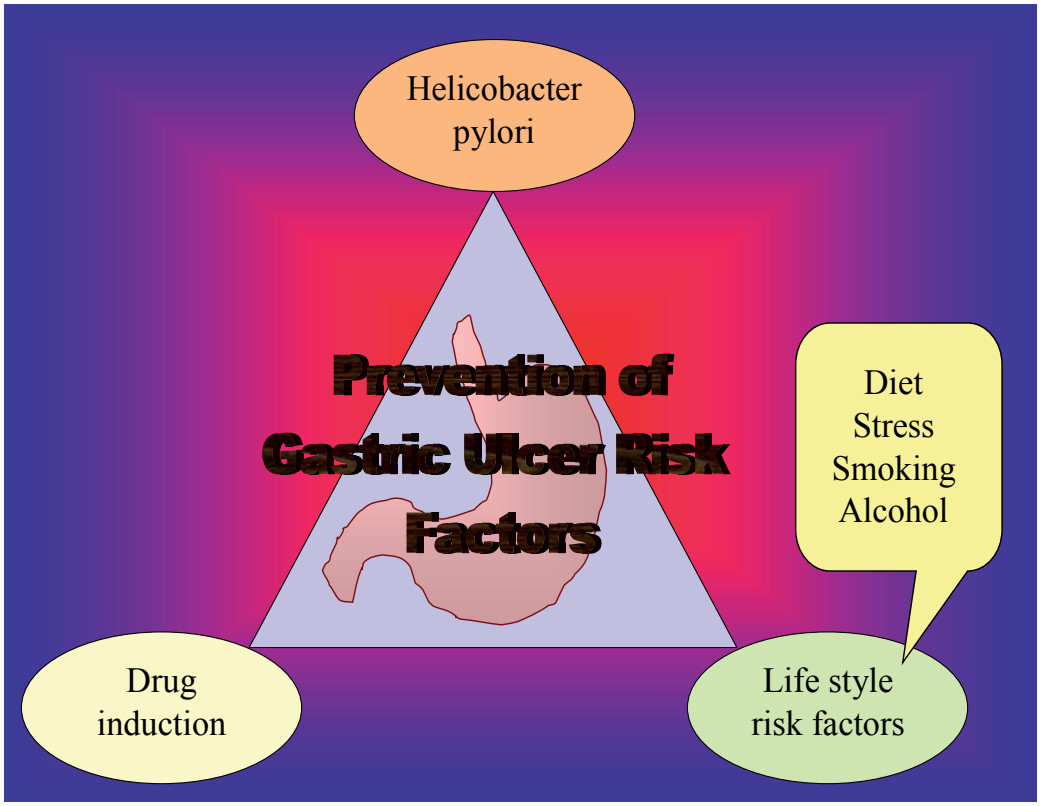

Fig. 1. Methods of prevention of gastric ulcer: Avoiding risk factors as Helicobacter pylori, drug-induced ulcer by medications as non-steroidal anti-inflammatory drugs and performance of life style changes.

levels which is usually very high, reaching above 1000 pg/ml (normal level is < 100 pg/ml). Diarrhea may occur before ulcer symptoms. Gastro-esophageal reflux disease may occur and its complications may include narrowing due to strictures of the esophagus. Ulcers associated with this syndrome are usually persistent and difficult to treat. In the past, removing the stomach was the only option for treatment. Nowadays, treatment includes removing the tumors only and therapeutic suppression of acid secretion.

Other unavoidable factors associated with higher incidence of gastric ulcer include sex, as there is higher prevalence of the disease among women then men. People over age 60 years old are also more prone to gastric ulcer disease. In addition, ethnic backgrounds as African-Americans or Hispanics have 2-fold higher risk in developing gastric ulcer. Furthermore, patients suffering from other diseases as congestive heart failure have higher incidence of having gastric ulcer as well. Type O blood group has also been associated with increased incidence of the disease. Genetics is another unavoidable risk factor of gastric ulcer. Pepsinogen C gene polymorphism, for example, is significantly associated with development of gastric ulcer (Sun et al., 2009). Other relatively rarer predisposing factors to development of gastric ulcer includes Crohn's disease of the stomach, eosinophilic gastritis, systemic mastocytosis, radiation damage and viral infections by cytomegalovirus or herpes simplex (Malfertheiner et al., 2009).

2.1 Helicobacter pylori as a risk factor for gastric ulcer

Infection with Helicobacter pylori is the most well-defined risk factor for the development of peptic ulcers. The two Australian scientists who identified Helicobacter pylori as the main cause of stomach ulcers in 1982 were awarded the Nobel Prize in Medicine in 2005 for this discovery. Helicobacter pylori bacteria are found in about 50% of people with gastric ulcer disease. Inflammation of the stomach and stomach ulcers result from the infection by these bacteria, as their corkscrew shape enables them to penetrate the mucus layer of the stomach so that they can attach themselves to the lining. The surfaces of the cells lining the stomach contain a protein, called decay-accelerating factor, which acts as a receptor for the bacterium.

Helicobacter pylori can survive in the highly acidic medium of the stomach by producing urease, an enzyme that generates ammonia to neutralize the acid. These bacteria then produce a number of toxins causing inflammation and damage to the stomach, leading to ulcers especially in predisposed individuals. The bacteria also alter certain immune factors that allow them to evade detection by the immune system and cause persistent inflammation. Even if ulcers do not develop, the bacterium is considered to be a major cause of active chronic inflammation in the stomach (gastritis). Helicobacter pylori together with unavoidable risk factors as genetics and concomitant diseases may contribute in gastric ulcer formation and the subsequent metaplasia and dysplasia leading to gastric cancer (Fig. 2). Avoidance of risk factors, therapeutic intervention and some protective herbs can be employed to prevent the initiation of this sequence.

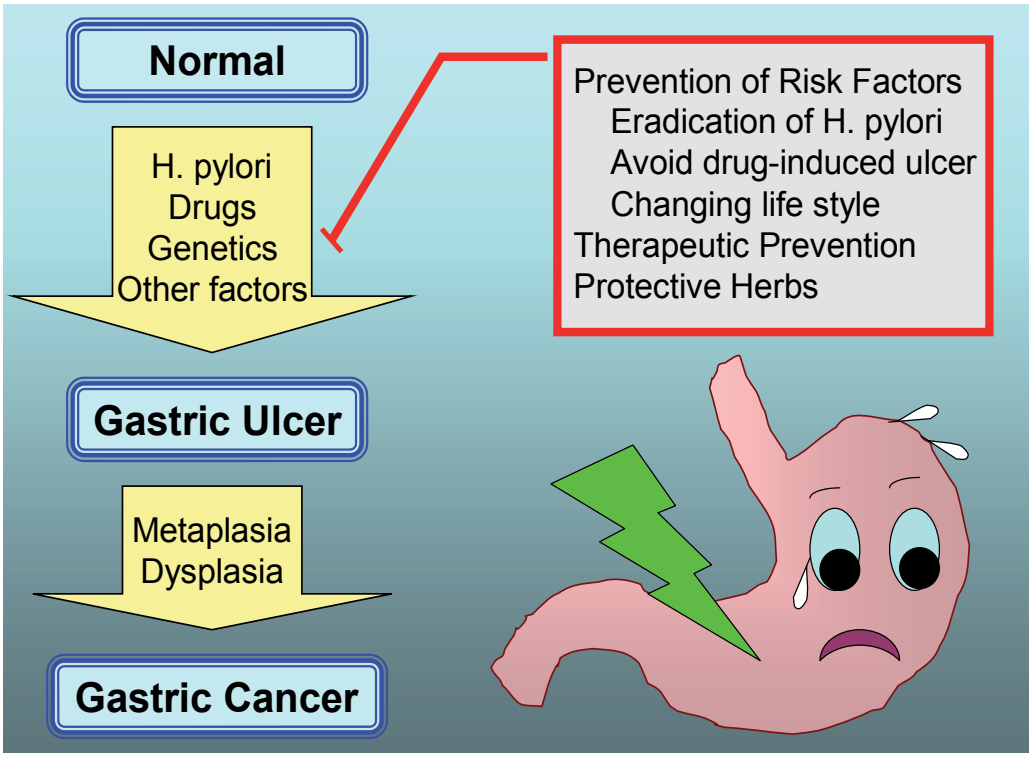

Fig. 2. Prevention of gastric ulcer formation is a step in preventing the development of gastric cancer.

Less than 15% of people infected with Helicobacter pylori develop gastric ulcer. Factors that trigger gastric ulcers in Helicobacter pylori carriers include genetic factors, which explain the higher incidence of development of ulcers in certain ethnicity. Another factor is abnormal immune response, which allows the bacteria to injure the stomach lining. Lifestyle factors as chronic stress, drinking coffee and smoking were long believed to be primary causes of gastric ulcer; it is now thought that they only increase susceptibility to ulcers in some Helicobacter pylori carriers. Interrupted sleep may be another trigger as people who work at night shifts have a significantly higher incidence of ulcers than day workers. Frequent interruption of sleep is thought to weaken the immune system's ability to protect against harmful bacterial substances.

Using certain medications as non-steroidal anti-inflammatory drugs or corticosteroids may contribute to higher infection rates of Helicobacter pylori. Patients with prior gastric ulcer, Zollinger-Ellison syndrome, congenital stomach malformations, malignant diseases such as mastocytosis and basophilic leukemia, head trauma, severe traumatic injuries, burns, radiation, or recently had major surgery are also more prone to Helicobacter pylori infection. Increased risk of Helicobacter pylori infection is seen among people who live in crowded places with unsanitary conditions. Some genetic predispositions for Helicobacter pylori infection cure rate may exist. One example is cytochrome P450-2C19 polymorphism that seems to predict the cure of Helicobacter pylori infection and predisposition to gastric ulcer (Lay and Lin, 2010). Another example is cytokine genes polymorphism that was significantly associated with persistent infection (Abdiev et al., 2010). Polymorphism of multidrug resistance protein 1 also was reported to influence Helicobacter pylori-induced gastric inflammation (Tahara et al., 2011). Such genetic predisposition gives us hope that the infection predisposing to peptic ulcer and gastric cancer may some day be a target for preventive gene therapy in the near future.

Therapeutic interventions to eradicate Helicobacter pylori are needed to prevent ulcer formation and its transformation to gastric cancer, one of the major complications of chronic gastric ulcer. Helicobacter pylori eradication therapy comprises a combination of two or more drugs including antimicrobials, proton pump inhibitors and gastro-protective agents. Several eradication methods were suggested. Dual eradication therapy using proton pump inhibitor with amoxicillin was tried (Graham et al., 2010). Triple eradication therapy employing 2 antimicrobials together with proton pump inhibitor also showed some success, but not enough to be considered first-line treatment. Quadruple Helicobacter pylori eradication was also successfully tried and consisted of 2 antimicrobials, proton pump inhibitor and the gastro-protective agent colloidal bismuth subcitrate (Zheng et al., 2010).

Nowadays, the first line of Helicobacter pylori eradication therapy is a regimen of 7 or 14 days consisting of a proton pump inhibitor as omeprazole (20 mg 12 hourly), in combination with clarithromycin (500 mg 12 hourly) and metronidazole (400 mg 12 hourly). A second regimen that is equally effective is by using omeprazole as previously mentioned, together with less dose of clarithromycin (250 mg 12 hourly) and substituting metronidazole with amoxicillin (1 g 12 hourly). Omeprazole can be replaced with other proton pump inhibitors. Despite that the prevalence of Helicobacter pylori is decreasing in developed countries, as a result of improvements in living standards and hygiene, Helicobacter pylori is still a common cause of gastric ulcer in developing countries. Attempts to develop effective vaccination against this bacterium reached phase I and II clinical trials, and may present effective preventive strategy in preventing gastric ulcer formation and, more importantly, preventing gastric cancer in the future (Majumdar et al., 2011).

2.2 Avoidance of drug-induced gastric ulcers

Patients receiving medications as non-steroidal anti-inflammatory drugs, the anticoagulant drug warfarin, corticosteroids or the anti-osteoporotic drug alendronate may be more prone to gastric ulcer. Non-steroidal anti-inflammatory drugs are valuable therapeutics that act not only as anti-inflammatory, but also as analgesics and antipyretics. They are used in a wide variety of clinical scenarios, including arthritis and other musculoskeletal disorders. Unfortunately, their use has been limited by their gastric ulcer-inducing effects. Nearly 25 % of chronic users of these drugs develop gastric ulcer disease (Lanza et al., 2009).

The rate of non-steroidal anti-inflammatory drugs-induced gastric ulcers is increasing, as more people are taking these drugs regularly as over-the-counter self-therapy. In general, the possibility of gastric ulcer initiation of a non-steroidal anti-inflammatory drug with non-selective cyclooxygenase inhibition actions correlates with its anti-inflammatory activity. Non-steroidal anti-inflammatory drugs with a high analgesic effect at doses with low anti-inflammatory activity, such as ibuprofen, are less ulcerogenic than those that have adequate analgesic effects only at doses with high anti-inflammatory activity, as in case of piroxicam. Ibuprofen appears safer compared to other members of this drug group in part because it is frequently prescribed for short durations in a low dose to control temporary mild painful conditions. However, when full anti-inflammatory doses of ibuprofen are given, the risk of gastric ulceration with ibuprofen is comparable with other non-steroidal anti-inflammatory drugs.

One member of this group is indomethacin, which is a frequently clinically used and is also applied to induce experimental animal model of acute gastric ulcer. Indomethacin induces gastric injury by suppressing the formation of prostaglandins, which control many of the components of mucosal defense system, as they stimulate mucus and bicarbonate secretion, elevate mucosal blood flow, increase the resistance of epithelial cells to injury induced by cytotoxins and suppress the recruitment of leucocytes into the mucosa. Prostaglandins can also inhibit the release of a number of inflammatory mediators, such as tumor necrosis factor-α from macrophages and interleukin-8 from neutrophils. Tumor necrosis factor-α promotes gastric epithelial cell apoptosis and triggers activation of adhesion molecules and leucocyte recruitment, leading to microvascular perturbations. Other mechanisms by which indomethacin induce gastric injury involves gastric hypermotility and the increased production of reactive oxygen species, as well as lipid peroxidation (Morsy et al., 2010).

Physicians prescribing these drugs face two problems; one problem is identification of high-risk patients and the second is selection of appropriate strategies to prevent gastric ulcer. Risk factors of these drugs-induced gastric ulcers include older age, concomitant use of anticoagulants, corticosteroids, other non-steroidal anti-inflammatory drugs including low-dose aspirin, and chronic debilitating disorders, especially cardiovascular diseases. Helicobacter pylori infection increases the risk of this drugs-induced gastric ulcer. Eradication of Helicobacter pylori infection, if present, in patients requiring long-term therapy by these drugs is recommended.

Patients who require long-term non-steroidal anti-inflammatory drug therapy can reduce their risk of inducing ulcers by concomitantly taking conventional anti-ulcer therapy. Proton pump inhibitors and/or histamine H_2 receptor antagonists can significantly reduce these drug-induced gastric ulcers. The synthetic prostaglandin E_1 analog, misoprostol, is also very effective in preventing the development of gastric ulcers in patients taking these medications. Unfortunately, its use is limited by its gastrointestinal adverse effects.

Avoiding unnecessary intake of ulcer-inducing over-the-counter medications may help in reducing the prevalence of gastric ulcers. When it is mandatory to use such therapeutics, their replacement with less irritating drugs may reduce ulcer formation. Non-steroidal anti-inflammatory drugs which are selective cyclooxygenase-2 inhibitors show similar anti-inflammatory, analgesic and antipyretic efficacy compared to non-selective inhibitors. However, these selective drugs are associated with lower incidence of gastric ulcers. Unfortunately, there use is limited due to their association with myocardial infarction and thrombosis. Unexpectedly, experiments using cyclooxygenase-1 knockout mice showed that these animals do not develop gastric ulceration at higher rate and have some reduced inflammatory response.

Some studies tried to find a safer replacement for non-steroidal anti-inflammatory drugs as regards their gastric ulcerogenic effect. In one study, a safer anti-inflammatory drug as regards its gastric toxicity was developed (Shoman et al., 2009). A number of nitric oxide donating pyrazoline derivatives were synthesized and they showed equivalent anti-inflammatory effect to the anti-inflammatory drug indomethacin, with significantly less development of gastric ulceration. Other similar trials have been made by other investigators, for example testing the effect of cyclodextrin combination with non-steroidal anti-inflammatory drugs on gastric ulcer formation which resulted in gastro-protective effect (Alsarra et al., 2010).

2.3 Life style risk factors

Several studies implied that modulating life style factors as dietary factors, controlling stress, reducing smoking and alcohol intake may directly prevent the initiation of gastric ulcers, especially in predisposed people. Some even suggested certain physical exercises to reduce the risk of ulcer formation or recurrence. Such exercises were seen to directly improve psychological and cardiovascular conditions and thus may be indirectly related to decreasing gastric ulcer development.

2.3.1 Diet

Diet rich in fibers may decrease the risk of developing gastric ulcers by about 50%. Fiber found in fruits and vegetables is particularly protective, as vitamin A contained in many of these foods may increase the benefit. Milk, previously thought to aid in decreasing ulcer symptoms, actually encourages the production of acid in the stomach, although moderate amounts (2-3 cups/day) appear to do no harm. However, yogurt may protect against gastric ulcer, as it contains probiotics. Coffee (caffeinated and decaffeinated), soft drinks and fruit juices with citric acid increase stomach acid production. Although no studies have proven that any of these drinks contribute to ulcers, consuming more than 3 cups of coffee per day may increase susceptibility to Helicobacter pylori infection (University of Maryland Medical Center website).

Studies conducted on spices and peppers have yielded conflicting results. In general, these substances should be used moderately, and should be avoided if they irritate the stomach. Some studies suggest that high amounts of garlic may have some protective properties against stomach cancer, although a recent study concluded that garlic offered no benefits against Helicobacter pylori and, in large amounts, can cause considerable gastrointestinal distress. Studies have shown that phenolic compounds in virgin olive oil may be effective against Helicobacter pylori infection. Although no vitamins have been shown to protect

against Helicobacter pylori-induced ulcers, Helicobacter pylori appears to impair the absorption of vitamin C, which may play a role in the higher risk of stomach cancer.

2.3.2 Psychological factors: stress

As a body response to stress, many diseases may develop. There is debate as to whether psychological stress can influence the development of gastric ulcers. Some studies still suggest that stress may predispose a person to ulcers or prevent existing ulcers from healing. Some even believe that the relationship between stress and ulcers is so strong that people with ulcers should be treated for psychological conditions. Stress causes the digestive tract to slow down and more gastric acid is allowed to accumulate in the stomach. Increased stomach acidity may predispose to or aggravate an already present ulcer. Stress can also cause change in appetite, leading to over-eating or lack of appetite. Overeating causes the stomach to produce more acid while lack of appetite will subject the stomach mucosa to the acid produced in an empty stomach. Although psychological stress is no longer considered a direct cause of ulcers, it surely can delay the healing and aggravate already existing gastric ulcers. Physical stress, however, is definitely a risk factor for developing gastric ulcers, as in patients with injuries such as severe burns or patients undergoing major surgeries.

2.3.3 Smoking

Cigarette smoking appears to be a risk factor for the development and recurrence of gastric ulcer. The incidence of gastric ulcer is higher among smokers than non-smokers. Compared with non-smokers, people who smoke cigarettes are twice as likely to develop gastric ulcer. Smoking may lead to initiation of ulceration, slow ulcer healing and an increased risk of gastric ulcer recurrence. Smoking may have an inconsistent effect on gastric acid secretion; however it reduces prostaglandin and bicarbonate production, reduces mucosal blood flow, interferes with the action of histamine H_2 receptor antagonists and accelerates gastric emptying of liquids. Cessation of smoking or reducing it is usually associated with the prompt relief of already existing gastric ulcer symptoms.

2.3.4 Excess alcohol intake

Alcohol increases the production of acid in the stomach, which may irritate an existing ulcer. Alcohol also relaxes the lower esophageal sphincter, allowing stomach contents to reflux back up into the esophagus, increasing the discomfort associated with gastric ulcer. Patients suffering of gastric ulcer should, thus, avoid taking alcohol. People predisposed to gastric ulcer may dilute alcoholic beverages to reduce their concentration, restrict the number of drinks to one or two a day, replace red wine with white wine of less toxic content, or better, have drinks which are non-alcoholic.

3. Endogenous protection against gastric ulcer

Astonishingly, despite of the presence of one or more risk factors as smoking, alcohol intake, non-steroidal anti-inflammatory drugs consumption and/or Helicobacter pylori infection, some people still do not develop gastric ulcer. For example, non-steroidal anti-inflammatory drugs induce clinically significant gastric ulceration in 17% of patients receiving these drugs. This is due to the strong natural endogenous gastric cyto-protection that spares the vast

majority of patients at risk. Gastric mucosal barrier together with endogenous mediators comprise a strong defense mechanism against gastric ulceration. Understanding these naturally occurring defense mechanisms is crucial to try to enhance them to prevent gastric damage and ulceration in more vulnerable patients.

3.1 Physiological gastric mucosal barrier

The gastric mucosal barrier is considered the main defense system against gastric ulcer formation. Several luminal factors contribute to this barrier (Fig. 3). These factors include secretion of bicarbonates, mucus, phospholipids and immunoglobulins. The gastric epithelial barrier also represents part of the defense system that is remarkably resistant to acids or irritants and has the capability of rapid repair. The mucosal microcirculation, together with sensory innervations, harmonically defends the mucosal barrier. Sensing acidic diffusion into the gastric mucosa results in neural system-mediated induced endogenous mediator release and hormonal responses leading to increase in mucosal blood flow, which is a critical step in preventing damage and facilitating repair of gastric mucosa. The mucosal immune system represents another gastric mucosal protective method. Mast cells and macrophage generate immune signals of inflammatory response that contributes to prevention of gastric damage.

3.1.1 Luminal gastric protection

The mucus-bicarbonate-phospholipid barrier comprises the first line of mucosal defense mechanism. This barrier is formed of mucus, bicarbonate and phospholipids. Mucus presents a layer that contains secreted bicarbonate and surfactant phospholipids. Mucus that acts as a physical barrier against luminal digestive enzymes, bicarbonate that maintains an almost neutral pH at the epithelial surface, together with phospholipids of high hydrophobic properties can naturally protect against mucosal damage. Disruption of this mucus-bicarbonate-phospholipid barrier by ulcerogenic substances, as bile salts or non-steroidal anti-inflammatory drugs causes elevated diffusion of acid into the mucosa and mucosal damage (Allen and Flemstrom, 2005). Helicobacter pylori release phospholipase enzymes and ammonium ions that can reduce the strength of this single and only barrier existing between the epithelium and the lumen. Other protective mechanisms may then interfere to protect against this bacterial induced injury.

3.1.2 Gastric epithelial barrier

Mucosal surface is formed of a continuous layer of surface epithelial cells that secrete components of the mucus barrier as well as endogenous protective mediators as prostaglandins, heat shock proteins and cathelicidins (see below; section 3.2). These surface epithelial cells form a physical barrier preventing back-diffusion of gastric acid and digestive enzymes. Basolateral membrane of epithelial parietal cells, that secrete hydrochloric acid in high concentrations into the lumen of the stomach, contains transporters responsible for maintaining intracellular homeostasis. These transporters efflux large amounts of bicarbonate to prevent cell alkalinization. The effluxed bicarbonate, known as alkaline tide, is an integral constituent of mucus-bicarbonate barrier (Tulassay and Herszenyi, 2010). Continuous and rapid cell renewal enhances the resistance of epithelial barrier to damage. Mucosal progenitor cells in gastric epithelium promote cell renewal by continuously replacing surface cells that undergo apoptosis. Proliferation of progenitor cells is controlled by endogenous growth factors' mediators.

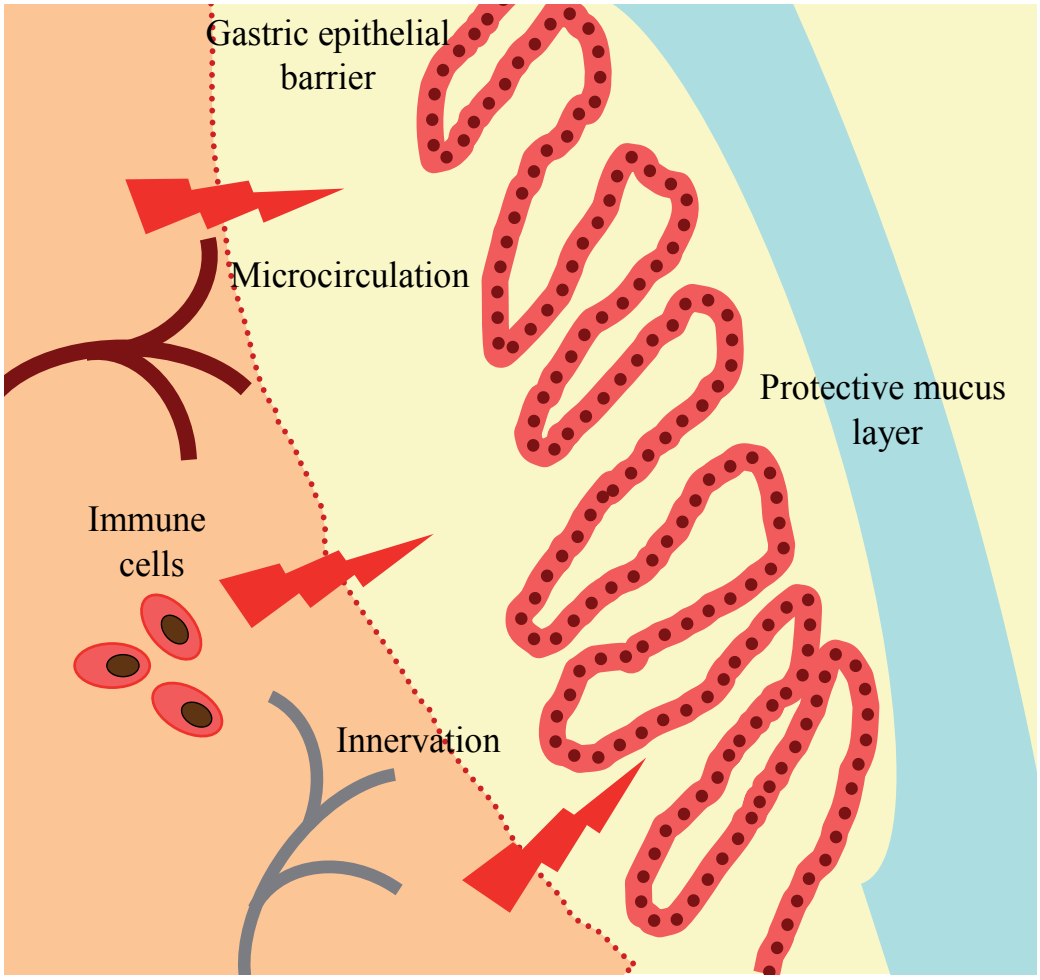

Fig. 3. Physiological gastric mucosal barrier. It is composed mainly of protective luminal mucus layer, gastric epithelial barrier, immune cells, gastric microcirculation and sensory gastric innervation.

3.1.3 Mucosal microcirculation

Mucosal ischemia triggers gastric ulcer by inducing tissue necrosis, free radical formation and cessation of nutrient transport, all resulting from vascular and microvascular injury such as thrombi, constriction or other occlusions. Mucosal blood flow thus provides gastric lining with adequate vascular perfusion that prevents epithelial damage from progressing to necrosis of deeper layers of the mucosa. Increase in mucosal blood flow occurs as a response to gastric mucosal exposure to an irritant or when acid back-diffusion occurs. Potent vasodilators such as nitric oxide and prostaglandin I_2 generated by endothelial cells protect the gastric mucosa against injury and damaging action of vasoconstrictors such as leukotriene C_4, thromboxane A_2 and endothelin. These potent vasodilators prevent platelet and leucocyte adherence to endothelial cells, maintain the integrity of the gastric epithelium and the mucus barrier and protect the gastrointestinal tract by inhibiting gastric acid

secretion from parietal cells. Endogenous mediators that affect mucosal microcirculation as nitric oxide and hydrogen sulfide are further discussed below (section 3.2).

3.1.4 Gastric sensory innervation

Gastric mucosal defense is also regulated by the central nervous system innervation. Gastric mucosa and submucosal vessels are innervated by primary afferent sensory neurons. When gastric mucosa gets exposed to damage by gastric acid or other irritating chemicals, afferent neurons are activated and directly start controlling the tone of the submucosal arterioles, which regulate mucosal blood flow. When sensory afferent nerves of the superficial mucosa detect gastric acid, they respond by releasing neurotransmitters as substance P and calcitonin gene-related peptide. These mediators cause relaxation of smooth muscle surrounding gastric mucosal arterioles, resulting in an elevation of mucosal blood flow. In addition, vagal activation increase mucus secretion, while nervous response to stress control central corticotropin-releasing factor signaling pathways. Furthermore, the transient receptor potential vanilloid 1 agonists are effective in protecting gastric mucosa against various experimentally induced ulcer models (Morsy and Fouad, 2008).

3.1.5 Mucosal immune system

The mucosal immune system is a key factor of mucosal defense against exogenous and endogenous irritants. Impairment of this immune system can lead to mucosal injury and to impairment of endogenous cyto-protective repair mechanisms. The mucosal immune system is coordinated by innate and adaptive immune response regulated by several mediators released from immuno-regulating cells. Neutrophils and macrophages infiltrate into the gastric mucosa as a response to Helicobacter pylori infection. These cells release lysosomal enzymes, leukotrienes and reactive oxygen species which impairs mucosal defense and drives the immunopathogenetic process of ulcerogenesis. T and B lymphocytes activated by bacterial antigens and pro-inflammatory cytokines regulate the local and systemic immune response with release of further cytokines and antibodies. The type of T-cell response can change the outcome of this infection, as more mucosal damage results from T-helper predominant response, whereas a high regulatory T-cell response with interleukin-10 release confers gastric ulcer protection (Malfertheiner et al., 2009).

3.2 Endogenous gastro-protective mediators

Some endogenous mediators can work through cyto-protective mechanisms reducing gastrointestinal injury induced by topical irritants, thus preventing the initial steps of gastric inflammation. These endogenous mediators may be inhibited by causative risk factors, leading to gastric ulceration and thus provide a mechanism through which these risk factor contribute in gastric damage. On the other hand, therapeutic modulation of endogenous gastric mediators can provide a target to improve gastric protection against ulceration.

3.2.1 Mediators of cyclooxygenase pathway: prostaglandins and lipoxins

Prostaglandins are fatty acids produced from arachidonic acid via cyclooxygenase enzyme. It is known that suppression of prostaglandin synthesis is a major mechanism of action of aspirin and other non-steroidal anti-inflammatory drugs, which is probably one of the mechanisms by which these drugs cause gastric ulcers. Prostaglandins modulate a number of components of mucosal defense as they stimulate mucus and bicarbonate secretion,

promote mucosal blood flow, increase the resistance of epithelial cells to cytotoxins-induced injury and suppress the recruitment of leukocytes into gastric mucosa. Prostaglandins can also down regulate the release of a number of other inflammatory mediators that may contribute to the generation of gastric ulcer (Martin and Wallace, 2006). Prostaglandin E receptors have a prominent role in mucosal protection and gastric ulcer healing (Takeuchi, 2010). Prostaglandin E_2 has been shown to be a potent inhibitor of tumor necrosis factor-α and interleukin-1 release from macrophages and of leukotriene B4 and interleukin-8 release from neutrophils.

Lipoxins are the resultant of consequent conversion of arachidonic acid by cyclooxygenase-2 and 5-lipoxygenase enzymes. Lipoxin-A_4 is an endogenous mediator contributing to resolution of the inflammatory state and, thus, has an important role in mucosal defense. Lipoxin A_4 protects the stomach from aspirin-induced damage via suppressing leukocyte adherence within gastric micro-circulation. In addition, Lipoxin A_4 can inhibit inflammatory pain processing and regulate trans-epithelial electrical resistance. Antagonism of Lipoxin A_4 receptor can significantly exacerbate gastric ulcer (Lim et al., 2009).

3.2.2 Nitric oxide

Oxidization of arginine by nitric oxide synthase yields the volatile gas nitric oxide, which has numerous physiologic properties including regulation of inflammation. Nitric oxide is an important factor in modulating gastrointestinal mucosal defense mechanisms. Some of nitric oxide actions overlaps with that of prostaglandins, as it modulate the activity of mucosal immunocytes and reduce leukocytic endothelial adhesion. In addition, it modulates mucosal blood flow and reduces epithelial permeability, resulting in enhanced mucosal resistance to ulceration. Nitric oxide also prevents adherence of leukocytes to the vascular endothelium. This gaseous mediator has a role also in modulating gastric mucus and bicarbonate secretion. Suppression of nitric oxide synthesis renders the gastric mucosa more susceptible to injury, while administration of nitric oxide donors can protect the stomach from injury. Agents that release nitric oxide in small amounts over a prolonged period have been shown to greatly reduce inflammation and to accelerate ulcerative healing (Martin and Wallace, 2006).

Some studies showed that dietary nitrate and pretreatment with nitric oxide donor protected against drug-induced gastric ulcer. Furthermore, the use of nitric oxide-donating agents concomitantly with non-steroidal anti-inflammatory drugs as aspirin also resulted in reduced risk for gastric ulceration and bleeding. This lead to the development of cyclo-oxygenase inhibiting/nitric oxide donating drugs, in which nitric oxide is chemically linked to a non-steroidal anti-inflammatory drug, which showed effective anti-inflammatory capabilities together with less gastric injury. Examples of such drugs include nitric oxide-flurbiprofen, nitric oxide-ketoprofen, nitric oxide-diclofenac and nitric oxide-naproxen. These drugs are suitable therapeutic options for patients with diseases requiring long-term non-steroidal anti-inflammatory drugs therapy (Lanas, 2008).

Despite all evidence that nitric oxide contribute in mediating mucosal defense under normal conditions, under different circumstances, as in case of already inflamed mucosa, it is suggested that nitric oxide may contribute to tissue injury. In this case, nitric oxide reacts with superoxide anion, produced by activated neutrophils, to form peroxynitrite, which is another potent oxidant. Peroxynitrite is known to produce widespread gastrointestinal injury and inflammation. Although the role of nitric oxide is still controversial, most studies suggest a net protective effect of this molecule in the gastrointestinal tract.

3.2.3 Hydrogen sulfide

Hydrogen sulfide is another gaseous mediator generated endogenously that causes vasodilatation, decreases adhesion of leukocyte to vascular endothelium, inhibits non-steroidal anti-inflammatory drugs-induced gastric mucosal injury and inhibit tumor necrosis factor-α expression (Tulassay and Herszenyi, 2010). The enzymes responsible for hydrogen sulfide generation in the gastric mucosa are cystathionine β-synthase and cystathionine γ-lyase.

Despite the protective role of this gas against mucosal injury, it suspected that hydrogen sulphide may contribute to the pro-inflammatory actions in Helicobacter pylori infection. Nevertheless, with non-steroidal anti-inflammatory drugs, hydrogen sulfide provide gastric protection by inducing up-regulation of anti-inflammatory and cyto-protective genes, including hemeoxygenase-1, vascular endothelial growth factor, insulin-like growth factor receptor and several genes associated with the transforming growth factor-β receptor signaling pathway (Lim et al., 2009).

A number of therapeutic possibilities combining hydrogen sulfide with non-steroidal anti-inflammatory drugs are considered in early stages of development. This new class of combination is based on that non-steroidal anti-inflammatory drugs reduce hydrogen sulphide production in gastric mucosa, which may contribute to these drugs' inducing mechanisms of gastric ulcer. In return, sodium hydrogen sulfide prevents the reduction of mucosal blood flow induced by non-steroidal anti-inflammatory drugs. Furthermore, this gaseous mediator reduces non-steroidal anti-inflammatory drugs-induced leukocyte adhesion to vascular endothelial cell. The combination causes reversal of the increased expression of tumor necrosis factor-α and improvement prostaglandin E_2 synthesis impaired by non-steroidal anti-inflammatory drugs (Lim et al., 2009).

3.2.4 Cytokines

Cytokines are important in mucosal defense and play a pivotal role in the regulation of the mucosal immune system. Interleukin-1β and tumor necrosis factor-α release comprise the early inflammatory systemic response to inflammation or infection. Various types of cells produce interleukin-1β, including monocytes, macrophages, neutrophils, endothelial cells and fibroblasts. Interleukin-1β increases the resistance of gastric mucosa to injury and reduces the severity of ulcerative damage. This is through its action as a potent inhibitor of gastric acid secretion, stimulator of prostaglandins and nitric oxide release and inhibitor of ulcer-promoting mediators as platelet-activating factor from mast cells (Tulassay and Herszenyi, 2010). Tumor necrosis factor-α is another key cytokine that contribute in producing gastric mucosal injury. Still, by stimulating cell proliferation, tumor necrosis factor-α may also promote mucosal repair after damage associated with Helicobacter pylori infection and the use of non-steroidal anti-inflammatory drugs. Tumor necrosis factor-α reverses gastric mucosal injury via stimulation of epithelial cell proliferation.

3.2.5 Proteinase-activated receptors

Proteinase-activated-2 receptors are expressed throughout the gastrointestinal tract, especially in the epithelial cells and sensory afferent neurons. In the stomach, the activation of these receptors triggers mucus secretion and reduces the extent of stomach endothelial damage induced by non-steroidal anti-inflammatory drugs. This may be through modulating sensory afferent nerves and regulating the release of vascular endothelial

growth factor from platelets, which affect new blood vessel angiogenesis that promote ulcer healing (Yoshida and Yoshikawa, 2008).

3.2.6 Proteolytic enzymes

Proteolytic enzymes have important functions in gastric ulcer prevention and healing. It has been shown that impaired fibrinolysis occurs due to alteration of the proteolytic enzymes formed through tissue-type plasminogen activator-inhibitor system. Intramucosal proteases; as cathepsins, are also involved in protection against gastric ulcer initiation and promotion of healing. Cathepsins act as antimicrobial peptides expressed by the gastric epithelium preventing bacterial colonization and accelerate ulcer healing. The proteolytic enzymes urokinase-type plasminogen activator and plasminogen activator-inhibitor type-1 are involved in angiogenesis process, and thus has a direct role in cell proliferation, inflammation and ulcer healing. Matrix metalloproteases are involved in extracellular matrix reconstitution and tissue remodeling and thus may have an impact in gastric ulcer healing (Tomita et al., 2009). Secretory leucocyte protease inhibitor exerts antimicrobial and anti-inflammatory effects. Its expression is induced during inflammation. However, the expression is significantly decreased during Helicobacter pylori-mediated gastritis. This is due to local down-regulation of this proteolytic enzyme in gastric mucosa in response to Helicobacter pylori infection (Tulassay and Herszenyi, 2010).

3.2.7 Heat shock proteins

Heat shock proteins are important mediators of cellular homeostasis during normal cell growth. They also promote cell survival during various cellular stresses, as they are generated by gastric epithelial cells in response to oxidative stress, cytotoxicity and high temperature. Heat shock proteins generated play an important role in cellular recovery. This is done through acting on enzymes related to cyto-protection, gastric inflammation and gastric ulcer healing. Heat shock proteins act by refolding these partially damaged functional enzymes or increasing delivery of their precursor proteins to important organelles such as mitochondria and endoplasmic reticulum. This results in improvement of mucosal defense, protection against gastric ulcer and promotion of healing of existing damage (Choi et al., 2009).

3.2.8 Growth factors

Growth factors are considered a pivotal stimulus for cell proliferation, division, migration and re-epithelization. Cell proliferation and repair of injured gastric mucosal epithelium are controlled by a number of these growth factors activated as a response to tissue injury. Growth factors such as epidermal growth factor, hepatocyte growth factor, platelet derived growth factor and basic fibroblast growth factor activate epithelial cell migration and proliferation and accelerate ulcer healing by binding to their specific receptors on the cell surface, triggering a number of intracellular signaling events that result in cell migration and proliferation.

In the stomach, epidermal growth factor triggers mitogenic response and is important for epithelial cell proliferation, migration, re-epithelization and reconstruction of gastric glands. Vascular endothelial growth factor is important for angiogenesis, vascular remodeling and mucosal regeneration. Transforming growth factor-α protects against gastric mucosal injury and promotes wound healing. Receptors for epidermal and transforming growth factors are

expressed in gastric progenitor cells and are trans-activated by gastrin and prostaglandin E_2 that trigger cell proliferation and repair of gastric mucosa (Tulassay and Herszenyi, 2010). These growth factors are mainly derived from platelets, macrophages and injured tissue. Ulceration also triggers induction of genes encoding these growth factors in cells lining mucosa of the ulcer margin. These locally produced growth factors activate epithelial cell migration and proliferation via actions on autocrine and/or paracrine systems.

3.2.9 Peroxisome proliferation-activated receptor

Peroxisome proliferation-activated receptors (α, β and γ) are members of the nuclear response family of transcription factors. These receptors are expressed in the gastrointestinal tract, liver, skeletal muscle, heart, adipose tissue, breast and skin. Stimulation of peroxisome proliferation-activated receptors plays an important role in the mechanism of non-steroidal anti-inflammatory drugs action. Peroxisome proliferation-activated receptors cause subsequent inhibition of nuclear factor-κB and other transcription factors. These receptors regulate transcription of target genes involved in lipid and lipoprotein metabolism, glucose homeostasis and cell differentiation. In addition, peroxisome proliferation-activated receptors inhibit the activation of certain inflammatory response genes. Thus, activation of peroxisome proliferation-activated receptors during non-steroidal anti-inflammatory drugs administration blocks the production of inflammatory response markers, such endothelin-1, vascular cell adhesion molecule-1 in endothelial cells and tissue factors as matrix metalloproteinase-3 and tumor necrosis factor-α in macrophages. These anti-inflammatory actions are mediated by inhibition of pro-inflammatory transcription pathways as nuclear factor-κB, activator protein-1 and nuclear factor of activated T cells (Lim et al., 2009).

3.2.10 Neuropeptides

Several neuropeptides as cholecystokinin, gastrin 17, bombesin, corticotrophin-releasing factor, peptide YY and intragastric peptone are involved in gastro-protection. Ghrelin is also a neuropeptide associated with gastro-protective effects with important effects on energy homeostasis and gastrointestinal motility. Ghrelin is effective against ethanol-induced gastric ulcers. This protective effect is dependent on cyclooxygenase-1-derived prostaglandin E_2. Ghrelin mediates its gastro-protective effects also via stimulation of nitric oxide production and calcitonin gene related peptide release from sensory afferent nerves, enhancing gastric mucosal blood flow. Orexins are another family of neuropeptides having gastro-protective role, especially orexin-A. Orexin-A prevents mucosal injury and gastric ulceration through several mechanism including increasing gastric blood flow, elevating luminal nitric oxide, reducing lipid peroxidation, generating prostaglandin E_2 and enhancing vagal and sensory nerve activity (Nayeb-Hashemi and Kaunitz, 2009).

3.2.11 Hemeoxygenase-1 enzyme

Hemeoxygenase-1 is the rate-limiting enzyme of heme catabolism that catalyzes the breakdown of heme into carbon monoxide, iron and biliverdin. Hemeoxygenase isoform 1 is a phase II drug detoxifying enzyme. It is highly inducible as a response to stress, as oxidative stressors, ultraviolet irradiation, inflammatory cytokines, heavy metals and non-steroidal anti-inflammatory drugs. Up-regulation of hemeoxygenase-1 infers anti-apoptotic resistance to the cells due to potent antioxidant effect of bilirubin, biliverdin and carbon

monoxide formed. Hemeoxygenase was shown to protect gastric mucosal cells against non-steroidal anti-inflammatory drugs (Aburaya et al., 2006).

4. Therapeutic interventions in prevention of gastric ulcer

Several therapeutic interventions may aid in preventing gastric ulcer. Enhancement of normal physical barriers and physiological protective factors can aid in prevention of gastric ulcer. Some endogenous gastro-protective factors (see above) may be enhanced to decrease the risk of gastric ulcer formation. Conventional medications used in treatment of gastric ulcer can also be used in prevention as well, especially in predisposed people. Several investigations also tested drugs not conventionally used in treatment of gastric ulcer for having possible ulcerogenic protective effects. The main aim of most of these studies is to decide which drug to preferentially use in treating conditions presenting concomitantly with high risk of development gastric ulcer.

4.1 Prevention of gastric ulcer by conventional anti-ulcer drugs

Most anti-ulcer drugs target gastric acid secretion and mucosal defense mechanisms (Table 1). Successful classes in treating gastric ulcer include Helicobacter pylori eradication therapy, prostaglandin analogs, cyto-protective drugs, histamine H_2 receptor antagonist and proton pump inhibitor groups. In terms of acid inhibition, proton pump inhibitors possess higher acid inhibitory potency. Histamine H_2 receptor antagonists have, thus, been gradually replaced with the more potent class of acid inhibitory drugs, the proton pump inhibitors. Current ulcer therapy consists of Helicobacter pylori eradication in Helicobacter pylori-positive gastric ulcer and proton pump inhibitors for healing and preventing peptic ulcers induced by drugs.

Proton pump inhibitors selectively block the H^+/K^+ ATPase of the parietal cells. These proton pump inhibitors are the most popular group of drugs used in Helicobacter pylori eradication regimens (see before; section 2.1). Misoprostol, a prostaglandin analog, has been the most widely used but its application is limited by abdominal side-effects as abdominal cramps and diarrhea. Sucralfate and bismuth salts improve mucosal repair. Sucralfate also acts by reducing acid secretion and suppressing Helicobacter pylori infection. Bismuth salts, having mild anti-Helicobacter pylori activity, are used in treatment of gastric ulcer therapy in combination with antibiotics (Malfertheiner et al., 2009).

All of these drugs have been used successfully to treat gastric ulcers and prevent remittent attacks. Nevertheless, their efficiency in prevention of gastric ulcers in individual predisposed groups is still controversial. Histamine H_2 receptor antagonist is one example, as their standard dosage succeeded only in reducing the risk of duodenal ulcer, but not gastric ulcer induced by non-steroidal anti-inflammatory drugs. The benefit from histamine H_2 receptor antagonists was limited to preventing the risk of ulcers induced by Helicobacter pylori infection (Chan and Graham, 2004). Contrarily, in another study, histamine H_2 receptor antagonists were effective for prevention of low dose aspirin-induced ulcers and showed similar potency as proton pump inhibitors (Nakashima et al., 2009). Another example is the use of cyto-protective drugs (as in Table 1) for prevention of gastric ulcer, whose efficacy is still controversial.

Using these conventional anti-ulcer drugs in prevention of gastric ulceration is, thus, dependent on the type of predisposing risk factor. Risk factors used in assessment are old age, presence of cardiovascular diseases, use of high dose or multiple non-steroidal anti-

inflammatory drugs, concomitant use of low-dose aspirin and other anti-platelet drugs, corticosteroids or warfarin. When one or two of these factors are present, presenting a moderate risk, an anti-secretory agent or misoprostol may be used. If three or more risk factors are combined, presenting a high risk, switching from non-selective, to selective cyclooxygenase inhibitors is recommended. In addition, misoprostol can be used for prevention of aspirin- or warfarin-induced gastric ulcers. In very high risk patients, who have been subjected to previous ulcer complications, avoidance of non-steroidal anti-inflammatory drugs and intake of proton pump inhibitor and/or misoprostol is recommended.

Drug group	Examples	Mechanism of action
Helicobacter pylori eradication therapy	Proton pump inhibitor with two antibiotics	Treatment of Helicobacter pylori infection and prevention of ulcer formation
Proton pump inhibitors	Omeprazole, pantoprazole, lansoprazole, rabeprazole, esomeprazole	Most potent acid inhibition
Histamine H_2 receptor antagonists	Cimetidine, ranitidine, famotidine, nizatidine, roxatidine, lafutidine	Less potent acid inhibition
Prostaglandin analogs	Misoprostol	Weak acid inhibition and increase mucosal resistance
Cyto-protective drugs	Rebamipide, azulensulfonate, teprenone, polaprezinc, sofalcone, alginate sodium	Very weak effect in cyto-protection and enhancement of natural defense mechanisms
Bismuth salts	Subcitrate, subsalicylate	Weak antibacterial effect and increase mucosal prostaglandin synthesis

Table 1. Drugs used in prevention and treatment of gastric ulcer and their main mechanism(s) of action.

4.2 Non-conventional gastro-protective drugs

A number of drugs, other than traditional anti-ulcer medications, were investigated and showed an effect in prevention of gastric ulcer formation. Stress causing hypertension may concomitantly predispose to gastric ulcer. The effect of antihypertensive drugs, namely angiotensin II T_1 receptor blocker; telmisartan, was investigated for its effect as gastro-protective agent. The results showed that telmisartan and candesartan can prevent gastric ulcer formation, with higher potency of telmisartan than candesartan. Telmisartan's protection of gastric mucosa from non-steroidal anti-inflammatory drugs-induced ulceration is possibly through its anti-oxidant action and may also be ascribed, at least in part, to its peroxisome proliferator-activated receptor γ agonistic properties (Morsy et al., 2009).

Gastric ulcer is also commonly seen concurrently in type 2 diabetic patients. Moreover, peptic ulcers related to the diabetic state are more severe and are often associated with complications. The possible gastro-protective effects of insulin sensitizers

thiazolidinediones; rosiglitazone and metformin were tested. Both drugs have the ability to ameliorate oxidative stress and inflammation, rendering them attractive candidates for the prevention of gastric ulcer in patients with type 2 diabetes. Both rosiglitazone and metformin prevented indomethacin-induced gastric ulcer in diabetic rats. Their gastro-protective effects were probably due to anti-secretory actions, enhanced mucosal protection and anti-oxidant activity. This was reflected on their ability to increase mucin concentrations and gastric mucosal nitric oxide levels. In addition, rosiglitazone increased gastric juice pH, providing superior gastro-protection to metformin (Morsy et al., 2010).

Other investigations tested the effect of another anti-diabetic drug; pioglitazone as a gastro-protective drug. Pioglitazone has an agonist of peroxisome proliferator-activated receptor γ and exerted strong effect in both preventing the formation of gastric ulcers and healing of already existing ones. This gastric ulcer preventing/healing effect of pioglitazone is, at least in part, mediated by endogenous nitric oxide. Astonishingly, under diabetic conditions, pioglitazone gastro-protective effect decreased. The attenuation of pioglitazone action is possibly due to reduction in nitric oxide, angiogenesis and increased expression and release of pro-inflammatory cytokines under diabetic conditions (Konturek et al., 2010).

Organoselenium compounds were tested in naproxen- and Helicobacter pylori-induced gastric ulcers and showed not only gastro-protective and ulcer healing effects, but also they possessed antibacterial effect against Helicobacter pylori (Santhosh et al., 2010). When tested on indomethacin-induced gastric ulcer in mice, melatonin demonstrated gastro-protective effects via having angiogenic properties through up-regulation of matrix metalloproteinase-2; an important regulator of angiogenesis (Ganguly et al., 2010).

5. Gastric protection by herbs

Fig. 4. Herbs, unlike traditional drugs are natural, safer, cheaper and with less side effects.

The need for more effective and cheaper management and prevention of gastric ulcer has attracted an increasing interest for herbal products because of their effectiveness, less side effects and relatively low costs (Fig. 4). For long, some herbal tea constituents and food additives have been known for their gastro-protective effects. For example, liquorice has been used as gastro-protective agent. Eugenol, a compound extracted from clove oil, has also protective effect against the formation of indomethacin-induced gastric ulcer. This effect was mediated by its anti-oxidant activity, decreasing acid-pepsin secretion and increasing mucus production (Morsy and Fouad, 2008).
Similarly, curcumin demonstrated protective effect against gastric ulcer via inhibiting gastric acid secretion, relieving oxidative stress and ameliorating apoptosis. A number of Chinese naturally occurring phytochemicals were reported to have gastro-protective action with potent anti-Helicobacter pylori effects (Li et al., 2005). Lysophosphatidic acid, which is a component of soybean lecithin and antyu-san, has a protective effect against gastric ulcer induction in an animal model, suggesting that daily intake of lysophosphatidic acid-rich foods or Chinese medicines may be beneficial for prevention of gastric ulcer in humans (Adachi et al., 2011). In the ongoing search for bioactive natural products of herbal origin that have ulcer protective activity, crude plant extracts and plant-derived compounds are tried in different experimental models.

5.1 Herbal extracts

Several studies on the gastro-protective effect of crude plant extracts have been undertaken. Although the pathogenesis of gastric ulcer is multi-factorial, secretion of gastric acid is still recognized as a central component of this disease; therefore the main therapeutic target is the control of this secretion using the anti-secretory drugs. On the other hand, many plant extracts, which significantly decrease the ulcer index in experimental animals, have no clinical effects owing to their deficient anti-secretory activity. Accordingly, a large number of studies have been addressing the relationship between plant extracts and their anti-secretory activity on animal experimental models.
We conducted a PubMed search to identify the most relevant articles to these crude plant extracts and focused on those related to the anti-secretory properties, published between January 2010 and December 2010. The number of articles retrieved in a search with such tight limitations reflected the increased scientific interest in using plant extracts in prevention and treatment of gastric ulcer. These natural herbal extracts that has gastro-protective effect include methanol extract of Abarema cochliacarpos bark, a plant that mainly grows in Brazil (da Silva et al., 2010). Another herb, celery (Apium graveolens), which is widely used as food additive worldwide, was also tested, and ethanol extracts of it showed anti-secretory properties (Al-Howiriny et al., 2010).
Extracts of herbs that mainly grow and are widely used in India were tested for their gastro-protective effects as aqueous extract of Pedalium murex leaves (Banji et al., 2010), methanol extract of Hedyotis puberula (Joseph et al., 2010), methanol extract of Punica granatum (Alam et al., 2010), aqueous extract of Myrtus communis (common myrtle) berries (Sumbul et al., 2010), extracts of Cinnamomum tamala leaves (Eswaran et al., 2010) and extracts of Xylocarpus granatum fruit (Lakshmi et al., 2010). Roxb (Ailanthus excelsa bark) (Melanchauski et al., 2010) and camelthorn (Alhagi maurorum) (Shaker et al., 2010) that are widely used in Egypt also showed gastro-protection against ulcers. Hot water extract of Trichosanthes cucumerina Linn that is mainly used in Sri Lanka also showed similar effects (Arawwawala et al., 2010).

5.2 Pure compounds

Purified compounds may have the privilege of specifying the exact compound that is exerting gastro-protective effects. Unlike total herbal extract, pure compounds may lack the presence of several combined components that may contradict each other's action or add an undesired adverse effect.

5.2.1 Flavonoids

Flavonoids represent a highly diverse class of secondary metabolites that constitute the largest and most important group of polyphenolic compounds in plants. The pleiotropic actions of natural compounds are important for developing new drugs for multifactorial diseases. This is particularly true with regards to flavonoids as they display several pharmacological properties in the gastro-protective area, acting as anti-secretory, cyto-protective and antioxidant agents. Besides their action as gastro-protective, flavonoids also can be alternatives for suppression or modulation of gastric ulcers associated with Helicobacter pylori. Flavonoid fraction extracted from Mouriri pusa leaves, a plant from Brazilian cerrado also known as manapuçá or jaboticaba do mato which is commonly used in the treatment of gastrointestinal disturbs in its native region, shows beneficial effects in prevention and reversal of gastric ulcer (Vasconcelos et al., 2010).

Quercetin has an anti-secretory mechanism of action. It has antihistaminic properties, therefore, decreases histamine levels, as well as preventing the release of histamine from gastric mast cells and inhibiting the gastric H^+/K^+ proton pump, diminishing acid gastric secretion. On the other hand, the gastro-protective effects of chalcones involve increasing the mucosal blood flow, stimulating the synthesis of mucus in the gastric mucosa and increasing prostaglandin levels. Nevertheless, the most important mechanism of action responsible for the anti-ulcer activity of flavonoids is their antioxidant properties, seen in garcinol, rutin and quercetin, which involve free radical scavenging, transition metal ions chelation, inhibition of oxidizing enzymes, increase of proteic and nonproteic antioxidants and reduction of lipid peroxidation. In addition, sofalcone (a chalcone) and quercetin (flavonol) have anti-Helicobacter pylori activity (Mota et al., 2009).

5.2.2 Alkaloids

Alkaloids represent a diverse group of low molecular weight nitrogen-containing secondary metabolites that have gastro-protective activity. For examples, the isoquinoline alkaloid isolated from Coptidis rhizome, coptisine; the quinolizidine alkaloid isolated from Sophora flavescens, matrine which decreases the acid secretion and inhibits the gastric motility; the piperidine alkaloid piperine, which protects the stomach against ulceration by decreasing the volume of gastric juice, gastric acidity and pepsin-A activity; the phenylakylamide alkaloid capsaicin, which inhibits the acid secretion, stimulates the alkali/mucus secretions and mainly increases the gastric mucosal blood flow; the steroidal alkaloid pachysandrine A obtained from Pachysandra terminalis; and the indole alkaloid nigakinone found in Picrasma quassioides, which decreases gastric acid/pepsin secretions and protects the mucous membrane (de Sousa et al., 2008).

5.2.3 Terpenoids

Terpenoids are a large and diverse class of naturally-occurring organic chemicals similar to terpenes. Gastro-protective terpenoids have been isolated from several plants, including

sesquiterpenes from Artemisa douglasiana, triterpenes from Fabiana imbricate and carbenoxolone from Glycyrrhiza glabra. Most of the work on the gastro-protective activity has been focused on the clerodane diterpenes from Croton cajucara. Other diterpenes with anti-ulcerogenic effect include cordatin from Aparisthmium cordatum and trichorabdal A from Rabdosia trichocarpa (Schmeda-Hirschmann and Yesilada, 2005).

6. Conclusion

Gastric ulcer is a multi-factorial disease that has become a real socio-economic burden and opposes a great challenge in its treatment. Prevention is better than cure, as they say. Usage of medications designed for treatment of gastric ulcer as a means for its prevention is faced by several drawbacks; as limited effectiveness of these drugs in ulcer prevention, numerous side effects of available anti-ulcer drugs and the cost of gastric ulcer medications. Consequently, separate line of research has been devoted to investigate preventive measures of gastric ulcer. Despite of the size of investigations done on this subject, prevention of gastric ulcer is still a challenge especially in predisposed groups. Herbal compounds can provide an alternative preventive means for gastric ulcer as they are safer, cheaper and usually having limited, if any, side effects. For reaching the optimal remedy that can prevent gastric ulcer formation, more investigations are definitely still needed.

7. References

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Part 5

Peptic Ulcer Management in Animals

25

Association Between Nonsteroidal Anti-Inflammatory Drugs and Gastric Ulceration in Horses and Ponies

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1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely employed in equine medicine to treat acute and chronic inflammation in tendon, ligament and musculoskeletal injuries, as well as after surgery (Cunningham & Lees, 1994; Lees et al., 2004; Dirikolu et al., 2008). These drugs are used because of their analgesic, anti-inflammatory, and anti-pyretic properties; they are also used as adjuvant therapy in the treatment of endotoxemia and to suppress platelet aggregation (Johnstone, 1983; MacAllister, 1994; MacAllister & Taylor-MacAllister, 1994; Mathews, 2002).

An ideal anti-inflammatory drug is potent and has few adverse effects. In fact, several of the commonly used NSAIDs have a narrow safety margin. It is imperative, therefore, to administer a correct dose at adequate intervals. Thus, use of these drugs for controlling pain in equine is recommended for well-hydrated animals aged over six weeks with normal oncotic pressure. Kidney and liver function should be normal, there should be no signs of gastric ulcers, and the animals should not be taking corticosteroids. Furthermore, two or more NSAIDs should not be given at the same time (Mathews, 2002).

It is essential to study in depth the adverse effects, the pharmacokinetics and pharmacodynamics of NSAIDs because of their side effects. The half-life of substances differs among species as a function of biotransformation pathways, drug metabolization time, associated disease (especially renal and hepatic conditions), age (younger animals have immature hepatic enzyme systems, whereas older animals have less efficient kidneys and livers), binding of NSAIDs to food components in the gastrointestinal tract, and association of NSAIDs with other drugs.

Studies on the relation between NSAIDs and gastric ulcers in equid species are complex because several factors may cause gastric injury: the physiological status of the stomach; a pH often below 2 (Murray, 1997, 1999); prolonged fasting (where the pH may be as low as 1.55) (Murray & Schusser, 1993); intense exercising in sports animals [which increases abdominal pressure, decreases stomach volume, and results in reflux of small intestine acids into the nonglandular mucosa (squamous mucosa) of the stomach] (Vatistas et al., 1999a; Lorenzo-Figueira & Merritt, 2002; McClure et al., 2005); diseases that cause loss of appetite

or anorexia (Murray, 1999), and stress (confinement, administration of drugs, different environments, weaning), which may increase the level of circulating corticosteroids, in turn inhibiting the synthesis of prostaglandins and other chemical mediators, thereby generating favorable conditions for ulcers (MacKay et al., 1983; MacAllister et al., 1992; Andrews & Nadeau, 1999; Murray, 1999; Andrews et al., 2005; McClure et al., 2005; Pinto et al., 2009).

2. Types and mechanism of action of NSAIDs

There are several classifications of NSAIDs. These fall into five major chemical groups: carboxylic acid derivatives, enolic acid derivatives, specific cyclooxygenase 2 (COX-2) inhibitors, inhibitors of COX-2 with weak anti-inflammatory effect, and other nonsteroidal anti-inflammatory drugs. Carboxylic acid derivatives may be further subdivided into salicylic acids (e.g., aspirin and diflunisal), acetic acids (e.g., indomethacin, diclofenac, sulindac and eltenac), propionic acids (e.g., naproxen, ibuprofen, fenoprofen, flurbiprofen, ketoprofen and carprofen), aminonicotinic acids (e.g., flunixin meglumine), and fenamic acids (e.g., meclofenamic acid, sodium meclofenamate and mefenamic acid). Enolic acids may be subdivided into pyrazolones (e.g., phenylbutazone, monophenylbutazone, oxyphenbutazone, isopirin and apazone), and oxicam derivatives (e.g., piroxicam, droxicam, tenoxicam, and meloxicam). Selective COX-2 inhibitors are: celecoxib, etoricoxib, lumiracoxib, valdecoxib, parecoxib, firocoxib and nimesulide. Meloxicam and eltenac may be considered selective COX-2 inhibitors because of increased hepatic, renal and gastric tolerance in horses. Cyclooxygenase inhibitors with a weak anti-inflammatory effect include paracetamol and dipyrone. Other anti-inflammatory drugs not included in the above mentioned groups are dimethyl sulfoxide and glicosaminoglicans (Kore, 1990; Tasaka, 2006; Doucet et al., 2008; Burke et al., 2010).

After absorbing over 90% of NSAIDs bind to plasmatic proteins; the unbound fraction is biologically active (Tobin et al., 1986; Kore, 1990; Vicente, 2004). Most of these substances bind to albumin until saturation, at which point the concentration of the unbound fraction increases rapidly, which explains the relatively rapid onset of action of NSAIDs (Kore, 1990). According to Gerring et al. (1981), at least 98% of phenylbutazone is bound to plasma protein following administration at therapeutic doses.

Although NSAIDs are administered by several routes, they are generally metabolized by mixed function oxydase enzymes in the liver. A number of conjugated reactions are involved in eliminating these drugs. Excretion is primarily renal – glomerular filtration and tubular secretion – although some conjugates may be eliminated by the biliary tract. The excretion rate is often related with the pH; other weak acids may competitively inhibit secretory paths (Tobin et al., 1986; Kore, 1990; Vicente, 2004).

Effective plasmatic levels of NSAIDs administered orally are reached within an hour (Mathews, 2002). Several factors, however, may affect the absorption rate, such as the gastric pH, the presence of food, gastrointestinal motility, drug concentration, and the animal species (Kore, 1990; Mathews, 2002).

Phenylbutazone, an enolic acid pyrazolone derivative, is one of the most commonly used NSAIDs in equine medicine (Snow et al., 1979; Tobin et al., 1986; MacAllister et al., 1993; Kawcak, 2001; Dirikolu et al., 2008; Sabaté et al., 2009). This drug was synthesized by Stenzl in 1946 (Tasaka, 2006) and introduced into human medicine in 1949 for the treatment of

rheumatoid arthritis, ankylosing spondylitis, and several other musculoskeletal conditions (Shearn, 1984). Because of its efficacy and low cost, it has been used mainly in horses since the 1950s (more specifically in 1952) for treating lameness caused by articular conditions, soft tissue diseases, and gastrointestinal colic (MacAllister et al., 1993; MacAllister, 1994; Vicente, 2004; Erkert et al., 2005; Tasaka, 2006). It is excreted unmetabolized in urine and as a metabolite of glucuronic acid oxidation and conjugation; the most relevant metabolites are oxyphenbutazone (active metabolite), γ-hydroxyphenylbutazone and γ-hydroxy-oxy-phenylbutazone (inactive metabolites) (MacAllister, 1994; Vicente, 2004; Igualada & Moragues, 2005).

Bioavailability studies have shown that the plasmatic kinetics of phenylbutazone is dose-dependent (Tobin et al., 1986). Sullivan & Snow (1982) compared in horses and ponies the intramuscular (2.5 mg/kg bwt) and enteral (5 mg/kg btw) routes for administering phenylbutazone and found that the absorption rate and bioavailability were slowed with intramuscular injections. These authors suggested that the drug precipitated in the neutral muscle pH. This property precludes intramuscular use because of binding to muscle proteins, which delays absorption and causes pain (Tasaka, 2006).

The plasmatic half-life of intravenously administered phenylbutazone in horses may range from 3.5 to 7.0 hours (Tobin et al., 1986; Lees et al., 1987; Vicente, 2004); it is about six hours in ponies (Snow et al., 1981). When administered orally, the phenylbutazone presents a variable, but longer half-life (3 to 10 h) (Tobin et al., 1986).

Regarding the mechanism of action of NSAIDs, it is known that following tissue damage (by trauma, hypoxia, toxins, endotoxins, etc.) short-chain fatty acids (such as arachidonic acid) are released from the cell membrane by phospholipase A_2 (Cunningham & Less, 1994; MacAllister, 1994; Lees et al., 2004, Tasaka, 2006). This enzyme works on cell membrane phospholipids to make arachidonic acid available for the enzymatic cascade involving cyclooxygenase or lipoxygenase in the cytoplasm (MacAllister, 1994; Tasaka, 2006). Cyclooxygenase 1 (COX-1) and COX-2 are the two cyclooxygenase isoforms that have been investigated in greater depth; there is an enzymatically active variant of the COX-1 gene named COX-3 (Smyth et al., 2010).

The COX-1 catalyzes the conversion of arachidonic acid into prostaglandins, which are involved in gastrointestinal, renal, and vascular physiological processes. COX-2 isoform produces an inflammatory response based on cytokines and inflammation mediators; the lipoxygenase cascade reaction yields primarily leukotrienes (Cunningham & Less, 1994; MacAllister, 1994; Jones & Blikslager, 2001; Lees et al., 2004; Tasaka, 2006; Smyth et al., 2010). COX-1 isoform is present in most tissues, and COX-2 is upregulated in monocytes, fibroblasts, synoviocytes, as well as chondrocytes in response to inflammatory stimuli (Johnston & Fox, 1997).

The majority of anti-inflammatory drugs block COX-1 and COX-2 to a greater or lesser extent (Tasaka, 2006; Burke et al., 2010). Studies have underlined the difficulty in separating the roles of COX-1 and COX-2 (Jones & Blikslager, 2001; Fitzpatrick et al., 2004); thus, the selectivity of these compounds is still controversial. Furthermore, some drugs may appear selective for an enzyme relative to another, but not potent. In fact truly selective or specific COX-2 inhibitors licensed for veterinary use are rare. Evidence suggests that phenylbutazone, flunixin meglumine and ketoprofen are not selective (Fitzpatrick et al., 2004; Burke et al., 2010; Pozzobon, 2010). Vicente (2004) has argued that phenylbutazone

inhibit the COX-1 isoenzyme more than COX-2, where the inhibitory power of prostaglandin endoperoxide H synthase-1 (PGHS-1) is one to five times that of PGHS-2, resulting in adverse effects such as erosion or ulcers of the mucosa in the mouth and gastrointestinal tract, diffuse gastritis, hemorrhagic gastroenteritis, venous thrombosis, nephritis, and chronic renal injury, which have been widely discussed in the literature (Snow et al., 1981; MacAllister, 1983; Mathews, 2002; Fitzpatrick et al., 2004).

Price et al. (2002) argue that veterinarians working with small animals may be more concerned about the adverse effects of NSAIDs than those working with horses. The former prefer using carprofen and meloxicam, which appear to cause fewer side effects. These authors applied a questionnaire to 400 veterinary practitioners in the UK about pain management in horses. Of these 93 were used for data analysis; the data indicated that the four most frequently used analgesics in order of preference were: phenylbutazone (92%), flunixin meglumine (90%), butorphanol (89%), and dipyrone (75%). Phenylbutazone was preferred because of lower cost compared to other licensed NSAIDs. The analgesic potential was the most important criterion when choosing between NSAIDs or opioids.

Considering the analgesic potential of NSAIDs, the intravenous administration of single doses of phenylbutazone (4 mg/kg bwt), flunixin (1 mg/kg bwt) or carprofen (0.7 mg/kg bwt) to 63 horses for post-surgical pain was effective, but the mean required times for further analgesia were 8.4 h (phenylbutazone), 11.7 h (carprofen), and 12.8 h (flunixin) (Johnson et al., 1993). Erkert et al. (2005) compared the analgesic effect of phenylbutazone (4.4 mg/kg bwt at 24 h intervals) and flunixin meglumine (1.1 mg/kg bwt at 24 h intervals) in horses with the navicular syndrome and found similar responses among these drugs.

Sabaté et al. (2009) assessed the analgesic efficacy of suxibuzone and phenylbutazone for the treatment of pain caused by lameness in 155 horses aged from 2 to 25 years and body weight from 350 to 540 kg. All animals had acute or chronic nonspecific single limb lameness. The drugs were administered orally as follows: phenylbutazone (4.4 mg/kg bwt every 12 h) for 2 days, followed by phenylbutazone (2.2 mg/kg bwt every 12 h) for 6 days (n=79), and suxibuzone (6.6 mg/kg bwt every 12 h) for 2 days, followed by suxibuzone (3.3 mg/kg bwt every 12 h) for 6 days (n=76). The authors found no difference (P=0.113) between these treatments for pain relief in horses.

3. Gastric ulcers

Equine gastric ulceration is a highly prevalent multifactorial disease with vague and non-specific clinical signs. Abdominal pain, weight loss, and loss of performance may be seen. On the other hand, asymptomatic cases (Murray et al., 1987, MacAllister et al, 1992; MacAllister & Sangiab, 1993; Andrews & Nadeau, 1999; Murray et al., 2001; Murray & Pipers, 2001; Murray, 2002) diagnosed by gastroscopy have been described. Reports have shown a poor correlation between ulcer severity and clinical signs (Murray et al., 1987; MacAllister & Sangiab, 1993; Murray, 2002; Marqués, 2007; le Jeune et al., 2009); thus, animals with deeper lesions may have relatively mild signs, while others presenting with more significant abdominal discomfort may have only superficial erosions (Murray, 2002).

Murray et al. (2001) found asymptomatic gastric ulcers in 18 horses out of 209 animals that underwent gastroscopy. The practical experience of the authors of this chapter supports the above mentioned informations about clinical signs; monitoring ten ponies with untreated

gastric ulcers (diffuse or localized hemorrhagic lesions), kept in free paddocks for eight months, revealed that 90% had no signs of bruxism, sialorrhea, decrease in appetite, rough hair-coat, diarrhea, abdominal discomfort, colic or any other sign of gastrointestinal tract involvement.

Studies have shown that foals may also develop gastric ulcers without apparent clinical manifestation (Murray et al., 1987; Marqués, 2007); thus, silent gastric ulceration is a common condition in these animals (Andrews & Nadeau, 1999). Léveillé et al. (1996) also reported a lack of clinical signs in three foals aged from 7 to 10 days that were given phenylbutazone 5 mg/kg bwt orally every 12 h during 7 days. On the other hand, necropsy revealed multifocal gastric ulcers.

Sports horses, such as performance and racehorses, have a high prevalence and severity of gastric ulcers (Hammond et al. 1986; Murray et al. 1989, 1996; Vatistas et al. 1999b; Pellegrini, 2005; Jonsson & Egenvall 2006; Orsini et al., 2009). A study conducted by Pellegrini (2005) showed that almost all performance horses have some kind of ulcer and that at least 60% of them have colonic ulcers. On the other hand, le Jeune et al. (2009) described the gastric ulceration syndrome in pregnant females (66.6%) and non-pregnant females (75.8%) kept free in irrigated pastures with alfalfa and grain supplements, but with no controlled physical activity. Luthersson et al. (2009) also reported this condition in nonracehorses.

Gastric ulcers may be found throughout the stomach of horses; the most commonly affected area is nonglandular mucosa – lined by stratified squamous epithelium – along the *margo plicatus* (Hammond et al., 1986; Murray et al., 1989, 1996; Andrews & Nadeau, 1999; Sandin et al., 2000; Ferrucci et al., 2003; Bruijn et al., 2009; le Jeune et al., 2009). The pathophysiology of ulcers consists of loss of equilibrium between aggressive factors (hydrochloric acid with or without synergistic action from volatile fatty acids, lactic acid, bile acids, and pepsin), and protective factors (mucus/bicarbonate barrier; prostaglandin $E_{2;}$ adequate mucosal blood flow; cellullar restitution, and the epidermal growth factor) (Murray, 1992; Jeffrey et al., 2001; Andrews et al., 2005; Morrissey et al., 2008; Nadeau & Andrews, 2009).

Parietal cells produce a 10^6-times higher hydrogen ion concentration in gastric juices compared to plasma, a process that requires carbonic anhydrase, which catalyzes the reaction between water and carbon dioxide. Sodium bicarbonate – resulting from dissociated carbonic acid (H_2CO_3) – is transferred into the plasma from parietal cells; this process involves its exchange for chloride ions (Cl^-) by means of an HCO_3^-/Cl^- carrier protein in the basolateral membrane. The absorbed Cl^- moves to the apical membrane, exits through canaliculi, and enters the intestinal glands. Carbonic anhydrase-generated hydrogen ions are actively secreted by the membrane in apical cells into the lumen of the gland. This ion exchange process makes it possible for parietal cells to maintain a constant pH and at the same time a highly acid solution in the gastric lumen (Randall et al., 2000).

Gastrin, histamine (H_2 receptors), and acethycholine (vagus nerve) stimulate the H^+,K^+-ATPase enzyme, which in turn causes parietal cells in gastric glands to secrete chloridric acid (Andrews et al., 2005; Videla & Andrews, 2009). The stomach of adult horse secretes about 1.5 l/h of gastric juices, which contains 4–60 mMol of hydrochloric acid. The feeding regimen and the region of the stomach that is measured alter the pH of the gastric content (Luthersson et al., 2009). Andrews & Nadeau (1999) found that the pH was stratified, being neutral in the dorsal portion of the esophageal region, more acid (from 3 to 6) close to the *margo plicatus*, and even lower (from 1.5 to 4.0) close to the pylorus. The pH of the gastric

content in continuously fed equines may remain around 3.1; in fasting animals, the pH may reach 1.6 (Murray & Schusser, 1993).

Studies have shown that freely grazing horses continuously produce large amounts of bicarbonate-rich saliva as a response to chewing, which has an important gastric acid buffering effect (Murray et al., 1996; Andrews & Nadeau, 1999; Andrews et al., 2005; le Jeune et al., 2009; Martineau et al., 2009; Videla & Andrews, 2009). On the other hand, the prevalence of ulcers did not differ significantly in full-time stabled horses, part-time stabled horses, or animals kept full-time on pastures (Bell et al., 2007).

Several ulcer-classifying systems based on the number and severity of lesions have been developed (Hammond et al., 1986; Murray et al., 1987; Johnson et al., 1994; Vatistas et al., 1994; Murray & Eichorn, 1996; MacAllister et al., 1997; Anon, 1999). Murray et al. (1987) characterized ulcers by location (nonglandular surface, *margo plicatus*, glandular surface) and severity. Lesions were graded from 0 to 4 (0=normal, 1=1-2 localized lesions, 2=3-5 localized ulcers or 1 diffuse lesion, 3=1-5 localized lesions with visible hemorrhage or multiple diffuse lesions with apparent mild to moderate loss of surface epithelium, and 4=greater than 5 localized ulcers or multiple diffuse lesions with apparent extensive loss of surface epithelium and/or hemorrhage).

Risk factors associated with this disease include diet, stress (moving horses from pasture to stall confinement, hospitalization, intense exercise, feed and water deprivation, among others), and administering NSAIDs (le Jeune et al., 2009, Luthersson et al., 2009; Nadeau & Andrews, 2009; Videla & Andrews, 2009) (the topic of this chapter). Reported factors related with disease prevalence in racehorses include a high-concentrate diet, low intake of hay, meal feeding, prolonged fasting, the type and intensity of training, as well as the use of NSAIDs (Vatistas et al., 1999a; Merritt, 2003; Roy et al., 2005; Jonsson & Egenvall, 2006). Studies on the relationship between NSAIDs and equine gastric ulcer are complex because of these many factors. Use of these drugs in human patients increases 3- and 5-fold the risk of peptic ulcers respectively in *H. pylori*-positive and *H. pylori*-negative patients (Voutilainen et al., 2001).

NSAID-induced gastric ulceration in horses was described in the late 1970s; phenylbutazone has been studied in greatest detail (Snow et al., 1979, Snow et al., 1981; MacAllister, 1983; Collins & Tyler, 1984; Tobin et al., 1986; Vicente, 2004). Studies describing the side effects of flunixin meglumine, ketoprofen and phenylbutazone started to be published in the 1980s (Trillo et al., 1984; MacAllister et al., 1992; MacAllister, 1994; MacAllister & Taylor-MacAllister, 1994). Other drugs, such as suxibuzone (a prophenylbutazone drug), firocoxib, monophenylbutazone (phenylbutazone-derivate), acetylsalicylic acid, eltenac, nimesulide and meloxicam, have also been studied (Prügner et al., 1991; Goodrich et al., 1998; Monreal et al., 2004; Villa et al., 2007; Andrews et al., 2009; Sabaté et al., 2009; Videla & Andrews, 2009; Pozzobon, 2010). Nevertheless, studies of phenylbutazone (or derivatives) have not been abandoned, possibly because of ulcerogenic effect and therapeutic efficacy (Vicente, 2004; Driessen, 2007). As mentioned previously, the nonsteroidal anti-inflammatory drugs are widely employed in equine clinical practice to treat acute and chronic inflammatory conditions, especially of the locomotor apparatus (Prügner et al., 1991; Jones & Blikslager, 2001; Sabaté et al., 2009; Videla & Andrews, 2009).

Gastric injury usually occurs when NSAIDs are given at high doses or prolonged treatments (Snow et al., 1979, 1981; MacAllister, 1983; MacKay et al., 1983); nevertheless, therapeutic doses have been known to cause ulcers in horses. The most widely accepted hypothesis for

the association between NSAIDs and gastric ulcers is cyclooxygenase inhibition (See item 2 – Types and mechanism of actions of NSAIDs), in which conversion of arachidonic acid into prostaglandins is blocked (MacAllister, 1983; MacAllister et al., 1993; Murray, 1999). The physiologic vasodilating effect of prostaglandins (in particular PGE_2) on the stomach mucosa generates a bicarbonate buffering system that attenuates the corrosive action of hydrochloric acid contained in gastric secretions (Andrews & Nadeau, 1999; Murray, 1999; Morrissey et al., 2008). These substances increase gastric mucosa blood flow and mucus secretion, and reduce gastric acid production. They also facilitate basal cell migration towards the lumen for repairing the mucosa and maintaining the integrity of nonglandular and glandular mucosa; this takes place by stimulation of active surface-protecting phospholipid production. Inhibition of prostaglandin synthesis may give rise to ideal conditions for ulcers in the gastrointestinal tract (Andrews & Nadeau, 1999; Murray & Pipers, 2001; Andrews et al., 2005). According to Andrews et al. (2005), gastric mucosal ischemia may lead to hypoxia-induced cellular acidosis, and release of oxygen-free radicals, phospholipases and proteases, which may damage the cell membrane and result in necrosis.

As mentioned previously, the majority of NSAIDs are poorly selective, inhibiting COX-1 and COX-2 equally (Fitzpatrick et al., 2004; Vicente, 2004). Drugs that inhibit COX-1 are considered the main causative of stomach lesions, because this enzyme is generally – but not exclusively – responsible for the above mentioned adverse effects on the gastrointestinal tract (Jones & Blikslager, 2001; Lees et al., 2004; Videla & Andrews, 2009). Although the ulcer-causing potential may vary among NSAIDs, a study of rat stomachs with normal mucosa after acid challenge showed that inhibition of both cyclooxygenases causes gastrointestinal injury; however, inhibition of only one of these enzymes did not have this effect (Gretzer et al., 2001). Furthermore, administering NSAIDs on an empty stomach may result in local gastric irritation. Therefore, these drugs should be administered with food when given orally (Mathews, 2002; Lees et al., 2004; Monreal et al., 2004).

The site of NSAID-induced ulcers in the stomach of horses remains controversial. Some authors have stated that the glandular mucosa is more commonly affected (Carrick et al., 1989; Vatistas et al., 1999a; Monreal et al., 2004; Marqués, 2007; le Jeune et al., 2009), while others have argued that the nonglandular mucosa is affected more frequently (MacAllister et al., 1992; Andrews et al., 2005). According to Mokhber Dezfouli et al. (2009), Persian Arab horses with history of long term treatment with NSAIDs have high prevalence of the gastric ulcer in the glandular mucosa. In addition, it has been documented that phenylbutazone can cause severe ulceration of the glandular gastric mucosa following administration at high dosages for as short as a few days (Collins & Tyler, 1985; Lees, 2003). Moreover, according to Andrews et al. (2005), 80% of ulcers induced by phenylbutazone are located in the nonglandular mucosa.

MacAllister et al. concluded 1992 that flunixin meglumine (1.5 mg/kg bwt intramuscularly every 8 hours for 6 days) may result in ulcers of the nonglandular mucosa of ponies. In 1993, MacAllister et al. reported ulcers in the nonglandular and glandular mucosa of horses. The authors compared the adverse effects of phenylbutazone (4.4 mg/kg bwt), flunixin meglumine (1.1 mg/kg bwt) and ketoprofen (2.2 mg/kg bwt) given intravenously every 8 hours in horses during 12 days. The phenylbutazone presented the highest ulcerogenic potential of these three drugs. Other studies of horses

and ponies revealed that the effect of NSAIDs on the nonglandular mucosa is less evident or undetected, and if there is pain, it is generally mild (Snow et al., 1979, 1981; MacKay et al., 1983).

The glandular region has adequate blood flow, cell restitution, mucus-bicarbonate layer, prostaglandin secretion, and growth factors (Murray, 1997; Andrews et al., 2005; Marqués, 2007; Nadeau & Andrews, 2009). The nonglandular mucosa has a thinner layer, no mucus-bicarbonate layer, and often desquamation in foals aged over 35 days, and may remain in the first month of life (MacAllister et al., 1992; Murray et al., 1987; Andrews & Nadeau, 1999; Andrews et al., 2005, Murray, 1997). This region is constantly exposed to chloridric acid, pepsin and bile acids (Andrews & Nadeau, 1999; Murray, 1999). Besides the stomach, the phenylbutazone-induced ulcers may occur in the intestine – with reports in the duodenum (Snow et al., 1979; Snow et al., 1981; MacAllister et al., 1993), ceco, colon and rectum (MacAllister, 1983, Ruoff et al., 1987, Boothe, 2001).

Meschter et al. (1984) has stated that the primary target of phenylbutazone intoxication in horses is the wall of smaller veins. Other changes (ulcers on the tongue, stomach and intestine, as well as renal necrosis and venous thrombosis) should be interpreted as being secondary to vein lesions. In 1990, Meschter et al. suggested that phenylbutazone-induced gastrointestinal ulceration results from direct toxic injury to endothelial cells within the microvasculature of the mucosa. Vascular tumefaction, stagnation and cessation of blood flow, formation of fibrin, perivascular extravasation with subsequent edema, thrombosis and necrosis of the mucosa occur; finally, the mucosal epithelium breaks down. These authors argued that vasoconstriction is not the primary cause of mucosal necrosis; once formed erosions and ulcers, they could persist because of other non-prostaglandin-mediated processes, such as bacterial invasion (Nadeau & Andrews, 2009). Murray (1999) added that NSAIDs appear to cause neutrophils to adhere to the vascular endothelium of the gastric mucosa, thereby reducing mucosal perfusion and releasing chemical mediators that add further damage. Doherty et al. (2003) have suggested that phenylbutazone does not alter the baseline secretion of gastric acid in horses; rather, it decreases lipopolysaccharide-induced effects on the volume of secretions and on sodium production, and concentration in parietal cells.

4. Some experimental studies of NSAIDs

After identifying the types and mechanisms of cyclooxygenases, several studies aimed to discover NSAIDs with appropriate analgesic, antipyretic and anti-inflammatory properties and minimal ulcer-generating effects (MacAllister et al., 1993; Cunningham & Lees, 1994; MacAllister, 1994). However, these drugs should currently be used with caution in horses, as animal studies have shown varying results. There are several risk factors – especially stress – associated with gastric ulceration; these factors may potentiate the ulcerogenic effect of NSAIDs during experiments. A description is given below of selected experimental studies showing associations, or lack thereof, between nonsteroidal anti-inflammatory drugs and gastric ulceration in equid species.

Snow et al. (1981) conducted an experiment with horses and ponies in which oral phenylbutazone (8.2 mg/kg bwt) was administered every 24 h during 13 days to six horses; also, the same drug at 10 to 12 mg/kg bwt every 24 h was administered to nine ponies during 6 to 8 days. All horses remained apparently healthy, but five ponies developed

depression, hyporexia, weight loss, loose feces, and mouth ulcers; two ponies died. A biochemical analysis showed progressive decrease in total plasma proteins and albumin, a significant elevation of blood urea nitrogen, and a decrease in calcium and potassium concentrations. The authors suggested that ponies were more susceptible to the adverse effects of phenylbutazone.

The manufacturer's daily recommended dose of phenylbutazone for horses is 4.4 to 8.8 mg/kg bwt orally, and 2.2 to 4.4 mg/kg bwt intravenously (MacAllister, 1994). The risk of intoxication may increase when phenylbutazone is administered at daily doses above 8.8 mg/kg bwt for more than four days (MacKay et al., 1983). According to Hu et al. (2005), considering the toxicity of phenylbutazone, the higher dosage (8.8 mg/kg) may not be beneficial in chronically lame horses, because this dose was not associated with greater analgesic effects compared to 4.4 mg/kg dose in quarter horse-type breeding studied by Oklahoma State University (USA). In fact, the most commonly used analgesic dose for equine in the clinical setting is 2-4.4 mg/kg bwt given intravenously or orally every 12 h (Robinson & Sprayberry, 2009), for 5 to 7 days.

Although the dose of 4.4 mg phenylbutazone/kg bwt is considered safe to use in horses (Taylor et al., 1983; Tobin et al., 1986), the oral administration of this dosage every 12 h with concurrent intravenous administration of flunixin meglumine (1.1 mg/kg bwt every 12 h) for 5 days resulted in acute necrotizing colitis, with lesions most severe in the right dorsal colon in one of 29 adult horses (Keegan et al., 2008). According to the authors, considering that the drugs were lower than those that reportedly cause toxic effects, it is likely that it was the combination of NSAIDs, as well as the total increase in concentration irrespective of type, that was responsible for these abnormalities. On the other hand, neonatal foals (two days old) treated with recommended dosage of flunixin meglumine (1.1 mg/kg bwt/day) for five days, did not have clinicopathological or pathological differences compared to treatment with physiological saline, but the dose of 6.6 mg/kg/day increased total gastrointestinal ulceration, gastric ulceration and cecal petechiation (Carrick et al., 1989).

Administrating high doses of phenylbutazone (10 mg/kg btw) daily for ponies (Snow et al., 1979) and foals (Traub et al., 1983), and 8 mg/kg bwt daily for adult horses (Ruoff et al., 1987) resulted in ulcers in different parts of the gastrointestinal tract (from lips and tongue to the rectum), and marked edema and inflammation of the small intestine, colon, and rectum. In addition, MacAllister (1983) administered phenylbutazone 10 mg/kg bwt orally every 24 h to ten ponies during 14 days and found that seven animals were intoxicated, characterized by anorexia, oral ulcers, soft feces, and depression; six animals died, one of which by euthanasia. Necropsy revealed gastrointestinal ulcers, enteritis, necrotic colitis, peritonitis, and renal papillary necrosis.

Monreal et al. (2004) found ulcers on the glandular gastric mucosa in 100% of mix-breed horses (aged from 2 to 16 years, and body weight from 288 to 527 kg) treated with high doses of phenylbutazone (10.5 mg/kg bwt every 12 h, for two days, followed by 5.25 mg/kg bwt every 12 h, for 12 additional days); the same findings were present in only 40% of suxibuzone-treated animals (15 mg/kg bwt every 12 h, for two days, followed by 7.5 mg/kg bwt every 12 h, for another 12 days); the ulcers were significantly larger and deeper in the animals that were given phenylbutazone. Conversely, histopathology studies revealed similar inflammation when comparing these two drugs; there was severe neutrophilic inflammatory infiltration and signs of a healing reaction in both groups of animals. According to these authors, ulcers in the oral cavity, softened feces, anorexia, weight loss,

hypoproteinemia and hypoalbuminemia, considered classical signs of phenylbutazone toxicosis, were seen in one horse only. Further, small mouth ulcers were encountered in two animals in the suxibuzone group.

Andrews et al. (2009) evaluated the gastric ulcerogenic effect of a top-dress formulation containing suxibuzone or phenylbutazone for 18 adult horses aged from 3 to 14 years and body weights ranging from 294 to 467 kg in study conducted at the Louisiane State University (USA). There were three groups: a control group, a group given phenylbutazone (2.6 mg/kg bwt), and a group given suxibuzone (3.5 mg/kg bwt), during 15 consecutive days. Gastric ulcers in the phenylbutazone-treated group were not more severe than those in the suxibuzone-treated group, suggesting that suxibuzone has no advantage over phenylbutazone in preventing gastric ulcers at recommended label doses.

Prügner et al. (1991) reported that intravenous administration of eltenac (1 mg/kg bwt at 24 h intervals) during three days was more effective ($P<0.001$) in reducing pain caused by lameness of several causes (tendinitis, pododermatitis, navicular disease, non-infectious arthritis, etc.) in 32 horses compared to placebo controls (n=32). Goodrich et al. (1998) studied this same drug in four groups of six horses given different doses (0.5, 1.5, 2.5 mg/kg bwt or sterile saline solution every 24 h for 15 days), and found that it was not toxic for the gastrointestinal tract at a dose of 0.5 mg/kg bwt; the authors concluded that eltenac might be beneficial for horses.

Videla & Andrews (2009) reviewed firocoxib, an NSAID approved for controlling pain and inflammation due to osteoarthritis in horses. This drug (0.1 mg/kg bwt orally every 24 hours, for 30 days) did not cause ulcers in the study sample. These authors suggested that the efficacy of firocoxib in horses with abdominal pain is unknown, and that it should not be administered to animals with abdominal discomfort and gastric reflux or dysphagia, as there is currently no systemic formulation of the drug. According to Doucet et al. (2008), firocoxib appears to be a safe alternative to the long-term use of phenylbutazone in horses.

Loew et al. (1985) stated that monophenylbutazone is five to six times less toxic than phenylbutazone, but it is less effective when given at the same dose. Pinto et al. (2009) studied whether monophenylbutazone was associated or not with gastric ulcers in ponies in a two-step experiment conducted at the Universidade Federal de Viçosa (Brazil). The first step consisted of three groups of two healthy ponies each, treated with daily intravenous doses (3, 4.5 or 6 mg/kg bwt during 12 days) of the drug. One pony in each group was given omeprazole (3 mg/kg bwt orally every day). The second step, conducted six month after the first, consisted of two groups, each with two healthy ponies; the first group was given monophenylbutazone 4.5 mg/kg bwt intravenous daily for 12 days, and the second group was given 5 mL of 0.9% NaCl intravenously. All ponies underwent endoscopy before and after the trial. At the end of the first step, endoscopy revealed nonglandular gastric mucosa ulcers along the *margo plicatus* (Fig. 1) only in the two animals that were given the highest dosage (6 mg/kg bwt) of the drug. However, the occurrence of ulcers was unrelated with the dose ($P>0.05$). The authors suggested that individual variation, confinement stress, daily handling, and administration itself of the drug, may have contributed to the number and severity of ulcers, a situation that has already been reported by other authors (MacAllister et al., 1992; Murray, 1999; Andrews et al., 2005). le Jeune et al. (2009) have suggested that stress may be a major contributing factor to ulcer development.

In the second step of Pinto et al.'s (2009) study, two animals developed gastric ulcers, one of which had been given monophenylbutazone (4.5 mg/kg bwt during 12 days) (Fig. 2); the

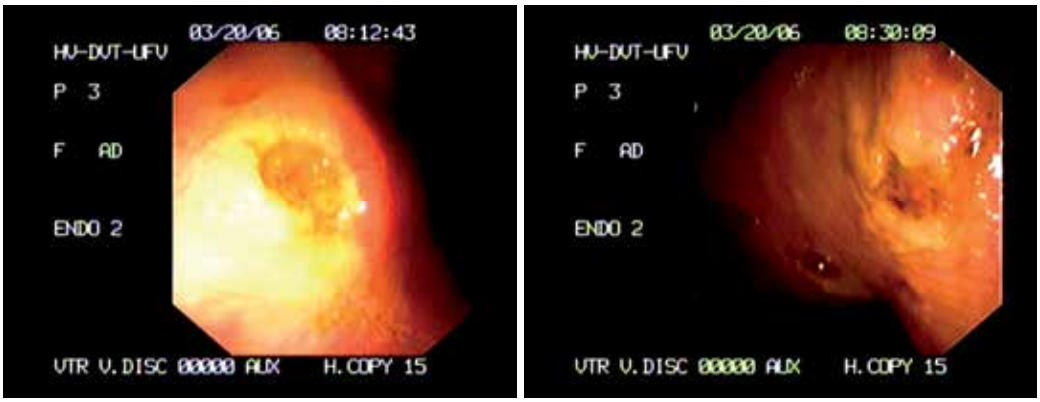

Fig. 1. Ulcers on the nonglandular gastric mucosa of a pony treated intravenously during 12 consecutive days with monophenylbutazone (6 mg/kg bwt every 24 h).

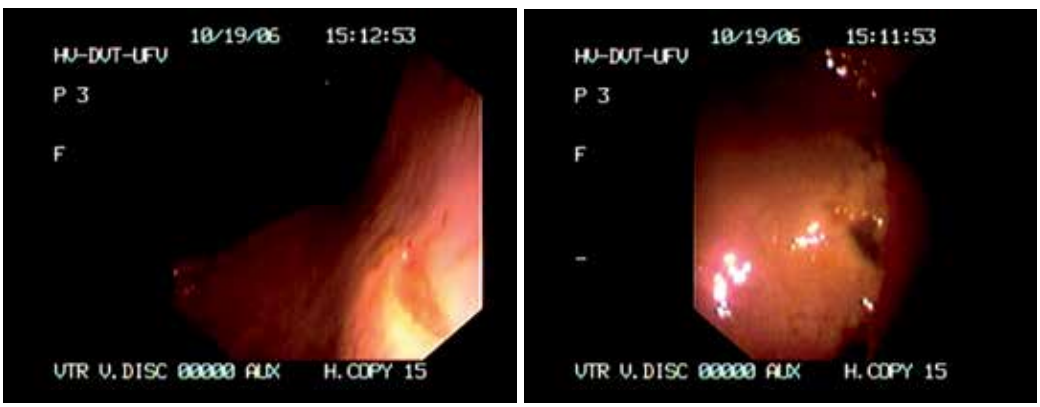

Fig. 2. Ulcers on the nonglandular gastric mucosa of a pony treated intravenously during 12 consecutive days with monophenylbutazone (4.5 mg/kg bwt every 24 h).

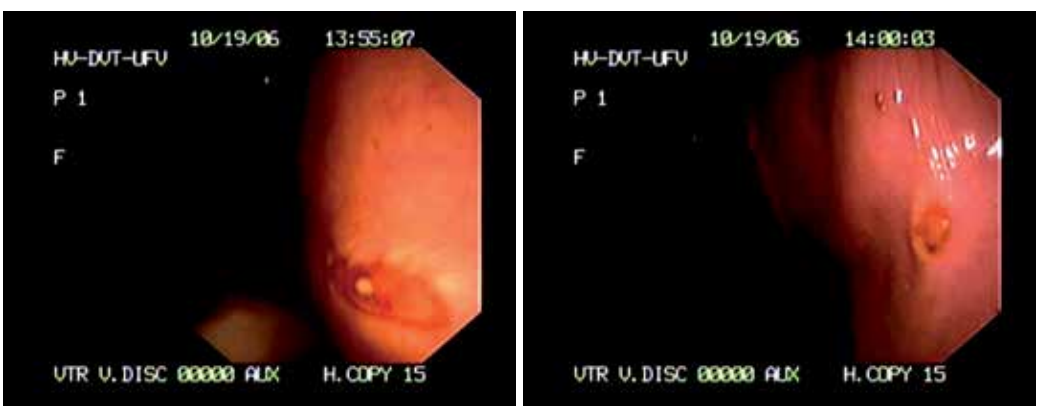

Fig. 3. Ulcers on the nonglandular gastric mucosa of a pony treated daily during 12 consecutive days with 5 mL of 0.9% NaCl intravenously.

other pony that presented gastric ulcers had been given only 5 mL of 0.9% NaCl intravenously, during 12 days (Fig. 3). Monophenylbutazone did not influence the occurrence of ulcers ($P>0.05$). The authors suggested that the discomfort associated with daily intravenous injections of saline solution may have generated enough stress to cause ulcers. MacAllister et al. (1992) also suggested an association between stress and application of medication, in a study where flunixin meglumine (1.5 mg/kg bwt every 8 h during 6 days) was given intramuscularly to ponies. MacAllister et al. (1993) encountered similar results when comparing the occurrence of ulcers following administration of flunixin meglumine and 0.9% NaCl to horses; there were no significant differences between these two groups.

Vatistas et al. (1999a) studied stress in thrirty mature Thoroughbred horses and suggested that the following situations could raise serum cortisol concentrations: road transport, exposure to a new environment, abrupt weaning in foals, physical restraint, anesthesia, nasogastric intubation, and diseases in general. Costa el al. (2007) have argued that the pathophysiology of stress-induced gastric mucosal injury remains controversial; the main suggested causal factor has been decreased blood flow in the mucosa due to splanchnic vasoconstriction associated with increased sympathetic tonus and an increased level of circulating catecholamines. Furthermore, increased endogenous corticosteroid concentrations during stress may inhibit prostaglandin synthesis. As mentioned previously, decreased prostaglandin levels result in loss of balance in mucosal protective factors; this is commonly stated as the primary cause of ulcers in foals and adult horses (Andrews & Nadeau, 1999; Andrews et al., 2005).

Villa et al. (2007) evaluated the pharmacokinetics and pharmacodynamics of nimesulide in 15 healthy horses aged from 3 to 6 years. The animals were divided into three groups. Group A was given nimesulide (1.5 mg/kg bwt) orally and intravenously; groups B and C were given nimesulide (1 mg/kg bwt) orally once. According to the authors, a 1.5 mg/kg bwt dose may yield the desired effects when administered every 12 or 24 h, depending on the severity of the animal's condition. However, as this dose exceeds the *in vitro* IC_{50} for cyclooxygenase 1 and 2 isoforms (see item 2 – Types and mechanism of action of NSAIDs), the selectivity is lost, which results in side effects due to COX-1 inhibition. Thus, the authors suggest that nimesulide should be used with caution in equid species.

Pozzobon (2010) assessed the side effects of meloxicam on the gastric mucosa and semen of six healthy ponies at the Universidade Federal de Santa Maria (Brazil). Two animals were treated with meloxicam (0.6 mg/kg bwt, orally) for 30 days, two were treated with ketoprofen (2.2 mg/kg bwt, orally) for 30 days, and two were not given anti-inflammatory drugs. The experiment was repeated three times, alternating the ponies per groups according to a Latin square design; thus, all animals were given all treatments. The study lasted 15 weeks, with a one-week interval between treatments. Gastroscopy done at the end of the study did not reveal gastric mucosal disease, even though the concentration of total prostaglandins in the seminal plasma was decreased ($P<0.05$) and the quality of semen was negatively affected; the findings suggested a physiological effect of COX-2 on the reproductive tissues of stallions.

Gastric ulcers have also been reported in donkeys from South West of England. Burden et al. (2009) examined at necropsy 426 non-working aged donkeys, and found that 41% of these animals had gastric ulcers. The mean age of the animals was 30.5 years; the study took two

years. The majority (n=96; 49%) were medium-sized ulcers (> 2 cm^2; < 10 cm^2), located mainly on the nonglandular mucosa along the *margo plicatus* (n=155; 89%). Information on NSAID use (e.g., phenylbutazone, flunixin meglumine, meloxicam, etc.) was available for 418 animals (98%); 214 donkeys (50.2%) in the study had been given NSAIDs for at least 7 days immediately prior to death, and the majority of animals had been given these drugs for months or years. The authors, however, found no relation (P=0.9) between the risk of having gastric ulcers on the glandular mucosa and use of NSAIDs in these animals.

There is an ongoing search for new analgesic and anti-inflammatory drugs because of the adverse effects of NSAIDs. Videla & Andrews (2009) have recommended xylazine (0.2-0.4 mg/kg bwt) or detomidine (20-40 µg/kg btw) as alternatives to NSAIDs, since these drugs are good analgesics and have minimal effects on the gastrointestinal tract. However, these drugs have other side effects or may increase the cost of therapy. These same authors have suggested that the choice of NSAIDs for horses should take into account the following criteria: minimal side effects on the gastrointestinal tract, the minimal pain-controlling dose, and use of an anti-ulcer drug together with the anti-inflammatory medication.

Drugs for treating ulcers in equid species have been investigated; this is a complex topic, to be addressed in another chapter of this book. However, the authors of the current chapter believe it is important to report their study with omeprazole, a drug that binds irreversibly to the H^+, K^+ -ATPase enzyme of gastric parietal cells (which secrete hydrogen ions into the stomach in exchange for K^+ ions), thereby inhibiting the production of chloridric acid. Omeprazole also selectively inhibits carbonic anhydrase, which adds to its acid suppressive properties (Daurio et al., 1999; MacAllister, 1999). Although this drug is considered the most effective inhibitor of gastric secretions (90% in 24 hour at 4 mg/kg bwt daily), it has a low bioavailability after oral intake (14-16%) (Andrews et al., 1992; Téllez et al., 2005). Murray et al. (1997) showed that the healing time of gastric ulcers was significantly shorter in horses given omeprazole (1.5 mg/kg bwt orally) daily during 28 days. MacAllister (1999) suggested that healing of ulcers using this drug appears to depend on the dose and duration of therapy; an oral dose of 4 mg/kg bwt daily during 28 days appears to have the highest success rate.

Pinto et al. (2008) administered omeprazole 4 mg/kg bwt orally for 31 consecutive days to verify its efficacy for healing gastric ulcers (score 1 to 4 in Murray et al., 1987) on the nonglandular mucosa of three ponies. Three other ponies with ulcers were controls, and were managed similarly except for therapy. At the end of the treatment, gastroscopy showed that the three controls no longer had ulcers, but two animals treated with omeprazole had marked granulomatous tissue over the ulcerated area (Figs. 4-5). Histopathology revealed tissue necrosis, fibrinous-leukocyte exudates, and exuberant granulation tissue. One of the fragments had hyperplastic squamous epithelium within the ulcer. In addition, filamentous structures similar to bacteria and spores of *Candida sp* were observed. The animals remained symptom-free throughout the treatment. Local *Candida sp* colonization may have been due to nearly complete omeprazole-induced inhibition of gastric acid secretion. Prim & Vila (2002) described a case of oropharyngeal candidiasis in a patient aged 65 years given omeprazole 20 mg daily. There are no reports of granulomas following the use of omeprazole in equid species, but findings suggesting enterochromaffin-like cell hyperplasia has been noted, and gastric carcinoid tumors has

been observed in rats (Hoogerwerf & Pasricha, 2001). The exuberant granulomatous tissues were regressing gradually until complete disappearance from 60 to 100 days after their identification (Fig 6).

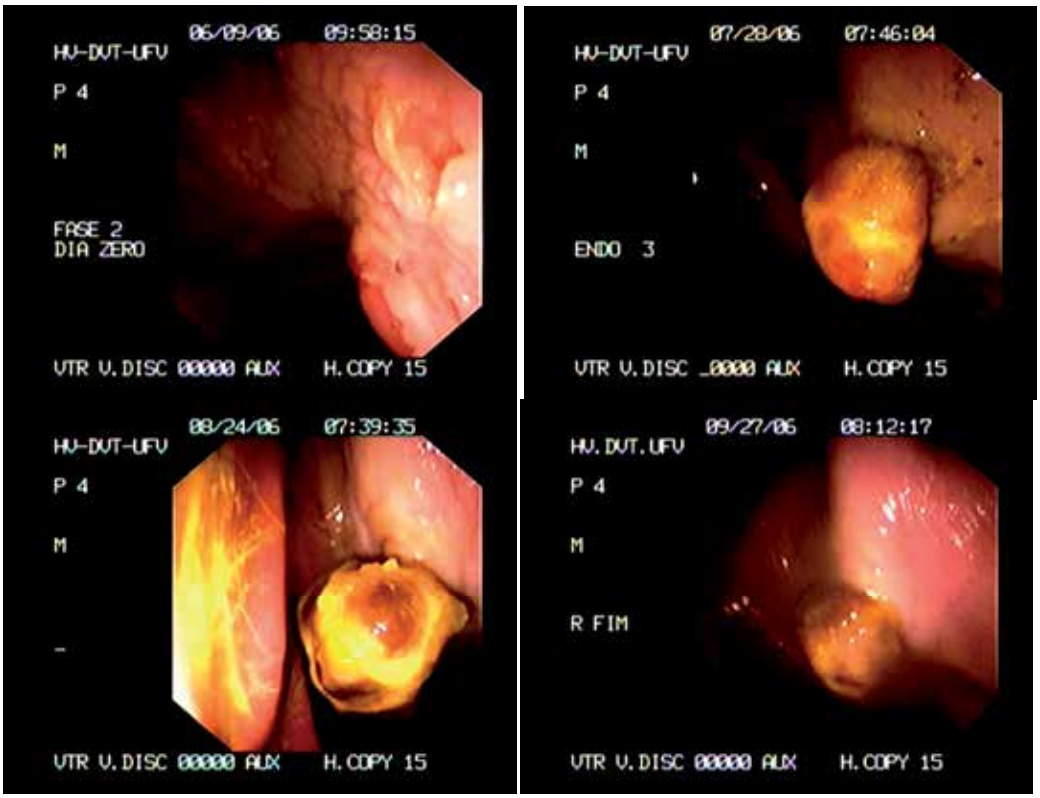

Fig. 4. Exuberant granulomatous tissue in a pony with gastric ulcers treated with omeprazole 4 mg/kg bwt orally for 31 days. The images show the monthly monitoring of the injury.

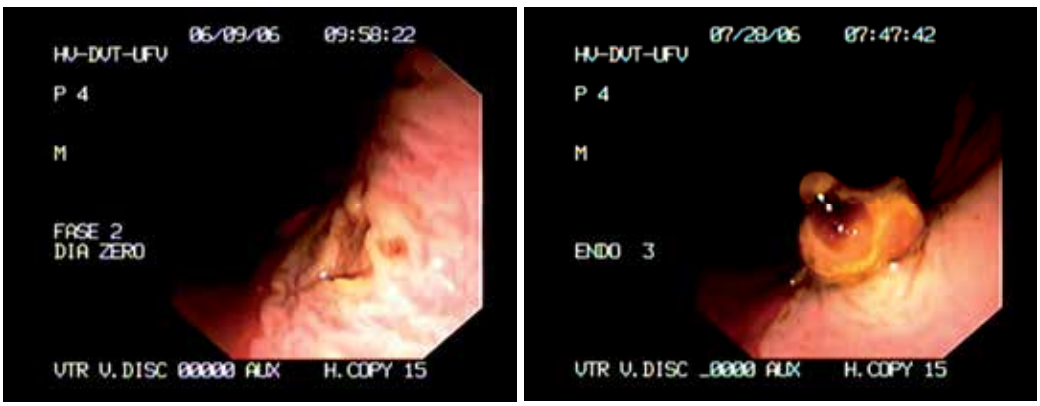

Fig. 5. Another granulomatous tissue at the ulcer on the nonglandular gastric mucosa, in pony treated with omeprazole (4 mg/kg bwt orally for 31 days).

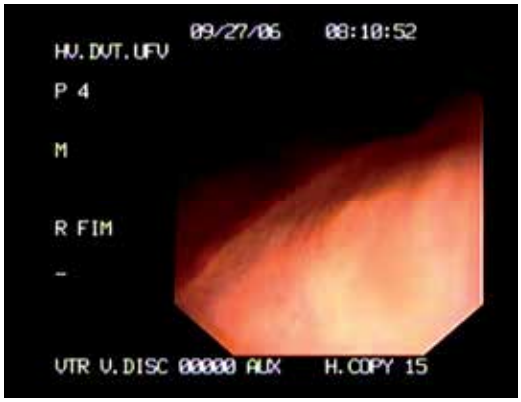

Fig. 6. Aspect of nonglandular gastric mucosa shown in Fig. 5, with complete disappearance of the granulomatous tissue about three months after its identification.

5. Conclusion

Nonsteroidal anti-inflammatory drugs are very useful for treating many clinical and surgical conditions in horses and ponies. Despite the significant amount of research, there is no single NSAID that is considered completely safe. Therefore, while the ideal drug is not discovered, careful measures (dose, application interval, and duration of treatment) should be taken when using these drugs, which are considered relevant risk factors for the gastric ulceration syndrome.

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