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Environmental Health Risk

Hazardous Factors to Living Species

Edited by Marcelo L. Larramendy and Sonia Soloneski

ENVIRONMENTAL HEALTH RISK - HAZARDOUS FACTORS TO LIVING SPECIES

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Notice

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Meet the editors

Marcelo L. Larramendy, Ph.D., serves as Professor of Molecular Cell Biology at the School of Natural Sciences and Museum (National University of La Plata, Argentina). He has been appointed senior researcher of the National Scientific and Technological Research Council of Argentina. He is also former member of the Executive Committee of the Latin American Association of Environmental Mutagenesis, Teratogenesis and Carcinogenesis. He is author of more than 470 contributions, including scientific publications, research communications and conferences worldwide. He is recipient of several national and international awards. Prof. Larramendy is a regular lecturer at the international A. Hollaender Courses organized by the IAEMS and is former guest scientist at the NIH, USA, and University of Helsinki, Finland. He is expert in genetic toxicology and is, or has been, referee for more than 30 international scientific journals. He is also member of the International Panel of Experts at the International Agency for Research on Cancer (IARC, WHO, Lyon, France) in 2015 for the evaluation of DDT, 2,4-D, and Lindane. Presently, Prof. Dr. Larramendy is Head of the Laboratory of Molecular Cytogenetics and Genotoxicology at the UNLP.

Sonia Soloneski is Ph.D. in natural sciences and professor assistant of molecular cell biology at the Faculty of Natural Sciences and Museum of La Plata, National University of La Plata, Argentina. She became a member of the National Scientific and Technological Research Council (CONICET) of Argentina in genetic toxicology field. Presently, she is member of the Latin American Association of Environmental Mutagenesis, Teratogenesis and Carcinogenesis (ALAMCTA), the Argentinean Society of Toxicology (ATA) and the Argentinean Society of Genetics (SAG). She has authored more than 290 scientific publications in the field, including scientific publications in research papers, reviewed journals and conferences worldwide. She is a referent for issues related to genetic toxicology, mutagenesis and ecotoxicology field.

Contents

Preface

Nowadays, health problems associated with the environment continue to be a major source of concern worldwide. Our global society needs to establish actions that will considerably reduce the real and potentially hazardous factors in the environment that can result in health risks for humans and other living species. Despite the progress in science, technology, and industrialization making immense positive contributions to health, the interaction between environmental risk and health is often intricate and can involve a variety of not only social and economic but also lifestyle factors. Public health depends on the good quality of environmental factors such as air, water, soil, and food, among others. However, the principal or even the ultimate challenge in environmental public health is to understand the risks that modern societies possess. They are exposed to heterogeneous xenobiotics, which are continuously released into human habitats, deliberately, inadvertently, or by nonregulated industrial discharges.

Environmental health, as a concept, is not easy to define. In this regard, according to the World Health Organization, the definition of *environmental health* is "all the physical, chemical, and biological factors external to a person, and all the related factors impacting behaviors. It encompasses the assessment and control of those environmental factors that can potentially affect health. It is targeted toward preventing disease and creating health-supportive environments…" However, for the National Environmental Health Association, this concept refers to "the protection against environmental factors that may adversely impact human health or the ecological balances essential to long-term human health and environmental quality, whether in the natural or man-made environment." Finally, the definition of *environmental health* according to the National Institute of Environmental Health Science also involves the criterion that "the social environment encompasses lifestyle factors like diet and exercise, socioeconomic status, and other societal influences that may affect health."

In general terms, our health and the health of many other species are negatively affected by five broad categories of environmental hazards, namely electromagnetic fields (produced by power lines, electrical wiring, appliances, cell phones, computers, and televisions), radiation (including nuclear fallout from weapons testing, fission materials from nuclear power plants, leaking radioactive disposal sites, flying at high altitudes, and mammograms and x-rays), toxic chemicals (such as some organochlorines, phthalates, polybrominated flame retardants, perfluorinated substances, bisphenol-A, and several toxic metals, among others, which have been shown to have endocrine-disrupting properties), and, finally, soil mineral depletion as a complex environmental hazard.

By definition, *risk assessment* can be considered the quantitative and/or qualitative determination of a risk related to both a well-defined situation and a recognized threat (i.e., a hazard), and thus, health risk assessment includes variants such as risk as the type and severity of response, with or without a probabilistic context. In this context, risk-based methods play a strategic role in identifying and ranking the adverse responses or the structure of the effects of exposure against environmental factors.

This book, *Environmental Health Risk - Hazardous Factors to Living Species*, is intended to provide a set of practical discussions and relevant tools for making risky decisions that require actions to reduce environmental health risk against environmental factors that may adversely impact human health or ecological balances. We aimed to compile information from diverse sources into a single volume to give some real examples extending concepts of those hazardous factors to living species that may stimulate new research ideas and trends in the relevant fields.

Although we are dealing with many diverse topics, we have tried to compile this "raw material" into three major parts in search of clarity and order. First, in General Background and Remarks, readers will find two chapters with background information about the nature of pesticides, their history, their classification, their risks, and their effects on health and environment, as well as general aspects of environmental pollution by metals as a serious problem in many areas of the planet, with a special emphasis on the assessment of metal bioaccumulation and toxicity. Second, in Validated Methods and New Models of Evaluation, we have included two chapters on, first, problems with the use, analysis, and interpretation of results obtained through the comet assay in human buccal cells and, second, a new system of hierarchical limit values to protect soil environments, food chains, and human health against contamination that reveals the areas where the soil does not meet the soil quality standards and where the human health is at risk. Finally, Specific Evaluation of Some Environmental Toxicants encloses six chapters describing specific examples of toxicants with potential risks to living species, such as humans. The first chapter describes the genotoxic properties of bisphenols and mycotoxins, which are prominent environmental contaminates and potential carcinogens. The second chapter reviews the toxic effects of different phthalate esters and bisphenols and their availability in the environment, mechanisms and modes of actions, biotransformation, and reproductive effects. Then, the next two chapters describe, respectively, the relationship between chronic exposure to coal and coal ash particles and cancer and a novel procedure for the early detection of previous asbestos exposure, its relation with mesothelioma, and chemoprevention of asbestos-related cancers. The last two chapters review, respectively, the accumulation of organic pollutants in the environment, with a special reference to the mechanism in the causation of type 2 diabetes mellitus, and risks that amoxicillin poses to the environment, focusing on detailed ecotoxicity testing using a wide range of aquatic organisms to fully understand the environmental toxicity of this antimicrobial product, and how it may affect both aquatic and terrestrial environments.

Many researchers have contributed to the publication of this book. Given the fast pace of new scientific publications shedding light on the matter, this book will probably be outdated very soon. We regard this as a positive and healthy fact. The editors hope that this book will continue to meet the expectations and needs of all those interested in the environmental risk assessment field of study.

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Section 1

General Background and Remarks

Chapter 1

Pesticides, Environmental Pollution, and Health

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Additional information is available at the end of the chapter

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Abstract

In recent years, people have been exposed to several types of substances with broad spectrum due to the rapidly evolving technology. One of these chemical substance groups are pesticides. Pesticides have been an essential part of agriculture to protect crops and livestock from pest infestations and yield reduction for many decades. Despite their usefulness, pesticides could pose potential risks to food safety, the environment, and all living things. Concern about the environmental impact of repeated pesticide use has prompted research into the environmental fate of these agents, which can emigrate from treated fields to air, other land, and water bodies. The importance of agricultural pesticides for developing countries is undeniable. However, the issue of human health and environmental risks has emerged as a key problem for these countries in accordance to a number of studies. In the last five decades, pesticide usages increased the quantity and improved the quality of food. However, with the increasing amounts of their usage, concern about their adverse effects on nontarget organisms, including human beings, has also grown. The purpose of this publication is to explain the nature of pesticides and their history, classification, risks, and effects on health and the environment.

Keywords: organic pollution, health concern, environment, pesticides, environmental pollutant

1. Introduction

In the last three decades, there has been an increasing global worry over the public health impacts attributed to environmental pollution. It was the industrial revolution that gave birth to environmental pollution as we realize it today. Populations of developing countries are particularly vulnerable to toxic pollution resulting from industrial processes.

Pollution is the introduction of contaminants into the environments that cause harm or discomfort to other living organisms or damage the environment, which can come in the form of chemical substances or energy, such as heat, light, or noise. Pollutants can be naturally occurring energies or substances but are considered contaminants when in excess of the natural levels. Santos divided environmental pollutants into biodegradable and nonbiodegradable ones. Biodegradable pollutants can be broken down and processed by living organisms, including organic waste products, phosphates, and inorganic salts. Nonbiodegradable pollutants cannot be decomposed by living organisms and therefore persist in the ecosphere for extremely long periods of time. They contain metals, plastics, glass, pesticides, and radioactive isotopes [1].

In recent years, people have been exposed to several types of substances with broad spectrum due to the rapidly evolving technology. Technology has brought us clear conveniences, and thousands of chemicals produced in different areas are up on the market every year. One of these chemical substance groups are pesticides [2,3].

1.1. Pesticides

Through the ages, it seems increasingly that people find a need to minimize the damage of pests with the use of pesticide chemicals and by other means [4]. Of the many examples of how pests have impacted human society, one of the most infamous is the Black Plague in Europe in the 14th century, when millions of people died from mysterious diseases. At that time, the diseases were believed to be because of God's punishment. A number of reports in the literature, art, and public statues certify the fear and destruction of those epidemics. Many years later, scientific data proved that a bacterial disease spread by rat fleas was the cause of the plague, which ruined almost the whole of Europe. Today, this disease, known as bubonic plagues, can be easily treated if it is properly diagnosed. Hence, controlling rodents including rats as well as fleas can reduce the relative frequency of the occurrence of diseases [5].

Ireland's potato crop destruction by a pest in the 19th century is another story. At that time, late blight, a plant disease, wasted potatoes in Ireland. Up to 1 million Europeans starved to death during the Great Irish Famine of 1845 to 1847. Late blight is still one of the major potato pathogens that chemists aim to synthesize new pesticides against [5].

Pesticides are chemical substances used on agricultural land but also in private gardens, along railways, and in other public areas [6]. The use of pesticides for crop protection is expected to increase based on a growing world population and the need for more food supplies. While pesticides increase agricultural production, bioaccumulation through the food chain can eventually become a risk to mammals because pesticides induce certain negative effects [7–10]. Some parts of pesticides sprayed on crops will remain in farmland, but some of them will enter the surrounding soil, water, and air [11,12]. As artificial organic compounds, pesticides can remain in the environment for many years and may be transported over a long distance [13]. Pesticide residues in soil and water are significant environment threats and have been classified as carcinogen pollutants in many countries [14,15]. Hence, the excessive application of these compounds over the past half-century has posed serious risks to human health [16,17].

There have been numerous reports regarding pesticide residues detected in grains [8], milk [18], vegetables [19], and fish [20].

Although the benefits of pesticides have been immense, humans and other living organisms are often exposed to them in the environment [21]. Several epidemiological studies reported in the last two decades suggest harmful effects of pesticides on human health, including a possible relationship between pesticide use and cancers, such as non-Hodgkin's lymphoma, leukemia, and various types of solid tumor [22–24]. Public health concerns regarding the improper use of pesticides and poison have increased in recent years. To date, certain countries, regions, and international organizations have established maximum residue limits (MRLs) for foodstuffs. Additionally, national food monitoring programs for pesticides have been enacted worldwide [7–9] to ensure consumer health, improve the management of agricultural resources, and prevent economic losses [10].

Despite the adoption of the International Code of Conduct on the Distribution and Use of Pesticides (Code of Conduct) [25], the strict control of banned/legacy pesticides has been proven difficult in many developing countries. This could be attributed to weak regulations on importation and use of dangerous substances and the activity or absence of control agencies at international borders [26]. This scenario has led to the proliferation of banned chemicals in local markets located in agricultural areas, making large quantities of pesticides available to rural farmers, which eventually could pose potential threats to the environment and the health of the people [27–29].

The existence of persistent chemical substances in the environment and their effects on the wildlife and mankind has raised a serious global concern. In this case, we need to mention the risks of pesticides.

Pesticides are poisons and can be hazardous. Fortunately, people are becoming more aware of their danger, and even producers are trying to produce safer chemicals and better application methods. Even the awareness is improving for risk-benefit ratio side; the job has not been completed yet. Misuses of pesticides still occur. On the contrary, even if they are used correctly, some pesticides can harm nontargeted living organisms and the environment. Just as the benefits of pesticides are real, so are the risks. The purpose of this publication is to explain the nature of pesticides and their history, classification, risks, and effect on health and the environment [4].

2. Historical perspective

Pesticides are used for a number of decades. People have been fighting with pests for centuries [5]. Chemical experiments during the late 19th and early 20th centuries allowed human beings to develop modern pesticides. Producing new mixtures with a right proportion made it possible to control unwanted organisms. Paris green was one of the first chemical pesticides produced, marking the beginning of chemical insecticide use in the United States in 1867 [30]. By the late 19th century, U.S. farmers were using calcium arsenate, nicotine sulfate, and sulfur to control insect pests in field crops, except Paris green [4]. Since the middle of the 20th century,

these chemicals have been widely used to control pests [31,32]. Ancient Romans controlled weeds with salt and killed insect pests by burning sulfur [4]. Sulfur, also known as brimstone, was used by pagan priests 2000 years before the birth of Christ. Additionally, sulfur was used to purify a sick room and cleanse its air of what was believed to be evil. In the 1600s, ants were controlled with mixtures of honey and arsenic. Early plant-derived insecticides included nicotine to control aphids, hellebore to control body lice, and pyrethrins to control a wide variety of insects [5].

The availability of dichlorodiphenyltrichloroethane (DDT), starting in 1945 for civilian/agricultural usage, opened a new era of pest control, leading to not only its extensive usage but also the development of numerous other synthetic organic insecticides. DDT was especially favored for its broad-spectrum activity against insect pests of agriculture [4]. Unfortunately, its properties of persistence, along with its broad-spectrum biological activity against pests and beneficial insects alike, made it a poor choice for use in agriculture after World War II [33]. Except DDT, aldrin, BHC, endrin, dieldrin, and 2,4-D began to be used after World War II. These new chemicals were effective, inexpensive, and enormously popular [34]. However, with continuous usage of pesticides, some pests developed resistance to them. As a result, nontarget plants and animals were damaged; surprisingly, pesticide residues were observed to be present in unexpected places. Rachel Carson's book, *Silent Spring* in 1962, shook public confidence in pesticide usage. Carson presented a harsh picture of environmental consequences of careless pesticide employment. Although her report has been strictly criticized, Carson pointed out the risks of pesticides more than anyone [4].

As chemical controls became more and more common in agricultural, public health, and nuisance applications throughout the first half of the 20th century, a myriad of problems were being discovered. Chemically reliant methods had quickly resulted in pesticide resistance within the target species, harm to nontarget species, food contamination, water contamination, overall ecological degradation, and public health problems [30].

3. Classification of pesticides

The word "pesticide" is an umbrella term for all insecticides, fungicides, herbicides, rodenticides, garden chemicals, wood preservatives, and household disinfectants that may be used to kill some pests. Pesticides have different identities and physical and chemical properties. Synthetic pesticides are classified based on various ways. In general, there are three main ways to classify them: classification based on the (i) mode of action, (ii) targeted pest species, and (iii) chemical composition of pesticides [35].

3.1. Classification based on the mode of action

Pesticides are classified based on the way they act to bring about the desired effect in this classification. Under this type of classification, pesticides are classified as nonsystemic and systemic pesticides. Nonsystemic pesticides are those that do not appreciably penetrate plant tissues and consequently not transported within the plant vascular system. On the contrary,

systemic pesticides are those that effectively penetrate plant tissues and transported within the plant vascular system to bring about the desired effect [36].

3.2. Classification based on the targeted pest species

Classification by target pest is perhaps the most familiar. For example, insecticides are pesticides that target insects, and herbicides target plants. The others are rodenticides, fungicides, acaricides and miticides, molluscicides, bactericides, avicides, and virucides.

3.3. Classification based on the chemical composition

In this type of classification, pesticides are characterized regarding their chemical nature and active ingredients. This is the most useful one for researchers studying the field of pesticides and the environment, because it is this kind of classification that gives the clue of the efficacy and physical and chemical properties of the respective pesticides and precautions that need to be taken during application and the application rates, the knowledge of which is important in the mode of application [37].

According to chemical properties, pesticides can be generally divided into about seven types, including organochlorines, organophosphorus, carbamates, pyrethroids, amides, anilins, and azotic heterocyclic compounds. Organochlorine chemicals are organic compounds with five or more chlorine atoms. Organochlorines were the first synthetic organic pesticides to be used in public health and in agriculture. These pesticides generally have a steady chemical structure and often accumulate and persist in the environment. Most of them are widely used as insecticides for the control of a wide range of insects. Organochlorine insecticides act as nervous system disruptors leading to convulsions and paralysis of the insect and its eventual death. They can cause serious endocrine disorders in mammals, fish, and birds, so most of them have been banned in agriculture worldwide [36,38]. Organophosphates are another type of highly toxic pesticides that contain a phosphate group and occupied up to 48.6% of all pesticides in 1997 [39]. The importance of synthetic organophosphates increased considerably during World War II with their use as warfare materials. Since then, these pesticides have been used in agriculture, industry, cosmetics, medicine, and many other areas [40,41]. These chemical compounds inhibit the acetylcholinesterase enzyme, which hydrolyses acetylcholine in the nervous system of a number of species, including humans [42]. Although they are easier to be degraded than organochlorines, organophosphate pesticide residues is one of the biggest threats to the ecosystem and food industry because their acute toxicities are irreversible [43].

Many people are exposed to pesticides occupationally, and pesticide self-poisoning is a major public health problem [44]. Annually, 3 million cases of acute poisoning have been reported from pesticide exposure, resulting in the deaths of 250 to 370,000 people every year [45,46]. Therefore, the usage of organophosphates has been restricted or banned all over the world [43].

Carbamates are organic pesticides, reversibly inactivating the enzyme acetylcholinesterase; these pesticides are derived from carbamic acid. The cholinesterase inhibition of carbamates differs from that of organophosphates in that it is species specific and is reversible [35,47]. Organochlorines, organophosphates, and carbamates are three generations of traditional

highly toxic pesticides, and the later developed pyrethroids, anilines, amides, and azotic heterocyclic compounds are generally less toxic [48].

Pyrethroids are synthetic analogues of the naturally occurring pyrethrins, a product of flowers from pyrethrum plant (*Chrysanthemum cinerariaefolium*), and were detected in the 1980s to mimic the insecticidal activity of the natural pyrethrum. Pyrethroids are acknowledged for their fast knocking down effect against insect pests, facile biodegradation, and low mammalian toxicity [37]. These pesticides are nonpersistent sodium channel modulators and are much less toxic than carbamates and organophosphates to mammals. Therefore, the usage of pyrethroids has been increased greatly in the last 30 years. Unfortunately, pyrethroids are highly toxic to aquatic organisms such as mollusks, fish, and arthropods [49,50].

Amide herbicides, such as acetochlor, butachlor, and metolachlor, are widely used in recent years. However, butachlor can persist in the environment for up to 10 weeks, and what's even worse is that butachlor and metolachlor have been identified as mutagens. Another type of pesticides is aniline and dinitroaniline. Trifluralin and pendimethalin are widely used in this group of pesticides. These pesticides show high toxicity to aquatic organisms and they can impair the thyroid gland and liver. Hence, these two aniline herbicides have been banned in many European countries. Nitrogen-containing heterocyclic compounds, especially for imidazole and triazole heterocyclic chemicals, have become the hotspot for new pesticide development. In the last 10 years, they occupied no less than 70% of all the newly developed chemical pesticides [48].

Except for these classifications, pesticides are classified according to the mode of formulation, activity spectrum, and toxicity level. According to the mode of formulation, pesticides are classified into six groups as wettable powders, emulsifiable concentrates, baits, granules, dusts, and fumigants. In active spectrum, pesticides are classified into two groups as broad-spectrum pesticides and selective pesticides. Broad-spectrum pesticides are designed to kill a wide range of pests and other nontarget organisms. On the contrary, selective pesticides are designed to kill only specific pests. In toxicity level, the World Health Organization (WHO) has developed a classification system that group pesticides according to the potential risks to human health and they are grouped into the following classes: class Ia=extremely hazardous, class Ib=highly hazardous, class II=moderately hazardous, class III=slightly hazardous, and class IV=products unlikely to present acute hazards in normal use [37].

4. Pesticide pollution

Since the middle of the 19th century, pesticides have been commonly used to control pests [31,32] causing a widespread release of these xenobiotics into the environment [51]. The intensive use of pesticide leads to an increased risk of contamination of the environment and harmful effects on biodiversity, food security, and water resources [52,53].

Pests, such as insects, weeds, and plant diseases, are an ongoing challenge to agricultural producers. Oerke [54] reported that, globally, an average of 35% of potential crop yield is lost

to preharvest pests. With the expected 30% increase of world population to 9.2 billion by 2050, there is a projected demand to increase food production by 70% according to Popp et al. [55]. Although nonpesticidal tools have a vital role, there will be a continuing need for pesticide-based solutions to pest control and food security in the future [55,56]. **Figure 1** shows the average pesticide use intensity (kg ha^{-1} yr^{-1}) on the cultivable and permanent cropland worldwide. High use intensity countries above 10 kg ha^{-1} yr^{-1} include Surinam, Malta, Columbia, Palestinian, Japan, Korea, Chile, and China [57]. **Figure 2** presents that pesticide sales are increasing in Europe, Asia, and Latin America [58,59].

Figure 1. Average annual pesticide use intensity (kg ha^{-1} yr^{-1}) on arable and permanent cropland from 2005 to 2009. Data are from FAO [57].

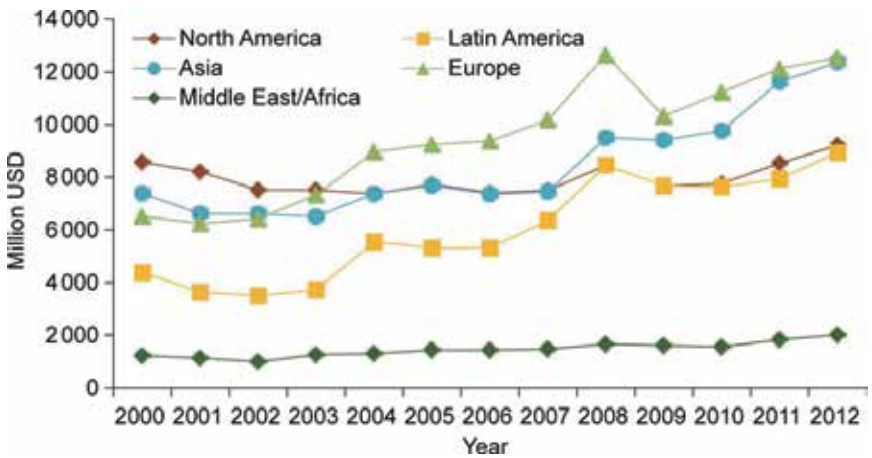

Figure 2. Annual pesticide sales by geographic regions. Data are from FAO [58].

More than 500 different pesticide formulations are being used in our environment, mostly in agriculture [60]. In the past five decades, pesticide usages increased the quantity and improved the quality of food. However, due to their usage with increasing amounts, the concern about their harmful effects on nontarget organisms, including human beings, has also been growing. Nontarget pesticide poisoning has been reported from fish, birds, and humans [61]. Although it is estimated that less than 0.1% of pesticide applied to crops actually reaches the target, the rest of it enters the environment [62]. Additionally, many pesticides can persist for long periods in an ecosystem; organochlorine insecticides, for instance, are still detectable in surface waters 30 years after their use and had been banned [63]. In the food chain, they meet with nontarget organisms, including mankind. They accumulate in the body tissues of organisms and cause a number of health problems [64,65].

Pesticides and herbicides are heterogeneous chemicals used widely in agriculture. Their design as bioactive molecules to exterminate different animal, vegetal, or fungal species implies that they are toxic by definition. Due to this toxicity, their use is regulated in the European Union. Depending on the water solubility and polarity of each specific pesticide, they can follow different pathways to reach water bodies once applied in the crop fields. In the case of surface waters, the most common entry pathway for these pollutants is runoff from agriculture lands after precipitation or irrigation [66]. Pesticides could influence biological communities in lakes, forcing changes from a clear-water, macrophyte-dominated state to a turbid state due to their effect on zooplankton or macrophytes [67,68].

As explained above, both point and diffuse pollution sources of pesticides, herbicides, and polycyclic aromatic hydrocarbons (PAHs) are usually anthropogenic. Therefore, it is expected that the concentration of some of these compounds in surface water is related to human activities that take place in the surroundings. The proportion of cultivated lands around the lake and the agricultural pressure and intensity are especially relevant, as certain substances such as herbicides and pesticides have a close relationship with agriculture. In fact, it has been observed that land uses are strongly related to nutrient concentrations in surface waters [69] and PAH concentrations in wetland sediments [70]. On the contrary, the distance between lakes and point or diffuse pollution sources such as urban areas, thermal power plants, industries and roads could also be related to the amount of these chemical compounds detected in aquatic ecosystems [68].

Pesticide fate in the environment is characterized by a number of complex processes occurring in different environmental compartments, such as air [71], soil [72], plant [73], and surface and groundwater [53,74].

Pollution due to the uncontrolled use of pesticides has become one of the most alarming challenges when pursuing sustainable development. Although pesticides are directly applied in soils and plants, only 1% of pesticide sprayed is delivered to the intended target. An accidental release of pesticides due to leaking pipes, spills, waste dumps, underground storage tanks, and groundwater may lead to their persistence in the environment for a long time (due to long half-lives). For proper management of pesticides, one needs to accurately assess the status of their contamination in soil, water, and air [75,76].

Soil is a major reservoir for a variety of pollutants [77] and is a secondary emission source of contaminants to surface water, groundwater, and air [78]. Multiclass environmental endocrine disruptor compounds (EDCs), such as organochlorine pesticides (OCPs) phthalate esters (PAEs), and polybrominated diphenyl ethers (PBDEs) may coexist in soils and accumulate in crops and human bodies through food chains, posing risks to human health and the ecosystem [79]. In addition, soil plays an important role in pesticide residue in plants. There are two pathways for pesticide transfer between the plants and their planted soils. First, most of pesticides could shift or fall onto the soil when pesticide is applied onto plants. Next, most of the deposited pesticides on the plant could be washed off by rainfall to the soil. Second, the residues of adsorbed pesticides in soil, especially for organochlorine pollutants, remain as contaminants in the environment because of their long-term persistence and mobility, and they could enter into food again via the plant uptake effect [10,80,81].

Persistent organic pollutants (POPs), such as OCPs, are ubiquitous contaminants in different compartments of the environment [82,83]. Although a number of countries have been removed from the circulation of the usage of POPs for nearly 30 years, these synthetic chemicals are found in nature at considerable levels worldwide due to their persistence. These substances are mainly generated by anthropogenic processes and can be introduced into the environment through various routes. These pesticides are toxic, carcinogenic, and mutagenic features. They are extremely hazardous for the both biota and environment [84]. Hence, the investigation of POPs in aquatic environments is needed to provide relevant information on the anthropogenic impact on the environment, and concentrations serve as an indicator of contaminant load [85, 86].

Pesticides are major components of the modern agricultural production because of their reliability and high capability for crop protection against pests [87]. Approximately 5 billion kilograms of pesticides are applied worldwide per year, which can have serious effects on biodiversity, nontarget organisms, and the food chain, posing high risks to the environment and human health [88]. In rural areas of developing countries, 3 million farmers suffer annually from serious pesticide poisoning and 25 million farmers suffer from mild poisoning, resulting in approximately 180,000 fatalities among agricultural workers annually [89] because of incorrect perceptions, lack of knowledge, regulation, and education among farmers [90,91].

Unsafe pesticide use or misuse in developing countries includes the use of pesticides banned by the local government [92], lack of self-protection [93], incorrect pesticide storage [94], overspraying [95], improper handling of pesticide containers [96], and, in extreme cases, reuse of washed pesticide containers as containers for food and drinking water (as reported by 35.4% and 77.2% of farmers in Nigeria and Ethiopia, respectively) [97]. The local authorities, the WHO, the Food and Agriculture Organization (FAO), and various nongovernment organizations that focus on low- and middle-income countries (e.g. China, India, Vietnam, and African countries) have taken initiatives to improve the protective behaviors of farmers in pesticide use, including personal and environmental protection through education [98] and legislation and community intervention [99], although the results were often unsatisfactory [100]. The factors that affect farmers' behavior in pesticide use are far more complex than expected.

Pesticide use can be influenced by age [101], gender [102], perceptions [90,91], level of knowledge, pesticide retailers [103], and even cultural or planting differences [92].

The chemical pesticide provides a necessary guarantee for the output increase, but pesticide abuse has led to daily worsening of the ecosystem of agricultural lands [104,105]. The use of large amount of pesticide is the main reason for agricultural pollution [106].

5. Effect of pesticides on health and the environment

The importance of agricultural pesticides for developing countries is undeniable. However, the issue of human health and environmental risks has emerged as a key problem for these countries in a number of studies [107–112]. Attention to the impacts of pesticide use on the environment and ecosystems has grown since the book *Silent Spring* was published in 1962. Extensive published literature has well documented the impacts of pesticide use to the ecosystem and human health [55]. Pesticides can move off-site to contaminate surface water and leach to groundwater. Damage to nontarget organisms and pollution to the soil and air are well documented [59].

The released pesticides into the environment and their impacts on many species have been known for a long time. The senseless and widespread use of OCPs between the 1960s and the 1970s caused a striking decrease in wildlife populations nearly all over the world [113]. DDT, dieldrin, and other toxic OCPs affected birds and other wild species during that time and have been finally banned from agricultural use. Since then, however, decline in birds, wild bees, and aquatic organism populations have been continuing [114]. This could be linked to usages of newly synthesized pesticides that are present in every kind of habitat on the world. Pesticides are still being discovered in marine, freshwater, and terrestrial communities [115].

Insecticides may kill not only the target species but also other invertebrates on which birds rely on for their food. In addition, herbicides are designed to control weed species and they can also kill many other plant species in fields, including the essentially beneficial species, which give both shelter and food for the members of wildlife. Amphibians are now considered the most threatened and rapidly decreasing species on Earth. Brühl et al. [116] suggested that frogs are sensitive to the toxicity of pesticides that are currently used in agriculture.

In addition, pesticide factory workers and agricultural farm workers have high risk to pesticide direct exposures [88]. In recent years, pesticide residues in food have become a focus for food safety and trade. Quarantine regulations sometimes require pesticide treatment of food shipments to prevent the establishment of exotic pests. Nonetheless, local consumers and international trading partners increasingly demand food that is free from unsafe pesticide residues. Therefore, many countries have initiated programs to monitor pesticide residues in food. In addition, many countries are implementing programs to reduce the use of pesticides and thereby minimize pesticide impacts [59].

Intensively used pesticides, despite their ability to protect crops, threaten the environment and human health [88,117]. Besides, the use of pesticides also results in residue problems. Pesticide

residue is defined by the WHO as any substance or mixture of substances in the food of either humans or animals that is caused by the use of pesticides and any specified derivatives, such as degradation and conversion products, reaction products, metabolites, and impurities that are considered toxic [118].

5.1. How are we exposed to pesticides?

People who live in agricultural areas have a high disclosure to pesticides by inhalation of pesticide spray blow in urban areas and parks or in the houses after breathing contaminated air. Farmers and their families can have a higher exposure to pesticides than the general population. Besides, when nursing mothers and pregnant women are exposed to pesticides, their children may also be exposed. Some pesticides can pass through the placenta to the developing fetus in the womb and through breast milk to the nursing infant [119].

These "poisons by design" are prevalent and serious occupational hazards faced by farmers and agricultural workers [117]. The high levels of occupational exposure to pesticides are correlated with low educational levels, which would preclude the ability of farmers to follow the hazard warnings developed by the chemical industries and agencies [120]. Tragedies, such as acute and chronic intoxication and, in some extreme cases, suicide, have frequently been reported, especially in rural regions [117,121]. The lack of a legislative framework regulating the use of pesticides also contributes to the high incidence of poisoning in developing countries [117].

The present data seem to be too limited to analyze the full health effects of pesticide referable chronic exposures. On the contrary, suicide commitments in 2002 using pesticides resulted in 258,000 deaths [122]. In 2002, intentional poisoning from pesticides accounted for approximately one third of the world's suicides, and in 2004, 71% of the unintentional poisonings were considered preventable by improving chemical safety methods [45]. The groups most at risk from unintentional pesticide poisoning are children, especially those between ages 0 and 4 years [123]. Human deaths induced by insecticides were mainly because of ingestion of OPPs. OPP poisoning is evident for "cholinergic syndrome". The symptoms in this syndrome are headache, slurred speech, coma, blurred vision, convulsions, blockage of the respiratory center, and delayed neuropathy [124]. In this sense, survivors of acute OPP poisoning may suffer long-term adverse effects to the nervous system [119].

Data show that there is a positive relationship between high pesticide exposures and occurrence of several types of cancer (e.g. prostate and lung) as well as the increase of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease. There are also evidences that pesticides may impair endocrine function and the immune system. Although the mechanisms of such failures are not completely comprehended, there are some clear evidences showing the disruptions in enzymatic function and signaling mechanisms at cellular levels. DNA-based toxicity studies also indicate that pesticides affect gene expression and this may transferred to generations through epigenetic inheritance [119].

Organophosphate compounds (OCs) and OCPs have been widely used as pesticides in agricultural productivity. However, they have been proven to be extremely hazardous for

human health. OCs and other pesticides may persist on Earth for a long time, adverse to the ecology. Therefore, pesticide residues in vegetables, fruits, water, and on Earth are drawing more and more attention [125–128]. Applied pesticide residues may persist within the tissues or on the surface of the crops when we buy from market. Scientists have developed a variety of techniques to both determine and quantify the pesticide levels in food. Data obtained from these studies suggested that nonstop monitoring is needed to ensure that pesticide residues do not exceed their acceptable values [99]. Most countries, on either a regional or a national basis, maintain a threshold maximum residue level (MRL) for each substance, above which the foodstuff is thought unacceptable for human consumption [119].

OCPs are among the substances restricted or banned globally under the Stockholm Convention on Persistent Organic Pollutants [129]. These compounds are environmentally persistent [130], toxic, and apt to bioaccumulation [131] and have adverse effects on animals and humans [132]. Some developing countries are still using these compounds because of their low cost and versatility in industry, agriculture, and public health [133]. Consequently, environmental problems associated with toxic contamination in these countries are of great concern [134]. As a result, organochlorines in various environmental media have received much attention [133]. They are well-known anthropogenic and lipophilic pollutants due to their high bioaccumulation potential in fatty tissues of living organisms [135]. Although these substances are generally stored in the fat and muscles of the animals, some can also be found in the brain, lungs, liver, and other offal. Additionally, because milk and other dairy products contain a range of fat, these foods may also contain a number of pesticides. This is important because cow's milk is one of the indispensable components of human diet [119,136,137]. OCPs can enter animal tissues through different pathways of ingestion, dermal contact of dust, and inhalation [138].

In the past decades, attention was focused on the determination and pollution levels of OCPs in human blood serum, maternal and cord serum, adipose tissue, human milk, and hair and other available tissues to study human exposure and assess health risk [139]. Human exposure to OCPs is through many routes: breathing OCP-contaminated air, working in or living beside OCP factories, drinking and taking a bath with OCP-polluted source water, eating vegetables and grains containing OCP residues, and eating especially fish and animal meats [140]. OCPs accumulated in the human body could cause various negative effects such as immunological function damage, endocrine disruption, female spontaneous abortions and preterm, and children neurodevelopmental delays [141].

Several studies showed that cancer risks could be induced by OCP exposures [142]. At the same time, OCPs could be transferred from maternal to fetal tissues through placenta and from mother to infant through breast milk. Exposure to OCPs could also lead to some adverse effects on human productivity, including spontaneous abortions and preterm [143], delayed neurodevelopment during childhood [144], and reproductive disorders of man [145] and other negative effects. In the fetus, as the rapid growth and development occur during early development, the organs of the baby can be sensitive to the toxic substances; especially, the brain is more susceptible to neurotoxicants [146]. Increasing evidence suggests that prenatal pesticide exposure may have a permanent effect on children's behavior and intelligence. Besides, organophosphates are also hazardous compounds in the environment and public

health. When children are exposed to pesticides in various ways at a young age, there is an observed negative effect on the development of the central nervous system [147]. Developmental impacts were mainly described as behavioral or cognitive, particularly those related to attention-deficit disorders and motor skills [119].

Experimental research has shown that many pesticides are endocrine disruptors that can disturb the functioning of various hormones throughout the body [148]. The production of thyroid hormone is thought to be inhibited by substances such as cyhalothrin, amitrole, pyrimethanil, and fipronil. Other pesticides may also alter thyroid hormone levels and potentially cause thyroid disease. Experimental studies *in vitro* support observations that the balance of sex hormones can be disrupted by exposure to certain pesticides. There is also evidence that fertility of both women and men may be decreased with increased pesticide exposure [119].

Studies showed that there are evidences of pesticide exposure and disorders in both hormonal regulation imbalance and immune system activities. The statistical results are associated with pesticide exposure and occurrence of some diseases. This finding cannot be ignored. The mechanisms of pesticide-induced diseases are not yet fully understood, but we now know that some key enzymatic activities in main metabolic pathways and/or the permeability of the ion channels are affected by them [149].

Moreover, some people carry susceptibility genes to the health effects of pesticides, and for that reason, they are likely to be more at risk than others. The questions on these epigenetical differences and developing policy approaches to ensure a high level of protection for mankind may remain insurmountable for a long time. In the meantime, people will continue with the routine application of pesticides to get more crops. On the contrary, the next generations, even they if are not exposed to pesticides, may also be at risk to these diseases due to epigenetical inheritance [119].

As mentioned by Allsop et al., many synthetic pesticides used in agriculture are persistent and pervasive in the environment. As a result, mankind is exposed to the mixture of pesticides via the food consumed and the environment around. Evidences suggest that more exposure means more toxic effect we will face. Although assays have been made to describe the toxicity of these kinds of interactions, there are no validated international guidelines in assessing these risks. In this case, we need to essentially rethink and change our systems to get rid of the exposure of pesticides. We must protect the health of vulnerable groups as well as the general population and whole ecosystems [119].

Reducing the use of pesticide strategies will not help us protect human health, because there are enormous kinds of pesticides in the market to be sold. In this case, people need to go towards ecological farming. This is a critical act in avoiding all risks. Protecting crops via a multilevel approach will help us increase the heterogeneity of the agricultural areas and this will provide a natural habitat for pollinators and natural pest control species. Thus, a functional biodiversity can be created if we can achieve an active vegetation management. A variety of crop types and cultivars increase both the fertility of soils and resistance to pests. Natural

control agents, such as beneficial bacteria, viruses, insects, and nematodes, can be used in improving crop protection successfully [150].

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Chapter 2

Modelling in Metal Risk Assessment

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Additional information is available at the end of the chapter

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Abstract

Unique properties of metals that are largely different from the characteristics of organic substances should be considered in risk assessment. The bioavailability and toxicity of metals depend on their chemical speciation, that is, physical-chemical forms, in the environment, which is largely influenced by the environmental chemistry. Since metals in the environment are not always available while organisms have developed different processes to actively regulate the body burden, assessment of metal bioaccumulation might provide a better understanding of potential risks. Metal bioaccumulation is a prerequisite for metal toxicity, but is not the only determinant of metal toxicity. In addition to metal accumulation, metal toxicity is influenced by the subcellular partitioning of metals, which is controlled by the capacity of organisms to sequester and to detoxify metals. Different modelling approaches have been developed to address some of these factors. Both empirical and mechanistic equilibrium models have been developed and applied for characterising metal speciation in the environment. Metal bioaccumulation has been predicted by biodynamic models. The ability of organisms to detoxify metals has been taken into account in assessment based on the induction of metallothionein (MT) or subcellular partitioning. In addition, the interactions between organisms and metal ions have been taken into consideration in assessment of metal toxicity based on the accumulation of metal ions at biological surfaces.

Keywords: Metal, modelling, risk assessment, speciation, bioaccumulation, subcellular partitioning, toxicity

1. Introduction

Environmental pollution with metals is a serious problem in many areas in the world, and assessment of metal bioaccumulation and toxicity is of high concern. In such assessment, unique properties of metals that are largely different from the characteristics of organic substances

should be considered. The bioavailability and toxicity of metals are controlled by their chemical speciation, which is heavily influenced by environmental chemistry [1–5]. Metals are an intrinsic component of the environment, naturally occurring with varying background concentrations [6, 7]. Several metals are essential elements, that is, the elements that are present in living organisms and able to interact with the living system, and an insufficient amount of these elements leads to preventable or reversible effects on certain biological function, which do not occur at a physiological amount of the elements [8]. There is a limit to the regulation by the homeostasis mechanisms. Biological functions of organisms are affected by either the deficiency (below the limit) or toxicity (above the limit) of the essential metals. Because of these unique properties, organisms have developed various regulatory mechanisms for uptake, metabolism, distribution, storage, and elimination in response to metal exposure and to maintain metal homeostasis. The responses of organisms depend on a number of factors, from environmental conditions, bioaccumulation, to internal detoxification [9–11]. Metal bioaccumulation, a prerequisite for metal toxicity, is determined by the chemical speciation of metals in the environment, which depends on environmental conditions. In addition to metal accumulation, metal toxicity is influenced by metal subcellular partitioning. Different modelling approaches have been developed to address some of these factors as described in more detail in the following sections.

2. Metal availability in the environment

The bioavailability and toxicity of metals depend to a large extent on the chemical speciation of metals [12–15]. A widely recognised definition of the chemical speciation is that the chemical speciation of an element is the identification and quantification of the different, defined species, forms, or phases in which the element is present [16, 17]. Each form of the element is defined by its isotopic composition, electronic or oxidation state, or molecular structure [18, 19]. The mobility of metals in soil depends on the partition of metals between the solid and solution phases [20], which is determined by properties of the metals as well as composition of both solid and solution phases [21]. The most important soil properties include the total metal content, pH, cation exchange capacity (CEC), contents of soil organic matter (SOM), clay, oxides, and exchangeable Ca and Mg [22–26]. Besides the chemical extraction for direct determination, the solid-solution partitioning of metals in soils can be characterised by equilibrium models, which allow for predicting the speciation of a metal based on soil properties. Generally, two main approaches have been applied for modelling the solid-solution partitioning of metals in soils. They are based on: (1) empirical relations or transfer functions between the concentration of metals in soil solution or in a specific form and soil and solution properties [22, 24, 26, 27] or (2) complex process-based (mechanistic) multi-surface complexation models incorporating a detailed simulation of soil surface complexation and solution chemistry [23, 26, 28–31].

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2.1. Transfer functions

The partition of metals between the solid and solution phases in soil has been expressed by adsorption isotherms or a distribution coefficient (K_d; L/kg):

$$k_d = \frac{c}{c_{ss}} \tag{1}$$

where K_d (L/kg) is the distribution coefficient; C (mg/kg or mol/kg) is the total or reactive concentration of the metal in soil; C_{ss} (mg/L or mol/L) is the solution concentration.

In aerobic conditions, metals occluded in mineral particles and organic matter that are not readily available are included in the total pool, whilst the reactive fraction (extracted with 0.43M HNO_3) represents the metals available for exchange with soil solution with a particular time span [32]. Therefore, the reactive concentration has been used to substitute the total concentration in the above equation [32, 33]. The partitioning of metals in soil is usually expressed by sorption isotherms as described by a Freundlich equation:

$$C = k \cdot C_{ss}^{n} \tag{2}$$

where k and n are the Freundlich parameters. The exponent n reflects the variation in the binding strength with varying total pools. Equation 2 can be rewritten and extended to take into account the influence of soil properties [22, 32, 34–36]:

$$\log C = \log k + n \cdot \log C_{ss} + \sum a_i \cdot \log\left(X_i\right) \tag{3}$$

where a_i is the coefficient that reflects the influence of soil parameter X_i on the partitioning of metals between the solid and solution phases. The distribution coefficient has also been related to soil properties:

$$\log K_d = a_0 + a \cdot \log C_{ss} + \sum a_i \cdot \log\left(X_i\right) \tag{4}$$

Empirical relationships have been established between the distribution coefficient or the dissolved concentration, on the one hand, and the total/reactive concentration and soil properties, on the other. Different equations have been derived, depending on the empirical data that they have been based on, whilst a full description of soil properties is usually not available. Among soil properties, pH is the most commonly integrated parameter in transfer functions, followed by soil organic carbon (SOC), while other soil properties are rarely included in. This reflects the importance of pH and organic matter in determining metal speciation as well as the partitioning of metals between the solid and solution phases.

2.2. Mechanistic multi-surface complexation models

Substantial progress has been obtained in simulating the sorption of metal ions to soil and dissolved organic matter (DOM) with the development of different models, for example, MINEQL [37], GEOCHEM [38], NICADonnan [39], and WHAM V and VI [40, 41]. Metal speciation in soil has been characterised by various models calculating metal movement and available fractions and simulating impacts of physicochemical properties on metal dynamics in soil. The movement of different metal species in an environment is characterised by fate and transport models, while the partitioning of metals into dissolved, colloidal, and particulate phases is predicted by speciation/complexation models. Assemblage models have been formed by incorporating various surface complexation models. These process-based models provide a detailed description of metal complexation on different sorption surfaces [39, 42]. Surface complexation models based on thermodynamic processes have been developed for describing reactions of metals on the surface of soil reactive phases, such as SOM [39, 41], oxides [42, 43], and clay minerals [44]. Multi-surface complexation models have been formed from individual surface complexation models, assuming that the adsorption properties of soil can be characterised by the sum of the individual adsorption properties of individual components. Metal concentrations in soil solution have been predicted by using mechanistic geochemical models accounting for interactions of metals with various reactive surfaces [23, 24, 26, 29, 30].

Originally developed to characterise metal speciation in oxic waters [40, 41], WHAM has been applied to simulate the solid-solution partitioning [23, 45]. This model consists of several submodels: Humic Ion-Binding Model V and models of inorganic solution chemistry, adsorption-desorption reactions of fulvic acids, precipitation of oxides, and cation exchange reactions on clays. In the Humic Ion-Binding Model V, humic substances are represented by molecules containing proton-dissociating groups that can bind to metal ions [40]. The binding of metal ions to humic substances is described in terms of complexation at discrete sites while taking into account both electrostatic interactions and competition among ions. In the application to soil systems, a fixed charge mineral cation exchanger was included to describe the presence of clays [46].

Weng et al. [29, 47] have proposed another multi-surface or mechanistic model for characterizing metal speciation in which soil is considered a set of independent sorption surfaces, that is, organic matter, clay silicates, and iron (hydr)oxides. Metal binding to these surfaces is simulated using advanced adsorption, cation exchange models, and default binding parameters. Humic acid is considered an analogue for SOM. The site density of SOM is assumed to be equal to 31% of the density of humic acid [47], while metal binding to SOM is described by using the non-ideal competitive consistent Adsorption (NICA)-Donnan model [39]. Clays are represented by illite because of their importance in Dutch soils [48]. The average charge of illite is assumed to be pH dependent (0.25 mol/kg; [49]). An electrostatic Donnan model is used to describe metal binding to clay. For amorphous iron (hydr)oxides, their amount is predicted as the oxalate-extractable Fe, and their surface area is considered similar to that of hydrous ferric oxices (HFO; 600m^2/g) [42]. For crystalline iron (hydr)oxides, their surface area is assumed to be the same as that of goethite (100 m^2/g), and their amount is calculated as the difference between total (aqua regia) and oxalate-extractable Fe. Metal binding to the oxides is simulated

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using the two-site surface complexation diffuse double layer model [42]. The complexation of metals with carbonate and dissolved organic matter (DOM) is also taken into account in the model. DOM is assumed to consist of 30% humic acid and 30% fulvic acid [29].

The ORCHESTRA (Objective Representing CHEmical Speciation and TRAnsport) modelling framework [50, 51] is a combination of different sub-models. Thermodynamic data from the MINTEQA2 [52] were used to calculate solution speciation. The sorption of ions to SOM and DOM was simulated with the NICA-Donnan model [39, 53, 54] using generic binding parameters and constants derived by Milne et al. [55, 56]. When data on DOM were not available, DOM was assumed to consist of 50% reactive humic substances. In addition, SOM and DOM were represented by humic acid assuming that humic acid consists of 50% carbon [56]. The sorption of ions on the surface of (hydr)oxides was described by the generalized two layer model (GTLM) of Dzombak and Morel [42]. Site densities of the amorphous iron and aluminium (hydr)oxides were calculated from the assumed specific surface area of 600 m^2/g for hydrous ferric oxide [42]. Crystalline iron (hydr)oxides were represented by the hydrous ferric oxide, assuming the same reactivity. Moreover, a specific surface area of 100 m^2/g was assumed for these (hydr)oxides [43]. The nonspecific sorption of ions to permanently charged clay surfaces was modelled with a Donnan model. The illitic clay mineral with an average charge density of 0.25 eq/kg and a fixed Donnan model of 1 L/kg were used to represent the clay mineral [49].

3. Metal bioaccumulation

Since metals in the environment are not always available, assessing metal accumulation in sentinel species is one of the most effective methods for evaluating effects of metals on biota. The tendency of chemicals to be accumulated in organisms is usually expressed by the bioaccumulation factor (BAF) and the bioconcentration factor (BCF). These factors represent the ratio of the residue in the organisms versus the concentration in water at equilibrium, including or excluding the uptake from dietary sources, respectively. For metals, BCF and BAF vary widely and are inversely related to the external water concentration [57–59]. These characteristics of the accumulation factors for metals are attributed to the complex mechanisms of metal uptake and accumulation [60]. In a number of studies, uptake via saturable kinetics-exhibiting mechanisms has been shown to be more common and toxicologically relevant than passive diffusion [61–65]. The concentration of metals accumulated in organisms is further influenced by different physiological and anatomical mechanisms in response to metal exposure [6]. For essential metals, organisms are able to actively regulate metal bioaccumulation and maintain homeostasis over a range of exposure via exclusion or increased elimination [11, 57, 65]. Organisms have also developed different mechanisms to sequester, detoxify, or store excess metals [11, 62, 66]. The dependence of BCF and BAF on exposure conditions as well their inverse relationship with the exposure concentration invalidates the use of these factors in risk assessment [6, 57, 59].

Biodynamic models have been recommended as an alternative to single and generic values of BCF or BAF in the assessment of metal bioaccumulation [67]. Such models allow for integrating

and distinguishing different exposure routes (e.g., water vs. diet) and the dynamic nature of bioaccumulation processes [6, 68, 69]. The models provide a better understanding of the extent of and the contributors to the variability in the bioaccumulation among metals in various species and at different conditions [67, 70]. The biodynamic models are based on the concept of biodynamics that the accumulation of chemicals occurs as a result of a balance of different fluxes. The models assume that the rate of the fluxes can be realistically determined in controlled experiments with varying concentrations and conditions [67]. Uncertainties are inherent in the application of these empirical rates to different conditions, especially those that are extremely different from the conditions in which the rates have been derived. This limitation can be eliminated by integrating inverse phenomena into unifying concepts. The biokinetic model based on a unifying concept is considered 'mechanistic' from the perspective that physiological rates are estimated from chemical-specific properties of substances and species-specific physiological characteristics of organisms [71, 72]. This method facilitates extrapolation to a wide range of chemicals, organisms, and environmental conditions, without the necessity for case-specific calibration. Quantitative relations between uptake and elimination rate constants, on the one hand, and metal-specific properties and species-specific physiological characteristics, on the other hand, are required for the development of a mechanistic model. Some potential for meeting this requirement has been revealed from the findings during the last decades. Specifically, metal absorption and elimination rates have been related to the filtration rate and species weight, respectively [71, 73]. The affinity of metals for proteins, which are intensively involved in trafficking processes of metals, may explain the uptake rate constant from the dissolved phase [72]. The affinity of metals for the proteins depends on metal charge and atomic radius and reflects the preferences for coordination and ligands [72, 74, 75]. The suggestion on a potential correlation between uptake kinetics and the affinity of metals for protein has been substantiated by the reported relationship between the absorption efficiency and the binding of metals to membrane transport proteins [76]. Efforts have been put in developing bioaccumulation and toxicity models based on the affinity of metals for biological ligands [69, 72, 77]. Different indicators of metal binding to biological ligands and bioaccumulation have been related to various chemical properties of metals [78–82].

The accumulation of metals in organisms occurs as a result of a balance of the uptake from food as well as water and losses. Moreover, metal concentrations in organisms are affected by the growth dilution. Taking these factors into consideration, the concentration of metals accumulated in organisms (C; μg/g dw) can be expressed by the following equation:

$$\frac{dC}{dt} = \left(k_u \times C_w\right) + \left(IR \times AE \times C_f\right) - \left(k_{ew} + k_{ef} + g\right) \times C \tag{5}$$

where k_u (L/g dw/d) is the absorption rate; C_w (μg/L) is the dissolved metal concentration; IR (g/g dw/d) is the ingestion rate; AE (/) is the assimilation efficiency; C_f (μg/g) is the metal concentration in food; k_{ew} (1/d) is the excretion rate; k_{ef} (1/d) is the egestion rate; and g (1/d) is

http://dx.doi.org/10.5772/62911

the growth rate. The instant concentration of metals in organisms can be solved from Equation 5:

$$C = \frac{\left(k_u \times C_w\right) + \left(IR \times AE \times C_f\right)}{k_{ew} + k_{ef} + g} \times \left(1 - e^{-\left(k_{ew} + k_{ef} + g\right) \times t}\right) \quad (6)$$

The first factor in Equation 5 represents the uptake from the dissolved phase and can be further elaborated based on the absorption efficiency and the filtration rate [69]. In the study of Le et al. [69], the filtration, ingestion, and growth rates were related to the species weight while the absorption and assimilation efficiency was considered metal specific. In addition, elimination rates were parameterised based on both chemical properties of metals and mussel size.

Such a mechanistic model has shown good potential for predicting metal accumulation in the zebra mussel with different size and from various sites. A mechanistic model, which is based on chemical properties of metals and physiological characteristics of organisms while taking site-specific contamination levels into consideration, facilitates a wide extrapolation to different conditions and metals. However, a number of disadvantages are inherent in the current model. The modelling is based on the dissolved metal concentrations without a specification of chemical species and forms of metals in the environment. Although different uptake pathways are included in the model, a distinction of tissue-specific accumulation of metals is not accounted for. Another disadvantage of the model is related to the derivation of the relationship between the rate of physiological processes and the chemical properties of metals. Despite the potential correlation between the uptake kinetics and some chemical properties of metals shown recently, quantitative relationships established between these two factors have hardly been validated. Moreover, relationships between uptake kinetics and chemical properties of metals have usually been derived based on limited experimental data, thereby leading to intrinsic uncertainties in the application of the derived relationships.

4. Metal subcellular fates and partitioning

4.1. Subcellular partitioning

Bioaccumulation is a prerequisite, but not necessarily a reliable indicator of metal toxicity due to the species-specific capacity of organisms to detoxify the metals accumulated [9, 59, 82]. The detoxifying mechanisms have been suggested to account for the tolerance of organisms [83]. Besides metal uptake, metal toxicity is determined by the subcellular partitioning of metals in organisms [83]. Metals accumulated are distributed to different cellular components and sequestered by binding to proteins or peptides (e.g., metallothionein and glutathione) and granules [10, 83–86]. Therefore, only parts of meals are accumulated in sensitive cellular fractions [9]. From a toxicology perspective, metals accumulated have been divided into two fractions, that is, metal-sensitive fraction (MSF) and biologically detoxified metal (BDM) [83, 87, 88]. The former consists of metals in mitochondria, associated with heat-denaturable

proteins (HDPs), lysosomes, and microsomes. The latter includes metals in association with heat-stable proteins (HSPs) or metallothionein (MT)-like proteins and metal-rich granules.

It has been suggested that sub-lethal toxicity is accompanied with changes in subcellular partitioning, especially when the threshold is exceeded, that is, saturation of detoxification mechanisms [83]. Eyckmans et al. [89] showed the relationship between the tolerance of three freshwater fish species to excess Cu and the subcellular partitioning of Cu. The subcellular partitioning of metals may divulge potential mechanisms of toxicity as well as the fate of accumulated metals [90]. For instance, the binding of metals to cytosolic proteins such as MT has widely been demonstrated to alleviate toxic effects [91]. In contrast, the association of metals such as Cd to mitochondria modulates oxidative phosphorylation, followed by decreases in ATP production [92]. Similarly, the binding of Cd to the nucleus is potentially toxic because it can lead to DNA damage and stimulate mutagenesis [93, 94]. Different ligands are included in the HSP fraction, that is, amino acids, glutathione, and metallothioneins [95]. This fraction determines the tolerance and resistance of organisms to metals [83, 84, 87]. The relationships between subcellular partitioning and metal toxicity are complicated because of the different physiological functions of each fraction. The accumulation of metals in the lysosomes and microsomes might reflect the storage for eventual elimination if metals are mainly in the lysosomal fraction [85, 96]. In contrast, metals associated with microsomes can indicate toxicity because of the presence of fragmented endoplasmic reticulum, which is involved in the synthesis and transport of proteins [85, 87]. In addition, mitochondria is the most sensitive fraction [85]. Because of these factors, subcellular partitioning has recently been included in the assessment of metal toxicity.

4.2. Assessment based on metallothionein induction

The induction of MT has been integrated in various monitoring programmes and ecotoxicological assessment. However, recent reviews on the use of MT induction as biomarkers of metal exposure and toxicity raise questions on the validity of this method [11, 97]. The main concern comes from the lack of well-described time- and dose-dependent MT induction, while the induction of MT widely varies, depending on metals, species, and environmental conditions [97]. Further concern results from the lack of well-described relationships between MT induction and metal exposure, metal accumulation, and biological effects [11]. The validity of the use of MT induction as a biomarker is questionable because of the lack of enhancement in MT induction in response to metal exposure and/or the insignificant relationships between metal and MT concentrations [11]. As explained in the reviews by these authors, these results are attributed to different factors determining the cytosolic free metal ion concentrations, the presence of different MT isoforms in different tissues with various physiological functions, and turnover kinetics of MT as well as the high variability of MT induction and the basal level [11]. These factors should be taken into consideration in the assessment of metal exposure and toxicity based on MT induction. Another concern is related to the selection of organs/tissues as the target in the assessment. The digestive gland has widely been used as the target tissue [98, 99] from the point of view that this is the long-term storage organ. In addition, this organ plays an important role in different processes such as immune defence, homeostasis, xenobiotic

mechanisms, elimination, and detoxification [100–103]. Another reason for the use of the digestive gland is that this organ has the highest amount of proteins, including MT [104]. However, the suitability of the digestive ligand is doubtful because of the influence of natural factors and physiological changes on the induction of MT in this organ [104, 105]. As a major tissue for metal uptake, the induction of MT in gills is expected to represent the response of organisms to the ongoing exposure or accidental pollution [106]. Because of these issues, a single value of MT concentrations in the whole soft tissue or in a specific tissue is not always good indicator of metal exposure or effects on biota. As suggested by Le et al. [11], the induction of MT needs to be considered in relation to a number of factors as briefly mentioned above.

4.3. Assessment based on subcellular partitioning

Binding to MT is not the only mechanism for organisms to detoxify and to sequester excess metals. Metals can be associated with insoluble complexes in granules or lysosomes as mentioned above. The proportion of metals bound to MT is not always the dominant fraction and so MT induction is not necessarily a reliable indicator of metal exposure and biological effects. According to Adams et al. [107], the kinetic partitioning of metals into MSF and BDM is the key factor for an understanding of the exposure-toxicity relationship. This has been demonstrated by the shift of Cu accumulated in gills of common carp from MSF to BDM in the first period of the Cu exposure [89]. The 'Spillover' hypothesis has been used to simulate the relationship between metal bioaccumulation and toxicity. This term indicates the situation when the capacity of organisms to detoxify excess metals is overwhelmed, thereby resulting in adverse effects [66]. From a broad perspective, spillover is assumed to occur when the uptake rate exceeds a combination of the detoxification and elimination rates, leading to the accumulation of metals in the MSF fractions [9, 108]. This hypothesis has been used in some approaches for assessing metal toxicity.

The first approach is based on the ratio between MSF and MDP fractions or the relative contribution of the MSF and MDF fractions in other words [89, 90]. This approach is simplistic and does not provide a full description of toxicity [90]. There is not constant partitioning between MSF and BDM. Metals can be exchanged between the two fractions, and only a small fraction of metals occurs as free ions [90]. The use of the relative distribution between these two pools may lead to misinterpretation of spillover [90, 92, 109]. For instance, the increase in the amount of metals bound to MSF might be hidden by the lack of changes in the relative proportion, while the accumulation in all compartments increases. This has been illustrated by increases in metal concentrations in both BDM and MSF fractions in yellow eels [110], mummichogs [111], or yellow perch [85].

The second approach is to develop a relationship between MSF and toxic effects [112], assuming that adverse impacts on organisms increase with increasing amounts of metals in MSF. This method is based on the hypothesis that spillover occurs when certain detoxification mechanisms are overwhelmed and excess metals are accumulated in MSF, leading to adverse effects.

The third approach is based on the kinetics of metal accumulation in MSF [108, 113, 114]. The accumulation of metals in MSF can be described as a balance of uptake (e.g., from the dis-

solved), losses via elimination, and the partitioning of the metals to BDM, expressed by the detoxification rate:

$$\mathrm{MIT} = \left(k_u \times C_w\right) = \left(k_{detox} + k_e\right) \times C_{IT} \quad (7)$$

where MIT (mol/g/d) is the metal influx threshold ; k_u (L/g/d) is the uptake rate constant; C_w (mol/L) is the dissolved metal concentration; k_{detox} (1/d) is the detoxification rate constant; k_e (1/d) is the elimination rate constant; C_{IT} (mol/g) is the metal accumulation in MSF at the influx threshold.

This approach is supported by a negative correlation between elimination and detoxification [113, 115]. However, disadvantages are inherent in the assumption of this method that spillover occurs when the uptake rate exceeds combination of the elimination and detoxification rates, that is, no metal is accumulated in MSF below the threshold. Previous studies have indicated that spillover does not happen at low-exposure concentrations. In other words, there is no threshold below which the accumulation of metals in the sensitive fractions does not occur [84, 85].

5. Biological responses to metal exposure

Available approaches based on the induction of MT or taking into account subcellular partitioning of metals have shown some limitations in predicting metal toxicity as mentioned in the previous section. Moreover, the target organ or tissue is species specific, depending on the kinetics of metal accumulation, that is, the differences in absorption, distribution, and excretion [6]. A method that has been demonstrated to be more applicable to different species is to predict metal toxicity based on the accumulation of metals at biological surfaces, which allows for taking into account interactions between organisms and metals at biological surfaces.

5.1. Biotic Ligand Model

Interactions at the water-organism interface have been integrated in the Biotic Ligand Model (BLM). The conceptual framework of the BLM originates from two models: the gill surface interaction model and the free ion activity model (FIAM) [116–118]. The FIAM model assumes that free ions are the main reactive species of metals, determining metal bioavailability and toxicity [116, 117]. The FIAM model has then been extended to take into account the interactions of metals with organisms. One example is the fish gill surface interaction model [119], which has been developed by integrating conditional metal-gill surface binding constants to a geochemical speciation model. On the basis of the fish gill surface interaction model, the BLM has been developed to facilitate the application to various species, metals, and exposure conditions as well. According to the concept of the BLM, environmental geochemistry as well as toxicology principles are taken into consideration in determining the fraction of metals that

provokes effects on biota [119–121]. Initial toxicology bases of the model were effects of trace metals on ionoregulation. Apical (e.g., Na^+ and Ca^{2+} channels) and basolateral (e.g., Na^+/K^+- and Ca^{2+}-ATPase) transport channels, exchangers, and enzymes are negatively charged proteins that potentially bind to metal cations [122].

With the principles mentioned above, the BLM has shown substantial advantages in assessment of metal bioavailability. By including interactions of metal ions with both biotic and abiotic ligands, the BLM might provide more reliable estimates of metal bioavailability and toxicity. Moreover, the accumulation of metal ions at the physiological active sites of toxic actions, which determines toxic effect, is, in principle, distinguished from the total body burden [6]. Another advantage of the BLM is the inclusion of environmental conditions in estimating metal bioavailability and subsequent application to site-specific assessment [6]. Furthermore, the BLM provides a mechanistic understanding of metal-organism interactions as well as a mechanistic interpretation of metal toxicity, as illustrated by the results in the study of Le et al. [123]. The BLM has potential for assessing the toxicity of metal mixtures [123, 124]. Norwood et al. [124] suggested that metal-metal interactions can be predicted based on known stability constants. If two metals compete for the same binding site, the total amount of the metals bound to the biotic ligands determines combined effects [123]. Alternatively, the accumulation of individual metals at the biotic ligands can be used as inputs to the response addition model for estimating toxicity of metal mixtures [124, 125]. This suggestion has been demonstrated by the increasing application of the BLM for predicting joint toxicity of metals. Liu et al. [126] predicted the toxicity of metal mixtures based on the simple sum of the fraction of biotic ligands occupied by individual metals. This approach allows for taking into account the metal-specific affinity for binding sites of biotic ligands, but not the metal-specific toxic potency. In other studies, the BLM concepts are usually combined with conventional concepts of mixture toxicity such as concentration addition. As such, the specific toxic potency of metals is taken into account in the estimates. For instance, the toxicity of metal mixtures has been related to the toxic equivalent quotient (TEQ), which is based on the accumulation of metal ions at the binding sites of biotic ligands while giving consideration to the metal-specific toxic potency [123, 126]. The toxic unit (TU) is the concept mostly integrated in the BLM-based approaches for estimating joint toxicity of metals.

5.2. The electrostatic model

The electrostatic model originates from the reported differences between the ion concentration at the root plasma membrane surface and the ion concentration in the external medium, which is induced by the negative charge at the plasma membrane surface [127, 128]. The surface potential affects the activity of ions at the plasma membrane surface via electrostatic attraction or repulsion. In addition, the potential influences the difference in the electrical potential across the membrane, which stimulates the transport of ions through the membrane. A number of studies have shown the effects of electrical potential at the plasma membrane surface on cation uptake [127, 128]. The principle effect of the electrical potential at the plasma membrane surface is to control the ion activity at the surface [129].

The model allows for integrating plant-ion interactions in predicting metal toxicity [130–132]. In addition, the model gives consideration to the interactions among ions while estimating metal toxicity [132]. The electrostatic model has been applied to simulate effects of major cations on the toxicity of trace metal ions [131–134]. According to the principle of the electrostatic model, additions of cations to the bulk medium reduce the negativity of the electrical potential at the plasma membrane surface, thereby leading to decreases in the negativity of the electrical potential at the plasma membrane surface accompanied by reduced accumulation of trace metal ions at the plasma membrane surface and subsequent alleviation of toxic effects [135]. The electrostatic approach therefore might provide additional explanation for interpreting ion-ion interactions as well as effects of major cations on the toxicity of trace metal ions besides the competitive binding assumed in the BLM. According to the electrostatic principles, three mechanisms have been suggested to account for ameliorative effects of Ca^{2+} on metal toxicity [128]. The first mechanism is the electrostatic displacement of trace metal ions at the plasma membrane surface by Ca^{2+}. The second is the restoration of Ca^{2+} at the cell surface in response to low levels of surface Ca^{2+}. This mechanism is mediated in order to avoid the inhibition of the plant growth induced by the low contents of Ca^{2+} at the cell surface. The third mechanism covers other types of interactions between Ca^{2+} and trace metal ions, such as the blockade of the ion channel [136]. Despite the toxicant- and major cation-specific relative importance of these mechanisms, mechanism 1 generally occurs in all cases while the contribution of mechanism 2 is minor [128]. The significance of mechanism 3 is trace metal ion specific. The relative influence of major cations such as Ca^{2+} and Mg^{2+} on the toxicity of trace metal ions varies, depending on the trace metal ions and species [128]. For instance, Ca^{2+} has stronger effects on the toxicity of Al^{3+} to soybean than Mg^{2+} [137, 138]. In wheat, Mg^{2+} is more effective than Ca^{2+} in inhibiting Zn^{2+} toxicity, whereas Mg^{2+} does not have effects on the toxicity of Al^{3+} [128].

5.3. WHAM-based bioavailability model

Originally developed for determining and quantifying chemical forms and species of metals in different phases in the environment, speciation models such as WHAM have recently been used for estimating metal bioavailability and toxicity (hereafter referred to as the WHAM-based model). In this method, humic acid is considered a surrogate to biological surfaces [139–144]. As such, the amount of metals bound to humic acid computed by WHAM is used for estimating metal toxicity for different species. In the application of the WHAM-based model to plants, humic acid is used as a surrogate for the root surface [144]. In other words, the interactions of metal ions and the root surface are simulated by the interactions with humic acid.

The WHAM-based model was initiated from the similarities in terms of chemical structure between the root surface and humic acid. The root surface is a heterogeneous mixture of various metal-binding functional groups [145–147]. Similarly, humic acid possesses a heterogeneous mixture of functional groups, mainly carboxylic and phenolic acids [141]. Furthermore, the ratio of 1:2 between the site densities of phenolic and carboxylic groups assumed in WHAM [40, 41] lies in the range from 0.49:1 to 1:1 reported for root cell walls of different plant

species [148]. Because of this similar structure of the root surface and humic acid, metal accumulation on the root surface and metal binding to humic acids are influenced by similar factors. These factors include electrostatic interactions and chemical heterogeneity [127, 128, 149, 150]. The relevance of using metal binding to humic acid to represent metal accumulation at biological surfaces is further substantiated by the nature of the sorption of metal cations to the biological surfaces [151].

The WHAM-based model allows for the interactions between metal ions to be integrated in modelling metal toxicity [144]. Specifically, interactions between ions are considered in modelling metal binding to humic acid in WHAM and therefore accounted for in estimating metal accumulation at the biological surfaces. Another advantage of the WHAM-based model is related to the availability of binding constants in WHAM, which facilitates a wide application to different metals [144]. Moreover, previous studies have shown small variations in the binding constants among different species [145, 147, 152, 153]. For instance, the logarithm of the binding constants of Cd to the carboxylic group on the membrane surface of bacteria, fungi, and plants were in the range 3.3–3.5. Moreover, the cell walls of algae, maize, soybeans, and higher plants have similar titration curves [154–156]. These results indicate the applicability of a single set of binding constants to different organisms.

In the study of Le et al. [144], the amount of metals bound to humic acid was computed with WHAM in which metal sorption to humic substances is simulated by using a structured formulation of discrete, chemically plausible binding sites for protons and metals. This allows the creation of regular arrays of bidentate and tridentate binding sites for metals. Metal aquo ions compete with their first hydrolysis products, protons, and other metals for binding sites. In addition to the intrinsic metal binding strength to uncharged molecules, electrostatic effects were taken into consideration while predicting metal binding to humic acid. Le et al. [144] showed the potential of the WHAM-HA model for predicting both total and internalised metal concentrations in roots. Another example of the approaches based on the geochemical equilibrium in WHAM is the WHAM-F_{TOX} model for estimating metal toxicity [140, 143]. In this approach, mixture toxicity was simulated as a function of the metal-specific toxic potency and the amount of metal ions bound to the biological surfaces computed with WHAM.

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Section 2

Validated Methods and New Models of Evaluation

Chapter 3

Soil Contamination Health Risks in Czech Proposal of Soil Protection Legislation

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Additional information is available at the end of the chapter

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Abstract

A new system of soil contamination limit values proposed for Czech legislation is described. The system is based on the hierarchical limit values system with two levels. The first one—prevention limit—defined background values of risk elements (REs) and persistent organic pollutants (POPs) in Czech agricultural soils supported by the data from soil monitoring system. The second one—indication limit—is defined for human health protection by two principles, the protection of food chain and the protection of direct human health risks by inhalation, dermal and oral intake of RE and POPs in soil particles on the field. The practical application of limit values proposal was applied in the project focused on soil contamination influence on health and environmental risks in fluvial zones of Czech important river basins. The floodplain soils belong to the most contaminated soils in Europe generally and the project defined the potential fluvial areas with increased human health risks.

Keywords: soil contamination, health risks, risk elements, persistent organic pollutants, soil protection legislation

1. Introduction

The one of the important way of contamination risk elimination is the existence of legislative norms of contaminants in the environment. The soil is medium where the load from other environments can concentrate and interact. The limit values of main contaminants (risk elements (REs) and persistent organic pollutants (POPs) predominantly) were set in most of developed countries worldwide including the Czech Republic. The limits of REs and POPs concentra-

tions in agricultural soil are set by the Decree No. 13/1994 Coll. in the Czech legislation [1]. These limit values have a status of maximum tolerable values in agricultural soils. The criteria were derived from available data in the Czech Republic at the beginning of 90th and the data were corresponding with the load of Czech agricultural soils and also of some European countries. The REs limit values stated in the decree were derived as rounded 90 percentile of the background values in soil (pseudototal content in extract of Aqua regia). Some authors [2, 3] published the data concerning the total content of REs in the Czech soils before the proposal of background values of REs in Czech agricultural soils [4] was given. The history of POPs limit values assessment was different. The POPs limits were derived from available external data (especially from the Netherlands) since no relevant data for the Czech soils were available in 1994. As a result, limit values of some individual polycyclic aromatic hydrocarbons in the Decree No. 13/1994 Coll. are lower than their real background values in Czech agricultural soils proposed later [5]. This situation is misapplied by subjects demanding appropriation of agricultural land for construction purposes because there are assessed lower levies for the appropriation in the cases where the limit values are exceeded.

The described limit values were derived statistically and do not represent any specific risk in fact. The delimitation of soil suitability for agricultural use by the existence of one value of risk substance concentration is very questionable. For these reasons, the presented version of limit values can be considered as behind the time. The new version of limit values was proposed [6] and it is based on the principle of hierarchical limit values, differentiated in three levels. These individual levels present specific risks. The first one is derived from the background values of RE (POPs, respectively) in agricultural soils and the principles of limit construction follow German experiences [7], Regulation BGBl I, No. 36/1999 [8]. The principles of the assessment of nationals' soil background values of REs presented by [9] include following steps: The assessment of natural background given by the geology—REs contents in rocks and parent materials and REs contents in organic matter of soils; the assessment of diffusion load given by atmospheric deposition especially (determined the background values of organic pollutants) and the definition of practical questions connected with soil use and its relationship to environmental protection level. The suitable statistical methods for the assessment of element background levels in soils (defined as the first level) and of the higher levels of soil limits were described in detail in previous study [10].

The second level of limit values can be defined for specific risks (transfer into plants, transfer into ground water, or microbial activity inhibition for example). Considering the limits for transfer into plants, the Czech legislation proposal follows the German approach [11] using single extraction methods (1 mol/L NH_4NO_3, 0.01mol/L $CaCl_2$) which were scientifically verified by several studies [12–15].

The third level of limit values is directly connected with an impact on human health (Maximum Permissible Concentrations—MPC in the Netherlands, Contaminated Land Exposure Assessment in Great Britain) or the threat of ground water contamination (US EPA) generally. The applications of soil decontamination technologies must be used when these limit values are exceeded. The limit for Czech legislation was based on the US EPA methodology [16]. The protection of direct human health risk by inhalation, dermal, and oral intake is based on the

http://dx.doi.org/10.5772/62456

fact that zootoxic RE and POPs can cause the kind of mentioned risks to farmers spending the time on the field during agro technical activities. The zootoxic RE (As, Cd, Hg, Pb, and Tl) and POPs substances (sum of PAHs, benzo(a)pyrene, sum of 7 PCBs, sum of DDTs, HCB, HCH, and PCDDs/Fs) were chosen for their known negative impact on human health. The EPA methodology was applied for limit values calculation based on the toxicity of individual RE or POP substance (defined carcinogenic risk by WHO), the general soil properties and expected time period spending by the farmer on the field.

The limit values system is in legislative process in current days and validity is presumed since 2016. The practical application of limit values proposal was verified in the project focused on soil contamination influence on health and environmental risks in fluvial zones of Czech important river basins. The floodplain soils belong to the most contaminated soils in Europe generally and the project defined the potential fluvial areas with increased human health risks by the methodology described above. The results of project are presented as the practical application of proposed limit values in the methodology of selected risks in fluvial zones. The Fluvisols are soil group with specific soil properties and soil vulnerability by contamination (the sources of soil contamination) developed on fluvial sediments. The floods are the most serious way of soil contamination and soil properties show a high heterogeneity and variability. The heterogeneity is influenced by nature water stream dynamic (the gradient of erosion-deposition properties) with increased influence of neolitisation, it means acceleration of erosion-accumulation processes as the result of vegetation cover change and husbandry development in the landscape. The Fluvisols belong to fertile soils that has been used in agriculture historically. The husbandry in contaminated fluvial zones could cause increased risk and our study defines the risks on the most important fluvial zones in the Czech Republic.

2. Material and methods

Two levels of soil limit values were proposed for the Czech legislation, so-called prevention and indication limit. Their general characterisation is as follows:

Prevention limit was derived from the background values of REs and POPs in Czech agricultural soils when real data were calculated. The indication limits reflect two kinds of the risks. The first one is focused on increased REs transfer from soil into agricultural plants (POPs transfer was not calculated). The second one calculates direct impact on human health via their inhalation, dermal or oral intake on contaminated land for selected POPs and REs.

2.1. The prevention limits of RE and POPs

The prevention limit was derived from background values of RE and POPs in Czech agricultural soils proposed by [4] and [5]. REs background values are depending strongly on geochemical properties of the soil substrates and were proposed for 13 soil-lithological groups originally. The reduction into two groups was realised for pragmatic reasons. The background values are not valid for geochemically anomalous soils (mafic rocks, metallogenic zones of acid

rocks, etc.). The RE background values were calculated for pseudototal REs contents (Aqua regia extract, ČSN EN 13346 [17]) finally.

The POPs background values were calculated by [5]. The research of 560 soil samples of agricultural soils from the area of the Czech Republic was utilised. The background values were statistically calculated as two multiples of the standard deviation of geometric means or 90% percentiles—GM.GD^2) for both groups (RE and POPs). The background values were set for every individual substance of observed POPs groups. Clearly, the simplification of limit values for legislative process was necessary in result of which summary limits were calculated for some POPs groups.

The sum of PAHs—calculated as the sum of 12 substances concentration (anthracene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, phenanthrene, fluoranthene, chrysene, indeno(1,2,3-cd)pyrene, naphthalene, pyrene).

The polychlorinated hydrocarbons—limits for sum of seven indication congeners of polychlorinated biphenyls—PCB_7 (28 + 52 + 101 + 118 + 138 + 153 + 180) and sum of DDTs (DDT, DDE, and DDD).

The hexachlorbenzene and hexachlorcyclohexane ($(\Sigma\ \alpha + \beta + \gamma)$ and polychlorinated dibenzo-p-dioxines and dibenzofurans (PCDDs/Fs) should be analysed only in the case of suspicion of their contents in soil.

The background value of PCCDs/Fs was calculated separately because of different collection of soil samples. The used statistic was identical and 102 soil samples taken in the areas of the Czech Republic with different source of the load [18] were taken into account. The value of International Toxic Equivalent (I-TEQ PCCDs/Fs) of 17 toxic congeners was calculated [19].

2.2. The indication limit values of food chain contamination and plant growth inhibition

The separation on phytotoxic and zootoxic REs should be accepted. The limits for plant growth inhibition were proposed for this reason. The limits for food chain contamination regulate the transfer of zootoxic elements from the soil into plant production. The limits were supported by the research of RE transfer from the soil into selected plants (triticale, radish) in experimental conditions and into fodder plants (clover, alfalfa, and grass species) in field conditions [20–23]) and the dependency of REs mobile contents and selected soil conditions (pH, Cox, soil texture) was evaluated by multidimensional statistical methods (factor analysis). The comparison of the selected RE total contents (As, Cd, Cu, Hg, Ni, Pb, Th, Zn) and the content in the extract of 1 mol/L NH_4NO_3 (As, Cd, Cu, Ni, Pb, Th, Zn) characterised as RE mobile fraction (ISO DIS 19730 [24]) was the principle of RE indication values assessment. The limit values were referred to RE critical values in eatable and fodder plants (the Decree No. 305/2004 Coll. [25]). The other legislative norms for plant contamination (European legislation) are shown in our practical study focused on the husbandry in fluvial zones.

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2.3. The indication limit values of human health protection

The limit values were derived from the direct risk of increased POPs and RE (As, Cd, Hg, and Pb) contents on human health by their inhalation, dermal, and oral intake on contaminated fields. The calculation corresponds with the US EPA methodology (US EPA 2002) and respects the toxicity of the selected substances or elements and the movement duration of farmers on the contaminated land (standard exposition scenario was applied). It is also supported by the experience following from the activities provided in Czech conditions [26].

2.4. The case study of human health risks assessment from soil pollution in flood affected areas in the Czech Republic

The evaluation of health risk was realised and verified in the research project focused on soil contamination of flood affected areas in the Czech Republic. The human health risk assessment is becoming relevant when the -proposed indication limit values are exceeded, because they are derived as "effect based" for worst-case scenario. The screening evaluation of exposition by Soil Screening Level (SSL) method was applied (see 3.3 of the chapter). The calculation approach is based on the application of exposition models of chemical substances inputs into human bodies followed by the comparison of this predicted chronical dose with referenced "effect-based" dose. This approach allows to assign individual exposition parameters to every locality and then calculate site-specific SSL values and risks following from the exposition. The calculation of human risk ($RISK_{HUMAN}$) has been done for individual chemical substances first and there has been calculated total sum of all evaluated substances including the calculation of percentage of individual substances contributions to total sum. The $RISK_{HUMAN}$ values should not be higher than 1. The values higher than 2 indicate the possible risk and in the dependency on detail evaluation of exposition scenarios up to significant. The other cases can be evaluated as non-significant considering selected exposition scenario.

The next step for the evaluation of contamination level in floodplain soils was a rigorous statistical evaluation of the results. The dataset for soil contamination of 100 floodplain soils in the Czech Republic was used to estimate the human health risks by presented methodology (Equations 1–3).[1] Relative contributions of each risky element/substance to an overall hazard index ($RISK_{HUMAN}$) were calculated. A matrix transformation of relative contribution of each analyte to total $RISK_{HUMAN}$ on each locality was undertaken before the statistical analysis. The similarity of the soil pollution profiles in individual floodplain samples was assessed by a hierarchical cluster analysis using the average linkage clustering. The results of hierarchical cluster analyses are presented using technique of heatmap, where the similarity among the objects in a cluster dendrogram is visualised by the colour intensity in a square matrix of coloured pixels (R Core Team, Library Gplot).

[1] There were sampled 100 floodplain soils in various catchments of the Czech Republic. For each sampling site, a mixed sample consisting of 10 individual samples from the area of 100 × 100 m was used. Samples are separated and homogenised by quartation. The sample depth was 0–10 cm for pastures and 0–30 cm for arable land. In the soil samples there was analysed a wide range of risky substances including seven indicator PCBs (28, 52, 101, 118, 138, 153, 180), 7 risky elements (As, Cd, Cu, Hg, Ni, Pb, and Zn), polycyclic aromatic hydrocarbons (29 PAHs compounds), and pesticides (DDT and metabolites; hexachlorcyclohexane isomers, HCHs; pentachlorbenzene, PeCB; hexachlorbenzene, HCB). The basic soil properties (e.g. total organic carbon, soil texture characteristics) were determined.

3. Results and discussion

3.1. Proposal of legislative limit values

3.1.2. Prevention limit

The prevention limits of the RE for two soil texture units are presented in **Table 1**. This separation includes light texture soils (loamy-sandy soils and gravel-sandy soils) and standard soils (all the other soil). The values show REs contents in the extract of Aqua regia (pseudototal contents). These values were derived from the background values of REs in Czech agricultural soils—the soil geochemical background plus the average diffuse anthropogenic load [4]. The prevention limits were derived from the soils developed on different soil substrates of the Czech Republic except of the soils developed on geochemically anomalous substrates. These causes including the substrates with increased REs contents of lithogenic or chalcogenic origin [27] must be under an individual evaluation.

	Prevention value (mg/kg of d.m.)												
Soil category	As	Be	Cd	Co	Cr	Cu	Hg	Mn	Ni	Pb	V	Zn	Tl
Standard texture soils[1]	20	2.0	0.5	30	90	60	0,3	1200	50	60	130	120	0.5
Light texture soils[2]	15	1.5	0.4	20	55	45	0,3	1000	45	55	120	105	0.5

[1]Soils except light texture soils.
[2]Sandy soils, loamy-sandy soils, gravel-sandy soils.

Table 1. Proposed RE prevention limits in agricultural soils.

The POPs prevention limits are shown in **Table 2**. The differentiation of the soil texture units has no relevant reason for POPs and was not done. The POPs limit values are given in the form of total POPs contents in the soil. The background values of POPs in soil were derived from the average diffuse anthropogenic load (the dependency of POPs soil contents on nature background values is marginal). The real Czech background values [5] were adopted for legislative proposal.

POPs	**Prevention value (mg/kg of d.m.)**
Polycyclic aromatic hydrocarbons	
Σ PAHs[1]	1.0
Chlorinated hydrocarbons	
Σ PCB[2]	0.02
Σ DDT[3]	0.075
HCB[4]	0.02
HCH[4] (Σ α + β + γ)	0.01

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POPs	Prevention value (mg/kg of d.m.)
PCDDs/Fs[5]	1.0*
Petroleum hydrocarbons	
Hydrocarbons C10–C40	100

[1]Σ PAHS—polycyclic aromatic hydrocarbons (anthracene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, phenanthrene, fluoranthene, chrysene, indeno(1,2,3-cd)pyrene, naphthalene, pyrene).
[2]Σ PCB congeners—28 + 52 + 101 + 118 + 138 + 153 + 180.
[3]Σ DDT, DDE, DDD.
[4]HCB and HCH (Σ α + β + γ)—analysed only by suspicion of their contents in soil.
[5]International toxic equivalent value (I-TEQ PCDDs/Fs) (ng/kg)—analysed only by suspicion of increased PCDDs/Fs contents in soil.

Table 2. Proposed POPs prevention limits in agricultural soils.

The exceeding of RE or POPs prevention limits signalises the increased anthropogenic soil load (over the background values). In the cases of prevention limits exceeding, the precaution measure is proposed: the use of sludge, dredged sediments, or biosolids on the field will be forbidden. This level of limit values has already been partially implemented in the Czech legislation, namely in the Decrees No. 382/2001 Coll. [28] and No. 257/2009 Coll. [29] for sewage sludge and dredged sediments [30] application on agricultural soils. The proposed prevention limits should be valid for all types of substances applied on the agricultural land generally.

The system of so-called background values is not absolutely unified and can be partially different in individual EU countries. The Czech one is derived from German methodology where the background values are characterised as the concentration resulting from geological and pedological processes and including diffuse source inputs. This method is described in ISO 19258 (2005) [31] for RE and POPs and has international relevance. This methodology is used for the background value assessment in France and United Kingdom. Belgium, Luxembourg, and Netherlands derive the REs and POPS background values only from clean reference areas without any anthropogenic inputs (concentrations found in soil unaffected by any human activity, respectively, soils possibly contaminated by line/point source are exceeded). Nevertheless, the approaches can be different not only between the member countries but between the regions of individual countries in some of them (LABO 1995 [32]) because of different geological and pedological processes and anthropogenic inputs influencing the values and the differences in legislation systems.

3.2. Indication limits

3.2.1. Indication limit of food chain contamination and plant growth inhibition

The indication limit values reflect the mobility of REs. The comparison of RE (pseudo) total contents and their mobile fraction analysed in the extract of 1 mol/L NH_4NO_3 are the principle of indication limits. The limits of zootoxic REs (As, Cd, Pb, Tl, Hg) were proposed for the

food chain protection purpose (**Table 3**). The mobility of REs dependency on soil properties complicates the limit values when indication values for Cd are most complicated because of Cd mobility dependency on soil texture and soil pH. The evaluation of REs pseudototal and mobile form must be done if the limit values are available. The exceeding of limit value of pseudototal or of mobile form means exceeding of indication limit. The proposal of this level of limit value was based on the testing of selected plant species (fodder plants, vegetables, and corns) in experimental and field conditions and general validity of proposed values was derived. The statistical probabilities of critical values exceeding in eatable or fodder plants can be resulted when RE indication limits in the soil are exceeded. The real exceeding of indication limit value in local field conditions must be confirmed by the testing on individual crop.

Element	Soil texture	pH/$CaCl_2$	Indication value (mg/kg of d.m.)	
			Aqua regia	1mol/L NH_4NO_3
As	–	–	–	1.0
Cd		<5	1	–
		5–6.5	1.5	–
	Standard texture	>6.5	2.0	0.1
	Light texture	>6.5	2.0	0.04
Ni		<5	90	–
		5–6.5	150	–
		>6.5	200	–
		–	–	1.0
Pb		–	300	1.5
Tl		–	10	0.2
Hg*		–	1.5	–

*Total content by AMA technique.
The exceeding of limit value is valid in the case of any exceeding, a) Aqua regia extraction, b) 1mol/L NH_4NO_3 extraction when both analyses must be done if the limit values are available.

Table 3. Proposed indication limits of food chain contamination.

The indication limit values of plant growth inhibition (**Table 4**) were proposed for phytotoxic REs (Ni, Cu, and Zn) because the phytotoxicity can result into significant yield reduction. The limit values proposal was supported by the testing on plant species identical with previous indication limit value and the exceeding of indication limit values must be confirmed by the testing on individual crop in field conditions as well. In the cases of exceeding of both indication limit values the suitable remediation techniques for REs immobilisation (the liming, the application of inorganic or organic additives [33] are recommended).

Element	Soil texture	pH/$CaCl_2$	Indication value (mg/kg of d.m.)	
			Aqua regia	1mol/L NH_4NO_3
Cu		<5	150	–
		5–6.5	200	–
		>6.5	300	–
		–	–	1.0
Ni		<5	90	–
		5–6.5	150	–
		>6.5	200	–
		–	–	1.0
Zn			400	–
			–	20

The exceeding of limit value is valid in the case of any exceeding, a) Aqua regia extraction, b) 1 mol/L NH_4NO_3 extraction when both analyses must be done if the limit values are available.

Table 4. Proposed indication limits of plant growth inhibition.

3.2.2. The indication limit values of human health protection

The limit was proposed for zootoxic REs (**Table 5**) and selected POPs (**Table 6**). The model calculation of exposition scenario (method US EPA [16] was used as the principle for limit values assessment. The scenario calculates the effect of individual element/substance, the input into human bodies by inhalation, dermal, and oral inputs and the time period of exposition (estimated number of days per year). The calculated value is maximum tolerable value and the exceeding of this level of limit values could cause human health risk. The precaution defined in the legislation is based on the risk analysis of the site confirmed or excluded human health risk. The similar approach is applied in some EU countries, for example, limit value for human health protection is defined as decontamination limit for chlorinated substances in the soils of Germany (Federal Ministry of Justice and Consumer Protection of Germany).

Element	Indication value (mg/kg of d.m.)
As[1]	40
Cd[1]	20
Hg[2]	20
Pb[1]	400
Tl	60

[1]Aqua regia extract—valid for all soil texture categories
[2]Total content by AMA method

Table 5. Proposed RE indication limits of human health protection.

Substance	Indication value (mg/kg of d.m.)
Σ PAHs[1]	30
Benzo(a)pyrene	0.5
Σ PCB[2)]	1.5
Σ DDT[3]	8.0
HCB[4]	1
HCH[4] (Σ α + β + γ)	1
PCDDs/Fs[5]	100*

[1]Σ PAHs—polycyclic aromatic hydrocarbons (anthracene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, phenanthrene, fluoranthene, chrysene, indeno(1,2,3-cd)pyrene, naphthalene, pyrene).
[2]Σ PCB congeners—28 + 52 + 101 + 118 + 138 + 153 + 180.
[3]Σ DDT, DDE, DDD.
[4]HCB and HCH (Σ α + β + γ)—analysed only by suspicion of their contents in soil.
[5]International toxic equivalent value (I-TEQ PCDDs/Fs) (ng/kg)—analysed only by suspicion of increased PCDDs/Fs contents in soil.

Table 6. Proposed POPs indication limits of human health protection.

3.3. The evaluation of health risks in floodplain soils in the Czech Republic

The project proposed the methodology for evaluation of health risks in contaminated flood-affected soils useful in practical conditions [34]. The method of SSL was proposed for first screening evaluation. The method is based on the approaches of risk evaluation by US EPA [35] and EPA [36]. The methodology uses the exposition models of chemical inputs into human body. The predicted chronic daily doses are then compared with reference "effect-based" doses mathematically. The partial values of chemical substances concentrations and parameters of chosen exposition scenario (used in limit values assessment) for the main three exposition ways together are used in calculation:

Dust particles inhalation entering into air as secondary dust in the vicinity of evaluated localities.

- Soil ingestion (by consumption of insufficiently washed crops/eatables).
- Dermal contact with soil.

The SSL model was adopted for estimation of the human intake of soil contaminants and consequent risks. This method is based on the risk assessment procedure developed by US EPA. SSLs represent the risk-based soil concentrations derived for the individual chemicals of concern from equations combining exposure assumptions with toxicity criteria.

http://dx.doi.org/10.5772/62456

For each chemical, SSL is back-calculated from the target risk level, whereas an excess lifetime cancer risk (ELCR) is 1×10^{-6} for the soil exposure. Following equations are used to calculate SSL values for a residential population exposed to hazardous chemicals via all three exposure pathways. Default exposure parameters are provided whenever site-specific data are not available. The site specific exposure parameters were set out according to typical conditions of an intensive agriculture (arable land in alluvial areas). The detailed methodology is also described [37].

A. SSL based on non-carcinogenic risks

$$C = \frac{THQ \cdot BW_c \cdot AT_n}{EF_r \cdot ED_c \left[\left(\frac{1}{RfD_o} \cdot \frac{IRS_c}{10^6 mg/kg} \right) + \left(\frac{1}{RfD_o} \cdot \frac{SA_c \cdot AF_c \cdot ABS}{10^6 mg/kg} \right) + \left(\frac{1}{RfD_i} \cdot \frac{IRA_c}{VF_s or PEF} \right) \right]} \quad (1)$$

where

C Contaminant concentration (SSL) (mg kg^{-1}) Chemical-specific

THQ Target hazard quotient 1

BWc Body weight, child (kg) 15

ATn Averaging time, non-carcinogens (days) ED × 365

EFr Exposure frequency, resident (day yr^{-1}) 250 (8 h/day)

EDc Exposure duration, child (years) 25

IRSc Soil ingestion rate, child (mg day^{-1}) 100

RfDo Oral reference dose (mg kg^{-1} day^{-1}) Chemical-specific

SA Dermal surface area, child (cm^2 day^{-1}) 3470

AF Soil adherence factor, child (mg cm^{-2}) 0.12

ABS Skin absorption factor (unitless) Chemical-specific

IRAc Inhalation rate, child (m^3 day^{-1}) 20

RfDI Inhalation reference dose (mg kg^{-1} day^{-1}) Chemical-specific

VFs Volatilisation factor for soil (m^3 kg^{-1}) Chemical-specific

PEF Particulate emission factor (m^3 kg^{-1}) Chemical-specific

B SSL based on carcinogenic risks

$$C = \frac{TR \cdot AT_c}{EF_r\left[\left(\frac{IFS_{adj} \cdot CSF_o}{10^6 mg / kg}\right)+\left(\frac{SFS_{adj} \cdot ABS \cdot CSF_o}{10^6 mg / kg}\right)+\left(\frac{InhF_{adj} \cdot CSF_i}{VF_s or PEF}\right)\right]} \quad (2)$$

where

C Contaminant concentration (SSL) (mg kg^{-1}) Chemical-specific

TR Target cancer risk 1E-06

ATc Averaging time, carcinogens (days) 25,550

EFr Exposure frequency, resident (day yr^{-1}) 250 (8 h/day)

IFSadj Age-adjusted soil ingest. factor ([mg yr^{-1}]/[kg day])$^{-1}$ 100

CSFo Oral cancer slope factor (mg kg^{-1} day^{-1}) Chemical-specific

SFSadj Age-adjusted dermal factor ([mg yr^{-1}]/[kg day^{-1}]) 361

ABS Skin absorption factor (unitless) Chemical-specific

InhFadj Age-adjusted inhalation factor ([m3 yr^{-1}]/[kg day^{-1}]) 11

CSFi Inhalation cancer slope factor (mg kg day)$^{-1}$ Chemical-specific

VFs Volatilisation factor for soil (m3 kg^{-1}) Chemical-specific

PEF Particulate emission factor (m3 kg^{-1}) Chemical-specific

In case of the exposure to multiple chemicals, total risk is calculated as an additive value according to following equation:

$$RISK_{HUMAN} = \frac{c_1}{SSL_1} + \frac{c_2}{SSL_2} + ... + \frac{c_i}{SSL_i} \quad (3)$$

Resulting ratio smaller than 1 indicates that the POP concentrations measured at the site are unlikely to result in an adverse health impact.

Following uncertainties must be taken into account in final result assessment:

- Other non-analysed substances can influence the real risk.
- Toxicological data of some substances are estimated from in vivo tests on animals or in vitro. Therefore, extrapolation for humans must be done; however, for some chemical substances, the indexes are not set out yet.
- The exposure coefficient can be a serious source of uncertainties.

http://dx.doi.org/10.5772/62456

4. The results of health risks assessment in floodplain soils in the Czech Republic

Since the magnitude of the total estimation for human health risks on individual sampling localities was calculated (Equations 1–3) and cartographically represented (see **Figure 2**), the regional differentiation of potential human health impacts of complex soil pollution can be determined for floodplains soils in the Czech Republic. An increase of human health risk estimation was recorded for the Elbe River below the industrial centres (Opatovice, Pardubice, Neratovice, the Ohře River inflow) confirming the spatial patterns of pollution of various environmental compartments in the Elbe basin reported by previous studies [38, 39]. The high PAHs contributions together with an above-average $RISK_{HUMAN}$ were surprisingly found in the upper reaches of the Elbe River and Morava River. This could only be explained by a high propensity of PAHs to atmospheric transport resulting in high concentration of airborne POPs in remote and unpolluted freshwater ecosystems [40]. The higher magnitude of $RISK_{HUMAN}$ was recorded in a consequence of some well-known hot spots in the Berounka catchment (the Litavka stream inflow [41, 42] or the influence of Ag-Pb-Zn deposit in Stříbro). Similarly, the elevated $RISK_{HUMAN}$ followed the Odra River with the regional rising near the Ostrava agglomeration where the long-term airborne pollution resulted in a higher PAHs and Cd contamination of agricultural soils [43]. The elevated level of quantified human risks was also recorded in soil samples near the confluence of the Morava and Dřevnice River below the Otrokovice-Zlín agglomeration as a regional centre of industry that involves especially plastic and rubber manufacturing and historically established chemical industries for secondary manufacturing (shoemaking tradition). Several local contamination rising were detected in a consequence of spatially confined pollution sources (industrial centre of Mladá Boleslav or the Svitava River near Boskovice). A cluster analysis was processed for the transformed data matrix of relative contributions of each analyte to the total estimation of human health risk to reveal patterns of pollution profiles of floodplain samples in the Czech Republic. The results proved high cophenetic correlation coefficient ($r = 0.92$) with the optimal number of 11 clusters in the cluster analysis. One substantial cluster (covered 71 from 100 sampling localities) and several regional pollution abnormalities were detected in our analysis (see **Figure 1**). The dominant cluster was formed by the localities characteristic in a high contribution of polycyclic aromatic hydrocarbons (and especially benzo(a)pyrene, benzo(a)anthracene and benzo(b)fluoranthene) and in an elevated contribution of lead to total estimation of health risks. Some regional pollution abnormalities were connected to higher contribution of organochlorine pesticides (the Berounka and Ohře River), elevated contribution of PCBs (the Elbe River), or geochemical anomalies connected to local metallogenic zones (deposits). When combining both the magnitude of estimated $RISK_{HUMAN}$ and structural characteristics of pollution profiles (the cluster analysis results), the highest estimated humanotoxicological risks proved only several localities with a high content of polycyclic aromatic hydrocarbons accompanied by higher lead contents (there are depicted the predominant pollution profiles for the localities with the elevated total $RISK_{HUMAN}$ hazard index in **Table 7**). The results of human risk assessment well correspond with the exceedance of indication limit values for human protec-

tion. The indication limits of human health protection for PAHs and Pb contents were exceeded for several localities of floodplain soils in our study.

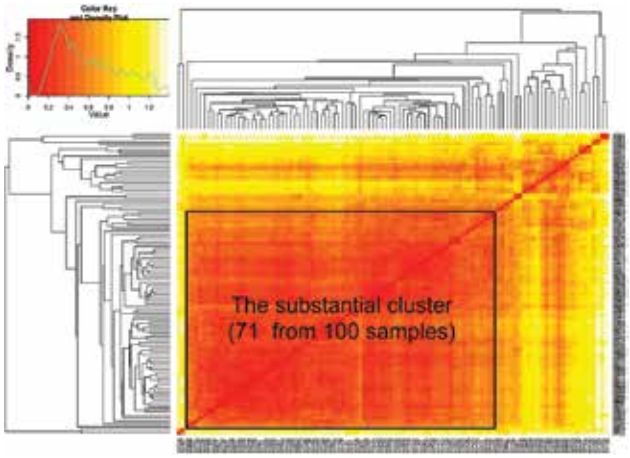

Figure 1. Similarity of the soil pollution profiles (relative contribution of pollutants to overall estimation of human risks—$RISK_{HUMAN}$) of individual floodplain samples in a cluster analysis presented by the heatmap and a projection of dominant cluster in our dataset. *Note—the more intense red color the more similar samples.*

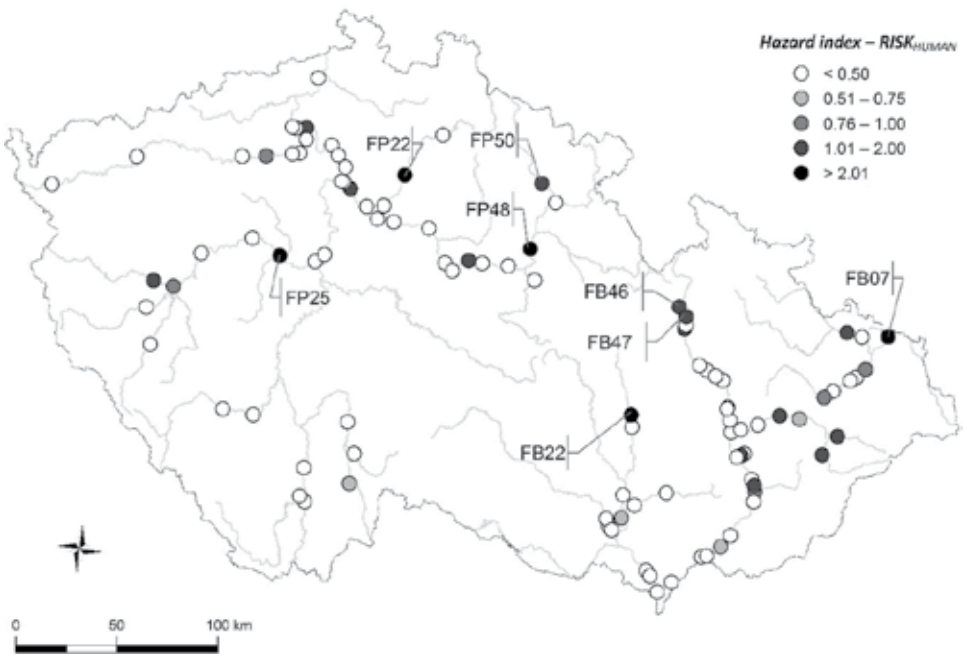

Figure 2. Spatial differentiation of magnitude of human health risks quantified using total $RISK_{HUMAN}$ (Equations 1–3) and visualisation of the regional hot spots (where $RISK_{HUMAN} > 1.5$).

http://dx.doi.org/10.5772/62456

Sample	$RISK_{HUMAN}$	Priority pollutants (relative contribution—%) [measured concentration—mg/kg]				
		1.	**2.**	**3.**	**4.**	**5.**
FB07	3.76	***B(a)P (74)[0.8]****	B(*a*)A (7.5) [0.82]	B(*b*)F (7.0) [0.77]	In(*cd*)P (4.9) [0.53]	DiB(*ah*)A (3.5) [0.04]
FB22	6.92	***B(a)P (74)[1.47]****	B(*b*)F (9.1) [1.82]	B(*a*)A (8.3) [1.67]	In(*cd*)P (4.8) [0.97]	DiB(*ah*)A (2.9) [0.06]
FB46	1.96	B(*a*)P (72) [0.41]	B(*b*)F (7.7) [0.44]	B(*a*)A (6.6) [0.38]	DiB(*ah*)A (5.3) [0.03]	In(*123*)P (4.9) [0.28]
FB47	1.61	B(*a*)P (71) [0.33]	B(*b*)F (8.2) [0.38]	B(*a*)A (7.9) [0.37]	In(*cd*)P (5.3) [0.25]	DiB(*ah*)A (5.1) [0.02]
FP22	2.15	B(*a*)P (75) [0.47]	B(*a*)A (6.9) [0.43]	B(*b*)F (5) [0.31]	DiB(*ah*)A (4.8) [0.03]	In(*cd*)P (4.4) [0.27]
FP25	2.03	B(*a*)P (52) [0.3]	***Pb (31.8) [516]****	B(*b*)F (4.4) [0.26]	B(*a*)A (4.1) [0.24]	In(cd)P (3.9) [0.23]
FP48	3.56	***B(a)P (78)[0.81]****	B(*a*)A (6.8) [0.7]	B(*b*)F (6.4) [0.66]	In(*cd*)P (4.6) [0.48]	DiB(*ah*)A (2.6) [0.03]
FP50	1.72	B(*a*)P (74) [0.37]	B(*a*)A (7.0) [0.35]	B(*b*)F (6.7) [0.33]	In(*cd*)P (4.9) [0.24]	Pb (3.4) [47]

Notes
B(a)P—benzo(a)pyrene; B(a)A—benz(a)anthracene; B(b)F—benzo(b)fluoranthene; In(cd)P—Indeno(1,2,3-cd)pyrene; DiB(ah)A – Dibenz(a,h)anthracene, Pb – lead.
*Exceeding of indication limit of human health protection for particular pollutant and locality.

Table 7. Priority pollutants for floodplain samples with topmost estimation of human health risks ($RISK_{HUMAN} > 1.5$) and their pollution profiles (predominant pollutant concentrations and their relative contribution to $RISK_{HUMAN}$).

5. Conclusion

The proposed system of hierarchical limit values helps to protect soil environment, food chain, and human health against the contamination and will improve the current version fundamentally. The currently valid principle of maximally tolerable values presenting no actual risk (but selected agricultural soils on two categories—useful and non-useful by the existence of one limit value level) will be replaced by the system of hierarchical limit values referred to an individual level of the risks and followed by appropriate measures in the cases of limit exceeding. The case study of floodplains research proved the operability of the established methodology and verified relevancy of the human health limits (indication limits of human health protection) in Czech proposal of soil protection legislation. The established methodology helped to reveal the areas where the soil does not meet the soil quality standards and where the human health risks were elevated. The characteristic pollution profiles of floodplain soils with elevated human health risks were defined on the basis of the results.

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Chapter 4

Assessment of DNA Damage by Comet Assay in Buccal Epithelial Cells: Problems, Achievement, Perspectives

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Additional information is available at the end of the chapter

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Abstract

DNA damage risk assessment in comet assay by the use of buccal mucosa cells has great advantages in comparison with other cell type sample due to more safely, easier, cheaper, and non-invasive method for in vivo studies. According to the OECD Guidelines, the in vivo mammalian alkaline comet assay is well-established and validated method for measuring DNA strand breaks in single eukaryotic cells. Considering exposure to xenobiotics and endogenous damage inductors, buccal mucosa cells are the first to be in direct contact after exposure and this makes them an ideal biomatrices in evaluation of the level of individual genotoxicity to several compounds already mentioned. Their clinical diagnostic applicability confers a potential use in patients across time. However, the number of publications referring to the human buccal comet assay is low in the last two decades. This low growing interest may be explained by several factors, including its relative technical problems. Different procedures have been used in collecting and processing the samples. In order to have widespread acceptance and credibility in human population studies, the comet assay in buccal cells requires standardization of the protocol, of parameters analyzed, and a better knowledge of critical features affecting the assay outcomes, including the definition of the values of spontaneous DNA damage. There is a need for further collaborative work as in the HUMN (micronucleus assay on lymphocytes) and HUMNxL (micronucleus assay on buccal cells) collaborative projects. The creation of a network of laboratories will allow more focused validation studies, including the design of a classic, historic, prospective cohort study in order to explore the link between measures of genetic instability in the buccal mucosa and the risk of cancer and other chronic-degenerative diseases. One such network connection will start in 2016 as a COST project under the name "hCOMET—The comet assay as a human biomonitoring tool" launched by Prof. Andrew Collins.

Keywords: SCGE assay, buccal mucosa cells, genotoxic risk assays, DNA damage, comet assay

1. Introduction

Human exposure to environmental chemical agents occurs as a result of contaminated air, water, soil, and food. Although many chemical agents are in use for more than two centuries, nowadays, it is known that a number of them can cause genetic damage. Chemicals that can cause this type of damage are specified and identified as mutagens, carcinogens, or teratogens based on the diverse type of investigations. It is estimated that chemicals play a predominant role in the etiology of a majority of human diseases. The possible genetic health hazards associated with chemicals are more difficult to evaluate in the human environment. There are tens of thousands of untested chemicals in the human environment, and some attempt must be made to identify the ones that are potentially hazardous to man. From 1972 when first UN Conference on the Human Environment was organized, World Health Organization and International Agency for Research on Cancer (IARC) have published many monographic editions categorizing dangerous chemicals based on collected in vitro and in vivo results of investigations [1,2]. Also, unique tools (methods) for assessing the potential effects of chemicals on human health, and the environment have been established under the name The OECD Guidelines for the Testing of Chemicals, methods, and guidelines internationally accepted as standard methods for safety testing [3] in which standardized and validated techniques are described that can estimate the level of DNA damage after the exposure.

During the past half century, the focus has been shifted from identification of these compounds in the environment to the risk assessment and minimization or prevention of unnecessary exposure in the first place. For this reason, along with an increasing understanding of mechanisms of action by which these chemicals can cause DNA or cell damage, and also cancer [4], a variety of hazard identification screening models have been developed and established. These models can serve in risk assessment studies. Risk is defined as the probability of a given toxicological hazard producing actual biological harm. This idea involves some form of mathematical relationship between exposure and toxicology. In the field of environmental toxicity assessment, the need for in-time risk management decisions requires setting up a battery of standardized and relatively easy to perform tests, allowing quick answers to pressing questions [5]. The use of diverse genotoxic bioassays is therefore unavoidable. Application of biomarkers in both qualitative and quantitative aspects of risk assessment has been eagerly anticipated for over a decade, since Hattis [6] first proposed their use in this process.

Numerous assays have been developed as screens for genotoxicity, beginning with the Salmonella mutagenicity assay. Genomic damage is probably the most important fundamental cause of developmental and degenerative disease. It is also well established that genomic damage is produced by environmental exposure to genotoxins, medical procedures, micronutrient deficiency, lifestyle, and genetic factors [7]. It is essential to have reliable and relevant minimally invasive biomarkers to improve the implementation of biomonitoring, diagnostics, and treatment of diseases caused by, or associated with, genetic damage.

Since methods in molecular epidemiology have been improved with the use of reliable biomarkers of exposure in analysis, population biomonitoring has become an extremely

powerful approach to determine the effect of environmental mutagens on human populations [8]. On this way, early effects may be highlighted in all accessible cell types, such as blood cells, epithelial cells and exfoliated buccal or urothelial cells; thus, genetic biomonitoring allows detecting adverse effects of mutagenic chemicals in human somatic cells [9].

Among different types of cells and especially of epithelial cells, the collection of buccal cells is arguably the least invasive method available for measuring DNA damage in humans, especially in comparison with obtaining blood samples for lymphocyte and erythrocyte assays, or tissue biopsies [7]. Without the need for cell culture establishing (cells do not divide, but just differentiate from basal cells), buccal cells analyzed by other techniques, such as micronucleus assay, have shown good correlation with the level of damage observed on lymphocytes after 72-h cell culture with DNA damage cytogenetic test called cytochalasin B blocked micronucleus (MN) assay [10]. Buccal micronucleus cytome assay can measure frequency of MN (its origin is either from chromosome breakage/loss of entire chromosome), nuclear buds and/or broken egg, binucleated cells, and various forms of cell death phase measured as condensed chromatin, karyorrhectic, pyknotic, or karyolitic cells [11]. Chronic exposure leads to a steady-state elevated expression level of MN regardless of the cell division rate if the period of exposure exceeds the time frame for one nuclear division, that is, 20–30 h. Carcinogens delivered primarily through blood stream influence equally DNA damage measured in buccal cells and lymphocytes. Since collection of buccal cells and their processing is easy, fast and low cost, and they do not divide just differentiate, they have potential to replace the tests that need cell culture establishment in order to estimate DNA damage. HUMNxL group (The HUman MicroNucleus project on eXfoLiated buccal cells group) has collected data from 30 different laboratories on 5424 subjects in order to evaluate the impact of host factors, occupation, lifestyle, disease status, and protocol features on the occurrence of MN in exfoliated buccal cells [12]. The results of this survey have shown high correlation of micronucleus detection in buccal cells with exposure for occupational groups reporting exposure to solvents, polycyclic aromatic hydrocarbons (PAHs) and gasoline, arsenic, and antineoplastic drugs. Also, significant association of higher MN frequency was found for oro-pharyngeal and respiratory cancers, and for all the other cancers pooled together. Although micronucleus assay in buccal cells does not need cell culture, it requires at least 3000 cells examined under the microscope. Since this can also be time consuming, one of the other methods for measuring DNA damage is alkaline comet assay, one of the newest OECD guideline tests (from 2014) for chemical exposure in vivo (No. 489), an easy and low-cost assay that measures primary DNA damage on any type of single-cell suspension sample [13]. The use of comet assay on buccal cells would be a potential new and reliable combination for chemical exposure and DNA damage assessment. The comet assay in buccal cell assay was first reported in 1996 [14]. Like in HUMNxL project, it will be necessary to develop and implement the results of an international collaborative validation group established to identify and quantify the key variables affecting the damage evaluation in buccal mucosa cells using the comet assay. In addition, an interlaboratory slide-scoring exercise could be undertaken to evaluate the intra- and inter-laboratory variability in the scoring of different parameters of comet assay in buccal cells, similar to the approach successfully used by the HUMN project for the MN assay in lymphocytes [15–17] and the HUMNxL project in buccal cells [7,12,17,18]. One such groups with prof. Andrew

Collins has started in 2016 a COST networking project under the name "hCOMET—The comet assay as a human biomonitoring tool", in order to give response to the questions discussed in this review.

1.1. Comet assay

The comet assay is a cheap, easy, fast, reliable, and sensitive method for measuring the level of primary DNA damage in single-cell suspension of any type and requires a small sample material. For these reasons, the comet assay in its various modifications (alkaline, neutral, and with lesion-specific enzymes to detect specific types of DNA damage such as 8OHdG, formamidopyrimidine DNA glycosylase, endonuclease III, T4 endonuclease. V.) has few serious competitors. The cells are embedded into agarose, and after lysis, denaturation, electrophoresis, and staining, the amount of DNA damage is measured either visually by dividing the damaged cells into five groups, or by the help of camera and software image program that analyses the image. Measured parameters are usually tail length (measured in micrometers), tail intensity or tail DNA percentage (when there is damage, DNA has a shape of a comet), and tail moment (combination of the first two parameters). It is recommended to use tail intensity parameter since the agents sometimes produce few small breaks that make comet tail long, but in fact, there is not a high percentage of DNA in the damaged part of the comet. When standardized and validated, the comet assay can provide valuable information in the areas of hazard identification and risk assessment of environmental and occupational exposure, diseases linked with oxidative stress (e.g., diabetes and cardiovascular disease), nutrition, monitoring the effectiveness of medical treatment, and investigating individual variation in response to DNA damage that may reflect genetic or environmental influences. The information obtained could lead to individual advice on lifestyle changes to promote health and especially on relative risks of genotoxic exposure to environmental pollution [19].

In human biomonitoring studies, the comet assay can provide crucial information on risk assessment of environmental, occupational, and lifestyle exposures. Earlier reviews have dealt with different aspects of the use of the comet assay in human biomonitoring studies [20–26], but without providing any specific, practical guidance for using the comet assay in human biomonitoring. Several general articles on biomonitoring are available [27–31] that can be helpful when designing biomonitoring studies using the comet assay. To avoid obtaining false-positive and false-negative results, certain basic principles should be respected and followed in study design and performing and these consider first of all matching of exposed and control group according to gender, age, alcohol, and smoking habits and their consumption, and also with other lifestyle and nutritional factors [19].

ComNet project group, established before last COST project that will make an effort in exposure type and DNA damage assessment, has made an effort to pool together data of all available comet assay biomonitoring studies, in order to establish baseline parameters of DNA damage, and to investigate associations between comet assay measurements and factors such as sex, age, smoking status, nutrition, lifestyle. Although this assay has been widely used in human biomonitoring for DNA damage measurement as a marker genotoxic agent's exposure or for investigation of genoprotective effects, single research studies had usually small

numbers of subjects, with sub-optimal design also in other critical respects already mentioned, and also with the use of significantly different comet assay protocols. For these reasons, the ComNet project has recruited almost 100 research groups willing to share datasets. Collins et al. [32] provided a background of the ComNet project, and the history of the comet assay itself, and the most important, he has pointed out important practical issues that can critically affect its performance. The survey pointed out comet assays diverse applications in biomonitoring studies (environmental, occupational exposure to genotoxic agents), genoprotection studies that were controlled by dietary and other factors and DNA damage assessment studies associated with various diseases and intrinsic factors that affect DNA damage levels in humans. The survey also analyzed the quality of data from a random study selection, using epidemiological and statistical point of view. Most of the studies have been done on lymphocytes or whole blood, and they can show damage of DNA caused by long term exposure or also exposure in the past, since lymphocytes circulate through the body and can live for up to 3 years. A new step will be also to established basal levels of DNA damage in relation to different exposure, diseases, and cell types used, and to correlate them with long-term and short-term exposure. Considering the short term or recent exposure, buccal mucosa cell comet assay would be ideal since those cells among epithelial cells are short living cells with no division and DNA damage found in them can demonstrate recent exposure or direct contact exposure with oral mucosa, so the DNA damage measured by comet assay on buccal cells would be indication of recent exposure and severity of that exposure [33].

1.2. Exfoliated oral mucosa cells

Buccal cells form the first barrier for the inhalation or ingestion route and are capable of metabolizing proximate carcinogens to reactive products [34–37]. About 92% of human cancers are derived from the external and internal epithelium, that is, the skin, the bronchial epithelium, and the epithelia lining the alimentary canal [7,38]. Therefore, it could be argued that oral epithelial cells represent a preferred target site for early genotoxic events induced by carcinogenic agents entering the body via inhalation and ingestion [7,39].

In the early studies from the 1980s, exfoliated buccal mucosa cells were used with the MN assay to evaluate the genotoxic effects of multiple factors including environmental and occupational exposures, radiotherapy, chemoprevention, vitamin supplementation trials, lifestyle habits, cancer, and other diseases (see [7] for review), with possibility of cell degeneration in form of condensed/fragmented chromatin, pyknotic nuclei, loss of nuclear material in form of karyolitic or "ghost" cells [18,40,41]. In rare cases, some cells can also demonstrate other forms such as binucleated stage with two nucleus in the same cytoplasm, form of nuclear bud or "broken egg" or form small micronuclei (MN) near nuclei in the same cytoplasm. These biomarkers of genome damage (e.g., MN, nuclear buds) and cell death (e.g., apoptosis, karyolysis) can be observed in both the lymphocyte and buccal cell systems, and thus provide a more comprehensive assessment of genome damage then only MN in the context of cytotoxicity and cytostatic effects [7,39,41].

2. The comet assay in mucosa buccal cells

DNA damage assessment in exfoliated cells (buccal epithelium) may be an innovative promising tool for genotoxicity studies since sampling is easy. Some results indicate that alkaline single-cell gel electrophoresis, using buccal epithelial cells, could be a good biomarker of early effects, and can be utilized for human monitoring, since, in some cases, this kind of cell is the first to interact with xenobiotics [14]. Comet assay can detect DNA single-strand breaks and alkali labile sites at pH 13 (alkaline version) or double-strand breaks under neutral conditions (neutral version) [42–44]. The relevance of SCGE lies in its requirement for very small cell samples, and in its ability to evaluate DNA damage in proliferating or non-proliferating cells [45].

While biomonitoring studies employing cytogenetic techniques are mainly done in lymphocytes, the SCGE technique can be applied to any cell population. Over the last years, exfoliated cells have been used for biomonitoring studies utilizing several genotoxicity endpoints [40]; however, there are few studies which apply SCGE on epithelial cells [14].

Over 90% of cancers are epithelial in their origin [47] and since crucial mechanism in cancer development is the level and amount of DNA damage [48], DNA damage assessment in buccal epithelial cells may prove as a good biomarker of early damage. In their work, Rojas et al. [14] established for first time, the conditions for using the comet assay in buccal epithelial cells.

The use of surrogate cells, other than lymphocytes, such as exfoliated cells from epithelial tissues is of particular interest due to the ability to be collected with non-invasive methods, and the cells are explored with the aim to evaluate their suitability in biomonitoring studies [7,49]. Beside the minimally invasive sample collection from the inner wall of the cheek, the cells have advantage in exposure assessment to inhaled or ingested genotoxic agents, and this all makes them a good model for large biomonitoring studies, and also in pediatric researches.

The application of the comet assay test in uncultured buccal exfoliated cells (since the test does not need cell culture conditions), started in the 1996, when Rojas et al. [14] by comparing DNA damage level between smokers and non-smokers group in exfoliated buccal mucosa cells, found that DNA tail length significantly increased in the smoker group (89.30 + 16.18 μm) vs. non-smoker group (52.01 + 10.43 μm), indicating that the SCGE assay could be applied to human monitoring using exfoliated buccal epithelial cells.

In that moment, Rojas et al. [14] indicated that alkaline single-cell gel electrophoresis assay, using buccal epithelial cells could be a good biomarker of early effects, and can be utilized for human monitoring since; in some cases, this kind of cell is the first to interact with xenobiotics. However, 20 years later, <40 articles have been published with this bioassay. **Table 1** represents the list of analyzed studies on buccal cells with comet assay with a point on sampling and preparation of slides for comet assay analysis. This table is extending the data collected in Rojas et al. [33] who only made observations in differences in preparing the slides, giving the highest impact on different lysis solution and enzyme digestion in preparation.

Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
With water	Small sterile spoon, kept in 1 mL of physiological solution at 37 °C	According with Singh et al. [100], Tice et al. [23], Speit and Hartmann [107], with some modifications	At 1000 rpm 10 min	50 µL of cell pellet in 50 µL LMP agarose (1% in PBS), sample carefully stirred, dropped on a slide, covered with a coverslip precoated with NMP agarose (1 % in PBS), and kept on ice during the polymerization of each gel layer		The coverslip removed, slides immersed in a freshly made lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10), 10 % DMSO, and 1 % Triton X-100 for 24 h at 4 °C	Horizontal electrophoresis chamber. Fresh electrophoresis buffer (300 mM NaOH and 1 mM Na_2EDTA, pH 13); 20 min	Electrophoresis was 20 min at 25 V and 300 mA	3 times rinsing in fresh neutralization buffer (0.4 M Tris, pH 7.5), fixed 5 min in absolute metanol, 75 µL EtBr (20 µg/mL) 10 min	[82]
Several times with distilled water	With a cytological brush, in a 20 circulare expanding rotations, from the center of the cheek, both left and right cheek sampled with separate brushes, cells in 20 ml PBS, 4°C until further process	Thomas et al. [11[, Szeto et al. [61]	At 1500 rpm 10 min, resuspended in 1 mL PBS in Eppendorf tube centrifuged (1000 rpm, 5 min)	10 µL cell suspension mixed with 0.75% LMP agarose, 75 µL immediately spread onto a glass microscope slide pre-coated with a layer of 1% NMP agarose. The LMP layer was allowed to set at 4°C for 5 min	0.25% trypsin solution was added, 30 min, 37 °C, after, cells were treated with proteinase K (1 mg/mL) 10 min.	Ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4°C for 1 week. This procedure removes cell proteins and leaves the DNA as 'nucleoids'	Horizontal electrophoresis unit, with fresh alkaline solution (300 mM NaOH, 1 mM EDTA, pH 13.0) 20 min at 4°C	20 min at 25 V and 300 mA (0.90 V/cm)	Slides were then neutralized (0.4 M Tris, pH 7.5), washed in distilled water, $AgNO_3$ staining protocol as described by Nadin et al. [108]	[59]
With water	Scraping the inner part of both cheeks 3 times with cytology brush, samples in sealed 1.5 ml Eppendorf tube with PBS, room temperature, no direct sunlight		At 2500 rpm 1 min	Mixing 500 µL of molten LMA with 50 µL of cells specimen at 37°C and immediately pipetting 75 µL of 1:10 (v/v) aliquot onto a comet slide, placed flat at 4°C in the dark 10 min		The slide was immersed in prechilled lysis solution for 60 minutes	Followed by immersion in freshly prepared alkaline solution, pH >13, for 45 minutes at room temperature in the dark	1 V/cm, 1 h	Slides rinsed by dipping several times into deionized water, fixation in 70% EtOH 5 min, 50 µL of dilute SYBR green	[81]

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Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
-	Scraping the buccal mucosa with a wooden spatula, in a tube containing 1 mL of minimal essential media, wrapped in aluminum foil to protect them from light, stored in refrigerator at 4°C and processed next day	Ostling and Johanson [109], Szeto et al. [61]	At 200 X g for 10 min, the cell pellet washed with 500 µL PBS and centrifuged	10 µL of suspension mixed with 85 µL of pre-warmed (40°C) LMP agarose 1% (w/v). Cells in LMP agarose were applied to a Trevigen comet slide and incubated at room temperature until the gel layer solidified	Layered with 50 µL trypsin solution (0.25% trypsin, 1 mM EDTA in Hanks balanced salt solution) and incubated for 30 min at 37°C, slides washed with PBS.	Cell lysis with proteinase-K (1 mg/ml) for 60 min	Alkaline solution for 20 min at room temperature in the dark	In eectrophoresis buffer (0.01 M NaOH, 1 mM EDTA, pH 9.1), 0.9 V/cm, for 18-20 min	Slides rinsed by dipping several times in distilled water. Fixation by immersing in 70% EtOH 5 min, then air dried. EtBr staining (50 mg/ml)	[72]
		Following the method outlined by Singh et al. [100]		The cells were embedded in agar on a microscope slide		Were lysed by detergents and salts at high concentrations		For 15 min under high pH , at 20 V and 400 mA	Stained with EtBr	[73]
Water	Scraping the inner part of both cheeks with a cytology brush, cells kept in 0.9% NaCl and PBS in separate microcentrifuge tubes, brought to laboratory	Eshkoor et al. [66]								[69]
	Exfoliated buccal mucosa cells were collected by gently scraping the mucosa of the inner lining of one or both cheeks	Rojas et al. [14], and modified based on standard procedures from comet assay kit								[85]

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Exfoliated Buccal Cells Sampling		Buccal comet assay technique							Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
Washing out the child's mouth with tepid water to remove exfoliated dead cells	Parents collected epitelial mucosa cell samples by gently brushing the inside of both cheeks with a cytology brush. The brush was then stirred in a PBS (pH 7.4).	Cells were processed in alkali conditions and underwent submarine electrophoresis accioni [51, 110]	Cell suspensions were washed twice with centrifugation at room temperature						Stained with Et-Br	[86]
Water	Scraping the inner part of the cheeks both sides with a cytology brush, cells kept in 0.9% NaCl and PBS in separate microcentrifuge tubes, brought to laboratory	Used the Trevigen Comet-AssayTM kit protocol		Cells + LMP agarose at 37°C at the ratio of 1:10, and 75 µL aliquots pipetted onto the slides and placed flat in a dark place at 4°C for 10 min.		The slides immersed in the pre-chilled lysis solution for 60 min	A freshly prepared alkaline solution, pH>13, at room temperature in the dark for 45 min	The slides placed flat on a gel tray, At 1 V/cm for 10 min	Stained with 50 ml of diluted SYBR Green	[67]
Water	The cells were collected by scraping the inner part of the cheeks both sides with a cytology brush. Then, the cells were gently mixed with 0.9% NaCl and PBS in separate microcentrifuge tubes and brought to the laboratory	Protocols described in a previous paper [66]								[68]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
Subjects rinsed their mouth thoroughly with saline solution to remove excess debris	Samples were obtained by scraping cells from both cheeks with a moist wooden spatula. The spatula was then vigorously shaken in a dark plastic tube containing 10 ml of cold PBS, pH 7.4, and immediately refrigerated		Within 1 h, exfoliated cells were processed by washing twice in PBS. After centrifugation at 800 x g for 3 min, the pellets were suspended in 40 µL PBS.	Samples were assayed in duplicate using 5 µL of the cell suspension for each spot	Under dim, indirect light. 8 µL of a 10 mg/mL proteinase K solution was added to 20 µL of cell suspensions and kept in PBS for 15 min at 40 °C. Microtubes were centrifuged 800 x g for 1 min. The pellets were washed in PBS and, after centrifugation at 6,000 x g for 2 min, suspended in 10 µL of PBS.	After slide preparation, lysis was performed overnight at 4°C in a cold solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, pH 10), where 10 % DMSO and 1 % Triton X-100 were added just before use	DNA unwinding and electrophoresis were performed in an alkaline solution (0.3 M NaOH, 1 mM EDTA, pH 13), for 20 min and 40 min, in a horizontal electrophoresis tank filled with an ice-cold alkaline solution.	At 300 mA and 25 V (0.86 V/cm)	After neutralization step, the slides were dehydrated with absolute ethanol and stored in dry air up to the end of sampling, slides were stained with EtBr (2 µg/mL in H_2O)	[56]
-	-	According to the alkaline single-cell gel electrophoresis method [100, 111]							Silver staining method [108]	[70]
Mouth washed with normal saline (0.9% NaCl) solution	Brushing in the morning before taking any tobacco or tea. Collected samples were taken in PBS	Using a standard protocol with some modifications [61]		Cell suspension mixed with 1% LMP agarose in 1:2 ratio and spread on a microscopic slide pre-coated with 1% NPM agarose.	Trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA) for 30 min followed by proteinase K (1 mg/ml) digestion for 1 h at 37°C		Slides were equilibrated in electrophoresis buffer (10 mM NaOH, 1 mM EDTA, pH 9.1) for 10 min	At 30 V for 20 min	Neutralization for 15 min in 400 mM Tris/HCl, pH 7.4 staining with EtBr (20 µg/ml)	[63]
Water	Scraping the inner part of both sides of the cheeks with a cytology brush. The cells were then gently mixed with 1.5 mL of 0.9 % NaCl and PBS in a micro-centrifuge tubes, taken to the laboratory	Used the Trevigen Comet Assay™ kit protocol		The cells were combined with LMP agarose at 37°C at the ratio of 1:10, and 75 µL aliquots were immediately pipetted onto the slides. The slides were prepared in duplicate and placed flat in a dark place at 4°C for 10 min		Prechilled lysis solution for 60 min	Freshly prepared alkaline solution, pH>13, room temperature in the dark for 45 min. After that, the slides were placed flat on a gel tray	1 V/ cm (measured from electrode to electrode) and applied for 10 min	Slides stained with 50 µL of diluted SYBR Green	[66]

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Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
Rinsing the mouth with temperate water to remove the exfoliated death cells.	A soft interproximal toothbrush was used to collect buccal cells by gently scraping the inside cheek (right and left) of the mouth		The toothbrush was vigorously agitated in 5 ml of cold PBS in a 15 ml plastic tube and the resulting buccal cell suspension centrifuged at 1500 rpm and 15°C for 10 min.	Conventional microscope slides were treated with two layers of agarose. The bottom layer was prepared by dipping the slides into 1.0% of NMP agarose, allowing the agarose to solidify at 4°C for a minimum of 5 min. Then, the top or cell-containing layer consisted of 100 µL of a buccal cell suspensión prepared in LMP agarose at 0.5%, (15 µL of cell suspensión and 85 µL agarose. After covering, the slide was kept at 4°C for 5 min	The cells were subjected to a lysis with 0.25% Trypsin in PBS (15 min, 37°C). Washed with 0.4 M Tris Base solution and subsequently treated with proteinase K (1 mg/ml) for 30 min.	The slides were rinsed, immersed in lysis solution (2.5 MNaCl; 0.1 M EDTA; 10 mM Tris Base; 1% Triton X-100; and 10% DMSO; pH 10) for another hour at 4°C and washed again with 0.4 M Tris Base solution.	Using an horizontal gel electrophoresis tank containing freshly prepared cold (4°C) electrophoresis buffer (1 mM Na,EDTA and 10 mM NaOH, pH 9) where the slides were submerged side by side in the gel tray and left for 20 min to produce single stranded DNA (unwinding).	Electrophoresis was run at 25 V and 300 mA for 20 min	Rinsed with Tris solution. The cells were stained with 75 µL of a 20 µg/ml solution of BrEt	[53]
Before the start of the study, all subjects were instructed to continue brushing but not to use toothpastes and mouthwashes containing fluoride or chlorhexidine	The cells harvested, according to Besaratinia et al. [112] by gentle scraping of the internal part of the right and left cheeks with a wooden tongue depressor. Each tongue depresor was stirred in a 2 mL tube prefilled with 1.5 mL of ice-cold PBS pH 7.4	According with Tice and Vasquez [113]			The lysis step included an additional step of 100 mL of 1 mg/mL of proteinase K for 45 min to enhance the lysis step as recommended by Szeto et al. [61]		Unwinding for 40 minutes in electrophoresis buffer with the pH above 13	Slides were electrophoresed in the alkali buffer at room temperatura at 20 V for 40 minutes, level of the buffer was adjusted until 300 mA	The slides were stained using 50 µL EtBr (20 µg/mL)	[54]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
-	Buccal mucosa cells were obtained by scraping the left inner cheek with a cervical brush		The cells were washed with PBS and centrifuged at 800 rpm for 10 min	Then, 20 μL of the pellet was resuspended in 80 μL of 0.75% LMP agarose		When the agarose solidified the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris; pH 10.0-10.5) containing freshly added 1% (v/v) Triton X-100 and 10% (v/v) DMSO for a minimum of 1 h and a maximum of 2 weeks	To allow DNA unwinding, slides were incubated in a freshly made alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA; pH > 13) for 20 min in a horizontal electrophoresis tank	20 min at 25 V (0.90 V/cm) and 300 mA	Slides were washed three times in a neutralization buffer (0.4 M Tris; pH 7.5) for 5 min, rinsed 3 times in distilled water, and left to dry overnight at room temperaturež Slides were stained with silver nitrate	[79]
Rinse their mouth thoroughly with water to remove unwanted debris	Gently rubbing the inside of both cheeks with an extra soft toothbrush for 1 min each. The participant then rinsed the mouth with 20 ml of 0.9% saline and expectorated into a 50 ml conical-based tube. The toothbrush rinsed in the tube and 30 ml saline was added before the cells were pelleted. The cells washed with PBS (pH 7.4)		The buccal cell suspension was centrifuged	The pellet obtained was mixed with 0.7% LMP agarose and placed on fully frosted roughened slides previously coated with 1% NMP agarose. To the solidified agarose, a third layer of 0.1% LMA was applied		Were immersed in freshly prepared ice cold lysis solution for 1 hour			Stained with EtBr	[80]

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Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
Women rinse their mouth with saline water to remove extraneous materials	Exfoliated buccal epithelial cells (BECs) were collected by scraping the inside of both sides of the cheek with a soft bristle toothbrush. The toothbrush was then agitated in 30 ml cold PBS in a 50 ml plastic tube	Szeto et al. [61]	Buccal cell suspensions was centrifuged at 2500 rpm at 4°C for 10 min	The supernatant was discarded and the cell pellet was resuspended in 100 µL of PBS. 10 µL cell suspension was mixed with 85 µl of pre-warmed (at 40°C) 1% (w/v) LMP agarose in PBS, and immediately applied to a microscopic slide already precoated with 85 µl of 1% (w/v) standard agarose in PBS. The slides were placed at room temperature until the gel layer solidified	50 µL trypsin solution (0.25% trypsin and 1 mM EDTA in Hanks balanced salt solution was layered onto the gel and left for 30 min at 37°C. the slides were washed with PBS, 50 µl of proteinase K solution (1 mg/ml of PBS) applied to each slide 1 h at 4°C	Slides were then immersed in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris and 1% Triton X-100, pH 10) for 1 h at 4 °C	Then the slides were transferred to a Coplin jar containing electrophoresis solution (0.01 M NaOH and 1 mM EDTA, pH 9.1) and left for 20 min (2 × 10 min) at 4°C	18 min at 12 V constant voltage, after which slides were removed and neutralized by immersing in three changes (3×5 min) with 0.4 M Tris at pH 7.5	Slides were then stained with EtBr (50 µL of a 20 g/ml aqueous solution)	[84]
Washed out the mouth three times with tepid water to remove dead exfoliated cells	Buccal swab taken by gentle brushing of the internal part of right and left cheek with a cytobrush. The brushes were stirred in 5 ml of RPMI 1640, liquid (with L-glutamine, 25 mM HEPES), fetal bovine serum, and penicillin-streptomycin solution and transported within 30 min to the laboratory	According with Singh et al. [100]	3 min/3,200 rpm; resuspended in PBS (pH 7.4)	8 µl of cell suspension was mixed with 100 µl of LMP agarose and added to a microscope slide pre-coated with 1.0% of NMP agarose.		Cells were lyzed (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Na-lauroyl-sarcosinate, 1% Triton X-100, and 10% DMSO, pH 10) for 72 h at 4°C and denatured (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0) for 10 min		Electrophoresis was performed at 0.66 V/cm, 300 mA for 16 min	Neutralization (0.4 M Tris-HCl, pH 7.5) staining with EtBr (20 µg/mL) was performed	[55]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
-	-	-	The buccal cell suspension was centrifuged	The pellet obtained was mixed with 0.7% LMP agarose and placed on fully frosted roughened slides previously coated with 1% NMP agarose.		To the solidified agarose, a third layer of 0.1% LMA was applied and were immersed in freshly prepared ice cold lysis solution for 1 hour			Stained with EtBr	[65]
Water	The interior surfaces of right and left cheeks gently scraped with a toothbrush. The cells suspended in 25 ml of Titenko-Holland buffer solution [114] and transferred within 2-8 hr, at 4°C and in the dark, to the laboratory	According with Singh et al. [100]	Cells were washed twice in the buffer solution and then suspended in about 100 µL of the same buffer, immediately before performing the comet assay	90 µl of 0.5% NMP agarose in PBS at 50°C layered onto bond gel film, immediately covered with a coverslip, and allowed to solidify at 4°C for 5 min. The coverslip was then removed, and about 40 µL of lymphocytes suspension or 80 µL of exfoliated cells suspension were mixed with 70 µL of 0.7% LMP agarose in PBS at 37°C, and layered on top of the film. A coverslip was added, and the film was left to solidify at 4°C for 5 min. The coverslip was then removed, and a second layer of 0.7% LMA was added and left to solidify		The coverslips were taken off, the films were layered onto glass slides, and bathed in freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris with 1% Triton X-100, and 10% DMSO added fresh) in the dark for 1 hr at 4°C	The slides were removed from the lysis solution, placed in a horizontal gel electrophoresis tank filled with fresh alkaline buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13) for 20 min at 4°C	Alkaline buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13) at 20 V and 300 mA for 20 min	Slides were then washed three times with 0.4 M Tris HCl for 5 min, and stained with 50 µL EtBr (10 µg/mL)	[76]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
	Buccal epithelial cells were collected by gently scraping the oral mucosa with a moist spatula. Suspended in phosphate buffered saline (PBS) and was processed for the comet assay	According with Singh et al. [100]	The buccal epithelial cell samples were washed with PBS, centrifuged and recovered from the pellet	On a clean, dry, plain slide 100 µL of 0.75% NMP agarose prepared in PBS was layered. These precoated slides were dried at 37°C. On top of this layer, 30 µL of PBL and buccal epithelial cells in PBS, mixed with 110 µL of 0.5% LMP agarose in PBS was layered. The third layer consisted of 100 µL of LMP agarose.		The slides were incubated in cold lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris; 1% sodium lauryl sarcosinate; 1% Triton X-100 and 10% DMSO added fresh) at 4°C overnight	Immersed in freshly prepared alkaline electrophoretic buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13) for 30 min	30 min at 300 mA, 0.67 V/cm	Neutralizing buffer (0.4 M Tris buffer, pH 7.5). The slides were then washed with distilled water and air dried. Silver staining	[64]
The buccal cells were collected three times from each subject at 3 alternate days after the work shift. Workers rinseed the mouth with distilled water	The buccal cells were collected using a toothbrush by scrapping the inside cheek of the mouth. The toothbrushwas agitated in 30 ml cold PBS buffer	Comet assay was performed as described by Szeto et al. [61]	The suspension was centrifuged at 2500 rpm at 4°C for 10 min. The cell pellet was resuspended in 100 µL PBS buffer	10 µL of the buccal cell suspension was mixed with 85 µL of pre-warmed (40°C) 1% (w/v) LMP agarose in PBS (0.137 M NaCl, 2.68 mM KCl, 6.4 mM Na_2HPO_4·7 H2O, 1.47 mM KH_2PO_4, pH 7.4), and immediately applied on a clean microscopic slide precoated with 85 µL of 1% (w/v) NMP agarose in PBS buffer. The slides were allowed to solidify in room temperature	A 50 µL of trypsin solution was layered onto the gel and left for 30 min at 37°C followed by washing with PBS buffer, proteinase K (1 mg/ml) treatment for 1 h at 37°C	Immersion in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h at 4°C	The slides were kept in electrophoresis tank filled with electrophoresis buffer (0.01M NaOH and 1 mM EDTA, pH 9.1) for 20 min	At 12 V for 18 min	Neutralized 3 times with 0.4 M Tris at pH 7.5. The slides were then stained with EtBr (50 µL of 20 µg/ml)	[78]
Washing out the mouth several times with tepid distilled water	Buccal cells were collected from each individual by gentle brushing of the inside part of the lower lip with a cytological brush. The brushes were stirred in 50 ml plastic tubes containing 20 ml of PBS	The alkaline version of the CA was employed in this study [51, 115]	Cells were washed twice, with centrifugation at 1500 rpm for 10 min at room temperature, and resuspended in PBS	Briefly, 10 µL cell suspension was mixed with 75 µL LMP agarose (0.7%) and added to a slide precoated with 100 µL agarose (1%)		Lysis was performed overnight at pH 10	Cells were placed in a electrophoresis chamber, exposed to alkali, pH 13, for 25 min	For 20 min at 25 V (0.86 V/cm) and 300 mA, at room temperature	The slides were neutralized, fixed, and stained with silver nitrate [108]	[52]

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Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
Prior to brushing subjects wash their mouth with 0.9% NaCl solution	Buccal squamous cells were collected from subjects by oral brushing	Comet assay was performed under alkaline conditions by using a standard protocol [100] with some modifications		Cells were embedded in LMP agarose on glass slide precoated with 1% NMP agarose		After solidification of gel the slide was submerged into cool lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10.0), 1% LSS lauryl sarcosine sodium salt to which 10% DMSO, 1% Triton X-100 were freshly added] and kept overnight at 4°C	The slides were then placed on the horizontal electrophoresis unit filled with fresh alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 30 min	At 25 V and 300 mA for 40 min	The slides were neutralized for ~60 min in 0.4 M Tris/HCl, pH 7.5 on ice and staining in EtBr (25 μg/ml in distilled water)	[62]
Wash their mouth with water	The interior surfaces of the right and left cheeks were gently scraped with a toothbrush. The cells were suspended in 25 ml of a buffer solution containing 0.01 M Tris-HCl, 0.1 M EDTA and 0.02 M NaCl (pH 7.0), and immediately sent to the laboratory where the comet assay was performed. The exfoliated buccal cells were washed twice in PBS and then suspended in about 100 μL of the same buffer	The procedure of Singh et al. [100] was used, with minor modifications	The exfoliated buccal cells were washed twice in PBS and then suspended in about 100 μL of the same buffer	90 μL of 0.5% NMP agarose in PBS at 50°C were layered onto gel bond film, immediately covered with a coverslip, and allowed to solidify at 4°C for 5 min. The coverslip was then removed and about 80 μL of exfoliated cell suspension were mixed with 70 μL of 0.7% LMP agarose in PBS at 37 °C, and layered on top of the film. A coverslip was added and the film was left to solidify at 4°C for 5 min. After this, the coverslip was removed and a second layer of 0.7% LMP agarose was added and left to solidify		The coverslips were taken off and the films were layered onto glass slides and bathed in freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, with 1% Triton X 100 and 10% DMSO added fresh) in the dark for 1 h at 4°C	Placed in a horizontal gel electrophoresis tank filled with fresh alkaline buffer (1 mM Na_2EDTA and 300 mM NaOH, pH 13) for 20 min at 4°C to allow denaturing and unwinding of the DNA, and the expression of alkali-labile sites	20 V and 300 mA for 20 min	The slides washed three times for 5 min each with 0.4 M Tris–HCl. Slides were stained with 50 μl of 10 μg/mL EtBr	[77]

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Exfoliated Buccal Cells Sampling		Buccal comet assay technique							Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
Rinsed their mouths with water	To collect exfoliated buccal cells, the right and left cheeks were gently scraped with a toothbrush. The cells were suspended in 25 ml of Titenko-Holland buffer solution [114]	Procedure of Collins et al. [116], with minor modifications	The exfoliated buccal cells were washed twice in PBS and then suspended in about 100 µL of the same buffer	Two gel bond films were prepared for each case (one to be treated with Fpg and the other left untreated) allowing the detection of oxidative DNA and direct DNA lesions (single-double strand breaks and alkali-labile sites), respectively [116]. About 80 µL of exfoliated cell suspension were mixed with 70 µL of 0.7% LMP agarose in PBS at 37°C and layered on top of each film		Then they were bathed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris with 1% Triton X-100 and 10% DMSO added fresh) and kept in the dark for 1 h at 4°C	The slides were washed 3 times in enzyme buffer (50 mM Na_3PO_4, 10 mM EDTA, 100 mM NaCl, pH 7.5), drained and incubated with 50 µL of either buffer or Fpg (1 µg/mL in enzyme buffer) in the dark for 30 min at 37°C. The slides placed in a horizontal gel electrophoresis tank filled with fresh alkaline buffer 1 mM Na_2EDTA and 300 mM NaOH, pH 13) for 40 min at 4°C	In the same buffer at 25 V and 300 mA for 30 min	The slides were then washed 3 times with Tris–HCl 0.4 M for 5 min and stained with 50 µL EtBr (10 µg/ml)	[75]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
Volunteers rinse their mouth thoroughly with filtered tap water	Cell suspensions were obtained by scraping the inner cheek with a wooden stick or with a disposable brush moistened with PBS. The first scraping from each side of the cheek was discarded. The cells from each of the next four scrapings were rinsed into ice-cold PBS using individual coded centrifuge tubes, and were kept on ice until processed (within 30 min).	According with Singh et al. [100] and Valverde et al. [83], with modifications	The cells were centrifuged at 89 x g, for 5 min. 10 μL were used to perform the SCGE assay	Briefly, aliquots of cell suspensions were suspended in 100 μL of molten 0.5% LMP agarose in PBS (cooled to 37°C). This mixture was layered onto a coded slide, precoated with a thin layer of NMP agarose [117]. The agarose layer was covered with a coverslip and left for 5 min at 4°C to solidify	Cell suspension was diluted into 150–300 μL PBS, and treated 1:1 with an enzyme "cocktail" (final concentration: 0.05 mg/ml DNAse I, 0.15 mg/ml collagenase I, and 0.125 mg/ml trypsin in 0.01% EDTA, pH 7.4) for 30 min at 37 (adapted from Olive et al. [118]. After lysis, the slides were treated with PK for 1 hr at 37°C, by layering 100 μL of 1 mg/ml PK in PBS (pH 7.4) onto the slide and adding a coverslip to achieve an equal distribution of solution. Afterwards, the slides were immersed in 400 mM Tris-HCl (pH 7.5) for 5 min to remove excess salt	Slides were immersed into either lysis solution I (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris (pH 10), and 1% sodium sarcosin°C ate, with 1% Triton X-100 and 10% DMSO added just before use) Or Lysis Solution II (1% SDS and 30 mM Na_2EDTA, pH 8) for at least 1 hr at 4°C	Different alkaline (pH > 13) unwinding times (5-40 min) and electrophoresis times (0.66 V/cm, 300 mA, for 5-40 min) were tested in the preliminary experiments. For the cross-sectional experiment, both pre- and postenrichment slides were randomized by location inside the electrophoresis box and by different runs.	Unwinding and electrophoresis for 20 and 10 min, respectively, In some experiments, the electrophoresis was performed without an unwinding step and under neutral conditions (300 mM sodium acetate, 100 mM Tris, adjusted to pH 9 with glacial acetic acid [119] for 1 hr at ~0.5 V/cm and 50 mA at 4°C so as to study the influence of alkali-labile sites on the migration of comets from cell samples	Slides were neutralized in Tris-HCl (pH 7.5) for 5 min, fixed with absolute ethanol, and stored. The slides were stained with 20 μg/ml EtBr	[92]

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Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Refer-ence
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
Distilled water	A soft bristle toothbrush was used to collect buccal cells by scraping the inside cheek of the mouth gently The toothbrush was then agitated in 30 ml cold PBS in a 50 ml plastic tube	Several	Buccal cell suspension was centrifuged at 2500 rpm 4°C 10 min. cell pellet was resuspended in 100 µL PBS	10 µL of this suspension were mixed with 85 µL of pre-warmed (40°C) 1% (w/v) LMP agarose in PBS, and immediately applied to a microscope slide which had been precoated with 85 µl of 1% (w/v) standard agarose in PBS. The slides were placed at room temperature until the gel layer solidified, and then put through the lysing procedure	In some cases lysis was followed by exposure of the cells to 0.1 mg/ml proteinase K (in lysis solution at pH 7.5 without Triton X-100) for up to 5 h in a Coplin jar at 37°C Also analyze the effects of trypsin treatment. The final (optimized) lysis protocol selected used cells pre-embedded in agarose on a microscope slide, with 50 µL trypsin solution layered onto the gel and left for 30 min at 37°C followed by ashing, proteinase K treatment.	Investigated the effect on buccal cells of immersion in standard lysis solution for up to 24 h at 4°C. cell lysis analize the effect of detergents	Slides were then were transferred to a Coplin jar containing electrophoresis solution at 4°C for 20 min (2×10 min). Electrophoresis solution comprised 1mM EDTA with various concentrations of NaOH (0.0003-0.3 M), with pH ranging from 5.9 to >13	Investigated the effect of electrophoresis at lower pH values. Electrophoresis was performed for 18 min at 12 V constant voltage,	Neutralized by immersing in three changes (3×5 min) of 0.4M Tris at pH 7.5. s were stained with EtBr	[61]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
Volunteers were required first to rinse their mouths with water, then to discard the water used in the mouthwash	Collect buccal epitelial cells directly from the inner cheek and sublingual region using a soft tooth brush. The cells collected on the toothbrush were transferred to a sterile PBS solution (pH 7). Cells were washed twice in PBS and then resuspended in RPMI-1640 medium			Slides were prepared in duplicate as follows: 120 μL NMP agarose (1% in PBS) were layered on to precleaned frosted microscope slides, immediately covered with a cover slip and allowed to solidify. Then, 20 μL of cell suspension was mixed with 75 μL 0.5% LMP agarose in PBS at 37°C, the cover slips were removed and the mixture was added to the slides. The cover slips were replaced and the slides were put on ice for 5 min. After solidification of the agarose, the cover slips were gently removed. Another top layer of 75 μL LMP agarose was added, the cover slips were replaced and the slides were again placed on ice	The slides with buccal epitelial and sublingual cells were treated at 4°C for 25 min in the lysis solution containing 10 μL proteinase K (10 mg/ml) in 100 ml lysis solution	Once the top layer had solidified, the cover slips were removed and the slides gently immersed in cold lysing solution (2.5M NaCl, 100 mM Na_2EDTA, 1% N lauryol sarcosine, 10 mM tris-HCl [pH 10], to which 1% Triton X-100 and 10% DMSO had been freshly added)	2 washings with distilled water for 10 min, in a horizontal gel electrophoresis unit containing fresh buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) to a level of 0.25 cm above the slides for 20 min.	20 min, 25 V, 300 mA (0.8 V/cm)	The slides were stained with 50 μL of propidium iodide (200 μm/mL in 50 ml PBS) for 10 min	[60]

http://dx.doi.org/10.5772/62760

Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	**Collecting**	**Followed protocol**	**Centrifuged**	**Slides preparation**	**Enzyme treatment**	**Lysis**	**Pre-electrophoresis**	**Electrophoresis**	**Neutralisation, fixation and staining**	
Rinse their mouths with tap water	Buccal epitelial cells were obtained by scraping the cheeks with a plastic spatula. The cells were added to 2 ml of RPMI-1640 medium		Buccal cells were washed with RPMI-1640 medium and centrifuged at 6,000 rpm for 1 min	Slides were prepared in duplicate, and 120 µl of NMP agarose 0.5%, in PBS was layered on to precleaned microscope slides, immediately covered with coverslips, and allowed to solidify. Then, cells were mixed with 75 µl of 0.5% LMP agarose in PBS at 37°C, the coverslips were removed, and the mixture was added to the slides. The coverslips were replaced and the slides were put on ice for 5 min. After solidification of the agarose, the coverslips were gently removed. A top layer of 75-µl LMP agarose was added, the coverslips were replaced, and the slides were again placed on ice	After lysis, buccal cells were treated with 140 µl of proteinase K (10 mg/ml) at 37°C for 2 h	Once the top layer had solidified, the coverslips were removed and the slides gently immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 1% N lauryl sarcosine, 10 mM tris-HCl [pH 10], to which 1% Triton X-100 and 10% DMSO had been freshly added). The slides were left at 4°C for at least 1 h	The slides placed close together in a horizontal gel electrophoresis tank near the anode. This was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) to a level of 0.25 cm above the slides, which were then left to soak for 40 min in the alkali	For 40 min at 19 V, 300 mA	Neutralising buffer (0.4 M tris-HCl, pH 7.5) was added dropwise to the slides 3 times, 5 min each time. Slides stained with 50 µl EtBr (40 mg/ml)	[50]

Exfoliated Buccal Cells Sampling		Buccal comet assay technique								Reference
Rinsing	Collecting	Followed protocol	Centrifuged	Slides preparation	Enzyme treatment	Lysis	Pre-electrophoresis	Electrophoresis	Neutralisation, fixation and staining	
-	Buccal epithelial cells were obtained by scrapping the internal part of the cheek with a wood stick and were added to 1 ml of cold RPMI-1640 medium.	Rojas et al. [14], with some modifications			After lysis (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-hydrochloride and 1% Nasarcosinate, pH 10) at 4°C for 24 hr, the cells were treated with 100 µL proteinase K (10 mg/ml) at 37°C for 1h		Slides were placed on a horizontal electrophoresis unit. The DNA was allowed to unwind for 20 min, in electrophoresis running buffer solution (30 mM NaOH and 1 mM Na_2EDTA, pH 13).	20 min at 25 V and 300 mA. After, the slides gently removed	Neutralized with 0.4 M Tris, pH 7.5; dehydrated in 2 steps with absolute ethanol for 10 min each, staining with 75 ml EtBr (20 mg/ml) with coverglass	[83]
-	Scrapping the internal part of the cheek with a wood stick	Tice et al. [120] with some modifications	The cells were added into 2 mL of RPMI-1640 medium and centrifuged at 6000 rpm for 1 min, approx. 50000 cells resuspended in 75 µL LMP agarose		After lysis (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% sodium sarcosinate, pH 10) at 4°C for 48 h, the cells were dropped into a new lysis solution with 140 µL of proteinase K (10 mg/ml) at 37°C for 2 h		Slides were placed on a horizontal electrophoresis unit. The DNA was allowed to unwind for 20 min, in electrophoresis running buffer solution (300 mM NaOH and 1 mM Na_2EDTA, pH 13).	For 20 min at 25 V and 300 mA	Neutralization with 0.4 M Tris-HCl, pH 7.5. staining with EtBr (75 µL of a 20 mg/ml solution) was added to each slide	[14]

Table 1. List of the articles and detailed methodology for sampling, slide preparation, lysis step, enzymatic digestion, electrophoresis, neutralization, fixation and staining.

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3. Use of comet assay in buccal cells

The comet assay in buccal cells has been used to evaluate DNA damage induced by different materials such as mouthrinses [50], metals released from orthodontic appliances [51–59], ionizing radiation [60], as well as assessment of DNA damage, and its modulation by life-style, dietary, genetic and healthy factors [61–74], occupational exposure [66–69,75–82], and environmental exposure [83–86]. Different procedures have been used in collecting and processing the samples that are presented and discussed in Rojas et al. [33]. The **Table 2** represents classification according to the type of population study based on exposure and lifestyle factors with the results of comet assay.

3.1. Mouthrinses and metal released from orthodontic appliances

The genotoxic properties of mouthrinses and metals from orthodontic appliances are essential for determining the biological safety of those materials in patients. Current in vivo human studies are aimed at representing the real condition of the oral cavity by sampling buccal cells, which are directly exposed to the appliances [51,52].

Eren et al. [50] evaluated the stability of buccal epithelial cells for SCGE assay after the use of chlorhexidine digluconate (CHX), a mouthrinse used by dentists as disinfecting agent for operation sites washing and for disinfection of root canals. A statistical increase was observed in the DNA damage after the CHX application. Considering orthodontic appliances, the first in vivo study was performed by Faccioni et al. [51], who conducted the alkaline comet assay in orthodontic patients. They reported genotoxic damage and found positive correlations between the concentrations of released cobalt and nickel and the number of comets as well as correlations between Co levels and comet tails. However, Westphalen et al. [52] did not find genetic damage after the placement of the orthodontic appliances.

According to Fernández-Miñano et al. [53], genotoxicity induced in buccal cells could be related to the composition of orthodontic appliances. Orthodontic apparatus made with titanium was not genotoxic for oral mucosa cells, whereas the stainless steel alloy and nickel-free alloy induced DNA damage in buccal mucosa cells. In contrast, Hafez et al. [54] observed that stainless steel brackets with stainless steel archwires produce the least damage, whereas titanium brackets with nickel–titanium archwires produced the highest amount of genotoxicity, assessed with the comet assay. Baričević et al. [55] assessed subjects with Co–Cr–Mo alloy and Ni–Cr alloy showed significantly higher comet assay parameters when compared with controls. Gonçalves et al. [59] showed the genotoxic effects of Hyrax auxiliary orthodontic appliances containing silver-soldered joints.

On the other hand, Hafez et al. [54] reported damage to the DNA in mucosa cells at 3 months of orthodontic treatment but not at 6 months. Thus, the difference in exposure period of prosthodontic and orthodontic appliances in oral cavity might explain discrepancies observed between results obtained by Faccioni et al. [51], and those of Westphalen et al. [52] and Baričević et al. [55].

Visalli et al. [56] found that both amalgams and resin-based composite fillings can induce genotoxic damage in human oral mucosa cells. They also report that lifestyle variables, including alcohol intake and smoking habits, did not affect the genotoxic response and did not act as confounding factors. Martín-Cameán et al. [57] observed induction of genotoxicity in buccal cells of subjects with orthodontic appliances and orthodontic appliances with micro-screws when compared with controls. In addition they found that damage was higher in women.

3.2. Radiation

Only one work that analyses and compares the DNA damage and repair following radiation challenge in buccal cells and lymphocytes using SCGE assay was found. The results suggested that baseline DNA damage in oral epithelial cells is greater than that in lymphocytes [60].

3.3. Life style, dietary, genetic and healthy factors

As mentioned above in the first work of this type, Rojas et al. [14] found a significantly increased tail length in a smoker group compared with a non-smoker group. Differences between genders either in the smoker or non-smoker group were not observed and were neither related to age or number of cigarettes smoked. Waterpipe smoking (a type of tobacco smoking) and its condensate have been examined for the genotoxic effects on buccal cells. The tail moment in buccal cells of smokers was found to be 186 ± 26, which is 371.9% higher than the tail moment in buccal cells of non-smokers. The other comet parameters such as tail length, % tail DNA, and fragmented DNA were 456 ± 71, 97.0 ± 19, and 32.0 ± 3.3, respectively, in buccal cells of smokers, whereas in control group (non-smokers), the values of tail length, % tail DNA, and fragmented DNA were extremely low [72].

Oral habits have also been associated with DNA damage. Khanna et al. [70] reported a case of a tobacco chewer in which the percentage of damaged cells was significantly higher than in the control. Also the effect of gutkha (a preparation of crushed areca nut, tobacco, catechu, paraffin wax, slaked lime, and sweet or savory flavorings) and pan masala (an herb, nut, and seed mixture that is commonly served in the Middle East countries) chewing along with and without smoking was studied in buccal epithelial cells using single-cell gel electrophoresis [71]. The increase in the mean comet tail length was observed as follows: non users < smokers < pan masala chewers < gutkha chewers < pan masala + smoking < gutkha + smoking. Like Rojas et al. [14], they conclude that these bioassay and biomarker are easier and safe methods to detect DNA damage among humans.

Assessment of DNA damage and its modulation by dietary and genetic factors in smokers using the comet assay has also been developed [87]. Pal et al. [62,63] analyzed the influence of regular black tea consumption on tobacco-associated DNA damage and human papilloma virus (HPV) prevalence in human oral mucosa. The increase in DNA damage was significantly associated with increase in age and tenure of tobacco habit. Reduced DNA damage was found to be significantly associated with increase in tea intake. In case of oral cancer patients, comparatively high frequency of DNA damage was observed. The frequency of DNA damage

and HPV infection was comparatively high in oral cancer patients than in the normal subjects. These studies indicated a chemopreventive role of black tea against reducing DNA damage risk of buccal cells due to tobacco exposure. Authors concluded that buccal cells could be used as cytological markers for detection of risk and risk reduction in normal population. Since, as mentioned above, more than 90% of human cancers arise from epithelial cells, it has been postulated that experiments with these cells may have particular relevance for the detection of cancer preventive effects [47].

On the other side, several polymorphisms in DNA repair genes have been reported to be associated with cancer risk [88]. The repair of DNA damage has a key role in protecting the genome from the insults of genotoxic agents. Tobacco-related compounds cause a variety of DNA damage, and DNA repair capacity plays an important role in agent-induced damage genotoxic. Several polymorphisms in genes that participate in different DNA repair pathways, such as XRCC1 399, hOGG1 326 [65], GSTP1 [66], CYP2E1 [67], CYP1A2 [68], and CYP1A1 [69], have been evaluated for their effects on different biomarkers [89], including comet tail length in buccal cells.

DNA damage effects of the used substances were confirmed in mechanical workshops workers, but with no confirmation of the influence of GSTP1 [66] or CYP1A1 [69] gene polymorphism on DNA damage, considering the comet assay performed on buccal cells. Conversely, workers with the wild genotype for CYP2E1 showed statistically significant higher comet tail length at the occupational exposure, while the mutated genotype did not have influence on this biomarker [67]. With CYP1A2 gene, the results showed that DNA damage in cells of workers carrying the mutated genotype was higher than workers carrying the wild genotype [68].

Sellappa et al. [65] found significant differences in the comet scores between smokeless tobacco users and control subjects when XRCC1399 and hOGG1326 polymorphisms and the frequencies of genetic damage among tobacco chewers were studied.

These findings provided evidence for the view that polymorphisms in DNA repair genes may modify individual susceptibility to genotoxic agents and justify additional studies to investigate their potential role in development of genetic damage.

4. The use of the comet assay in buccal cells in biomonitoring the effect of pollution

4.1. Occupational exposure

Cavallo et al. [75] suggested the use of comet assay on exfoliated buccal cells to assess the occupational exposure to mixtures of inhalable pollutants at low doses since these cells represent the target tissue for this exposure and are obtained by non-invasive procedure. In their study, tail moment values from Fpg-enzyme-treated cells (TMenz) and from untreated cells (TM) were used as parameters of oxidative and direct DNA damage, respectively, and

found in the exposed group a higher value in respect to controls of mean TM and TMenz. An oxidative DNA damage was found, for exfoliated buccal cells in the 9.7% of exposed in respect to the absence in controls. On the other side, in healthcare workers in oncology hospital regularly handling antineoplastic drug mixtures, comet assay showed an increase on exfoliated buccal cells, also when it was not statistically significant, of mean TM with respect to controls in day hospital nurses (the group handling the highest amount of drugs during the administration process), while ward nurses and pharmacy technicians did not show the differences [77]. Increased levels of DNA damage were also found among jewellery workers occupationally exposed to nitric oxide using buccal cell comet assay, and also a synergistic effect of DNA damage with the cigarette smoking habit was found among the jewellery workers [78]. On the other hand, Cavallo et al. [76] evaluated two groups of workers, one exposed to antineoplastic drugs and the other exposed to PAHs, but the comet assay on exfoliated buccal cells did not show significant differences between exposed and control groups for comet percentages, whereas the TM value was higher in workers exposed to PAHs. Occupational risk assessment of paint industry workers with the comet assay in epithelial buccal cells showed that the damage index and damage frequency observed in the exposed group were significantly higher relative to the control group [79]. In other study on biomonitoring of genotoxic effects among shielded manual metal arc welders, Sudha et al. [80] showed a significantly larger mean comet tail length values. Among paddy farm workers exposed to mixtures of organophosphates was observed that the tail length formation showed significant increase of tail length differences between farmers compared with the matched control group [81]. Age, smoking status, duration of smoking, and secondhand smoker factors pointed out the significant intragroup variations, among the study population. Smokers and secondhand smokers generally showed higher levels of DNA damage, with increase connected with age and smoking duration increase. The last finding in this study leads again to the hypothesis that occupational risk factors contribute to the main effect on DNA damage. However, Carbajal-López et al. [82] did not find significant effect on genetic damage as a result of age, smoking, and alcohol consumption when genotoxic effect of pesticides in exfoliated buccal cells of workers occupationally exposed in Guerrero, Mexico was evaluated. The study revealed that the tail migration of DNA increased significantly in the exposed group.

4.2. Environmental exposure

After the first publication with comet assay in buccal cells by Rojas et al. [14], the same group [83] with this bioassay investigated differences in the level of DNA damage between young adults from the southern and northern areas of Mexico City and compared its effects with the damage induced in leukocytes and nasal epithelial cells. They found an increased DNA damage in leukocytes and nasal cells from individuals who lived in the northern part; however, no differences were observed for buccal epithelial cells, highlighting that it is important to study the genotoxic effects in other cells besides lymphocytes, as well as in cells of those tissues which are the first sites of contact with toxic pollutants. Although in their first work DNA damage in smokers was reported, in this work, they reported that smoking habit did not significantly increase DNA migration when compared with the non-smoker group.

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A study of indoor air pollution from biomass burning was performed on Indian women engaged in biomass cooking (wood, dung, crop residues), and the group was compared with age-matched control women cooking with cleaner fuel liquefied petroleum gas. DNA damage was assessed on buccal epithelial cells (BEC) by comet assay and fast halo assay (FHA). Compared with control, BEC of biomass users showed higher comet tail % DNA, higher values for comet tail length, and olive tail moment, suggesting marked increase in DNA damage [84].

5. Clinical application of the comet assay in buccal cells

Significant stepwise increase in the DNA damage (basal/MNNG-treated/post-repair) was observed in buccal epithelial cells from control to pre-cancer patients and from pre-cancer to cancer patients. Considerable inter-individual and intercellular variability in DNA damage was observed, which also increased from control to pre-cancer patients and from pre-cancer to cancer patients [64]. Similar results were found in patients with oral squamous cell carcinoma (OSCC) and control group and suggested that comet assay may be used effectively to assess the prognosis of OSCC [73].

Among population studies regarding the health effects of air pollution, special attention should be given to children as a high-risk group, since some studies have shown significant correlation between early childhood exposure and development of chronic diseases in adulthood. Genotoxic biomarkers have been studied largely in adult population, but few studies so far have investigated children exposed to air pollution. Children are a high-risk group as regards the health effects of air pollution, and some studies suggest that early exposure during childhood can play an important role in the development of chronic diseases in adulthood. Genotoxic effects among farm children assessed with comet assay in buccal cells showed a significant increase in chromosome breakage and DNA strand breaks [85]. In other similar study, the exposure to pollutants was associated with markers of genotoxicity in exfoliated buccal cells of children living in a region with chipboard industries. The increase of outdoor formaldehyde was associated with a higher comet tail intensity and a higher tail moment [86].

6. Confounding factors in studies with the comet assay in buccal cells

A systematic and adequately powered investigation of key variables such as age, gender, genotype, season, diet, oral hygiene and dental health, life-style, smoking, alcohol, and other recreational drugs needs to be performed to identify the variables that have to be controlled [7].

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
MOUTHRINSES AND METAL RELEASED FROM ORTHODONTIC APPLIANCES										
9	4	9	4	Subjects filled in detailed questionnaires regarding confounding factors for DNA damage such as smoking, viral diseases, recent vaccinations, and radiodiagnostic examinations.	*Before CHX treatment* Undamaged 91.54 ± 6.75 Intermediate nuclei 6.00 ± 4.85 Tailed nuclei 2.46 ± 3.73 15.77 ± 4.6*After CHX Treatment* Undamaged 71.15 ± 7.12 Intermediate nuclei 13.08 ± 4.94 Tailed nucle 15.77 ± 4.6	Determine the degree of damage by grading the cells as undamaged, intermediate, and tailed.	Wilcoxon's test	DNA damage in peripheral blood cells	*Before CHX treatment* Undamaged 93.77 ± 7.65 Intermediate nuclei 5.62 ± 6.91 Tailed nuclei 0.62 ± 1.50*After CHX Treatment* Undamaged 82.62 ± 8.35 Intermediate nuclei 11.31 ± 7.93 Tailed nuclei 6.08 ± 4.44	[50]
55 orthodontic patients with fixed appliances in both arches: nickel-titanium alloy, stainless steel or chromium-cobalt-nickel alloy		30, no dental restorations		Smoking, drinking	TL: 10.54 ± 2.41 vs 15.56 ± 6.78TM: 0.46 ± 0.21 vs. 0.30 ± 0.09TI: 5.44 ± 1.89 vs. 4.72 ± 1.51	% DNA, tail length, TM	Mann-Whitney U test	Apoptosis, viability	Apoptosis: 3.15 ± 4.93 vs 1.00 ± 2.26,Viability%: 50.40 ± 13.55 vs 73.43 ± 12.29,	[51]
33 12-35	32 12-35	13 12-33	17 12-35							
14 16 + 2.5	6 16 + 2.5	14 16 + 2.5	6 16 + 2.5	Smoking or drinking or illnesses related to any genetic damage increase were not reported by any patient.	2.5 ± 3.08 vs 1.5 ± 1.05	Damage was visually scored according to five classes, based on tail size (from undamaged - 0, to maximally damaged - 4). Damage index (DI) was thus assigned to each individual, according to Hartmann et al. [121]. The DI is is a well-validated evaluation method as it is highly correlated with computer-based image analysis [122]	The one-tailed t-test with Welch's correction was used	Micronucleus assay	MN frequency (p = 0.0213)	[52]

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Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
15 patients 12-16 after treatment with metal apparatus for orthodontic treatment: 4 tubes and 20 brackets for 30 days: 5 with stainless steel, 5 with titanium, 5 with nickel-free		15 12-16 the same patients before treatment		The inclusion criteria were: absence of systemic diseases, need of orthodontic treatments in both dental arches, absence of cavities or any repaired treatment in the oral cavity, with good oral health and absence of any disability to impede a correct oral hygiene, and that the treatment does not generate in the patients any psychological alteration or difficulties in their everyday relationships.	Stainless steel 69.35 ± 11.68; Nickel-free 68.41 ± 26.63; H_2O_2 71.10 ± 5.15Titanium alloy and controlsOlive moment was similar	Olive moment	ANOVA, Tukey posthoc			[53]
22 20.2 ± 4.4	6 20.2 ± 4.4	10 21.5 ± 3.3	8 21.5 ± 3.3	Subjects were initially screened with a questionnaire to check whether they fit the criteria of the study. The eligibility criteria for subject selection included nonsmokers; no oral diseases, systemic diseases, oral restorations or prosthetics; clinically healthy oral mucosa; no previous orthodontic treatment; no occupational exposure to metals; not receiving any medications or supplements; no radiographic examination during the previous 6 months; and no known allergy to costume jewelry, watches, or sources of nickel and chromium.	DNA damage value, decreased from 125.6 ± 46.05 to 98.8 ± 33.70 at 6 months	Only nucleoids of the same size were chosen subjectively for scoring; A grade was given to each nucleoid according to DNA fragmentation in the comet tail. Also the damage frequency was calculated; this represents the number of comets per 100 examined nucleoids.	Normally distributed variables (composite score and damage frequency) were tested with paired t tests.			[54]

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
30 69.56		25 72.68		Exhaustive medical history was documented for all subjects. A prestructured questionnaire on dietary and smoking habits, alcohol and drug intake, as well as on systemic diseases and verified allergy to known allergens and medications has been filled for each subject..	Significantly increased tail length and percentage DNA in the tail values in subjects wearing metal appliances	DNA damage was evaluated as percentage DNA in the tail (% DNA) and tail length	ANOVA vMann–Whitney U test *t* Test Newman–Keuls test	Evaluate influence of general characteristics of the subjects (age, gender, dietary habits, pH of saliva, alcohol and drug intake) on parameters of comet assay.	None of demographic or lifestyle factors tested as possible predictors have exhibited significant influence on values of comet assay parameters	[55]
26 21.1 ± 0.30	17 21.1 ± 0.30	12 20.0 ± 0.55	8 20.0 ± 0.55	Collect information on age, gender, smoking, drinking, dietary habits, and previous drug intake. Moreover, chewing gum habits, toothbrushings per day, consumption of hot food and drinks, and bruxism behavior that could promote the release of restorative compounds	The DNA % was dose-dependently higher in subjects carrying dental fillings as compared with filling-free subjects. In subjects carrying at least two fillings	The results were expressed as percentage of DNA in the tail (TDNA %), measured by the automated image analysis system CASP (comet assay software project) (http:\\sp. sourceforge.net).	Mann–Whitney test and Poisson regression analysis	Morphological markers of cell death, including pyknosis (condensed chromatin), karyorrhexis, and karyolysis, were evaluated at the microscopic analysis of the same slides used for the MN test.	MN frequency higher in subjects with restorative fillings than in filling-free subjects	[56]
20 persons as positive control (smokers)20 patients with orthodontic treatment,20 patients with orthodontic appliances and microscrews		20			% DNA in tail significantly different between all fours groups, females with orthodontic appliances	% DNA in tail	-	-	-	[57]

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Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
16 (7-14)		16 (7-14). Each patient acted as his/her own control			*Damage frequency* 53.25 % vs 35.94 %*Damage indice* 75.69 vs 50.31	Cells were scored visually according to tail length into five classes: class 0: undamaged, without a tail; class 1: with a tail shorter than the diameter of the head (nucleus); class 2: with a tail 1 to 2X the diameter of the head; class 3: with a tail longer than 2X the diameter of the head and class 4: comets with no heads.	Wilcoxon's test	BMCyt	No significant differences were observed	[59]
RADIATION										
3 (34–45)	3 (34–45)	3 (34–45)	3 (34–45)	Participants were non-smokers, did not ever smoke and their weekly alcohol intake was less than 10 ml. Were healthy and had not received any medication for chronic/acute diseases were included in the study	DNA damage in oral epithelial cells is greater that in lymphocytes; There is no difference between the baseline DNA damage rate of buccal epithelial cells and sublingual cells;	A slide's visual score was converted to an arbitrary DNA damage score. The nuclei showing comets were categorized as follows: 1º (>80% DNA in comet head), 2º (50–79% DNA in comet head), 3º (20–49% DNA in comet head) and 4º (<20% DNA in comet head). Nuclei without DNA damage were listed in category 0	ANOVA Tukey's multiple comparison test Student's t-test Pearson correlation factor between parameters	Damage in lymphocytes	There is no difference between the baseline DNA damage rate of C0 and G1 lymphocytes; For all cell types there is a significant difference in baseline DNA damage rate between individuals.	[60]

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM		used		
LIFE STYLE, DIETARY, GENETIC AND HEALTHY FACTORS										
6 (24–43)	5 (32–63)	6 (19–43)	3 (25–34)		89.30 ± 16.18 μm vs52.01 ± 10.43 μm	DNA migration was measured with a scaled ocular as the total image length (including head and tail length).	Student's t-test			[14]
	1 (28)			Chewing tobacco since the last 17 years	59.16 ± 2.84 vs14 ± 1.87	DNA damage was quantified by visual classification of cells into categories of comets corresponding to the DNA damage [123, 124]		Chromosomal aberrations MN	CA 2.4 ± 0,69 vs1.2 ± 0.41 MN1,5 ± 0.5 % vs0,05%	[70]
50 gutkha chewers smokers (37.7 ± 1.50)50 pan masala chewers smokers (32.2 ± 1.18)50 gutkha chewers (32.5 ± 1.63)50 pan masala chewers (30.0 ± 1.42)50 smokers (40.1 ± 1.71)		50 with no addiction (29.7 ± 1.41)		Age, smoking habbit,consumation of guthka and pan masala	36.9 ± 3.6033.6 ± 3.5931.6 ± 3.5229.3 ± 3.4114.9 ± 0.79vs control:3.41 ± 0.41	TL, using comet score 1.5 software	Student's 't' test	-	-	[71]
	20 37.55		20		*Tail moment* 186 ± 26 vs 0.05 ± 0.001 *Tail length* 456 ± 71vs 9 ± 1.3 % *tail DNA* 97 ± 19 vs 1.12 ± 0.02 *Fragmented DNA* 32 ± 3.3 vs 3.4 ± 0.03	Analyzed for comet parameters using LAI Comet analysis system		Comet assay in human peripheral blood leukocytes	*Jurak smoke Condensate* Tail moment 12.61 ± 7.41 vs 0.01 Tail length 160.74 ± 47.66 vs 2.0 % tail DNA 22.36 ± 8.87 vs 0.31 Fragmented DNA 5.09 ± 1.41 vs 2.8 *Moassel smoke condensate* Tail moment 21.86 ± 13.33 vs 0.01 Tail length 213.10 ± 75.22 vs 2.0 % tail DNA 29.03 ± 9.77 vs 0.31 Fragmented DNA 5.23 ± 1.43 vs 2.78	[72]

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Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
Cancer patients 6	Cancer patients 31	Tobacco users 2 No users 14	Tobacco users 84 No users 31	Prior to the study all subjects gave informed consent in project participation. Oral cancer patients who had medical treatment or radiotherapy were excluded. Studied subjects were interviewed using a questionnaire to survey possible confounding factors.	*% DNA damage Oral cancer patients* 19.1 ± 9.14 *Tobacco Users* 7.10 ± 3.65 *Non-tobacco Users* 4.56 ± 2.68	DNA damage is represented as percentage data	T-test of unequal variance, Chi-square test Multivariate analysis.	Confounding factors	The evaluation of various confounding factors like age, tenure of tobacco habit and tea habit showed significant associations with DNA damage	[62]
21	125	93	69	Were screened using a questionnaire to find out the posible factors (age, tobacco habit, tea habit) that could affect ROS generation and DNA damage	*TD%* <65 59.58 ± 4.18 >65 61.86 ± 4.64 *Control* <65 40.46 ± 6.34 >65 46.09 ± 3.8	Tail DNA percentage (TD%) Olive tail moment (OTM) The Mean TD% and OTM for each group were compared with mean values of control subjects of respective age groups.	Student or to the study all *t*-test One way ANOVA	Intracellular ROS levels Apoptosis rate	In the <65 y age group percentage of apoptotic cells was low in the control subjects as well as in the subjects with/without tobacco and/or tea habit (7–9%). In case of >65 y age group, percentage of apoptotic cells was comparatively higher in the control subjects (17 ± 2.8%) and slight increase in apoptosis was observed in rest of the subgroups.	[63]
52 45.4 ± 10.2	104 45.4 ± 10.2	18 50.4 ± 8.7	52 50.4 ± 8.7	Questionnaires were completed to obtain detailed occupational, smoking, and medical histories.	Tail Length) <45 years 34.3 ± 1.12 vs 32.1 ± 1.14 ≥45 years 34.3 ± 1.21 vs 32.5 1.01	Tail length (TL) and tail moment (TM) were evaluated, with Comet Assay II	Student's t-test	MN chromosomal aberration assays	*MN Tobacco chewers* Male 2.2 ± 0.67 Female 2.0 ± 0.47 vs *Control* Male 0.86 ± 0.52 Female 1.2 ± 0.91 *Total Chromosomal Aberrations (CA) Tobacco chewers* 2.18 ± 1.31 vs *Control* 1.21 ± 0.91	[65]

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
78 wild genotype 42 mutant genotypes		66 wild genotype 54 mutant genotypes		The subjects were interviewed to evaluate their health status and lifestyles. None of the occupationally exposed subjects wore gloves. None of the workshops had proper ventilation	Workers with the mutated genotype (Ile-Val, Val-Val) had a significantly greater comet tail length than controls. The same is true for workers with the wild genotype Ile-Ile,	Tail length. The cells were analysed using commercial TriTek Comet Score (version 1.5) software.	ANOVA Non-parametric Mann-Whitney U-test. Independent t-test. Chi-square test	MN PCR Restriction fragment length polymorphism (RFLP). Telomero length	Workers with the mutated genotype (Ile-Val, Val-Val) had a significantly higher MN frequency, shorter telomere length than controls. The same is true for workers with the wild genotype Ile-Ile,	[66]
80 wild genotype 40 mutant genotypes		95 wild genotype 25 mutant genotypes		The subjects were interviewed to determine their health status and lifestyles.	c1c1 genotype 25.64 ± 9.35 vs 18.02 ± 8.40 c1c2 and c2c2 genotypes 24.09 ± 7.86 Men 15.42 ± 5.97 Mutated genotype (c1c2 and c2c2) not influenced significantly comet tail length	Tail length	Non-parametric Mann–Whitney U-test.	MN PCR RFLP Telomero length	Workers with the wild genotype showed statistically significant higher MN frequency, and shorter telomere length at the occupational exposure. The mutated genotype influenced significantly MN frequency in the workers, while the influence was not significant in relative telomere length	[67]
58 wild genotype 62 mutant genotypes		60 wild genotype 60 mutant genotypes		Subjects were interviewed about their health status, educational level, smoking habits, alcohol consumption, work history, duration of working at one occupation and other aspects relevant to the study	WW genotype 23.70 ± 8.59 vs 17.14 ± 7.81 MW and MM genotypes 26.46 + 9.01 vs 17.82 + 8.24 No statistically significant effect was found in wild (WW) or mutated genotypes (MW, MM)	Tail length	Non parametric Mann-Whitney U-test.	MNPCRRFLPTelomero length	Difference in MN frequency between workers and controls was statistically significant in both wild and mutated genotypes. In addition, the results showed that the mutated genotype significantly affected the relative telomere length in workers.	[68]

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Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
56	64	52	68	Subjects were interviewed about their health status, educational level, smoking habits, alcohol consumption, work history, duration of employment, and other aspects relevant to the study. In addition, duration of employment was assessed, and subjects were divided into 2 groups of more or less than 5 years of employment.	24.99 ± 9.14 vs 17.47 ± 8.40 Neither genotype showed any statistically significant effects	Tail length	Non parametric Mann–Whitney U test.	MNPCRRFLPTelomero length	The workers carrying wild or mutated genotypes showed a significantly higher MN frequency and shorter telomere length compared to controls	[69]
50 untreated patients with cáncer 50.42 (20-72) 20 untreated patients at precancer stage. 29.55 (17-50)	79 untreated patients with cáncer 50.42 (20-72) 118 untreated patients at precancer stage. 29.55 (17-50)	35 healthy 30.80 (16-67)	141 healthy 30.80 (16-67)	Case history and personal details were collected. Data included age and gender with similar smoking and tobacco use (chewing), dietary habits and socioeconomic status.	*Comet tail length Cancer* 28.64 ± 4.97 *Pre-cancer* 20.93 ± 5.58 *Control* 9.15 ± 3.83	Tail length was measured with an ocular micrometer fitted in the eyepiece	Student's t-test (paired and unpaired comparisons) and analysis of variance were carried out to evaluate various differences.	MN conducted on the buccal epithelial cells; Comet assay on peripheral blood leukocytes; The challenge comet assay on peripheral blood leukocytes.	% *MN Cancer* 0.48 ± 0.33 *Pre-cancer* 0.31 ± 0.24 *Controls* 0.21 ± 0.18 There was a significant stepwise increase in comet tail length from control to patients with precancer and then to cancer patients.	[64]
30 patients with oral squamous cell carcinoma (OSCC)		30 without OSCC		Patients who were diagnosed as having OSCC formed the study group	*OSCC* 3.874 ± 2.5205 μm vs *Normal subjects* 0.8616 ± 0.8142 μm	Total length and the diameter was measured.	Students' test. One way ANOVA "F"	To analize DNA damage, patients having OSCC were divided into four stages, namely stage I, II, III, and IV	Stage I 2.312 ± 0.366 Stage II 3.171 ± 1.439 Stage III 3.490 ± 1.971 Stage IV 6.890 ± 3.710	[73]

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
OCCUPATIONAL EXPOSURE										
41 43.0 ± 8.3		31 43.35 ± 9.4		All subjects gave informed consent. Anagraphic, clinical, working information and lifestyle habits (smoking, dietary habit, alcohol consumption) were obtained from a questionnaire administered by specialized medical personnel	*TM* 118.87 vs 68.20 *TMenz* 146.11 vs 78.32	Tail moment from Fpg-treated cells (TMenz) and Fpg-untreated cells (TM). Values TMenz and fromTM were used as parameters of oxidative and direct DNA damage, respectively. TMenz/TM ratio higher than 2.0 was used to indicate the presence of oxidative damage.	Student's t-test	MN and Fpg-modified comet assay on lymphocytes and exfoliated buccal cells, and by chromosomal aberrations (CA) and sister chromatid exchange (SCE) analyses	The exposed group showed a higher mean value of SCE frequency in respect to controls (4.6 versus 3.8) and an increase (1.3-fold) of total structural CA in particular breaks (up to 2.0-fold) and fragments (0.32% versus 0.00%), whereas there were no differences of MN frequency in both cellular types. Comet assay evidenced in the exposed group a higher value in respect to controls of mean TM and TMenz in lymphocytes (TM 43.01 versus 36.01; TMenz 55.86 versus 43.98.	**[75]**
Pharmacy technicians 2 35.8 ± 9.9 Day hospital nurses 10 37.6 ± 5.5 Ward nurses 11 32.7 ± 7.7	Pharmacy technicians 3 35.8 ± 9.9 Day hospital Nurses 2 37.6 ± 5.5 Ward nurses 2 32.7 ± 7.7	25 34.9 ± 8.5	5 34.9 ± 8.5	Data collection was by a questionnaire which included information on age, gender, life style, and habits (diet, smoking, alcohol consumption, chronic drug use), the types of antineoplastic drugs handled, and the number of mixtures prepared and administered	*Pharmacy technicians* 32.6 ± 18.2 *Day hospital nurses* 43.2 ± 36 *Ward nurses* 27.4 ± 13.9 *Controls* 28.6 ± 12.4	TM	ANOVA Chi square Student t test Kolmogorov-Smirnov non-parametric test Levene test	Comet assay in lymphocytes cells	*Pharmacy technicians* 20.8 ± 10.1 *Day hospital nurses* 15.5 ± 9 *Ward nurses* 14.7 ± 7.9 *Controls* 16.1 ± 8.1	**[77]**

http://dx.doi.org/10.5772/62760

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
				A questionnaire was used to collect the information on sex, age, duration of exposure, use of protective masks, general health status, smoking habits and exposure to drugs for each exposed and control subject.	The significant differences in the comet class between the controls and jewellery workers shows that the later group has increased DNA damage who are occupationally exposed to nitric oxide.	The comets were analyzed by visual classification and the damage was assigned to 5 classes [125]. The percentage of tail DNA was calculated according with Zhao et al. [126]	Student's t-test			[78]
87 (39.49 6 9.11) 30 (35.17 ± 7.4) workers exposed to antineoplastic drugs and 57 workers exposed to PAHs included 41 airport workers (43 ± 8.3) and 16 paving workers (38.62 ± 10.6.		76 (39.72 6 10.1)		Personal data, clinical and working information, and lifestyle habits (smoking, dietary habit, and alcohol consumption) were obtained from a questionnaire administered by specialized medical personnel.	% *Comets* 13.74 ± 10.9 vs 13.78 ± 9.80 *Tail moment* 48.01 ± 30.1 vs 32.31 ± 12.79	The percentage of comets on total cells was calculated. Measurements of comet parameters were: % DNA in the tail, tail length, tail moment	Students' t-test, Mann-Whitney U-test, ANOVA Kruskal-Wallis, and Bonferroni test	Comet and MN tests were performed on lymphocytes and exfoliated buccal cells.	The MN assay on lymphocytes did not show significant differences between exposed and controls, while the MN assay on exfoliated buccal cells showed higher values in workers exposed to antineoplastics as compared with controls (0.85 vs. 0.48). The comet assay on lymphocytes showed a higher comet percentage value (18.11 vs. 11.24 in controls) and mean tail moment (TM) value (21.84 vs. 16.72 in controls) in individuals exposed to PAHs as compared with controls; no significant differences were found in exposed to antineoplastics.	[76]

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
	58 29.03 ± 9.98		30 28.24 ± 10.99	According to the protocol published by the International Commission for Protection against Environmental Mutagens and Carcinogens [127] and participate in a face-to-face questionnaire which included standard demographic data (age, gender,) as well as questions relating to medical issues (exposure to X-rays, vaccinations, medications), life style (smoking, coffee, alcohol, diet,) and their occupation (number of hours worked per day, time exposed to organic solvents, use of protective measures).	*Damage Frequency* 22.38 ± 17.28 vs 13.56 ± 12.69 *Damage index* 33.43 ± 30.18 vs 18.81 ± 18.93	Cells were scored visually into five classes, according to tail size and shape (from undamaged – 0, to maximally damaged – 4), and a value (damage index (DI)) was assigned to each Comet according to its class [128]. DI thus ranged from 0 (completely undamaged: 100 cells×0) to 400 (with maximum damage: 100 cells×4. [22]. The damage frequency (DF) (%) was calculated based on the percentage of damaged cells (0–100%).	Non-parametric Mann–Whitney U-test	In peripheral blood lymphocytes and oral mucosa cells of paint industry workers	No significant difference was detected between the ontrol and paint industry workers. Comet assay data in peripheral blood leukocytes showed that both analysis parameters (DI and DF) were signifi cantly greater than that for the control group	**[79]**
85 35 (20–42)		76 34 (21–41)		During personal interview, each participant was requested to furnish information about age, education, family size and income, habit, cooking time per day, years of cooking, fuel and oven type, location and ventilation of kitchen, health problems in past 3 moths and last one year.	*Comet tail % DNA* 32.23 ± 8.51 vs 12.41 ± 3.87 *Comet tail length* (μm) 37.81 ± 11.21 vs 14.22 ± 3.89 *Olive tail moment in arbitrary unit* 7.08 ± 2.11 vs 3.15 ± 0.97	% Comets TL TM	Student's t-test Mann–Whitney U-test	Fast Halo Assay (FHA) Nuclear difusión factor (NDF)	There was 5-fold increase in DNA diffusion in BEC of biomass users, implying greater DNA damage than that of control. NDF	**[84]**

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Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
66 37.3 ± 7.45		60 38.7 ± 8.21		The selection criteria for the subjects were based on a questionnaire according to the protocol published by the International Commission for Protection against Environmental Mutagens and Carcinogens [127]	Welders showed a significant larger mean comet tail length.compared to controls. In exposed group, a significant difference was observed between smokers and non-smokers and between alcohol drinkers and never drinkers in relation to DNA migration. DNA damage was further found to be significantly higher in subjects with a longer duration of work	Tail length	Students 't' test.	MN	Welders showed a significant increase in micronucleated cells compared to controls	[80]
	160 40.13 ± 10.60		160 40.22 ± 9.79	Personal lifestyle, occupational, and residential information	TL 24.35 µm vs 12.8 µm	The cells were then analyzed by using the TriTek Comet Score (version 1.5) software. The tail length was measured (µm)		The effect of individual factors and levels of DNA damage by examining the significant differences in age,body mass index (BMI)), smoker and secondhand smoker, smoking duration, and number of cigarette per day (smoking frequency) among the study population	Age, smoker, smoking duration, and secondhand smoker highlighted the significant difference within groups, among the study population. Overall, smoker and secondhand smokers reported with higher levels of DNA damage, and this impairment increased with age and smoking duration.	[81]

Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
111 45.75 ± 3.5		60 37.55 ± 0.2		Complete a standardized questionnaire with personal data related to age; time of exposure, habits such as smoking and alcohol consumption, drugs, and diets; the type of work performed; and protective measures used. The questionnaire also included a history of recent illness and medical treatment, as well as of their knowledge about the pesticides used in these agricultural areas.	Tail migration of DNA increased significantly in the exposed group	Comet tail length (DNA distance	Kruskal-Wallis non-parametric test. ANOVA test. Tukey-Kramer multiple comparison test.	MN assay and other nuclear anomalies such as nuclear buds, karyolysis, karyorrhexis, and binucleate cells were also evaluated	Showed nuclear anomalies associated with cytotoxic or genotoxic effect. No significant effect on genetic damage was observed as a result of age, smoking, and alcohol consumption	[82]
ENVIRONMENTAL EXPOSURE										
South 32 19		North 16 19		Each student answered a self-applied questionnaire translated and validated from the American Thoracic Society (ATS) for respiratory tract symptoms	*South* 137.59 ± 55-88 vs *North* 121.96 ± 58.72	DNA migration (tail image length) Relative DNA damage index	U Mann-Whitney test	Alkaline SCG assay using leukocytes and nasal cells	*Leukocytes* South 13.97 ± 9.32 vs North 8.76 ± 3.80 *Nasal* South 40.07 ± 21.07 vs North 23.12 ± 10.36	[83]
54 10 ± 0.82	41 10.02 ± 0.80	43 10 ± 0.82	42 10.02 ± 0.80		8.45 ± 3.89 vs 4.38 ± 1.66	The level of DNA damage was measured using comet assay following the method described previously [14] and modified based on standard procedures from the comet assay kit	*t* statistic	MN	5.05 ± 2.45 vs 2.92 ± 1.54	[85]

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Exposed population		Control population		Interview	Results BCA	Parameters measured	Statistics	Other methods used	Results	Author
Females (n), age	Males (n), age	Females (n), age	Males (n), age		Exposure group vs control group	arbitrary units, TL, TI, TM				
231 9.4 ± 1.6	182 9.5 ± 1.6			The follow-up questionnaire is a short version of the baseline questionnaire on children's health and risk factors [129], with some additional items on oral hygiene	*Tail intensity (%)* 3.25 ± 0.88 *Tail length (μm)* 11.69 ± 2.11 *Tail moment* 0.20 ± 0.05	With Comet Assay II. DNA damage was quantified as: Tail intensity Tail length Tail moment The median of each parameter was used as the representative value for each subject [130]	Analysis of variance for quantitative variables and Pearson's chi-square test for categorical variables			[86]
9		10		Samples were obtained from volunteers among the laboratory staff	*Preenrichment* % Tail DNA 63.8 ± 70.2 vs 65.3 ± 13.9 Tail moment 25.8 ± 5.3 vs 23.1 ± 5.5 *Postenrichment* % Tail DNA 42.4 ± 20.4 vs 31.9 ± 10.5 Tail moment 15.4 ± 11-6 vs 9.1 ± 4.4	% Tail DNA Tail moment	Normality was tested by the Shapiro-Wilks W test. Student's t-test, paired and unpaired McNemar Chi^2 test	DNA damage in peripheral leukocytes	PK digestion increased the DNA migration and head diameter of leukocytes, regardless if they were untreated or treated with MMS. Therefore, PK digestion did not affect the ability of the assay to detect MMS-induced DNA damage	[92]

BCA: Buccal Comet Assay
BMCyt: Micronucleus Cytome Assays

Table 2. Information about exposure type, population studied, results, and statistics in observed articles with buccal comet assay.

None of demographic or lifestyle factors tested as possible confounding factors (age, gender, dietary habits, pH of saliva, alcohol, smoking habits, drug intake, and others have exhibited significant influence on values of comet assay parameters in buccal cells [55,56,64,66,67,76,82, 83,85]. On contrary, Pal et al. [62] in their evaluation of various confounding factors like age, tenure of tobacco habit, and tea habit showed significant associations with DNA damage. In the same line, Sudha et al. [80] showed that the combined exposure to cigarette smoke and Cr(VI) increased basal DNA damage in buccal epithelial cells of welders. How et al. [81] characterized potential risk factors that influence levels of DNA damage from exposure to mixtures of organophosphates, among all, age, smoking habit, smoking duration, number of cigarettes (per day); and secondhand smokers highlighted the significant differences between subjects and within groups. Martín-Cameán et al. [57] observed that DNA damage in buccal cells induced for orthodontic appliances was higher in women, and Jayakumar and Sasikala [78] found a synergistic effect of the habit of cigarette smoking among the jewellery workers.

7. Perspectives

The assessment of genotoxic risk in exfoliated buccal cells is a potentially useful and minimally invasive cytogenetic technique for measuring DNA damage in humans [7,12,17,18,46].

The comet assay is a widely used biomonitoring tool for DNA damage. The most commonly used cells in human studies are peripheral lymphocytes, harvested from venous or capillary blood. However, there is an urgent need to find an alternative target human cell that can be collected from normal subjects with minimal invasion [61].

Buccal cells are becoming an increasingly popular tissue source in human biomonitoring after exposure to occupational and environmental genotoxicants, particularly because they can be obtained non-invasively [50,61,90,91]. However, the number of publications referring to the human buccal comet assay is low in the last two decades. This low growing interest may be explained by several factors, including its relative technical problems.

A priority in this field should be to develop a protocol that could enable buccal cell lysis and DNA damage testing in the comet assay and to use the model to evaluate the potential of the buccal cells in human biomonitoring study [61].

Specialized cellular membranes, which make cell lysis difficult, contribute to making buccal mucosa cells a more complicated cell to SCGE assay [92]. As firstly mentioned in the review of Rojas et al. [33], there are studies that use proteinase K together with the lysis step in order to gain free nucleoids, and there are studies that do not use this enrichment, but only lysis solution, and it has been shown that results depend on this step. Szeto et al. [61] described the development of an improved protocol in which agarose embedded cells of epithelial origin from the mouth were digested with trypsin and proteinase K. Their early trials with buccal cells following the published protocol by Rojas et al. [14] were completely unsuccessful. They found that buccal cells sustained massive damage and disintegration at the high pH used, while at lower pH values, the cells were extremely resistant to lysis. According to these authors,

http://dx.doi.org/10.5772/62760

it is not possible to use earlier protocol developed as it leads to extremely high background levels. The adequate experimental design of SCGE trials in buccal cells is still a matter of debate, and the evaluation of the available data shows that there is an urgent need to develop guidelines [93].

Proper collection and storage of human (buccal) cells is essential step in order to preserve their integrity for later analysis by the comet assay [26,27]. After collection, more than 90% of the cells in a buccal sample are epithelial cells, a cell type with well-known low viability (10%) [91]. Although a prerequisite for using any cell type in the comet assay is that those cells must be viable [92,94], most of the reported studies did not consider this important factor. Failure in controlling of these confounding variables can lead to an over/under estimation of the DNA damage caused by exposure on work-place or in assessment of exposure to environmental genotoxicants [86]. Cell viability is expected to be low in epithelial tissue with terminally differentiated cell populations and a high renewal rate as buccal cells [95]. Dead or dying cells are extensively damaged (e.g., DNA fragmentation), and therefore, subjecting them to the alkaline conditions of the comet assay only increases DNA loss. Comet assay studies on epithelial buccal cell samples have reported high percentage of DNA "clouds" (>95%) [96]. Those clouds are excluded from the final quantitative analysis and that generally results in very low numbers of counted comets. Higher percentage of these atypical comets demonstrates that epithelial cells are not suitable for measuring DNA damage by the comet assay. Also enzymatic digestion such as proteinase K treatment is an essential step to enrich the number of epithelial viable cells, thus promoting necrotic cells destruction that are very numerous in the mucosa epithelium and have a very fast turnover. Enzymatic treatment with proteinase K caused degradation of leukocytes, mainly polymorphonuclear, which represent a great fraction of cells in the oral mucosa, due to migration from the blood through the gingival crevice [91].

Another problem in cell collection is that final cell suspension usually consists of mixture of epithelial cells and leukocytes with well-known fact that leukocyte fraction is more viable than epithelial cell fraction [91]. Pinhal et al. [92] investigated whether human buccal mucosa cells are suitable for use in the SCGE assay. After comparison of smoker/non-smoker group, there was no correlation of long-term smoking with the number of buccal cells that formed comets and represented damaged cells. They have also concluded that the cells that formed comets are probably leukocytes, and not buccal cells, and that the SCGE assay, used on a commonly performed way, without modifications, may not be useful for genotoxicity monitoring in human epithelial buccal mucosa cells. Similar conclusions were cited by Ribeiro [97].

In contrast, the uniform distribution of DNA within the heads of oral leukocytes and their greater viability indicates that this cell type is more suitable for assessing DNA damage in buccal samples [86]. Thus, recently McCauley et al. [98] and Kisby et al. [99] examined oral leukocytes of agricultural workers by the comet assay and demonstrated that DNA damage is greater in farmworkers who were exposed to pesticides.

As mentioned above, other alternative is to isolate lymphocytes from cells suspensions collected from the mouth and develop a technique for SCGE analyses, like it was followed by

Osswald et al. [91], and later, it was successfully implemented in an intervention trial with supplemented bread by Glei et al. [87].

The use of buccal epithelial cells to determine genotoxicity using the comet assay according to the procedure outlined by Singh et al. [100] was limited by the inability to obtain free nucleoids. In a recent review, Rojas et al. [33] showed that a broad variety of different protocols has been used in earlier investigations. No effort has been made so far to establish an international consortium which could develop and validate appropriate strategies for the use of SCGE assay in buccal cells. More information is required concerning the time and design of different phases, the duration of wash-out periods, the calibration of enzymes and other important factors which may influence the outcome of the experiments as has been proposed by Hoelzl et al. [93] for the use of SCGE assays for the detection of DNA-protective effects of dietary factors in humans.

8. Considerations

According to Rojas et al. [33], the use of alternative biomatrices to assess DNA damage in human populations has advantages and shortcomings focusing on the methodological characteristics of buccal mucosa cells and taking into consideration the sampling protocol, pre-processing, and post-sampling storage, as well as the possibilities of sample freezing and the need to adapt the classical alkaline comet assay protocol.

The use of buccal mucosa cells by comet assay in order to estimate DNA damage levels gives the possibility to obtain samples on cheap, safe, and non-invasive way in order to perform in vivo studies. Direct contact with xenobiotics and endogenous damage inductors makes this type of sample an attractive biomatrice for individual genotoxicity evaluation. Their applicability in clinical diagnostic confers a potential use in patients across time.

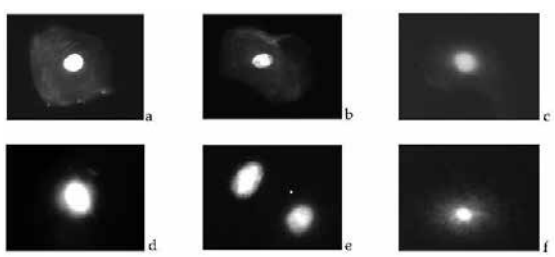

Figure 1. Picture of single buccal mucosa cells: (a) immediately stained after the solidification of agarose gel layer with sample cells, (b) the appearance of cells with cytoplasm after 1 h of classical lysis solution, (c) the appearance of the cells with cytoplasm after the combined treatment of lysis solution and proteinase K (1 mg/ml) for 1 h at 37°C, (d) the appearance of cells after 24 h of normal lysis, (e) the appearance of cells after 24 h of normal lysis and treatment with proteinase K 10 mg/ml for 1 h at 37°C, (f) 0.25% trypsin 30 min plus proteinase K 1 mg/ml 1 h, 37°C.

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The comet assay in exfoliated buccal cells has been used since the 1990s to demonstrate cytogenetic effects of environmental and occupational exposures, lifestyle factors, dietary deficiencies, and different diseases.

The general guideline to perform comet assay in epithelial cells requires the correct sampling procedure, to follow the alkaline version proposed by Singh et al. [100]. In this sense, Rojas et al. [33] proposed protocols specific to sampling protocol and sample storage and comet assay sample preparation for buccal mucosa cells. We have also performed the protocols suggested by Rojas, but there have been some confusing factors. Rojas recommendation did not give free DNA neither in first case of lysis treatment for 1 h or lysis treatment with proteinase K for 1 h (pictures represented in **Figure 1**). We have also tried the protocols that Szeto et al. [61] have done in order to established the best one, but in our case, we have demonstrated that although cells are embedded on agarose gel, treatment with 0.25% trypsin and then proteinase K for 1 h is too aggressive and still gives cloudy free nuclei. For us, the best results were with lysis and proteinase K 10 mg/ml 1-h treatment on 37°C. It seems that also high pH of alkaline denaturation and electrophoresis makes massive DNA damage, as already mentioned in Szeto et al. [61]. As Szeto et al. [61] already mentioned, buccal cells as a type of stratified squamous epithelium do not divide but undergo a terminal differentiation from basal cells on order to form a protective barrier (cell envelope rich in a small prolinerich protein) that will protect the buccal cell from very harmful environment in the mouth and also will give resistance of buccal cells to lysis. On **Figure 2**, we have represented some pictures of the buccal cells after lysis and electrophoresis in alkaline conditions (pH > 13). Szeto el al. [61] suggested that denaturation and electrophoresis in neutral conditions would be more appropriate. According to our

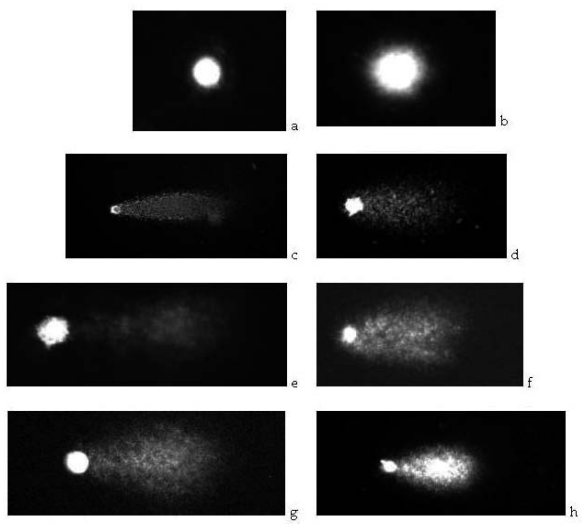

Figure 2. Pictures of buccal cells after different duration and type of lysis step, but all electrophoresis were at pH > 13: (a) treatment of lysis solution for 15 h 4°C, (b) lysis step for 20 h 4°C, (c) treatment with 0.25% trypsin for 30 min, and lysis for 30 min, both at 37°C, (d) 15 min of 0.25% trypsin a 37°C, 15 min of proteinase K 1 mg/ml, (e) 30 min of proteinase K 1 mg/ml at room temperature, 60 min of lysis at 4°C, (f) 24 h of lysis at 4°C, (g, h) 20 h of lysis at 4°C.

knowledge, alkaline conditions are also appropriate, but also this part needs further investigation.

A review of risk factors affecting background rates of parameters in the comet assay in cells of oral mucosa should be undertaken with a view to help in the interpretation of genotoxicity biomonitoring studies. Both endogenous factors and those due to methodological variation should be evaluated. Background variation of other indices of genotoxicity in buccal mucosa cells should be also considered as these data likely reflect overlapping causes of DNA damage and may provide some indicators for future research areas. A number of host risk factors, namely age, gender, smoking, vitamin status, alcohol consumption, disease conditions and infections, physical exercise, body mass index, and genotype should be identified, since there are evidences that they have an impact on background levels of genotoxicity biomarkers. Evaluation of these factors should be routinely included in genotoxicity biomonitoring studies [101].

However, important knowledge gaps remain about the methodologic procedures in laboratories around the world. To address these uncertainties, it will be necessary to develop similar projects as the HUMN and HUMNxL for validation of the lymphocytes and buccal cell MN assay, respectively [7,12,17,18]. Future research should explore sources of variability in the assay and resolve key technical issues, such as the method of buccal cell sample and sample storage, slide preparation, enzyme treatment, and optimal criteria for the classification of normal and degenerated cells. The harmonization and standardization of the buccal comet assay will allow more reliable comparison of the data among human populations and laboratories, evaluation of the assay's performance, and consolidation of its worldwide use for biomonitoring of DNA damage.

In order that comet assay in buccal cells has widespread acceptance and credibility in human population studies, standardization of analyzed parameters and protocol is necessary and also a better knowledge of critical features affecting the assay outcomes, including the definition of the values of spontaneous DNA damage. Developing the network of laboratories using this technique and performing and international collaborative studies would be an ideal solution. Result of connecting would be the assembly of large databases which would allow a more detailed analysis of the assays performance and study of the biological/clinical events associated with this biomarker.

The need for a careful consideration of factors affecting the comet assay in cells of oral mucosa exists, which, in turn, should aid in the interpretation of studies of environmental and occupational chemical exposures and health risk. There is a need for further collaborative work as in the HUMN collaborative project which has reported data on ~7000 individuals [15,16,102–104]. If these measures are achieved, then it would be possible to use the data from biomonitoring studies in risk assessments to derive risk management measures [95]. Based on the experience of the HUMN project [96], the Conference on Environmental Mutagens in Human Populations [105,106], and the HUMNxL project, design of the studies could be similar to (i) identify technical variables that affect the measurement of DNA damage of buccal cells assessed with comet assay, (ii) identify lifestyle variables affecting this damage, (iii) identify protocol variables that affect the recovery of buccal cells and their scoring in comet assay, (iv)

design intra- and inter-laboratory validation studies based on the results of information collected for the method and scoring criteria, and (v) determine the role of buccal genomic damage monitoring and the prediction of cancer and other degenerative diseases.

The creation of a network of laboratories will allow more focused validation studies, including the design of a classic, historic, prospective cohort study, to explore the link between measures of genetic instability in the buccal mucosa and the risk of cancer and other chronic-degenerative diseases [12]. ComNet project and new COST project are a great step forward.

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Section 3

Specific Evaluation of Some Environmental Toxicants

Chapter 5

Risks of Environmental Genotoxicants

Sabry M. Attia and Gamaleldin I. Harisa

Additional information is available at the end of the chapter

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Abstract

Humans have throughout their development been exposed to various environmental genotoxicants through food, air, water, and soil. Environmental exposure to genotoxic compounds may induce damage to human health and thereby increase risks of human cancers and other diseases. Environmental genotoxic chemicals have the ability to induce mutations. Such mutations can give rise to cancer in somatic cells. However, when germ cells are affected, the damage can also have an effect on the next and successive generations. Because of the potential health hazard represented by exposure to genotoxic chemicals, it is important that all chemicals for which there is possible human exposure be screened for genotoxic activity. If genotoxic hazard is detected, then the risks of exposure can be assessed and the use of the chemical controlled and when appropriate eliminated from the market and the environment. In this chapter, a general overview of the genotoxicity and the genotoxicity of some environmental genotoxicants are discussed. This is followed by a description of the genotoxic properties of some environmental genotoxicants such as bisphenols and mycotoxins, which are prominent environmental contaminates, and is believed to be genotoxic agents that contribute to the high incidence of carcinogenicity among populations.

Keywords: Environmental genotoxicants, mutations, carcinogenicity, mode of action, risk assessments

1. Introduction

DNA is constantly damaged by both endogenous and exogenous sources, and genotoxicity can be considered as an imbalance between DNA damage and DNA repair mechanisms. Maintenance of DNA integrity is essential for proper cellular and organismal function, and the capacity to withstand genotoxic challenge is important to avoid long-term genetic instability and population vulnerability. Unrepaired DNA damage can lead to mutations, cellular senes-

cence, apoptosis, progression of cancer, and the process of aging [1]. Mutation is a broad term covering a whole range of changes to the informational molecule, DNA packaged into chromosomes, of an organism from gene changes to modifications of the number and/or structure of chromosomes. Mutagenicity in normal cells is one of the most serious problems due to the possibility of inducing secondary malignancies and abnormal reproductive outcomes such as Down, Klinefelter, and Turner syndromes [2]. Such changes can be assessed directly by measuring the interaction of agents with DNA or more indirectly through the assessment of DNA repair or the production of gene mutations or chromosome alterations.

Genotoxicity covers a broader spectrum of endpoints than mutagenicity. For example, unscheduled DNA synthesis, sister chromatid exchanges, and DNA strand breaks are the measures of genotoxicity, not mutagenicity, because they are not themselves transmissible from cell to cell or generation to generation. Mutagenicity on the other hand refers to the production of transmissible genetic alterations. Although all cells of an organism contain the same DNA, somatic cells in different organs and tissues of the adult body become specialized to perform defined functions so that only some parts of the genome are expressed. A common feature of mutations in cancer-causing genes, such as those controlling cell division and proliferation, is that this results in genes being expressed in the wrong tissue at the wrong time. The effect of a mutation will depend upon the position of the mutation within the DNA and the location and activity of the particular gene in which the mutation has been induced. Mutations in the many genes that have been implicated in the multistage events leading to cancer can be produced by a variety of mechanisms and interactions and modifications of the genetic material [2, 3].

With the recent focus on environmental problems, increasing awareness of the harmful effects of industrial and agricultural pollution has created a demand for progressively more sophisticated pollutant and toxicity detection methods. In recent years, there has been a growing concern about the increasing number of environmental pollutants that may disrupt normal endocrine function in exposed humans and animals. Endocrine disrupting compounds comprise a large group of synthetic chemicals that mimic the actions of natural hormones, act as antagonist, or block their synthesis, release, or metabolism. The xenoestrogen bisphenols have received much attention due to their high production volume and widespread human exposure. Recent research in various animal models has shown the genotoxic activity of bisphenols using *in vivo* and *in vitro* assays. Nevertheless, notable differences have been reported, leading to opposite conclusions, which may well have been caused by differences in the screening test, the organisms used, and the exposure conditions assayed [4].

Many important agricultural products, especially those rich in carbohydrates, are attractive colonization sites for fungi. Some toxic secondary metabolites of fungal growth are identified as mycotoxins and may be found to contaminate agricultural products [5]. Mycotoxins are virtually ubiquitous at some concentration in the average human diet. Mycotoxins are able to resist decomposition or being broken down by mammalian digestion, even by ruminant livestock, allowing these compounds to persist in meat and even dairy products [6]. This gives rise to certain partially metabolized mycotoxins, such as aflatoxin M1, which are present in milk from cows or humans that consumed feed or food contaminated by aflatoxins. Even

temperature treatments, such as cooking and freezing, do not inactivate some mycotoxins. This section broadly discusses the genotoxic properties of the environmental genotoxicants bisphenols and aflatoxins, which are prominent environmental contaminates, and is believed to be genotoxic agents that contributes to the high incidence of genotoxicity and carcinogenicity among populations.

2. Bisphenols

2.1. Bisphenol A and its analogues

Bisphenols are a group of chemicals known as diphenylmethanes, which contain two benzene rings separated by one central carbon atom, usually with a 4-OH substituent on both benzene rings (e.g., bisphenol A, bisphenol F, bisphenol AF, and bisphenol Z). Bisphenol A is employed to make certain plastics and epoxy resins (**Figure 1**). In some bisphenols, the central carbon atom is replaced by a sulphone group (e.g., bisphenol S or bisphenol 1) or sulphide moiety (e.g., bisphenol 2). Some bisphenol A analogues seem to be safer alternatives to bisphenol A in industrial applications. For example, the production of bisphenol S, which is stable at high temperatures and resistant to sunlight, is increasing from year to year [7, 8]. The largest US manufacturer of thermal paper has been using bisphenol S as a replacement for bisphenol A since 2006. However, insufficient data are available to tell whether these bisphenol S-containing papers are safer than bisphenol A-containing papers. While bisphenol A is moderately susceptible to environmental breakdown, bisphenol S may be more persistent [9].

Figure 1. Some industrial applications of bisphenols.

From the viewpoint of biodegradability in the aquatic environment, bisphenol F is more biodegradable under aerobic and anaerobic conditions than bisphenol A and may replace bisphenol A to lower environmental risks [10]. Bisphenol AF also occurs as a monomer of phenol-formaldehyde resin. Bisphenol AF is a component of certain plasters and used as a rubber bridging material, while bisphenol A is a monomer that is polymerized to manufacture polycarbonate plastic products, epoxy, and polyester resins (**Figure 2**). Polycarbonate plastics have many applications including use in some food and drink packaging such as water and

baby bottles, compact discs, impact-resistant safety equipment, and medical devices including those used in hospital settings. Epoxy resins are used to coat metal products such as food cans, bottle tops, and water supply pipes. Bisphenol A can also be found in certain thermal paper products, including some cash register and ATM receipts. Some dental sealants and composites may also contribute to bisphenol A exposure [11, 12].

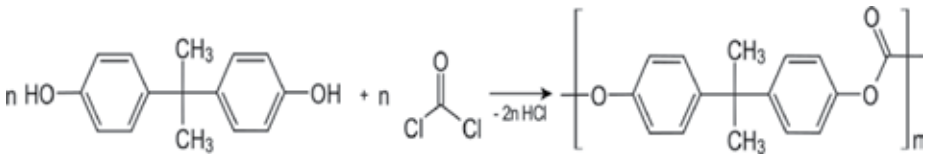

Figure 2. Synthesis of the polymer polycarbonate from bisphenol A and phosgene.

2.2. Human exposure to bisphenols

Human exposure to bisphenols may occur in the workplace through inhalation during production, but the most common route of exposure is by oral intake. Small amounts of bisphenol A are eluted from canned beverages, foods, and baby bottles, especially when heated [12]. At higher temperatures, longer contact with, and higher pH of the contact medium, bisphenol A monomer can hydrolyse and leach into food and beverages. Recent studies also suggest that the public may be exposed to bisphenol A by handling cash register receipts. In accordance with its widespread use in many applications, bisphenol A has been detected in dietary items [13] and human biological samples [14]. Moreover, bisphenol A was detected in environmental media as well [15].

2.3. Risks of exposure to bisphenols

In general, bisphenol A levels in humans have measured well below 50 mg/kg/day, which is the maximum acceptable dose set by the UA EPA [16]. Its ubiquitous presence and widespread distribution have provoked worldwide concerns about its possible association with human diseases such as obesity, diabetes, cardiovascular disease, reproductive disorders, and cancer [17, 18]. Despite its presence in human populations and its association with reproductive and developmental toxicity in animals, most countries have not imposed regulations on the manufacture, import, or sale of bisphenol A products. That has been due largely to conflicting scientific evidence for a direct association between low-level exposure and adverse health effects in humans. Some countries and regions, including Canada, Europe, Sweden, and the United States, on the other hand, have formally banned bisphenol A from infant and children's products, including, variously, cans of infant formula, baby bottles, and sippy cups. Current efforts are focused on replacing bisphenol A with safer food contact materials. All of these alternative materials need to be assessed for appropriate functionality and safety using state-of-the-art methodology and scientific knowledge.

Bisphenol A is a known endocrine disruptor compound. While initially considered to be a weak environmental estrogen, several recent publications have demonstrated that bisphenol

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A may be similar in potency to estradiol in stimulating some cellular responses. Furthermore, emerging evidence suggests that bisphenol A may affect multiple endocrine-related pathways [19]. In men, exposure to endocrine disruptors may be associated with decreased fertility and increased risk of testicular or prostate cancer [20]. In women, exposure may increase the risk of endometriosis, reproductive or other endocrine-related cancers, and impaired oocyte competence, ovarian function, or menstrual cycle [21]. Because females have higher levels of natural estrogens in their blood, the impact of estrogen-like compounds on females may be different from that on males. In women, high urinary bisphenol A levels were associated with reduced antral follicle counts in a cohort of 209 women undergoing infertility treatments [22], whereas no correlation was found between serum bisphenol A levels and antral follicle counts in another study on a smaller cohort of 44 patients [23]. Nevertheless, several data suggest a negative impact of bisphenol A on woman fertility. Urinary bisphenol A levels were negatively correlated with numbers and quality of oocytes retrieved in stimulated cycles for assisted reproduction [24]. Increased urinary or serum bisphenol A concentrations were also associated with decreased peak oestradiol levels [25]. Moreover, a study on 137 patients undergoing assisted reproduction suggested that high urinary bisphenol A levels might be associated with up to 50% higher chance of implantation failures, in comparison with patients with low or no evidence of bisphenol A exposure [26].

Because the chemical structure of bisphenol A is similar to that of diethylstilbestrol, which is carcinogenic to mammals, the possible genotoxicity of bisphenol A has been widely tested in a variety of *in vitro* and *in vivo* studies, but the results are controversial. Several studies have shown that bisphenol A can induce chromosome aberrations and DNA adducts formation in Syrian hamster embryo cells [27] or micronuclei formation in human MCL-5 cells [28]. Aneugenic properties were also observed in Chinese hamster V79 cells after bisphenol A exposure [29]. Moreover, in estrogen receptor-positive MCF-7 cells, bisphenol A caused DNA strand breaks that were estrogen receptor-dependent [30]. A recent study has reported that the genotoxic and cytogenetic effects of bisphenol A in Chinese hamster ovary cells were manifested in the form of DNA strand breaks, micronucleus formation, and chromosome aberrations [31]. Conversely, bisphenol A is considered non-genotoxic because it was negative to a set of basic genotoxicity tests. It was not mutagenic in the Salmonella/microsome assay [32], did not induce gene mutations [27, 32] or chromosomal aberrations [33] in mammalian cells *in vitro,* and failed to induce chromosomal aberrations and micronucleus formation *in vivo* in mice [34]. Bisphenol A is considered to lead to genotoxicity through oxidative stress. Bisphenol A-3,4-quinone, which is yielded by oxidative metabolism of bisphenol A, may cause genotoxicity by reacting with DNA [35].

By now, there is increasing evidence supports the notion that low bisphenol A concentrations adversely affect the epigenome of mammalian female germ cells, with functional consequences on gene expression, chromosome dynamics in meiosis, and oocyte development and quality [36]. An epigenetic impact of bisphenol A was demonstrated also on male germ cells. Male offspring of rats perinatally exposed to bisphenol A had reduced sperm counts and other changes in phenotypes not only in the first generation but also in the *F3* generation [37]. Induction of sperm epimutations and male-mediated trans-generational inheritance of obesity

and reproductive disturbances were also shown after bisphenol A exposure of rats [38]. When female mice were exposed during gestation and lactation to low bisphenol A doses deregulated, glucose homeostasis in the *F2* generation was observed; decreased global methylation and differential methylation of a specific CpG site in the glucokinase promoter in the *F1* sperm suggested that the *F2* phenotype could be caused by epigenetic alterations induced in the male paternal germline by bisphenol A prenatal exposure [39].

While comprehensive information is available about the adverse health impacts of bisphenol A, toxicological properties of alternative bisphenols are yet to be investigated. Alternative bisphenols are structurally similar to bisphenol A, and therefore expected to possess similar biological activities. However, most available toxicological information is limited to endocrine disrupting potentials, and only very little is known about the genotoxicity of alternative bisphenols [7]. In turn, bisphenol F has been reported to induce DNA strand breaks, but not micronuclei, in HepG2 cells [40]. In human HepG2 cells, bisphenol F induced histone H2AX phosphorylation, an indicator of DNA double strand breaks [41]. Moreover, bisphenol F induced metaphase arrest and micronucleus formation in V79 cells [29]. In Syrian hamster embryo cells, bisphenol F did not induce gene mutation or chromosomal aberrations, but induced aneuploidy and morphological changes [42]. Bisphenol A may cause oxidative stress, and induce DNA adduct and aneuploidy in rodents [43]. Nevertheless, eight bisphenols including bisphenol A showed no positive responses based on *umu*-test suggesting no genotoxicity [44]. Similarly, other alternative bisphenols are expected, but to date, very little efforts have been made on this aspect.

3. Aflatoxins

3.1. Sources of aflatoxins

With worldwide increases in population, the need for nutrient-rich food is rising. Contamination of foods by toxins, bacteria, viruses, parasites, allergens, and prions may lead to serious diseases; unhealthy foodstuffs are implicated in approximately one-third of cancer cases. Controlled storage conditions, improved packaging, and strict hygiene regulations for food production, preservation, and distribution are essential to diminish such problems. Aflatoxins are toxic metabolites produced by certain fungi in/on foods and feeds. They are probably the best-known and most intensively researched mycotoxins in the world. The occurrence of aflatoxins is influenced by certain environmental factors; hence, the extent of contamination will vary with geographic location, agricultural, and agronomic practices, and the susceptibility of commodities to fungal invasion during pre-harvest, storage, and/or processing periods. Aflatoxin B1 is a prevalent food pollutant, which is found typically in tropical countries. It imposes great costs on the world's economy and health [45]. Thus, it is important to eliminate aflatoxin B1 from food resources and prevent production of the toxin. Due to lack of infrastructure, poor and third world countries are the major victims of aflatoxin B1. The established carcinogenesis, teratogenesis, and severe multi-organ toxicity associated with aflatoxin B1 have made it a substantial challenge for scientists [46, 47].

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Figure 3. Some major sources of aflatoxins.

3.2. Human exposure to aflatoxins

Aflatoxins are a type of mycotoxin produced by *Aspergillus* species of fungi, such as *A. flavus* and *A. parasiticus*, which grow in soil, decaying vegetation, hay, and grains [48]. Aflatoxins have received greater attention than any other mycotoxins because of their demonstrated potent carcinogenic effect in susceptible laboratory animals and their acute toxicological effects in humans. Aflatoxins are regularly found in improperly stored staple commodities such as cassava, chili peppers, corn, cottonseed, millet, peanuts, rice, sorghum, sunflower seeds, tree nuts, wheat, and a variety of spices (**Figure 3**). Aflatoxin B1 is highly resistant to traditional detoxification protocols, such as heat, solvents, and radiation, which have consequences for food quality and safety. However, biological procedures using microbial or enzymatic tools that possess great specificity with minimal consequences are the appropriate choices for the treatment of contaminated foodstuffs; they also offer ease of application, affordability, and environmentally friendly behavior. The use of biological procedures requires optimized conditions, such as pH and temperature, for maximum efficiency. When contaminated food is processed, aflatoxins enter the general food supply where they have been found in both pet and human foods, as well as in feed stocks for agricultural animals [49]. Moreover, animals fed contaminated food can pass aflatoxin transformation products into eggs, milk products, and meat. The four major naturally produced aflatoxins are known as aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2 (**Figure 4**), which are based on their fluorescent color when exposed to ultraviolet light on thin-layer chromatography plates (B = blue fluorescence, G = yellow-green fluorescence), while the subscript numbers 1 and 2 indicate major and minor compounds, respectively. Aflatoxin M1 and M2 compounds are not found on cereal products themselves but are metabolites expressed in milk of mammals whose diet was contaminated by aflatoxins B1 and B2, respectively [50].

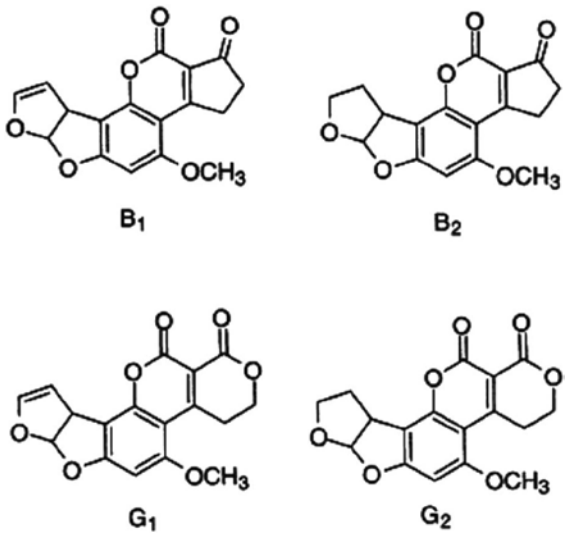

Figure 4. Chemical structures of the four major naturally produced aflatoxins: aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2.

3.3. Risks of exposure to aflatoxins

Aflatoxins have been reported to have several serious deleterious effects in humans and diverse animals with the species reacting differently to the toxicological effects. The target sites of this toxicant are also diverse and effects include hepatotoxicity, teratogenicity, immunotoxicity, hematological disorders, renal dysfunction, induction of chromosome aberrations, and mutations in somatic and germinal cells of animals and humans [51–53]. Aflatoxin B1, the most toxic, is a potent carcinogen and has been directly correlated with adverse health effects, such as liver cancer, in many animal species. Aflatoxin B1 is one of the major risk factors for the occurrence of liver injury and carcinogenesis, especially when it is combined with hepatitis B infection. Epidemiological investigations revealed that dietary contamination with aflatoxin B1 might be responsible for 5–28% of global hepatocellular carcinoma cases [54]. A great deal of evidence has demonstrated that aflatoxin B1 belongs to the indirect-acting carcinogens. Aflatoxin B1 is mainly metabolized in the liver to produce the genotoxic intermediate aflatoxin B1-exo-8,9-epoxide by the liver-specific cytochrome P450 enzymes, P4501A2, and 3A4. These epoxides can form aflatoxin B1-guanine adducts by binding covalently to DNA, thereby introducing GC-TA transversion that leads to DNA mutations and genomic instability [55]. For instance, a frequent hotspot mutation at codon 249 in the human p53 gene gives rise to a Ser to Arg substitution in the p53 protein that decreases its tumor suppressor activity [56]. Another major detoxification pathway of aflatoxin B1 in mammalian species is the glutathione conjugation of aflatoxin B1-8,9-epoxide, which is catalyzed by glutathione S-transferases [57]. Experimental studies conducted in rats have shown that rGSTA5, barely expressed in adult male liver, exhibits a greater activity toward aflatoxin B1-8,9-epoxide than other glutathione S-transferases subunits [58].

Aflatoxin B1 is a clastogen that has been tested for genotoxicity *in vivo* and *in vitro* and giving consistently positive results. It induces chromosomal aberrations, micronuclei formation, sister chromatid exchanges, and DNA strand breaks in several published works [53, 59–61]. An important mechanism responsible for the genotoxic potential of aflatoxin B1 is the formation of DNA adducts. Biotransformation plays a crucial role in the toxicity and carcinogenicity of aflatoxin B1. The enzymatic detoxification of aflatoxin B1 was studied [62], and the pro-oxidant properties and mutagenicity of the detoxification products were compared with those of aflatoxin B1. The results indicated that the metabolized aflatoxin B1 was more toxic than the non-metabolized form of it. A previous study also demonstrated that an epoxide metabolite had an important role in aflatoxin B1-mediated genotoxicity [63]. In the Ames mutagenicity test, the T98 strain exhibits a frame-shift mutation, and T100 exhibits a base-pair substitution. Virtually, all of aflatoxin B1 toxic and carcinogenic effects are attributable to the action of its reactive metabolites that are capable of reacting with cellular macromolecules such as DNA [64]. Furthermore, DNA repair activity and modulation of repair by aflatoxin B1 seem to be also major determinants of susceptibility to aflatoxin B1-induced carcinogenesis [61].

Since the discovery of these deleterious effects induced by aflatoxin B1, a large number of studies have explored the mechanisms and pathways involved in aflatoxin B1-mediated genotoxicity. However, few studies have focused on the epigenetic events involved in the induction of genotoxicity. Recently, several studies reported that cellular epigenetic aberrant changes, such as DNA methylation, histone modifications, and miRNA profiling alterations, also contributed to the hepatotoxicity and genotoxicity induced by chemical toxicants. A genome-wide miRNA-profiling analysis in an acute rat liver injury model induced by aflatoxin B1 predicted that several miRNAs and their potential targets were relevant to acute hepatotoxicity, although functional tests were not performed [65]. However, it is clear from gene expression profiling that the pathways involved in acute poisoning and chronic poisoning are not completely consistent. A recent study investigated alterations in miRNA profiles of rat liver tissues by Illumina deep sequencing and evaluated their roles in aflatoxin B1-induced hepatocellular genotoxicity and hepatotoxicity [66]. The authors demonstrated alterations in the miRNA profile in rat liver tissue, including rnomiR-34a-5p, rno-miR-200b-3p, rno-miR-429, and rno-miR-130a-3p, after aflatoxin B1 exposure. Functional tests showed that the increase in miR-34a-5p by p53 activation after aflatoxin B1 exposure led to cell cycle arrest *via* inhibiting cell cycle-related genes and affecting the micronuclei formation induced by aflatoxin B1, indicating that rno-miR-34a-5p played a critical role in aflatoxin B1-induced rat hepatogenotoxicity. Furthermore, the combination of circulating miR-34a-5p and the aflatoxin B1 level may be considered as a sensitive method for the detection of the genotoxic stress induced by chronic aflatoxin B1 exposure.

As it is realized that absolute safety is never achieved, many countries have attempted to limit exposure to aflatoxins by imposing regulatory limits on commodities intended for use as food and feed [67]. The FDA has established specific guidelines on acceptable levels of aflatoxins in human food and animal feed by establishing action levels that allow for the removal of violate lots from commerce. The action level for human food is 20 ppb total aflatoxins, with the exception of milk, which has an action level of 0.5 ppb for aflatoxin M1. The action level for

most feeds is also 20 ppb. However, it is very difficult to accurately estimate aflatoxins concentration in a large quantity of material because of the variability associated with testing procedures; hence, the true aflatoxin concentration in a lot cannot be determined with 100% certainty. However, the ability of aflatoxin-producing fungi to grow on a wide range of food commodities and the stability of aflatoxins in foods mean that control is best achieved by measures designed to prevent the contamination of crops in the field and during storage, or detection and removal of contaminated material from the food supply chain.

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Chapter 6

Environmental Effects of Endocrine-Disrupting Chemicals: A Special Focus on Phthalates and Bisphenol A

Pinar Erkekoglu and Belma Kocer-Gumusel

Additional information is available at the end of the chapter

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Abstract

Several environmental chemicals are classified as endocrine-disrupting chemicals (EDCs). Many of them have an impact on reproductive functions and sex hormones because of their estrogenic and/or antiandrogenic properties. Phthalates and bisphenol A (BPA) are two well-known EDCs. They are abundant in the environment. Phthalates are usually classified as antiandrogens, whereas BPA is considered as estrogen-like EDC and xenoestrogen. Other than their endocrine-disrupting effects, these two chemicals are also known to have genotoxic and epigenetic effects. Besides, they are hepatotoxic and have substantial effects on other organs/systems (thyroid, kidney, neuroendocrine system, immune system, etc.). In this chapter, we will mainly focus on the toxic effects of different phthalate esters and BPA by discussing their availability in the environment, mechanism and mode of actions, their biotransformation and reproductive effects, and their effects on other systems (hepatic, renal, etc.). Besides, we discuss epidemiological studies that are conducted to reveal their effects on the reproductive and endocrine systems. This chapter provides the readers a compact piece of knowledge on these abundant substances and helps them to understand the action of these substances at the molecular and cellular levels.

Keywords: endocrine-disrupting chemical, antiandrogen, xenoestrogen, phthalate, bisphenol A

1. Introduction

Exposure to environmental chemicals, particularly in early life, is among the substantial risks for developmental programming of different diseases in adult life of humans. In a report by

World Health Organization (WHO), it was estimated that more than 13 million deaths were caused by environmental exposures each year. Moreover, this report also proposed that nearly one third of mortality and morbidity can be due to environmental causes in underdeveloped or developing countries [1].

Many environmental exposures to different chemical, physical, or biological agents can interact with genetic and epigenetic mechanisms and affect the normal growth and development. Among those exposures, endocrine-disrupting chemicals (EDCs) are of particular concern, as humans are abundantly exposed to these chemicals by various means in every period of life. According to the U.S. Environmental Protection Agency (EPA), an EDC was defined as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural bloodborne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" [2]. Several well-known environmental chemicals are classified as EDCs. Many of them act on reproductive functions because of their estrogenic and/or antiandrogenic properties.

In the present chapter, we will mainly focus on the toxic effects of different phthalate esters and bisphenol A (BPA), which are the most abundant environmental chemicals. We will discuss their availability in the environment, mechanism and mode of actions, biotransformation, and effects on reproductive systems and other organs (hepatic, renal, etc.) in different periods of life. Besides, we will address the epidemiological studies that are conducted on these chemicals.

2. Availability of endocrine disrupting chemicals in the environment

EDCs are available in polyvinyl chloride (PVC) plastics, polycarbonate materials (type 7 plastics), epoxy resins, medical devices [intravenous (i.v.) bags, dialysis bags, surgical implants, dental fillings sealants], pharmaceuticals (enteric coatings of pharmaceutical pills and nutritional supplements), consumer products (make-up products, fragrances, nail polish, lotions, creams, baby products, soaps, liquid soaps shampoos, conditioners, hair sprays), children's toys, children products (modeling clay, waxes, paints), printing inks, paints, household products (detergents, softeners, surfactants), construction materials (including floorings and PVC windows, wood floor finishes, cements, caulking in buildings), insulating fluids (transformer oils) for transformers and capacitors, lubricating oils, stabilizing additives in flexible PVC coatings of electrical cables and electronic components, textiles (footwear, raincoats, picture printed shirts), vacuum pump fluids, pesticides (insecticides), and flame retardants and, most importantly, in food (packaging materials and in the inner lining of food cans) [3–5].

3. Classification of EDCs

From a toxicological perspective, EDCs can be classified according to their sources or their modes of action.

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In the first classification, EDCs can be grouped as [6, 7]:

a. natural (e.g., phytoestrogen: genistein and coumestrol) and

b. synthetic [e.g., phthalates, BPA, polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins; dichlorodiphenyltrichloroethane (DDT), vinclozolin, and diethylstilbestrol (DES)] compounds.

In the second classifications, EDCs can be classified as [8, 9]:

a. EDCs that effect reproductive system

b. EDCs that affect pancreas

c. EDCs that affect thyroid

d. EDCs that effect Central nervous sytem

e. EDCs that affect other systems

The signifacnt effects of EDCs on hormones are suggested to be:

a. Increasing or decreasing effect on the production of hormones (these substances may mimic naturally occurring hormones such as estrogens, androgens, or thyroid hormones or they may potentially cause the overstimulation of hormonal pathways within the body),

b. Increasing or decreasing effect on the transportation of hormones,

c. Increasing or decreasing effect on the metabolism of hormones,

d. Increasing or decreasing effect on the elimination of hormones,

e. Agonistic or antagonistic effect on the target cells of the hormones (by binding to a receptor within a cell and blocking the functions of endogenous hormones; i.e., acting as antiestrogens and antiandrogens), and

f. Altering the homeostatic systems of the organisms and causing their miscommunication or irresponsiveness to their own physiology and the environment.

4. Modes/mechanisms of actions of EDCs

a. Effect on hormone, nuclear, and nonnuclear receptors: Our understanding of the mechanisms by which EDCs exert their effect has grown. EDCs were originally thought to exert actions primarily through nuclear hormone receptors [i.e., estrogen receptors (ERs), androgen receptors (ARs), progesterone receptors, thyroid receptors (TRs), and retinoid receptors]. However, recent basic and mechanistic researches show that the underlying mechanisms of their toxicity are much wider than originally envisioned. Thus, other than nuclear receptors, EDCs may also act via nonnuclear steroid hormone receptors (e.g., membrane ERs), nonsteroid receptors (e.g., neurotransmitter receptors such as serotonin

receptor, dopamine receptor, and norepinephrine receptor), and orphan receptors [e.g., aryl hydrocarbon receptor (AhR)] [9–11].

b. Effect on enzymatic pathways: These chemicals can affect the enzymatic pathways that have substantial roles in steroid biosynthesis and/or biotransformation and several other mechanisms that militate sex-specific physiology/behavior and endocrine and reproductive systems. They can disrupt and inhibit the action of enzymes involved in steroidogenesis, particularly in the metabolism of estrogens. For instance, some PCB metabolites can inhibit sulfotransferase, resulting in an increase of circulating estradiol [12–14].

c. Effects on signaling pathways: EDCs can regulate many cellular signaling pathways. For instances, both BPA and phthalates were shown to induce epithelial-to-mesenchymal transition (EMT). These chemicals can also down-regulate or up-regulate the genes involved in the regulation of signal transduction [15, 16].

5. Toxicity mechanisms of EDCs

There are a number of toxicity mechanisms/pathways that are suggested to be caused by estrogenic and antiandrogenic EDCs [17, 18]. These mechanisms are associated with but are not limited to:

1. Genotoxicity,
2. Epigenetic mechanisms (hormonal dysregulation, peroxisome proliferation, cytotoxicity, oxidative stress, DNA methylation, histone modification, RNA interfering, apoptosis, and imprinting), and
3. Other mechanisms.

The effects of EDCs on cellular metabolism or functions, cellular organelles (peroxisomes, mitochondria, cytoskeleton), DNA damage, chromosomal aberrations, cell cycle checkpoints, translational control, cell death (apoptosis, autophagy, necrosis), immunology/inflammation response, neurological pathways, and development/differentiation are now being studied extensively. However, their effects were also shown to extend beyond these mechanisms/pathways and may include multiple functions, tissues, and organs such as the liver, kidney, and spleen. Thus, they may have broader impacts—most of them yet to be identified—on disrupting signaling webs and cellular communication [17, 18].

6. Phthalates

The word "plastic" originates from the Greek word "plasticos," which expresses "a material's being capable of molding into different shapes." The first plasticizer was synthesized in 1860. By the progression of technology and increase in the global population growth, plastic materials are now widely used and have very different application fields [19]. Phthalates are dialkyl or alkyl aryl esters of phthalic acid and are abundantly used to make plastic materials

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more flexible. They are synthesized by reacting phthalic anhydride with alcohol(s) [methanol and ethanol (C1/C2) up to tridecyl alcohol (C13)] either as a straight chain or with some branching. Their main use is for the softening of rigid plastics and polymers. Di(2-ethylhexyl)phthalate (DEHP), the most abundant phthalate derivative, is used 1% to 40% in plastics by weight [20]. Almost 90% of DEHP is used to soften PVC plastics, and in the European Union, 95% of DEHP is used in polymer products as a plasticizer [21].

Phthalates were first synthesized in the 1930s. DEHP was first synthesized in 1933 in Japan and in 1939 in United States in commercial quantities. DEHP was first used in 1949 in United States and has been the most abundantly used phthalate derivative in the Twentieth century. In 1999, the consumption of phthalates were 3.25 million tons and DEHP accounted for 2.1 million tons of the total production. The European Commission reported that 1 million tons of DEHP were used in 2000 [22].

6.1. Occurrence, uses, and exposure to phthalates

Phthalates migrate out PVC-containing items into food, air, dust, water, and soils and cause human exposure in various ways [23]. Several studies were conducted in different parts of the world, and human blood and urine (mostly spot urine samples) were used as biological fluids to evaluate the exposure to phthalates. The results of these studies revealed that humans are ubiquitously exposed to different phthalates, mostly in industrialized countries [24–27]. On the contrary, workplace inhalation is also of concern as phthalates; particularly, DEHP has low vapor pressure [28].

Diet is the main source of phthalate exposure in the general population. Particularly, fatty food (e.g., fish and oils) can cause high phthalate exposure if contaminated [29, 30]. One other major source is medical exposure by blood storage bags and blood transfusion equipment during receiving blood transfusion [31, 32] or hemodialysis (dialysis bags) [33, 34].

Based on the number of carbon atoms in their alcohol chain, phthalates are divided into two distinct groups, with very different applications, toxicological properties, and classification: high molecular weight (MW) phthalates and low MW phthalates. Their use largely depends on their MW. Higher MW phthalates, such as DEHP, are used in construction materials and in numerous PVC products, including clothing, food and beverage packaging, children products (toys, grip bumpers), and biomedical equipment (e.g., blood transfusion bags, dialysis bags, and umbilical catheterization devices), whereas relatively lower MW phthalates such as dimethyl phthalate (DMP), diethyl phthalate (DEP), and dibutyl phthalate (DBP), are mainly used as odor/color fixatives or as solvents and in cosmetics, textiles, and pharmaceuticals [35].

DEHP has a very low degree of acute toxicity, with oral lethal dose 50 (LD_{50}) values ranging from 26 to >34 g/kg in a variety of species. In a study by Lawrence et al. [36], the lethal effect of this compound appeared to be cumulative, because the LD_{50} value for intraperitoneal (i.p.) administration to mice five times weekly for 10 weeks was 1.36 g/kg in comparison to a single-dose value of 37.8 g/kg. Autian [37] concluded that this was because biotransformation was required before DEHP produces toxic effects. In rats, lethal concentration 50 (LC_{50}) by inhala-

tion (1 h) was found to be >23.670 mg/m^3 (1457 ppm) and inhalation LC_{50} (6 h) value was >600 mg/m^3 (37 ppm) [37]. The oral administration LD_{50} value for rats (Wistar, male) was suggested to be between 26.000 and 34.000 mg/kg body weight and the LD_{50} value for i.p. administration in rats was found to be between 30.600 to 49.000 mg/kg body weight [38].

6.1. Biotransformation of phthalates

Phthalates are not covalently bound to plastic products and therefore may leak out to contaminate blood or food products and can be ingested. When administered orally to humans and rodents, phthalates are rapidly hydrolyzed by esterases in the gut and other tissues to produce the corresponding active monoesters and their further oxidized metabolites. During phase I biotransformation, the relatively polar and low MW phthalates (e.g., DEP) are primarily metabolized to their hydrolytic monoesters by hydrolysis of one of the ester bonds. In contrast, the high MW phthalates are first metabolized to their respective hydrolytic monoesters and then, after enzymatic oxidation of the alkyl chain, to more hydrophilic, oxidative metabolites [39]. For example, DEHP is metabolized to its monoester metabolite, mono-2-ethylhexyl phthalate (MEHP), which is more toxic than the parent compound. MEHP is further metabolized to secondary oxidative metabolites [40]:

- Mono-(2-ethyl-5-hydroxyhexyl)phthalate (5OH-MEHP, MEHHP), which is then metabolized to mono-(2-ethyl-5- oxohexyl) phthalate (MEOHP, 5oxo-MEHP). MEHP can also be metabolized to different structural isomers of MEHHP, which also have further metabolism to different monoethylphthalate structures.
- Mono-(2-ethyl-5-carboxypentyl)phthalate (5cx-MEPP)
- Mono-[2-(carboxymethyl)hexyl]phthalate (2cx-MMHP)

Some phthalates are subject to phase II (particularly to glucuronidation and, to a lesser extent, sulfation) metabolic reactions. Glucuronidation not only provided higher urinary excretion of phthalate metabolites but also can reduce their biological activity. Monoesters and the oxidative metabolites of phthalates are excreted in urine (95%) and, to a very lesser extent, eliminated by feces (5%) [39].

6.2. Genotoxic effects of phthalates

The biological effects of phthalates are of major concern but so far elusive. Phthalates are shown to cause cytogenetic damage to animals and humans. In 1980s, phthalates were evaluated as epigenetic carcinogens because of their peroxisome proliferative effects. However, in the 1990s and in the Twenty first century, several studies confirmed their genotoxic effects [41–43].

Chromosomal aberration test, unscheduled DNA synthesis (UDS), Ames test, micronucleus test, and hypoxanthine guanine phosphoribosyltransferase (HPRT) mutation test were applied to evaluate the genotoxic potentials of phthalates. DEHP was shown to induce single chromatid aberrations and sister chromatid exchange (SCE) in human lymphocytes [44]. Besides, DEHP caused lymphatic mitotic inhibition after 4 h of exposure and caused an increase in the doubling time of human lymphocytes [44]. Later, Stenchever et al. [45] reported that DEHP

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caused chromosomal breaks in human lymphocytes; mitotic rate decreased and DEHP caused polyploidy and aneuploidy in human fetal lung cells.

Phillips et al. [46] reported that, in Chinese hamster ovary (CHO) cells, MEHP caused chromosome damage without affecting in the SCE and HPRT mutation test. However, after DEHP treatment in rat hepatocytes, Astill et al. [47] did not find a change in Ames test, mouse lymphoma activation assay, micronucleus test, UDS, and cell transformation tests. A study performed on both CHO cells and RL4 liver cells showed that MEHP caused chromosomal aberrations. However, S-9 mix (a mix of cytosolic and microsomal drug-metabolizing enzymes and cofactors) had no effect on the chromosome damage produced by MEHP in CHO cells [48].

A study using mouse hepatocytes evaluated the genotoxicity of these compounds (i.e., DNA repair or UDS). No changes were observed in DNA repair capacity. However, UDS of the hepatocytes obtained from mice treated with DEHP containing diet showed significantly higher UDS compared to control cells [49]. Lindahl-Kiessling et al. [50] showed that DEHP induced SCE in human lymphocytes, which were co-cultured with rat liver cells. Müller-Tegethoff et al. [51] observed that peroxisome proliferators (nafenopin, Wy-14,643) and DEHP did not induce any changes in micronucleus test in rat hepatocytes. Kim et al. [52] showed that DBP caused both chromatid and chromosomal type chromosomal aberrations (break and exchange) in the lymphocytes of B6C3F1 mice and this aberrations showed marked increases and these alterations show marked elevations dependent on the time of exposure. McKee et al. [53] reported that di(isononyl) phthalate (DINP) was not found to be mutagenic in Ames test, *in vitro* cytogenetic assay, and mouse micronucleus assay. Using Ames test, Lee and Lee [54] observed that the phthalic acid and terephthalic acid did not produce any mutagenic responses in the absence or presence of S9 mix on the *Salmonella typhimurium* strains in Ames test. Besides, phthalic acid and terephthalic acid did not show any significant cytogenetic effect on CHO cells in the chromosomal aberration test and in the mouse micronucleus test [54].

Many studies are performed on the genotoxicity of phthalates using Comet assay in the last 30 years. Anderson et al. [55] showed that both DEHP and its major metabolite MEHP induced DNA damage in human leukocytes as evidenced by increases in tail moment in Comet assay. Kleinsasser et al. [56] compared susceptibilities to DBP and di-iso-butyl-phthalate (DiBP) in nontumor patients to those in patients with squamous cell cancer (SCC) of the oropharynx or larynx using Comet assay and indicated that DBP and DiBP produced significant differences in the Olive tail moment (OTM) between oropharynx (TO), larynx (TL), and TO plus TL groups and the nontumor donors. The same researchers also determined the correlation between the genotoxic sensitivities to DBP and its isomer DiBP in mucosal epithelial cells or lymphocytes using Comet assay, and both phthalates showed significant genotoxicity on both cells and lymphocytes where the genotoxic effect of DiBP was higher than DBP in both cell types [57]. Biscardi et al. [58] reported that DEHP can leach out of polyethylene terephthalate (PET) bottles in time, especially after almost 10 months of storage, and this phenomenon can cause increases in both total tail length and number of cells in human leukocytes *in vitro*.

DEHP, BPA, nonylphenol, and paraquat dichloride were tested for their genotoxicity potentials on HeLa cells. DEHP showed genotoxicity (>90 μM) with significant increases in tail moment [59]. In a recent study performed on HepG2 cells exposed to various concentrations

of DEHP for 24 or 48 h, DNA damage increased significantly in a dose-dependent manner [60]. Throughout our studies on DEHP and MEHP, we determined that both DEHP and MEHP were both cytotoxic and genotoxic in LNCaP cells (human prostate cancer cell line) and Leydig cells (mouse Leydig carcinoma cell line). We also observed that selenium supplementation in either organic form (selenomethionine at 10 μM) or inorganic form (sodium selenite at 30 nM) was highly protective against the cytotoxicity and genotoxicity exerted by these particular phthalate derivatives [42, 43].

The correlation between urinary phthalate levels and sperm DNA damage is being investigated by several studies. Duty et al. [61] analyzed semen and urine samples of 141 subjects were for five phthalate metabolites using neutral Comet assay and DNA was only correlated with monoethyl phthalate (MEP) levels, although there was no correlation with other phthalate metabolites [monobenzyl phthalate (MBzP), mono-*n*-butyl phthalate (MBP), MEHP, and monomethyl phthalate (MMP)]. Hauser et al. [62] determined the urinary concentrations of phthalate metabolites among men (n=379) who were admitted to an infertility clinic. Sperm DNA damage was associated with MEP and MEHP. Recently, Ahbab et al. [63] determined the possible genotoxicity of two different phthalate esters, namely, di-*n*-hexyl phthalate (DHP) and dicyclohexyl phthalate (DCHP), at different concentrations using Comet assay in male rat pups from gestational day (GD) 6 to GD19 at different doses [0 (vehicle), 20, 100, and 500 mg/kg/day]. Male rats were allowed to grow until different ages (prepubertal, pubertal, and adulthood). The Comet assay was performed on blood lymphocytes and testes samples of adult male rats and the results showed that DHP significantly induced genotoxicity at doses of 100 and 500 mg/kg/day versus control; however, DCHP did not show the same effect [63].

6.3. Epigenetic effects of phthalates

The results of many studies strongly point out that that EDC exposure can caused by epigenetic mechanisms, which can lead to cumulative adverse effects on future generations. The epigenetic marks can induce up/down alterations in gene expression that may persist throughout a lifetime. These permanent changes will result in adverse health effects, such as neural and immune disorders, infertility, and late-onset complex diseases (cancers and diabetes) [64].

Phthalates are long suggested to be epigenetic carcinogens because of their peroxisome proliferator effects. The *in utero* and neonatal exposure to phthalates [particularly to DEHP, MEHP, benz-butyl phthalate (BBP), DBP, and MBP] may cause methylation changes in DNA at CpG islands near gene promoter regions, different histone modifications (acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and ADP ribosylation), and alterations in the expression of noncoding RNAs, including microRNAs (miRNAs) [65].

The treatment of human breast cancer MCF-7 cells with BBP led to the demethylation of ERα promoter-associated CpG islands, indicating that altered ERα mRNA expression by BBP can induce aberrant DNA methylation in the promoter region of this gene. Maternal exposure to DEHP was shown to induce DNA methylation and different DNA methyltransferase expressions in mouse testis. Fetal testis is suggested to be the main target for DEHP. DEHP can lead to testicular dysgenesis syndrome (TDS) due to a reduction in insulin-like hormone 3 (INSL3) expression and testosterone production [66]. During the period of embryonic sex

determination, transient exposure to a plastic mixture (BPA and phthalates) of gestating female rats was shown to promote early-onset puberty transgenerationally (F_3 generation) and decrease the pool size of ovarian primordial follicles in female pups. On the contrary, in male pups, spermatogenic cell apoptosis was also affected transgenerationally, and differential DNA methylation of the F_3 generation sperm promoter regions was also observed [67].

6.4. Carcinogenicity of phthalates

Phthalates are well-known peroxisome proliferators that can alter gene and protein expressions. This capability may result in the promotion of hepatic carcinogenesis in rodents [68]. On the contrary, there are data in the literature that indicate that phthalates increase oxidative stress in the rodent liver even before peroxisomal oxidases are induced. In addition, Kupffer cells have been suggested to be a potential source of oxidants in rodent liver after treatment with DEHP [69, 70]. It appears that molecular events, which may be a consequence of increase oxidative stress, could interact with other pathways activated by peroxisome proliferation in rodent liver [69, 70]. Although several studies including the studies by our group (in the last decade) pointed out that DEHP can induce reactive oxygen species (ROS) production and lead to increased cellular oxidative stress both *in vivo* and *in vitro*, there are no convincing data to prove whether the induction of ROS production is a one of the major pathways or whether ROS elimination is not efficiently achieved after a series of molecular events induced by phthalates, particularly by DEHP [42, 43, 70, 71].

In the 1980s, the hepatocarcinogenic effects of DEHP, due to its peroxisome proliferator effect, was shown by several studies. Back then, some concerns started rising about the safety of this substance. In 2000, the International Agency for Research on Cancer (IARC) classified DEHP as a Group III carcinogen (not classifiable as to its carcinogenicity to humans), as peroxisome proliferation has not been documented in human hepatocyte cultures exposed to DEHP nor in the liver of exposed nonhuman primates [71]. Therefore, the mechanism by which DEHP increases the incidence of hepatocellular tumors in rats and mice is not relevant to humans. However, later in 2013, DEHP was classified as a Group IIB carcinogen due to some concerns [72]. On the contrary, butyl benzyl phthalate (BBP) is classified as a Group III carcinogen in 1999 [73]. Blom et al. [74] showed that exposure to different phthalate esters, particularly to DBP, can lead to high proliferation of human breast cancer cell lines, which was explained in part by the potency of phthalates in terms of a "xenoestrogenic impact," although phthalates are usually classified as "antiandrogens" and not "xenoestrogens" [74, 75]. This effect is suggested to be related to a direct ER binding of some, but not all, phthalates [76, 77].

6.5. Reproductive toxicity of phthalates

Recent *in vivo* and *in vitro* studies on phthalates are mainly focusing on their reproductive toxicity potential. Phthalates were suggested to target mainly male reproductive system. The "TDS hypothesis" proposes that a proportion of the male reproductive disorders—cryptorchidism, hypospadias, decline infertility (or loss of fertility), and testicular cancer—may be symptoms of TDS, which is most likely a result of disturbed gonadal development in the embryo. There is a decline in male fertility and increase in the number of cases with TDS in

the last decades, and phthalates are suggested to be the major underlying factors. Several studies have shown that fetal exposures to DEHP or DBP induce TDS-like effects and reduce anogenital distance (AGD) in rodents [78].

In testis, Leydig and Sertoli cells are the main targets of phthalates. Many researchers observed that DEHP caused disruption in the function of both cell types. The administration of MEHP to Wistar rats at a single oral dose (400 mg/kg body weight) was toxic to Sertoli cells and caused detachment of germ cells [79]. In fact, Richburg and Boekelheide [80] demonstrated histopathological disturbances and alterations of cytoplasmic distribution of vimentin in Sertoli cells in testis of 28-day-old Fisher rats after a single oral dose of MEHP (2000 mg/kg). Tay et al. [81] also observed a correlation between the increase in TUNEL-positive cells and the vimentin disruption in treated mice. We also determined that DEHP exposure caused disruption and collapse of vimentin filaments and significantly induced apoptotic death of germ cells [82].

Exposure to phthalates, particularly to DEHP, resulted in decreased testicular testosterone production in rodents, and most of the reprotoxic effects are suggested to be related to their antiandrogenic potential [83, 84]. In our studies, we determined that DEHP caused abnormal sperm production, decreases in sperm count, and motility when administered to 10-week-old rats at 1000 ppm dose for 10 days. Moreover, we also observed that DEHP caused decreases in testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) levels [84]. Moreover, DEHP induced oxidative stress in rat testis, as evidenced by the significant decrease in GSH/GSSG redox ratio, marked increase in lipid peroxidation, and a significant decrease in GPx4 activity [85].

6.6. Hepatotoxic effects of phthalates

DEHP and other phthalates, such as di-(2-ethylhexyl) adipate (DEHA) and DINP, are shown to be hepatocarcinogenic in both sexes in mice and rats. These substances were shown to cause both hepatocellular carcinomas and adenomas [86–88]. There are a number of molecular events that underlie the hepatocarcinogenic potential of these substances: Their genotoxicity, peroxisome proliferative property, and epigenetic effects are the most studied mechanisms. Collectively, it appears that, in rodent liver, oxidative stress-related molecular events could interact with other pathways that can be activated by peroxisome proliferation. Previously, we have also shown that DEHP caused peroxisome proliferation, alterations in antioxidant enzyme activities (decreases in glutathione peroxidase 1, glutathione peroxidase 4, superoxide dismutase, and glutathione *S*-transferase activities; increase in thioredoxin reductase activity), and liver enzymes when administered to 10-week-old rats at 1000 ppm dose for 10 days. Besides, DEHP caused cellular disorganization, increases in catalase activity/immunoreactivity, and lipid peroxidation [89].

6.7. Effects of phthalates on other organs/systems

Other than testis and liver, phthalates were suggested to be toxic to kidneys and thyroid [90, 91]. Moreover, phthalates were also shown to affect the neuroendocrine system and the hypothalamus-pituitary-ovarian/testicular axis in rats [92–94].

6.8. Epidemiological studies concerning the effects of phthalates on reproductive/endocrine systems

In the last five decades, a gradual decline in global semen quality has been reported [95]. The effects of phthalate exposure on male fertility has been attracting the attention of researchers for a long period of time, although phthalates exert serious health effects on different organs/systems. Semen quality defines the sperm count, motility, and morphology. An early study described sperm concentration and DBP measured in the seminal fluid of American students (N=21) recruited and these students were classified as low metabolizers (n=12) and high metabolizers (n= 9). Seminal DBP levels were associated with decreased sperm concentration, and an overall analysis indicated a positive association between DBP and sperm concentration [95]. Total semen phthalates were higher among 21 men with an infertility diagnosis compared to 32 men without (p<0.05) in a cross-sectional study of infertility clinic patients in India. Among the infertile men, seminal phthalates were associated with increased sperm abnormality but not with sperm count or motility [96]. Later, in a cross-sectional study of 234 Swedish military conscripts aged 18 to 21 years, Jönsson et al. [97] observed that urinary MEP concentrations were associated with decreased sperm motility. No associations were determined between semen quality and MBP, MBzP, or MEHP. A positive association between sperm motility and phthalic acid was found, and this finding suggested that an increased ability to metabolize phthalates may be protective. Besides, the researchers reported a cross-sectional association between urinary MEP concentrations and decreased LH levels when comparing the quartile of highest exposure to the lowest [97].

Among 52 Chinese men attending a reproduction clinic in Shanghai, no associations were detected for sperm count or morphology and semen concentrations of DEP, DEHP, and DBP [98]. The majority of studies of phthalate exposure and semen quality have been conducted among infertile populations. In 2006, an American study was conducted on 443 men undergoing infertility therapy. Higher urinary MBP levels were associated with decreased sperm concentration and motility after adjusting for age, abstinence time, and smoking status. No associations with semen quality were suggested for MEP, MMP, MEHP, MEOHP, or MEHHP [99].

Pant et al. [100] measured the phthalate diesters levels in the semen of 300 Indian men, and correlations were detected between DBP, DEHP, and decreased motility in unadjusted analyses. Unadjusted associations were also reported between DBP, DEHP, DEP, and decreased sperm concentration and for DEHP and increased abnormal morphology. No associations were reported for semen DMP or di-*n*-octyl phthalate (DnOP) levels and sperm parameters [100]. Wirth et al. [101] determined the urinary phthalate levels in 45 men who were admitted to an infertility clinic. Urinary MEP levels were associated with decreased sperm concentration (after adjusting for race and for urine specific gravity) and abnormal morphology (after adjusting for urine specific gravity). Urinary mono-3-carboxypropyl phthalate (MCPP) levels were also associated with an increased proportion of morphologically abnormal sperm, and an association was suggested for urinary MEHP with low sperm concentration. However, no associations were indicated for MMP, MBP, MiBP, MBzP, MEHP, MEOHP, and MEHHP [101].

Another study on 349 men who were recruited from a German andrology clinic reported no associations between urinary DEHP metabolites (MEHP, MEHHP, MEOHP, and 5cx-MEPP) and any semen parameters after adjusting for age, smoking, abstinence period, and urine creatinine [102]. In another cross-sectional study, the median semen DBP concentration was higher in oligoasthenospermic men (*n*=65) compared to fertile men (*n*=50), and median DEHP concentration was also higher in oligoasthenospermic men versus in fertile men. In addition, sperm motility was inversely associated with DBP and DEHP in oligoasthenospermic (*n*=65) and asthenospermic (*n*=65) men, respectively. However, the associations were not adjusted for potential confounding variables [103]. Another cross-sectional study recruited 97 men undergoing infertility treatment in China and found the top tertile of urinary MBP to be strongly associated with decreased sperm concentration after adjusting for age, abstinence time, body mass index (BMI), smoking, alcohol consumption, and education. There were no associations reported for sperm parameters and MMP, MEP, MBzP, MEHP, or MEOHP [104]. A more recent work suggested that urinary MBP levels were inversely associated with sperm concentrations in a general population sample of 232 men residing in a heavily industrialized urban area in China. However, no such associations were found for MEP (median=3.10 ng/mL) or MEHP (1.10 ng/mL) or for motility and morphology [105]. A Polish study on men (n=269) under 45 years of age and attending a infertility clinic measured urinary MEHP, MEHHP, monoisononyl phthalate (MiNP), MBzP, MBP, and MEP levels. Inverse associations were detected between sperm motility and log-transformed MEHP, MEHHP, and MiNP levels after adjusting for age, smoking, abstinence period, past diseases, and creatinine as confounding covariates. No adjusted associations were detected between sperm concentration and morphology and urinary MBzP and MEP levels [106].

There are several studies that were conducted to understand the relationship between sex or reproductive hormones and different phthalate derivatives. One cross-sectional study in China quantified exposure using urinary metabolites in occupationally exposed workers (*n*=74) and unexposed referent workers (*n*=63) matched by age and smoking. Decreases in free testosterone, but not FSH, were associated with MBP exposure in all the men after adjusting for age and alcohol consumption [107]. An earlier work performed on 295 men reported an association between an interquartile range (IQR) increase in urinary MBzP concentrations and decreased FSH levels and a nonsignificant association between an IQR increase in MEHP and decreased testosterone after adjusting for age, BMI, and time of specimen collection [108]. Furthermore, MBP was nonsignificantly associated with increased inhibin B after adjustment for covariates. The results of this study are in contradiction with the results obtained from a study that recruited 118 men seeking infertility treatment in China. Sex steroid hormones were assessed in association with urinary MEP, MBP, MBzP, MEHP, MEHHP, and MEOHP levels among 425 men. The molar sum of DEHP metabolites and percentage MEHP were assessed, and an IQR increase for MEHP was associated with decreased serum estradiol and testosterone levels after adjusting for age, BMI, smoking, and time of specimen collection. A significant association between percentage MEHP and an increase in the testosterone/estradiol ratio was also determined. Adjusted decreases in the free androgen index [FAI; describes the ratio of testosterone to sex hormone binding globulin (SHBG)] were also reported for MEHHP, MEOHP, and the sum of DEHP metabolites. No associations were reported between MBP or

MBzP and serum prolactin, FSH, or LH. However, no associations between concentrations of the other phthalate metabolites and concentrations of the other hormones, including testosterone, estradiol, FSH, inhibin B, and SHBG, were found [109]. Another study performed on 425 men recruited through a U.S. infertility clinic found limited inverse association between MEHP and FAI by the proportion of DEHP metabolites in the urine measured as MEHP (MEHP%), a phenotypic marker of less efficient metabolism of DEHP to its oxidized metabolites. Finally, the ratio of testosterone to estradiol was positively associated with MEHP and MEHP%, suggesting potential relationships with aromatase suppression [110]. In a cross-sectional study of 363 fertile men participating in a multicenter U.S. study, inverse associations were described for urine DEHP metabolites (MEHP, MEHHP, MEOHP, and MECPP) and FAI after adjusting for age, BMI, smoking, ethnicity, urine creatinine, and time of sample collection. A positive correlation was also found between MEHP and SHBG, and the FAI/LH ratio after adjustment for covariates. However, no associations were reported for FSH, estradiol, or LH [111]. A recent study compiled data from two investigations of phthalate exposure and reproductive hormones, combining men from the Massachusetts General Hospital and multicenter U.S. studies. In a combined total of 783 men, there were no associations with urinary concentrations of MEP, MBP, or MBzP and any reproductive hormone measured. However, metabolites of DEHP (MEHP, MEHHP, and MEOHP) were associated with decreased free testosterone and increased SHBG levels [112].

A study performed by our group recruited 40 newly diagnosed pubertal gynecomastia cases and 20 controls. Plasma DEHP and MEHP levels were found to be statistically significantly higher in the pubertal gynecomastia group compared to the control group. There was a statistically significant correlation between plasma DEHP and MEHP levels (r=0.58; P<0.001). In the pubertal gynecomastia group, no correlation was determined between plasma DEHP and MEHP levels and any of sex hormone levels [24]. However, as a part of the Copenhagen Puberty Study, Mieritz et al. [113] did not find any difference between the urinary phthalate levels of Danish boys (n=555) versus control.

7. Bisphenol A

BPA is used to harden plastics and to manufacture polycarbonate plastics and epoxy resins. It has high abundance in the environment and considered as estrogen-like EDC or as a xenoestrogen. It was shown that BPA acts similarly to 17-β estradiol [114, 115]. BPA induces ERs (weakly to ERα and ERβ and strongly to ERRγ) but in the concentrations approximately 1000 higher (10^{-6}–10^{-4} M) in comparison to estradiol [116]. BPA was classified as a weak environmental estrogen before. However, newer studies determined that, even in very low concentrations (picomolar and nanomolar), BPA exerted divergent effects on the physiology of different cells and tissues and can bind to both nuclear and nonnuclear receptors [117]. Furthermore, most of BPA metabolites were shown to exert stronger estrogenic activities than the main compound [118]. Apart from the estrogenic effects, BPA was shown to bind to ARs, which can be related to excessive stimulation prostate and can lead to cancer. BPA was

suggested to modulate androgen-dependent prostate cancer cell proliferation even in the concentrations corresponding to its level determined in human blood [119].

Metal food and beverage cans have a thin coating of BPA on the interior surface, which is essential to prevent corrosion of the can and contamination of food. Moreover, fetuses and young infants are commonly exposed to BPA by transplacental transfer of maternal BPA and through ingestion of maternal milk or formula in BPA containing plastic bottles [120].

BPA exhibits moderate acute toxicity to vertebrates. Through the oral, i.p., and i.v. routes, LD_{50} doses of BPA in rats were found to be 3250, 841, and 35.26 mg/kg body weight, respectively [121, 122]. On the contrary, LD_{50} doses in mice were found to be 2400 and 150 mg/kg via oral and i.p. routes, respectively. Moreover, the U.S. EPA estimated the reference dose as 50 μg BPA/kg body weight/day [123]. Studies concerning the toxic effects of BPA suggested that humans are more susceptible than rodents to the deleterious action of this substance [124]. Recently, intensive investigations are being realized that refer to toxic, teratogenic, carcinogenic, and particularly estrogenic mechanisms of BPA action.

7.1. Biotransformation of Bisphenol A

BPA can be biotransformed in vertebrates, invertebrates, and plants and it is also biodegraded by microorganisms including bacteria, fungi, and algae. There are differences of species or strain in the metabolism of BPA. It was reported that, in primates, BPA can more easily be absorbed orally or subcutaneously (s.c.) compared to rats, and primates need a longer period of time to eliminate BPA from serum than rats [125]. Moreover, human liver microsomes do not have the ability to glucuronidate BPA as extensively as rat liver microsomes [126]. The metabolism of BPA is faster in female rats than in male rats [127]. It was suggested that gender differences in serum BPA concentrations of adult humans may be caused by differences in the androgen-related metabolism of BPA [128]. In addition, Kim et al. reported higher levels of BPA glucuronide in men than in women [129]; however, the levels of BPA sulfate were higher in women than in men [130].

BPA glucuronide is the major metabolite of BPA. Other metabolites (BPA sulfate conjugate, BPA diglucuronide, 5-hydroxy BPA, and the corresponding sulfate conjugate) were also reported. BPA glucuronide has lower estrogenic effect compared to the main compound [131–134]. 5-Hydroxy BPA is also less estrogenic than BPA [132, 133]. The sulfate metabolite of BPA does not show an estrogenic effect up to 1 mM [134].

7.2. Genotoxic effects of Bisphenol A

BPA did not show any mutagenic effect in Ames test even after metabolic activation [135]. However, BPA was shown to cause DNA damage in eukaryotic cells, that is, BPA induced DNA strand breaks in L5178Y mouse lymphoma cells [136] and induced aneuploidy and structural chromosomal aberrations in ER-positive MCF-7 cells and in CHO-K1 cells [132, 133, 137]. The genotoxic potential of BPA was also evaluated by Lee et al. [138]. In mutant chicken DT40 cell lines (deficient in DNA repair pathways), researchers determined chromosomal aberrations and double-strand breaks [138]. BPA was shown to induce ROS generation, which

in turn caused an induction of the production of DNA oxidative bases [139]. However, Audebert et al. [140] did not observe any genotoxicity when BPA was administered to three human cell lines [human intestinal cell line (LS174T), hepatoma cell line (HepG2), and renal cell line (ACHN)]. On the contrary, BPA metabolites (BPA-quinone) were shown to induce DNA damage by forming covalent adducts with DNA and adducts with deoxyguanosine [131, 141].

BPA was also suggested to alter gene and protein expression. Recently, Fernandez et al. [142] showed that BPA increased the expression of some genes (i.e., BRCA1, BRCA2, BRCC3, and BCL2L11) that are involved in DNA repair and apoptosis in human breast epithelial cells. The authors suggested that women who have BRCA1 or BRCA2 mutations may be more susceptible to such effects of BPA [142].

7.3. Epigenetic effects of Bisphenol A

In utero or neonatal exposure to low doses of BPA may cause alterations in DNA methylation, modifications in histones, and changes in the expression patterns of noncoding RNAs. These changes can up-regulate or down-regulate different gene expressions, which in turn may result in permanent health effects such as neural and immune disorders, infertility, and late onset of complex diseases. BPA induced permanent alterations in DNA methylation patterns of different genes that are responsible for cellular signaling [64, 143].

The epigenetic effect of BPA was clearly demonstrated in viable yellow mice [144]. By decreasing CpG methylation in the IAP retrotransposable sequence inserted upstream of the Agouti gene, the maternal exposure to BPA shifted the coat color distribution of viable yellow mouse offspring toward yellow. This effect was completely prevented by maternal supplementation with folic acid or the phytoestrogen genistein, both of which are sources of methyl group [145].

BPA was suggested to induce mainly breast and prostate cancer in both animals and humans. Keri et al. [146] suggested that BPA may increase the risk of such cancers by affecting various cell processes such as DNA methylation and chromatin remodeling during development. BPA exposure was shown to cause epigenetic alterations in rodent prostate and have been postulated to be the underlying cause of neoplastic development in later life [147]. Neonatal exposure of rats to BPA resulted in an increased incidence of prostate intraepithelial neoplasia, and the prostate tissues showed consistent methylation changes. For example, the phosphodiesterase type 4 variant 4 (Pde4d4) gene was found to be hypomethylated at the regulatory CpG island and started to have an elevated expression in the adult rat prostate [148, 149]. On the contrary, neonatal BPA exposure was also reported to alter the promoter methylation and expression of nucleosome binding protein-1 (Nsbp1) and hippocalcin-like 1 (Hpcal1) genes in rats [150]. Also, the neonatal exposure to BPA was shown to induce hypermethylation of ER promoter regions in rat testis. This phenomenon mediated epigenetic changes that in turn induced adverse effects on spermatogenesis and fertility [151].

Concerning miRNAs, BPA exposure of human placental cell lines has been shown to alter miRNA expression levels; particularly, miR-146a was strongly induced after BPA application.

Induction of miR-146a caused slower cellular proliferation rates and higher sensitivity to bleomycin, which strongly induces DNA strand breaks [152]. In mouse Sertoli cell line (TM4), 24 h BPA exposure leads to up-regulation or more often to down-regulation of 37 miRNAs [153].

7.4. Carcinogenicity of Bisphenol A

Currently, there is no evaluation of the IARC for BPA. However, "bisphenol A diglycidyl ether" is classified as a Group III carcinogen (not classifiable as to its carcinogenicity to humans) by the IARC [154].

At low doses, BPA was shown to alter mammary gland development and increased incidence of tumors in Sprague-Dawley rats [155]. Besides, at the comparable amounts to a reference dose, BPA caused development of breast, prostate, and nipple cancers in both mice and rats [146]. Acevedo et al. [156] recently evaluated the malignant potential of BPA in rats and showed that suggested that BPA acted as a mammary gland carcinogen at doses comparable to those present amounts in human urine and blood determined by epidemiological studies. BPA was also suggested to cause the development of hematopoietic cancers and induced testicular cancer in mice and rats [157].

It was also shown that BPA promoted proliferation of human epithelial ovarian cancer cells (OVCAR-3), increased the susceptibility of endometrial cells to the effects of estradiol, and inhibited the 17-β-estradiol-induced genomic activity of ERβ in DLD-1 colon cells [158–161].

7.5. Reproductive toxicity of Bisphenol A

Perinatal exposure to environmentally relevant BPA doses may predispose the tissue to earlier onset of disease, reductive fertility, and mammary and prostate cancers, as BPA may cause morphological and functional alterations in both male and female genital tracts and mammary glands. The estrogenicity of BPA has been shown by many rodent studies. When pregnant mice were exposed to environmentally relevant doses of BPA, earlier vaginal opening and earlier first estrous cyclicity in their offspring were observed. BPA elevated prostate weight in mice. An advanced reproductive aging was also observed [162]. BPA was also shown to disturb the development of reproductive organs, testosterone excretion, and sperm production in mice [163].

Some studies have suggested that BPA may not alter estrogenic function in rodents. Ryan et al. [164] showed that BPA exposure of male and female rats (2–200 μg/kg body weight/day) did not affect maternal pregnancy or weight gain or F_1 female birth weight as well as reproductive morphology, fertility, fecundity, or sexual dimorphic behaviors. Recently, Ziv-Gal et al. [165] suggested that BPA at low doses (110–438 μM) decreased estradiol levels and inhibited growth of follicles isolated from wild-type and AhR knockout mice and that AhR signaling pathways may be significantly involved in the modulatory effect of BPA on follicular growth.

7.6. Hepatotoxic effects of Bisphenol A

Atkinson and Roy [130] found that the BPA metabolite, bisphenol-*o*-quinone, could bind DNA *in vitro* and *in vivo*, leading to the induction of hepatotoxicity. Besides, BPA was also shown to cause damage to hepatocytes by oxidative stress. BPA markedly decreased antioxidant enzymes and glutathione *S*-transferase activities as well as depleted reduced glutathione levels in rats. Moreover, BPA (50 mg/kg) significantly increased the biochemical levels of liver enzymes and reduced the expression of hepatic antioxidant genes. The authors concluded that BPA generated ROS and reduced the antioxidant gene expression causing hepatotoxicity.

Huc et al. [166] observed that low BPA doses of BPA led to hepatic (HepG2) cell damage and this effect might be due to significant mitochondrial dysfunction including ROS production, mitochondrial transmembrane hyperpolarization, lipid peroxidation, and release of proinflammatory cytokines. Moon et al. [167] also observed that BPA exposure caused oxidative and proinflammatory damage in rat hepatocytes. The researchers observed a decrease in glutathione peroxidase activity, an increase in lipid peroxidation, and decreases in the proinflammatory cytokines [i.e., interleukin (IL)-6 and tumor necrosis factor-α]. Moreover, the researchers showed that, at low doses, BPA decreased the oxygen rate and ATP production and caused mitochondrial dysfunction. Based on these studies, they concluded that BPA induced hepatic mitochondrial dysfunction below the no observed effect (NOEL) value (5 mg BPA/kg body weight/day) [167].

7.7. Effects of Bisphenol A on other organs/systems

BPA was proven to affect not only estrogenic system but also functions of androgens, prolactin, insulin, and thyroid hormones [117, 168]. Gentilcore et al. [169] observed that BPA at low doses (10^{-9} M) affected the expression of the genes involved in thyroid hormone synthesis, thyroid follicular cells, and altered thyroid-specific transcriptional factors in zebrafish.

In vitro experiments showed that BPA may cause toxicity in nervous system cells. BPA in high concentrations (>100 μM) was shown to induce apoptosis of hippocampal neuronal cells by increasing calcium and ROS levels and then by activating caspase-3 and mitogen-activated protein kinases (MAPK) [170]. In rat embryonic midbrain cells, at relatively low concentrations (10^{-12} to 10^{-4} M), BPA caused S- and G_2/M-phase arrests and elevated the percentage of apoptotic cells. BPA also lowered the phosphorylation of c-Jun N-terminal kinase and increased the mRNA expression level of proapoptotic proteins (i.e., Bax and p53) [171].

BPA did not cause morphological and neurobehavioral changes in F_1 offspring of rats treated with different doses (0.15–2250 ppm) [172]. BPA (administrated 2–200 μg/kg/body weight/day by gavage) did not have any impact on the sensory system and neurobehavioral activity in Long-Evans rats [163]. However, some studies showed that BPA may have an effect on the neurotransmitter levels of rodents. Nakamura et al. (2010) observed that BPA (20 μg/kg s.c.) had increased brain levels of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), whereas the levels of serotonin and its derivative, 5-hydroxyindoleacetic acid (5-HIAA), decreased in pregnant mice [173].

BPA was suggested to both stimulate and inhibit the activity of immune system cells. It is postulated that BPA can modulate the immune activity by affecting ERs, AhR, and probably peroxisome proliferator-activated receptor (PPAR) [174]. Youn et al. [175] showed that BPA (in drinking water) caused increases in interferon-γ and decreases in IL-4 production in T lymphocytes of mice, whereas Lee and Lim [176] observed that BPA elevated IL-4 and IL-8 levels in mouse T lymphocytes. Exposure of mouse splenic lymphocytes to low BPA concentrations (1 μM) could inhibit mitogenesis, particularly the mitogenesis of B lymphocytes [177]. Goto et al. [178] observed that BPA produced lymphocytes with higher amounts of immunoglobulin A (IgA) and IgG2a in mice. Sugita-Konishi et al. [179] reported that BPA caused depletion in neutrophil activity and inhibited IL-6 formation in mice infected with nonpathogenic *Escherichia coli*.

Roy et al. [180] showed that offspring of female mice exposed to BPA were more susceptible to infection by influenza A virus because of the modulation of their innate immunity by BPA. However, the researchers did not observe impairment in antiviral adaptive immune response, which is a crucial response for virus clearance and survival.

7.8. Epidemiological studies concerning the effects of Bisphenol A on reproductive/endocrine systems

Most of the epidemiological studies on BPA mainly focus on its effects on endocrine and reproductive systems. Meeker et al. [181] analyzed urinary BPA concentrations of men (*n*=167) who were admitted to an infertility clinic. The researchers reported that their urinary BPA levels were inversely correlated with their estradiol/testosterone ratio [181]. In a cross-sectional study, Melzer et al. [182] determined the urinary BPA levels of subjects from Chianti, Italy (n=1453, age=20–102 years), and observed positive associations between higher urinary BPA concentrations and higher expression of two estrogen-responsive genes, encoding ERβ and estrogen-related receptor α (ERRα). Li et al. [183] examined the urinary BPA levels of 218 men with and without BPA exposure in the workplace. Increasing urinary BPA levels were statistically significantly associated with decreased sperm concentration, total sperm count, sperm vitality and sperm motility after adjustment for potential confounders using linear regression. Men with detectable urinary BPA concentrations had more than risk of decreased sperm concentration, sperm vitality, count, and motility compared to men who had undetectable urinary BPA levels. However, urinary BPA levels were not associated with semen volume or abnormal sperm morphology [183].

Takeuchi and Tsutsumi [127] investigated serum BPA concentrations of men (*n*=11), women (*n*=14), and women with polycystic ovary syndrome (PCOS; *n*=16). Serum BPA levels were significantly higher in normal men and in women with PCOS. There were significant positive correlations between serum BPA and total testosterone and free testosterone concentrations in all subjects and between serum BPA and total testosterone ($r \underline{5} 0.559$; $P<0.01$) and free testosterone concentrations in all female subjects; however, there was no association between serum BPA and other sex-related hormone concentrations in any group. The researchers determined that there were gender differences in serum BPA concentrations, where men had higher serum BPA levels (1.49 ± 0.11 ng/mL in men, 1.04 ± 0.10 ng/mL in women with PCOS,

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and 0.646; 0.10 ng/mL in women) possibly due to the differences in the androgen-related metabolism of BPA [27].

Miao et al. [184] conducted a cross-sectional study among female workers from BPA-exposed (n=106) and unexposed (*n*=250) factories in China. They observed a significant positive association between increased urine BPA concentration and higher prolactin and progesterone levels. Among exposed workers, a positive association between urine BPA and estradiol was also determined. In addition, a statistically significant inverse correlation between urinary BPA concentration and FSH was found among unexposed group. The researchers suggested that BPA exposure may lead to alterations in female reproductive hormone levels [184]. Mínguez-Alarcón et al. [185] conducted a prospective cohort study at the Massachusetts General Hospital Fertility Center, which included 256 women (*n*=375 *in vitro* fertilization cycles) who provided up to two urine samples before oocyte retrieval (total *N*=673) between 2004 and 2012. Urinary BPA concentrations were not associated with endometrial wall thickness, peak estradiol levels, proportion of high-quality embryos, or fertilization rates. The researchers did not observe any correlation between urinary BPA concentrations and implantation, clinical pregnancy, or live birth rates per initiated cycle or per embryo transfer. Women older than 37 years had thinner endometrial thickness across increasing quartiles of urinary BPA concentrations, whereas women younger than 37 years had thicker endometrial thickness across increasing quartiles of urinary BPA concentrations [185]. A cross-sectional study was carried out by Liu et al. [186] to determine the associations between urinary BPA levels serum reproductive hormone levels among male Chinese adults (*n*=592). A multiple linear regression and log-binomial model was used to examine the associations between urinary BPA level and hormone levels after controlling for age and smoking status. Increased urinary BPA levels were positively and significantly correlated with prolactin, estradiol, and SHBG levels and were negatively and significantly associated with androstenedione and free androgen index levels. The researchers suggested that high urinary BPA levels were associated with increased prolactin, estradiol, and SHBG level in males and these associations may contribute to male infertility [186]. Liu et al. [187] conducted a cross-sectional study to investigate the correlations between maternal phenolic exposure and cord sex steroid hormones and AGD in male newborns. Mother-infant pairs from each of two hospitals [one in a polluted town (Guiyu; *n*=77) and the other in a cleaner town (Haojiang; *n*=60)] were recruited in the study. Maternal urinary BPA concentrations ($\log_2$ transformed) were negatively correlated with testosterone levels and testosterone/estradiol ratio in male fetal cord blood samples (after adjustment for potential confounders in linear regression models). No significant associations between AGD or anogenital index (AGI) and BPA or cord hormone levels were found [187].

Another study by our group recruited nonobese girls newly diagnosed with idiopathic central precocious puberty (ICPP; *n*=28; ages 4–8 years) and 25 healthy age-matched girls with no history of ICPP or any other endocrine disorder. Urinary BPA levels were significantly higher in ICPP group compared to the control group. There were no significant associations between urinary BPA levels and serum LH, FSH, and estradiol levels [188].

8. Conclusion

EDCs are widely available in the environment. Although exposure to these chemicals can be reduced by not using plastics, the total prevention of exposure is not feasible. Serious measures must be taken to reduce the availability of EDCs and regulatory authorities must be aware of their toxic effects and the outcomes. Parents should avoid using plastic materials and EDC-containing materials (phthalate containing gum shields, toys, and paints; BPA-containing feeding bottles and water bottles) for children, particularly for newborns. Therefore, the reduction of the use of phthalates and BPA must seriously be taken into concern, and chemical companies must be encouraged to synthesize and produce nontoxic alternatives of these substances.

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Chapter 7

Occupational Exposure to Coal, Genotoxicity, and Cancer Risk

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Additional information is available at the end of the chapter

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Abstract

Coal is a heterogeneous mixture containing large quantities of organic and inorganic matter, including carbon, hydrogen, oxygen, sulfur, nitrogen, and organometallic forms. The presence of mineral matter in coal may result in a number of environmental and human health problems related to its mining, preparation, and combustion. During coal mining activities, large quantities of coal dust, ashes, polycyclic aromatic hydrocarbons (PAHs), and heavy metals are released into the environment, forming a complex mixture. This mixture becomes one of the most important occupational risks for the health and safety of workers due to its synergistic, additive, and enhancing effects. Once inside the organism, this cocktail-like mixture can interact with cellular mechanisms related to the production of reactive oxygen species (ROS) and can cause damage in important macromolecules such as DNA, lipids, and proteins. In this review, human populations exposed to coal and coal burning were analyzed. Data from different studies were evaluated in relation to the effect of complex mixture exposure on DNA damage and mechanisms, and the background factors, such as gender, age, or smoking habit. The high temperatures that occur in combustion processes affect the characteristics of the resulting particles. The coal fly ash is released by combustion and its composition varies depending on the coal type and the method of collection used such as electrostatic precipitators. Compounds such as PAHs once activated by the organisms have been shown to have mutagenic and carcinogenic activity due to its ability to form adducts with purines. Moreover, metals that commonly are evaporated during the cooling process increase its toxicity. The particles when inhaled can pass from the alveoli into the bloodstream and affect extrapulmonary organs. Several studies have described the inflammatory cascade that triggers exposure to coal and coal fly ash particles; they have a complex composition capable of generating a persistent inflammatory process, resulting in diseases widely described as emphysema, bronchitis, pneumoconiosis, asthma, and cancer. Several human biomonitoring studies have been conducted evaluating the inflammatory process and

the release of cytokines, polymorphisms involved in detoxification mechanisms, different biomarkers associated with occupational exposure, DNA damage, and the influence of oxidative stress in disease development. The relationship between chronic exposure to coal and coal ash particles and cancer is still widely debated. This review gave us a broad assessment about the associated mechanisms between cancer and exposure to coal and different findings around the world.

Keywords: coal, biomonitoring, DNA damage, ROS, PAHs, diseases

1. Introduction

In the last decades, the human population genetics integrity has been compromised by the great industrial activity, which exposes people to a variety of chemicals and genotoxic agents. As a result, it is important to determine what is considered as an "acceptable" level of genetic damage in a concrete population, carry out assay genotoxicity as a routine and also monitor those who, by their occupation or lifestyle, are more exposed or with a bigger risk of having alterations on their genetics stability [1].

One method to quantify the exposure to those substances, as well as its possible impact on the organism, is the use of biological monitoring procedures, or biomonitoring, through biomarkers. Biomonitoring studies try to establish a connection between the environmental factors and the diseases. They detect first alterations in nonmalignant phases, so as to prevent health problems by recognizing the environmental cause of them.

The biological markers, or biomarkers, are the measurable changes (biochemical, physiological, or morphological) that associate to a toxic exposure or any early biochemical alteration, whose study on the biological fluids, tissues, or exhaled air that allow to assess the health risk exposure intensity. The identification of genotoxicity markers believed to cause genome damage is useful, since it can define a prepathogenesis state, such as cancer. It is of vital importance for different diseases prevention, which is the final goal of biomonitoring. In order to achieve it, there must be two stages: 1) detecting human exposure to environment carcinogenic agents; 2) determining genotoxic effects *in vivo* [2].

The combined use of genetic biomarkers and classic epidemiology tools (clinic history and questionnaires) has enabled the identification of early effects to the occupational exposure to distinct pollutant around the world [2–4]. Many biomarkers are used to assess genotoxic effects on human populations exposed to complex mixtures of chemicals. Although there are different possibilities, micronuclei (MN) frequency, chromosomal aberrations (CAs), and comet assay are the most commonly chosen biomarkers. MN originates from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division [5, 6]. MN induction reflects clastogenic and aneugenic damage and is a predictive biomarker of cancer risk [7]. Comet assay detects DNA lesions in individual cells obtained under a variety of experimental conditions; the technique can also be used to evaluate DNA repair [8, 9].

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The large inter-individual variability in the capacity to activate or inactivate potential genotoxic and carcinogenic compounds is probably influenced by polymorphisms of the genes encoding the metabolizing enzymes. Genes and proteins involved in metabolization/detoxification of xenobiotics, as well as those involved in DNA repair, are usually used as potential markers of susceptibility for the development of several diseases in which the etiology is related to exposure to environmental hazards. Polymorphisms in such genes have been linked with an increased risk of cancer in several case-control studies [10].

Biomonitoring studies in populations exposed to complex mixtures of chemicals considering individual susceptibility are quite complicated due to inadequate toxicity data, and the unpredictable nature of interaction effects that may be synergistic, additive, or enhancers.

2. Occupational exposure to coal

The coal reserves in a worldwide level is up to 847.5 billion of tons, enough amount to serve the current production for 119 years. This prediction is different from the ones related to oil and gas, which have available supplies for less time [11]. According to data from the International Energy Agency (IEA), coal is the most used resource for energy generation in the world, responsible for 41% of the total production. Nowadays, the main application of mineral coal is to generate energy through thermal power plants. These reserves are considered to have a 109-year lifespan and their coalfields are located in 75 countries. The main world coal producers are China, the United States, India, Australia, Indonesia, Russia, South Africa, Germany, Poland, and Kazakhstan, which are responsible for 91% of the world's production [12]. If those projections are right, the consequences of coal mining and combustion will have large effects in the environment. Thus, the exposed populations monitoring is fundamental with the aim of contributing to the state of knowledge about the health risk and motivate the establishment of control, hygiene, and prevention strategies.

It is well known that coal mining activities are one of the biggest resources of contamination due to the large quantity of substances liberated in the environment. The content of the coal dust and ashes produced by burning are not always homogeneous and this depends on the source and rank of the coal [13, 14]. Coal dust is constituted from carbon, hydrogen, oxygen, nitrogen, quartz (crystalline silica), and inorganic minerals, such as beryllium, cadmium, cobalt, chromium, iron, boron, copper, nickel, antimony, zinc, aluminum, titanium, magnesium, manganese, mercury, and lead [15]. As observed, coal is a mixture of a variety of chemicals, including hydrocarbons, which may raise polycyclic aromatic hydrocarbons (PAHs). All technological processes associated with open fire or temperatures between 400 and 600°C, that may lead to PAHs, should be considered potentially hazardous [16, 17].

In relation to coal mining residues exposure, studies in which biomarkers of effect, susceptibility, and exposure are used as epidemiological tools remain rare and a big part of them come from studies on underground coal mining [18, 19]. The effects generated by open coal mining are little explored, though. In open coal mining, the residues pass directly to the atmosphere, where complex mixtures are formed, and the coal exposure to environmental factors such as

sunlight facilitates the processes of spontaneous combustion and, therefore, the release of PAHs [20].

Studies about the coal exposure and its harmful effects have been conducted around the world [21–23]. The main way for exposure of the coal mining workers to the potentially dangerous residues is through the inhaling of coal dust particles from mining and manipulation. It is a known fact that the coal mining continuous exposure can cause a variety of diseases, such as coal workers pneumoconiosis (CWP), silicosis, cancer, and chronic obstructive pulmonary disease (COPD), as emphysema and chronic bronchitis [24].

Many studies have established that some of those diseases could have been originated from the genotoxic damage generated by the inhalation of those mineral particles, able to interact with macrophages, epithelial cells, and other cells generating the production of large amount of reactive oxygen species (ROS) [24–26]. The continuous inhalation of coal dust and fly ashes particles is an important cell and non-cell source of ROS in the lung. This may be associated to the damage of target cells of that tissue and other cell lines, after spreading through the bloodstream [27].

Coal-induced DNA damage is related to macrophage activation and the recruitment of polymorphonuclear cells. This cell activation induces the release of inflammatory mediators, such as cytokines, ROS and reactive nitrogen species (RNS). The proinflammatory properties of ROS and RNS include endothelial cell damage, lipid peroxidation and oxidation, the release of chemostatic factors, the recruitment of neutrophils, and DNA damage [26, 28]. Interaction of ROS with DNA can result in DNA structural and transcriptional errors [29, 30]. Damage caused by ROS is recognized by DNA glycosylases, apurinic/apyrimidinic endonucleases of the base excision repair (BER) mechanism, and in some cases, by the nucleotide excision repair (NER) machinery, leading to DNA strand-breaks [31, 32].

Although chronic exposure may continue to damage the DNA, it has been suggested that inorganic elements can induce DNA single-strand breaks, possibly via the generation of ROS and that this type of damage is soon repaired. Metals are also known to modulate gene expression of enzymes [33]. In addition, PAHs can induce DNA lesions as single-strand breaks via DNA repair mechanisms, related with increased adduct formation and electrophilic metabolites [34–36]. Electrophilic metabolites covalently interact with the DNA [37, 38], and adducts are formed with purines, especially guanine, after metabolic activation by enzymatic complex P450 [39]. The International Agency for Research on Cancer (IARC) classified quartz, main constituent of coal, into IARC Group 1 (carcinogen), due to sufficient evidence for carcinogenicity in experimental animals and in humans [40, 41]. The other factor that could lead to different results in coal dust exposure, with positive and negative results, might be explained by the possible differences in composition, in which the proportion of the metals, PAHs, and silica (quartz) content may have an influence on the genotoxicity. Despite those findings, coal dust remains classified as non-carcinogen for human (Group 3) in IARC [40, 41]. The importance of coal as an energy source makes its characterization and estimation of risks of extreme importance to the safety of those individuals and the environment.

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Several factors may explain conflicting results among different studies with human exposed to coal, e.g. cigarettes smoked, age, gender, nutritional status, and individual polymorphisms [6, 42]. Susceptibility is critical to an understanding of coal diseases, including cancer, and many xenobiotic agents act to alter susceptibility. Unknown individual susceptibility, inadequate toxicity data, and the unpredictable nature of interaction effects make the implementation of a human biomonitoring assessment for complex mixtures of chemicals extremely complicated.

3. Oxidative stress and genotoxic damage related with coal exposure

One important aspect to consider about the coal exposure is the amount of products generated during the coal combustion. The burning of coal, in order to generate electricity, produces flue gasses and particulate materials like coal fly ashes and residues as scoria and bottom ash. The finer particles (coal fly ash) are obtained by mechanical or electrostatic precipitation of the dust in suspension in the gases produced by combustion, while the coarser particles fall to the bottom by gravity and are removed at the bottom of the boiler [43, 44].

The combustion temperature is an important factor that determines the physical properties of the particles. In the combustion of conventional high temperature (>1400°C), the main aluminosilicate melts and condenses to form spherical particles. The coal fly ash particles produced are mostly irregularly shaped and contain a complex mixture consisting of unburned carbon; oxides; quartz; elements such as aluminum, silicon, calcium, iron, nickel, arsenic, chromium, copper, lead, cadmium, zinc [45, 46], and PAHs [47].

The coal fly ash has a relatively low toxicity as compared with coal or quartz [45]. Studies have determined the role of coal fly ash particle size and the release of iron, which leads to generation of radicals and oxidative stress. In this context, it was demonstrated the ability of coal fly ash release of bioavailable iron, which triggers processes and redox oxidant production [48]. In addition, it was shown that interleukin 8 (IL-8) levels in human lung epithelial cells are increased in response to coal fly ash and vary with the bioavailability of iron, as a function of source of coal and particle size [49]. The smaller size fraction had more stimulatory activity, which may be related to the fact that iron is more concentrated in this fraction. Particle size is a critical factor because a larger surface area allows more significant transport of metal and other adsorbed components, increasing the pulmonary toxicity of particulate matter (PM) [50].

The particles are classified according to their aerodynamic diameter (in micrometer) in coarse (PM 10), fine (PM 2.5), ultrafine (PM 0.1) [51]. The smaller particles are more harmful with respect to health effects because of their very high alveolar deposition fraction, large surface area, chemical composition, ability to induce inflammation, and potential to translocate to the circulation to extrapulmonary organs [52–54]. These particles could trigger persistent lung inflammation compared to the coarse particles in addition to the exposure to genotoxic compounds, which are contained in the particles [26, 55].

Depending on the toxicity, the chemical properties, and the concentration in air, coal and coal fly ash particles can constitute a risk to exposed workers. When these particles are inhaled and

deposited in the lungs, they can lead to health risks due to the leaching of genotoxic compounds and altered immunological mechanisms affecting the lung parenchyma causing diseases [56]. These nanometric particles are very small, which allows them to penetrate the biological organs and affect its normal function. More specifically, as the particle load in the lung increases the alveolar macrophages and epithelial cells are activated, leading to the release of inflammatory mediators, ROS, enzymes (elastases, proteases, collagenases), cytokines [tumor necrosis factor alpha (TNF-α), interleukins], and growth factors (TGF-β) that control and stimulates the fibrosis, genotoxic events, and cell death [45, 57, 58].

Persistent inflammatory processes have been accepted as a crucial factor in the pathogenesis. In Zhai et al. [59], was investigated whether systemic TNF-α, soluble TNF-α receptors (p55, p75), IL-6, and soluble IL-6 receptor could be markers of biological activities of Chinese CWP. Interestingly, those results suggest that serum levels of TNF receptors and IL-6 are associated with the fibrotic process of CWP and serum cytokine levels may be correlated with the severity of CWP. In the pathogenesis of these respiratory diseases related with coal exposure, oxidative damage plays a key role. Either acting in association or independently, the chemical and physical characteristics can lead to the generation of ROS and oxidative stress [60, 61].

These particles are chemically heterogeneous and can be a source of oxidants by themselves ("acellular" mechanisms), due to their composition, such as oxides, metals, and PAHs [26]. Soluble metals (transition) associated to the particle can increase the generation of ROS by Haber-Weiss reactions. PAHs may be metabolically activated and induce ROS and oxidative stress, also forming bulky adducts or strand breaks on DNA [50, 62, 63].

Another way of generating oxidants is via cellular. Once in the lungs, alveolar macrophages are activated and generate large amounts of ROS, and chemoattractant factors of other inflammatory cells such as monocytes and neutrophils are released, which amplify this response generating more oxidants [64]. The particle size is a critical factor, because very large particles are difficult to phagocytose, leading to the process of incomplete or "frustrated" phagocytosis aggravating the response [65, 66].

Considering three different scenarios with respect to exposure to particles, the generation of oxidative stress, inflammation, and oxidative DNA damage, several authors questioned whether the lung inflammation may be related to secondary genotoxic effects. They also questioned if phenomena of oxidative stress, inflammation and DNA damage are independent or interrelated, whether oxidative stress stimulates inflammatory processes, or inflammation mediated by particles cause oxidative stress, or even if it is possible that particles may cause both phenomena of oxidative stress and inflammation but for different mechanisms of action [26, 61].

In normal physiological conditions, there is a balance between ROS generation and antioxidant defenses. However, the continuous inhalation of particles may interfere in this equilibrium leading to oxidative stress process in the lung. Consequently, a high loading of particles alters the oxidant-antioxidant balance, leading to oxidative damage and the beginning of pathological processes [67]. The most important effects of ROS in the lung include damage to cell

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membranes by lipid peroxidation process, protein oxidation, and DNA damage in target cells [27].

As seen in **Figure 1**, oxidative DNA damage can have many consequences, from cell death and tissue destruction to cell proliferation. Furthermore, ROS can also act as regulators in signaling pathways intracellularly and transcription factors of a variety of genes including those of proinflammatory cytokines, adhesion molecules, and proto-oncogenes [68].

In vitro effects induced by coal exposure have been described in different cells such as murine alveolar type II epithelial cells (C10) [69] and in 7TD1 cells [70]. ROS generation and oxidative damage by coal fly ash particles have been described in different cell lines, in human peripheral blood mononuclear cells [71], in rat alveolar macrophages (NR8383) [72], in BEAS-2B human lung epithelial cells [73], and in rat lung epithelial (RLE) cells [74].

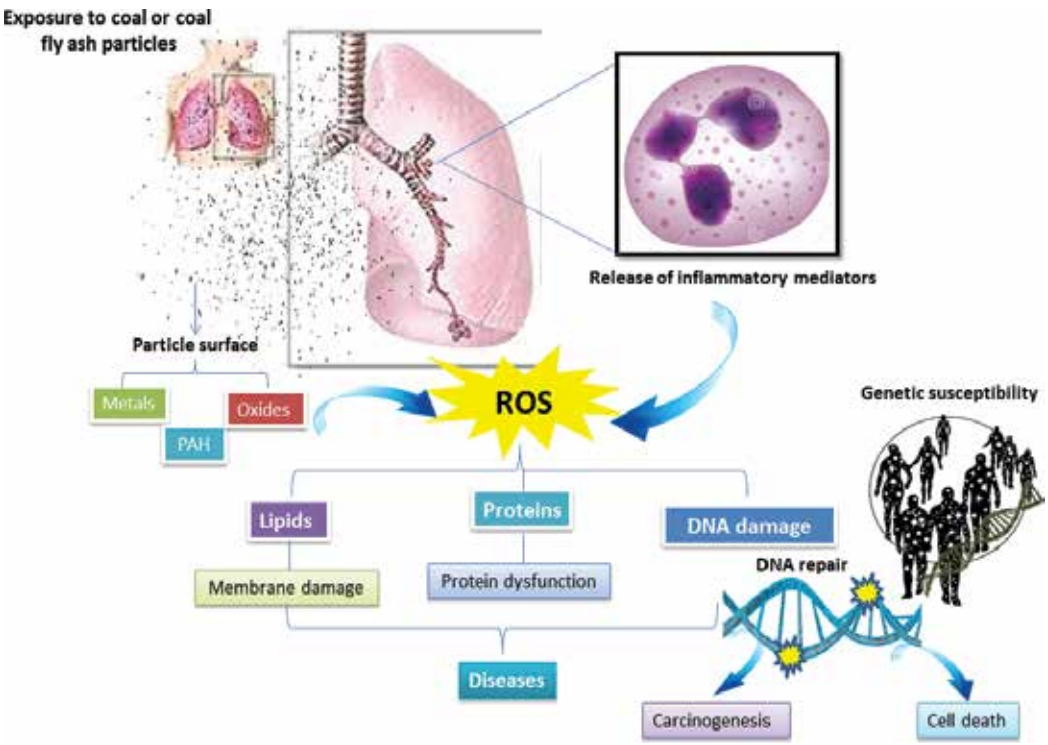

Figure 1. Main pathways associated with the generation of oxidative damage and the development of diseases induced by coal and coal fly ash particles.

ROS induce point mutations and CAs in cells. Many inhaled toxic substances contained in the particles contribute to oxidative modification that has as target of attack specific components of the cytoplasm and the nucleus. Such changes include DNA breakage, DNA oxidative modification, base modifications, alterations in the DNA sequence, poly-ADP ribosylation, activation of kinases, activation of proto-oncogenes, and inactivation of tumor suppressor

genes. Persistent generation of ROS generated by mineral particles indestructible or engulfed incompletely leads to damage to organelles keys [59, 61, 75]. The oxidation of C8 deoxyguanosine (dG), resulting in 7-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG), is the most common oxidative lesion generated by ROS. The proportion of 8-oxodG/dG has been considered as a biomarker of oxidative stress and has been studied in relation to exposure to mineral particles *in vitro* and *in vivo* [76].

Human biomonitoring studies about the effects of exposure to coal and residues using different biomarkers have been conducted around the world. In this context, our group has obtained interesting findings in workers exposed to coal mining in Colombia and Brazil. In Rohr et al. [77], was found that Brazilian workers with occupational exposure to coal had significantly increased genetic damage in peripheral blood lymphocytes compared with unexposed individuals. Exposed workers presented lower average levels of thiobarbituric acid reactive substances (TBARS) and catalase activity (CAT). In addition, DNA damage evaluated by human buccal micronucleus cytome (BMCyt) assay was observed in mine workers, which could be a consequence of oxidative damage resulting from exposure to coal residue mixtures [78].

In Colombia, DNA damage in lymphocytes of coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay were observed [79]. Also, in buccal mucosa samples, the micronucleus frequencies and nuclear buds were significantly higher in the exposed group than in non-exposed control group. Interestingly, blood samples of Colombian mining workers analyzed showed higher values of silicon and aluminum characteristic elements of coal particles, compared with the control group [80]. All these studies converge to a point: the compounds contained in the particles may be related to ROS generation, DNA damage, and formation of pro-mutagenic adducts.

These are important findings if we consider that oxidative DNA damage can lead to long-term risk of cancer and other diseases caused by air pollution by these particles. In **Table 1**, can be observed an overview of key studies on the genotoxicity in human population exposed to coal and coal combustion products. These studies demonstrated DNA damage using different methods, related with inorganic elements and oxidative stress.

References	Country	Exposure(s)	Biomarker	Outcome(s)
[81]	Slovenia	_	Sister-chromatid exchanges (SCE), unstable chromosome and chromatid aberrations and micronuclei in blood lymphocytes	Significantly higher levels of chromosomal aberrations, SCE and micronuclei in exposed group compared with the control group.
[82]	Brazil	Underground workers directly exposed, surface	Oxidative stress biomarkers (TBARS, GSH/GSSG, α-tocopherol, GST, GR, GPx, SOD, CAT).	The results showed that subjects directly and indirectly exposed to coal dust face an oxidative stress condition. They also indicate that people living in the

References	Country	Exposure(s)	Biomarker	Outcome(s)
		workers indirectly exposed, residents living near the mines.		vicinity of the mine plant are in health risk regarding coal mining-related diseases.
[22]	Turkey	Coal combustion products	Chromosomal aberrations (CAs), polyploidy, sister-chromatid exchanges (SCEs), and micronuclei (MN) in blood cells.	Significantly higher levels of CA, polyploidy, SCE, and MN in peripheral blood lymphocytes of workers compared with controls.
[83]	Turkey	Underground coal mining	SCE, CA, and micronuclei frequencies in peripheral lymphocytes.	Increase in sister chromatid exchanges, chromosomal aberrations, and micronucleus frequencies found in underground coal miners as compared to control group.
[21]	China	Indoor smoky coal emissions that contain high levels of polycyclic aromatic hydrocarbons (PAHs)	GSTM1 and GSTT1 genotypes. Expression of p53 protein in sputum samples.	The GSTM1 null genotype may enhance susceptibility to lung cancer due to these indoor coal combustion emissions. Smoky coal use was strongly associated with overexpression of p53 in tumor cells among highly exposed women.
[79]	Colombia	Open cast mining	(MN) frequency and DNA damage (comet assay) in lymphocytes.	The biomarkers evaluated showed statistically significant higher values in the exposed group compared to the non-exposed control group.
[80]	Colombia	Open cast mining	Micronucleus (MN) frequencies, nuclear buds, karyorrhectic and karyolytic cells in buccal mucosa samples and content of inorganic elements in blood samples by PIXE.	MN frequencies and nuclear buds in buccal mucosa samples were significantly higher in the exposed group than in the non-exposed control group. In addition, karyorrhectic and karyolytic cells were also significantly higher in the exposed group (cell death). Blood samples showed higher values of silicon (Si) and aluminum (Al) in the exposed group.
[84]	Russian	Underground coal	Chromosomal and chromatid type	A higher frequency of chromosomal aberrations in the exposed group

References	Country	Exposure(s)	Biomarker	Outcome(s)
		mining	aberrations in blood lymphocytes	compared with the control group.
[77]	Brazil	Open coal mining	MN and nucleoplasmic bridge frequencies in peripheral lymphocytes, damage index and damage frequency (comet assay).	Increased MN and nucleoplasmic bridge frequencies in peripheral lymphocytes, increased damage index and damage frequency (comet assay). Lower average levels of TBARS and catalase activity (CAT), while the mean superoxide dismutase activity (SOD) levels were higher in the exposed group.
[78]	Brazil	Open coal mining	Buccal micronucleus cytome (BMCyt) DNA damage, cell death, and basal cell frequency in buccal cells.	The exposed group presented a significantly higher frequency of basal cells, micronuclei in basal and differentiated cells, and binucleated cells compared to the non-exposed group. No correlation between DNA damage and metal concentration in the blood of mine workers.
[19]	Peru	Underground coal mining	Chromosomal aberrations in peripheral lymphocytes	Miners occupationally exposed to underground mining activity have an increased frequency of chromosomal aberrations compared with the controls.
[85]	_	Coal fly ash particles	SCE frequencies in peripheral blood lymphocytes.	No increased SCE frequencies were found in PBLs of workers potentially exposed to coal fly ash when compared to the control group. No differences were observed between the exposed and control groups for frequencies of gene mutations at the HPRT locus in PBLs, for micronucleus frequencies using the cytokinesis block method, or for urinary mutagen excretion measured with *Salmonella typhimurium* tester strains TA98 and TA97.
[86]	Germany	Underground coal mining	Structural chromosomal aberrations in peripheral lymphocytes	Coal miners had significantly higher frequencies of chromosomal aberrations compared with controls.
[87]	Turkey	Underground coal	Sister chromatid exchange (SCE) and	SCE and MN frequencies in CWP patients were found significantly higher than

References	Country	Exposure(s)	Biomarker	Outcome(s)
		mining	micronucleus (MN) frequency in lymphocytes of Turkish CWP patients.	in coal workers and unexposed groups.

Table 1. Overview of key studies on the genotoxicity in human population exposed to coal and coal combustion products.

4. Conclusions

The coal mining activities generate different types of compounds that are released into the environment. Once into the atmosphere, these compounds form a complex mixture that consists of metals, oxides, and PAHs. These compounds can interact with "acellular" and cellular mechanisms related with ROS production. The metals found in the coal fly ash and coal particles by different ways lead to the ROS formation. Important macromolecules as DNA, proteins, and lipids can suffer oxidative modifications. The PAHs contained in the particles also influence the particles toxicity. A second indirect way for excessive ROS formation is related to cellular mechanisms, which is consequence of oxidative burst of macrophages and neutrophils during phagocytosis of particles and inflammation produced.

If we think in exposed populations, we cannot ignore the social and environmental impact associated with coal mining. The continuous inhalation, the high load of particles in phagocytic cells, the oxidant-antioxidant imbalance which are linked to the origin of pathological processes; this whole scenario is worrisome to biologic level for these populations. In addition, in recent years, coal mining had a remarkable increase in demand; international mining companies have increased their investments in exploration around the world. For this reason, human biomonitoring studies in exposed populations become really necessary to contribute to knowledge state about the risk for those people in order to motivate the design of control, hygiene, and prevention strategies, besides epidemiological surveillance.

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Chapter 8

Immunological Risks Caused by Fibrous and Particulate Substances

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Abstract

The immunological risks caused by fibrous and particulate substances, especially the effects caused by asbestos fibers and silica particles, are discussed in this chapter. Patients with silicosis often suffer from autoimmune diseases, such as rheumatoid arthritis, systemic sclerosis, and antineutrophil cytoplasmic antibody–related vasculitis. Silica particles, SiO_2, may influence directly various immune cells resulting in the production of many autoantibodies and imbalance between responder and regulatory T cells. The core chemical content of asbestos fibers is Si and O, although the physical feature is different. Considering the complications in asbestos-exposed patients, malignant tumors, such as lung cancer and malignant mesothelioma, are the most important. To think about these situations, asbestos fibers may cause the reduction of antitumor immunity. The experimental findings and measurements of various immunological parameters in silicosis patients, as well as asbestos-exposed population, such as patients with pleural plaque and mesothelioma, are demonstrated and discussed in this chapter.

Keywords: asbestos, silica, autoimmune diseases, antitumor immunity, regulatory T cell

1. Introduction

Regarding environmental factors that cause health risks, exposure to fibrous and particulate substances, such as asbestos fibers and silica particles, represent classic examples, and the

investigation of other materials that lead to health impairment following exposure is ongoing [1–10]. In addition to pulmonary effects, such as fibrosis, chronic inflammations, and cancers, such as lung malignancies and pleural mesothelioma, in asbestos-exposed patients, there may be certain effects on immunological cells [11–16]. Among people who have been exposed to asbestos fibers or silica particles, people exposed to silica and have developed silicosis often suffer from complicated autoimmune diseases, such as rheumatoid arthritis, systemic sclerosis, and antineutrophil cytoplasmic antigen (ANCA)–related vasculitis [17–20]. The core chemical components of asbestos fibers are Si and O_2, and although the physical makeup of fibrous and particulate matter differs, asbestos fibers may affect the immune system. Therefore, we have been investigating the immunological effects of silica and asbestos [11–16].

Regarding silica particles, the mechanism of silica-induced dysregulation of autoimmunity is thought to involve silica acting as an adjuvant [21–24]. However, silica particles may also act by directly stimulating on circulating peripheral immune cells, which cause certain alterations in the cellular or molecular functions of these cells, since silica particles may remain in pulmonary lesions and lymph nodes after inhalation [11–16]. Since these direct effects may change the characteristics of immune cells and consequently facilitate the dysregulation of immune tolerance, clarification of these cellular and molecular mechanisms may be useful in the prevention of immune disorders that occur in silicosis patients (SIL), in addition to contributing toward an understanding of the etiology of various autoimmune diseases.

We have been focusing on the immunological effects of silica using human peripheral blood immune cells derived from healthy donors (HD) and SIL [11–14]. We will summarize our findings which indicate that silica is an environmental immune stimulator, and chronic activation of immune cells induced by recurrent and chronic exposure to silica causes an imbalance in the regulation of T cell responses.

Regarding asbestos fibers, asbestos-related cancers, such as malignant mesothelioma (MM) and lung cancer, have been a major global concern in Japan [25–29]. Given the conflict that has arisen due to economic considerations and the medical evidence, there is a confusion concerning the pathological mechanisms of asbestos-induced cancers, and in particular, an uncertainty concerning the dangers of iron-absent chrysotile (white) asbestos compared with iron-present crocidolite (blue) and amosite (brown) asbestos [30–33]. However, regarding the poor prognosis of MM, novel medical approaches to investigate the biological effects of asbestos and pathological mechanisms of asbestos-induced carcinogenesis, as well as clinical trials to detect early stages of MM, should be implemented to assist in the development of improved prevention strategies and cure of asbestos-related malignancies [34–36]. From this standpoint, our group has been investigating the immunological effects of asbestos with respect to the reduction of tumor immunity [11, 12, 15, 16]. In this chapter, cellular and molecular approaches to clarify the immunological effects of asbestos are described, and all findings indicate that a reduction of tumor immunity is caused by asbestos exposure and is involved in asbestos-induced cancers. In addition to confirming the well-known biological effects of asbestos, these investigations provide a basis for the development of a novel procedure for the early detection of previous asbestos exposure, mesothelioma and the chemoprevention of asbestos-related cancers.

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As shown in **Figure 1**, both silica particles and asbestos fibers cause pulmonary fibrosis known as pneumoconiosis, silicosis, and asbestosis. Additionally, both can affect various immune cells, such as B cells, CD4 T helper (Th1), regulatory T (Treg), cytotoxic T lymphocyte (CTL), natural killer (NK) cells, and other immune cells [11, 12, 15, 16].

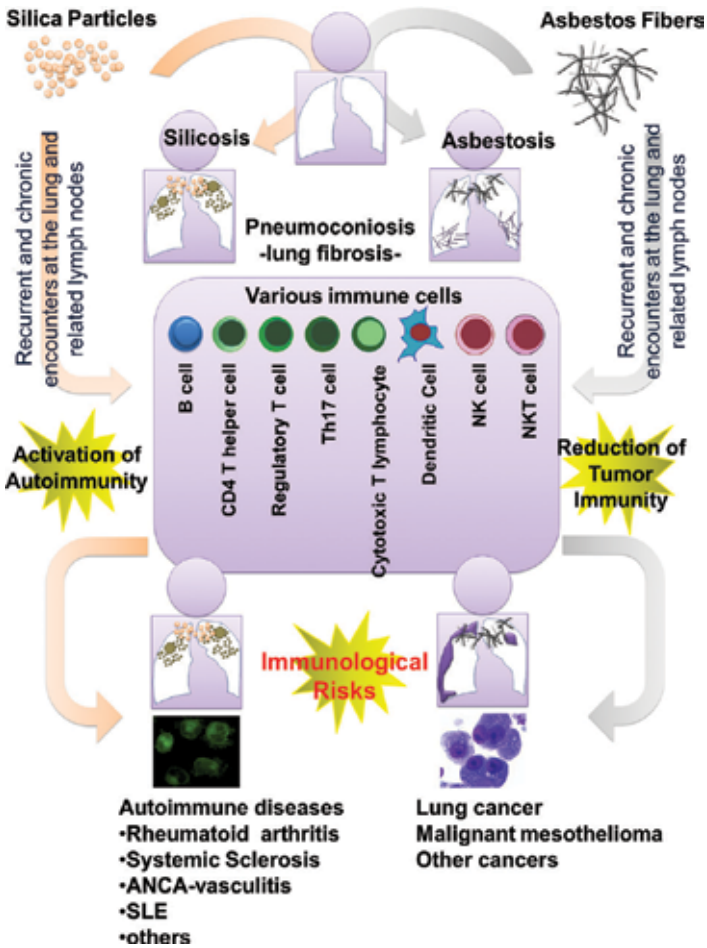

Figure 1. Schematic representation of immunological risks caused by exposure to silica particles and asbestos fibers. The immunological risks induced crucial complications, such as autoimmune diseases, in silicosis patients, as well as malignant tumors, such as lung cancer and malignant mesothelioma, in asbestos-exposed populations.

In this chapter, the immunological effects on various immune cells caused by silica particles and asbestos fibers as investigated in our laboratory will be presented and discussed with respect to the detection of immunological risks of particulate and fibrous environmental factors [11–16]. These summarized findings may be helpful in the development of future risk management strategies, including cases related to newly developed fibrous and particulate matter, such as nanoparticles and nanotubes.

2. Immunological risks caused by silica particles

As shown in **Table 1**, there are various immunological risks associated with exposure to silica particles. These findings were established by in vitro assays using peripheral blood mononuclear cells (PBMC) derived from HD cultured with silica particles as well as freshly isolated immune cells derived from SIL. Additionally, various autoantibodies (aAbs) were detected from SIL [11–14]. All SIL comprised Japanese workers of a firebrick factory located at Bizen City, Okayama Prefecture, Japan, diagnosed with silicosis according to the International Labor Organization (ILO) 2000 guidelines for pneumoconiosis and monitored at Kusaka Hospital or Hinase Urakami Iin/Clinic at Bizen City. All SIL showed no symptoms related to autoimmune diseases or cancers.

Risk manifestation	Target cells/molecules	Findings	References
Unusual autoantibody	B cell	Detection of autoantibodies against	
		➢ Fas/CD95	61
		➢ Caspase 8	62
		➢ Scl-70/Topoisomerase I	56–58
		◆ Specific HLA type	
		➢CENP-B/centromere	64
		➢Desmoglein	65
Dysregulated apoptosis	T cell	Increased level of molecules against	
		Fas-mediated apoptosis	
		➢ Soluble Fas	
		◆ Serum soluble Fas	69
		◆ mRNA expression in PBMC	70
		➢ Variant Fas, alternatively spliced variants	71
		➢ Decoy receptor 3	74
		◆ mRNA expression in PBMC	
		Chronic activation	80
		➢ Soluble IL-2 receptor	78
		➢ PD-1 expression	79
		➢ CD69 surface expression	
		Increase in Fas-mediated apoptosis	61
		➢ Autoantibody for Fas	75
		➢ Decreased expression of physiological inhibitors of Fas-mediated apoptosis	

Risk manifestation	Target cells/molecules	Findings	References
	Regulatory T cell	Chronic activation	
		➢ Excess expression of Fas/CD95	>78

IL, interleukin; PBMC, peripheral blood mononuclear cells; and PD-1, program death protein 1

Table 1. Immunological risks caused by silica particles.

2.1. aAbs detected in SIL

First, the risk of dysregulated autoimmunity assessed by the detection of particular aAbs will be discussed. Various aAbs have been detected in SIL, such as antinuclear antibody (ANA) [37–40], antismooth muscle aAb [41], antiglomerular basement membrane (GBM) aAb [41], antineutrophil cytoplasmic aAb (MPO-ANCA) [37, 42–48], rheumatoid factor (RF) [37–39, 49–53], anti-Scl-70/topoisomerase I aAb [37, 54–60], anti-Fas/CD95 aAb [61], anticaspase 8 aAb [62], anticentromere/CENP-B (centromere protein B) aAb [63], antidesmoglein aAb [64], anti-PL 12 (aminoacyl tRNA synthetase) aAb [65], and anticollagen aAb [39], as found in publications located via PubMed.

Of these aAbs, we investigated several Abs of interest, such as anti-Fas/CD95 Ab [61], anti-caspase 8 Ab [62], anti-Scl-70 Ab with respect to specific human leukocyte antigen (HLA) types [56–58], and anti-CENP-B Ab [63] and reported the case of antidesmoglein Ab-positive SIL [64].

We detected anti-Fas/CD95 aAb in approximately one-fourth of SIL [61]. Since T cells in SIL tend to be categorized into two classes, Fas/CD95-mediated apoptosis prone and resistant groups as described later in this chapter, it is important to determine whether the detected anti-Fas/CD95 aAb is functional in terms of the induction of Fas/CD95-mediated apoptosis. To examine this issue, we employed our established human sister myeloma cell lines, KMS-12PE and KMS-12BM. The former cell line was established from the pleural effusion of a myeloma patient, which showed high expression of Fas/CD95 on its surface as a result of apoptosis and growth inhibition caused by anti-Fas/CD95 agonistic antibody. The latter cell line was derived from bone marrow obtained from the same patients, who showed very low expression of Fas/CD95 and no apoptosis caused by Fas/CD95 agonistic antibody [66]. Following cultivation of both cell lines with anti-Fas/CD95 aAb-positive serum from SIL, the growth of KMS-12PE was reduced by apoptosis, whereas the growth of KMS-12BM was unaffected [61]. These results indicated that anti-Fas/CD95 aAb is functional. Additionally, epitope mapping employing 12-amino acid polypeptides with the SPOT system of anti-Fas/CD95 aAb was analyzed. As a result, a minimum of four and a maximum of ten epitopes were found, and several amino acid residues involved in binding Fas ligand, such as C66, R87, L90 E93, and H126, were identified [61].

As in the case of anti-Fas/CD95 aAb, anticaspase 8 aAb was investigated in terms of the dysregulation of Fas/CD95-mediated apoptosis of lymphocytes in SIL [62]. The association of anticaspase 8 aAb with HLA types was examined. As a result, the frequencies of HLA-DRB1*0406 were significantly higher in aAb-positive SIL (16.7%) compared with control individuals (3.0%, $p<0.001$). Additionally, HLA-DR4; DQB1*0302 was found in one-fourth of

positive SIL, and DPB1*0601 was also higher in positive SIL (5.9%) compared with controls (0.6%, $p<0.05$), whereas DQB1*0401 was lower in positive SIL (0%) compared with controls (13.3%, $p<0.001$). Furthermore, epitope mapping showed that a minimum of four and a maximum of thirteen polypeptides seemed to be involved. Among these, two important catalytic cysteine residues were found, cysteine Cys287 and Cys360, located in the unique pentapeptide motif QACQG [62].

Regarding the relationship between aAb and specific HLA type, we reported HLA types among anti-Scl70/topoisomerase I aAb-positive SIL [56–58]. Results indicated that the allelic frequency of HLA-DQB1*0402 was significantly higher in aAb-positive SIL (28.6%) than in aAb-negative SIL (1.5%, $p<0.001$), as well as in controls (0.8%, $p<0.001$). Additionally, DQDB1*0301, DQB1*0601, and DPB1*1801 were higher in aAb-positive SIL than in aAb-negative SIL, whereas no significant differences were found compared with controls [56–58].

In terms of anti-CENP-B/centromere aAb, the titer index (Log10) of anti-CENP-B autoantibody in SIL was higher than that of HV, and patients with systemic sclerosis (SSc) was higher than those of HV and SIL. This titer index was positively correlated with an assumed immune status for HV as 1, SIL as 2, and SSc as 3. Moreover, although the titer index of anti-CENP-B autoantibody formed the same factor with anti-Scl-70 autoantibody, the Ig G value, and age of SIL, the property of other factors extracted indicated that anti-Scl-70 antibody was positively related with the Ig A value, while the converse was true for anti-CENP-B from the results of factor analysis. Those results indicated that the titer index of anti-CENP-B autoantibody may be employed as a biomarker in identifying dysregulation in SIL cases.

Taken together, various aAbs found in SIL have indicated that dysregulation of autoimmunity was caused by chronic and recurrent exposure to silica particles that remained in lung and related lymph nodes of various human cells, especially B cells. Some of these aAbs may be related to Fas/CD95-mediated apoptosis of lymphocytes and cause further dysregulation of autoimmunity such as in the case of long-surviving self-antigen recognizing clones in T cells [11–14].

Furthermore, examination of HLA types seemed to be important in revealing several aAbs in SIL. Although it can be mentioned that repeated and continuous screening of aAbs as well as the initial screening of HLA types seems to be necessary among workers in contact with silica-related substances for the detection of dysregulation of autoimmunity, the use of genotyping, such as determining HLA types, is not permitted during employee selection procedures. However, a consideration of particular occupational health risks together with individual sensitivities is required in an effort to prevent occupational health hazards and associated future hardships.

2.2. Fas/CD95-mediated apoptosis–related molecules in SIL

Fas/CD95-related molecules analyzed in SIL are shown in **Table 1** [11–14, 67]. Regarding molecules that inhibit Fas/CD95-mediated apoptosis, the level of soluble Fas/CD95 was higher in the serum of SIL compared with HD, and similar to the level in systemic lupus erythematosus (SLE) [68], while higher mRNA expression, determined as the ratio of soluble to wild-

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type Fas/CD95, was present in SIL compared with HD in PBMC [69]. Additionally, higher amounts of various alternatively spliced variant messages of the Fas/CD95 gene were detected in PBMC from SIL compared with HD [70]. All of these variant messages, including soluble Fas/CD95, possess a Fas ligand-binding domain but lack a membrane-binding domain. Hence all of these translation products are secreted into the extracellular space and bind with Fas ligand, thereby protecting cells against membrane Fas-mediated apoptosis [70]. Furthermore, the expression of the protective molecule decoy receptor 3 (DcR3), which acts against the Trail molecule and similarly induces apoptosis via a Trail receptor and the same intracellular signaling molecules for apoptosis, such as caspase 8 and 10 [71, 72], was higher in SIL PBMC compared with HD [73]. These findings indicated that some types of T cells in PBMC from SIL provide protection against Fas/CD95- and Trail-induced apoptosis, which leads to long survival of these T cells and self-antigen recognizing clones [67].

However, several findings that showed accelerated Fas/CD95- and Trail-mediated apoptosis in PBMC of SIL were investigated. Messenger RNA expression in PBMC of several genes which act as physiological inhibitors of Fas/CD95- and Trail-mediated apoptosis, such as I-Flice (inhibitor of FADD-like interleukin-1β–converting enzyme), surviving, sentrin, and inhibitor of caspase-activated DNase (ICAD) was lower in SIL compared with HD [67, 74]. In addition to the aforementioned detection of functional anti-Fas/CD95 autoantibody, some types of T cells in PBMC from SIL possess enhanced Fas/CD95-mediated apoptosis [61]. Further studies revealed that this fraction may include Treg cells [13, 14]. Thus, a decrease in the number of Treg cells by apoptosis and an increase in the number of responder T cells caused by silica exposure may be the cellular biological mechanisms at work in SIL, which consequently impart susceptibility to autoimmune diseases in SIL.

We found higher expression of Fas/CD95 in Treg (CD4+, CD25+, and forkhead box P3 (FoxP3)+) [75, 76] and sensitivity to Fas-agonistic antibody–induced apoptosis in Treg cells from SIL [77]. Furthermore, when PBMC from HD were cultured with silica particles in vitro, Treg cell numbers were selectively reduced by apoptosis and the population of responder T cells was enhanced [77]. Thus, the aforementioned T cell population prone to Fas/CD95-mediated apoptosis seems to comprise Treg cells, and the imbalance that occurs as a result of a decreased Treg and surviving responder T cell population in SIL induces dysregulation of autoimmunity [13, 14, 77].

Moreover, there is evidence showing chronic activation of responder T cells. For example, CD69, an early activating marker of T cells, was gradually expressed in T cells when PBMC from HD were cultured in vitro with silica particles [78]. Expression of the program death protein 1 (PD-1) gene, another activation marker of T cells, in CD4+ CD25+ as well as in CD4+ CD25− T cell populations was higher in SIL compared with HD, which showed negligible expression [78]. Expression of serum soluble interleukin (IL)-2 receptor (sIL-2R) was also higher in SIL compared with HD [79].

Taken together, SIL possess a risk of developing dysregulation of autoimmunity. This risk can be detected using various markers mentioned above, such as serum soluble Fas, sIL-2R, and serum DcR3 (recently, the enzyme-linked immunosorbent assay (ELISA) kit is available for laboratory use), in SIL during their early clinical phases.

3. Immunological risks caused by asbestos fibers

As shown in **Figure 1**, the most important and critical complications that arise in asbestos-exposed patients concern the development of malignancies, such as lung cancer and MM [25–29]. Of course, asbestos fibers possess carcinogenic-related activities, such as oxygen stress caused by iron in the asbestos fibers, frustrated macrophages incapable of phagocytosing asbestos fibers, chromosome tangling, and the absorption of other carcinogenic substances inhaled in the lung, such as materials from tobacco smoke and other air pollutants [34–36]. However, given the long latency period that precedes the onset of MM following initial exposure to asbestos, it was considered that asbestos fibers cause alterations in antitumor immunity by recurrent and chronic encounters with various immune cells at the lung and related lymph nodes.

As shown in **Table 2**, our findings show altered immune cell function and manifestations from experimental settings as well as PBMC derived from pleural plaque and MM [11, 12, 15, 16, 80, 81].

Risk manifestation	Target cells	Findings	References
Innate Immune system	NK cells	Reduction of cytotoxicity	
		➢ Freshly isolated NK cells	
		◆ NK cells from asbestos-exposed patients (PP and MM)	83–84
		◆ NK cells from HD stimulated in vitro with	83
		asbestos	83
		➢ Human cell line cultured with asbestos	
		Reduced expression of NK cell activation receptor	83
		➢ Human cell line cultured with asbestos: NKG2D, 2B4	83,84
		➢ Freshly isolated NK cells	
		◆ NK cells from asbestos-exposed patients (PP and MM): NKp46	83,84
		◆ NK cells from HD stimulated in vitro with asbestos: NKp46	84
		Reduction of phosphorylation of ERK 1/2	
		➢ Human cell line cultured with asbestos	
MHC class I restricted killing System	CTLs	Suppressed differentiation and proliferation	
		➢ In vitro assay using MLR with asbestos	85
		Alteration of killing molecules (granzyme B, IFNγ, perforin)	
		➢ In vitro assay using MLR with asbestos	86
		➢ Freshly isolated and in vitro stimulated peripheral	

Risk manifestation	Target cells	Findings	References
		CD8+ cells from PP	86
		➢ Freshly isolated and in vitro stimulated peripheral CD8+ cells from MM	
MHC class II restricted killing system	Th1 cells	Decrease in CXCR3 expression, IFNγ	
		➢ Cell line model continuously cultured with asbestos	87
		➢ Freshly isolated and cultured in vitro with asbestos from HD	88
		➢ Freshly isolated CD4+ T cells from PP and MM	88
Regulation of T cell response	Regulatory T (Treg) cells	Enhanced function	
		➢ Cell line model continuously cultured with asbestos	
		◆ Increased suppressive function via cell-cell contacts	91
		◆ Excess production of soluble factors	
		✓ IL-10	91
		× Enhanced suppressive function	89
		× Phosphorylation of STAT3 with over-expression of Bcl-2 causing resistance against asbestos-induced apoptosis	
		✓ TGFβ	91
		× Enhanced suppressive function	90
		× Increased phosphorylation of p38 and SMAD3 causing resistance against TGFβ-induced growth inhibition	

CXCR3, CXC chemokine receptor 3; HD, healthy donor; IFN, interferon; IL, interleukin; MLR, mixed lymphocyte reaction; MM, malignant mesothelioma; NK, natural killer; PP, pleural plaque; TGF, transforming growth factor; SMAD, vertebrate homologues of *Sma* and *Mad* [Drosophila protein; mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA (from the sma gene for small body size)]; and Th1, T helper.

Table 2. Immunological risks caused by asbestos fibers.

3.1. NK cells

Regarding NK cells, cytotoxicity was reduced in peripheral NK cells from pleural plaque (PP) and MM, in NK cells from HD cultured in vitro with asbestos fiber, and in a human NK cell line continuously exposed to asbestos [82]. Additionally, the expression of various NK cell activating receptors, such as NKG2D, 2B4, and NKp46, was reduced in a human NK cell line

cultured continuously with asbestos, in freshly isolated NK cells from HD cultured in vitro with asbestos, and in fresh NK cells from PP and MM [82, 83]. Among these receptors, NKp46 was thought to be an important marker for impaired function of NK cells exposed to asbestos. Moreover, reduced cytotoxicity in NK cells exposed to asbestos was accompanied with reduced phosphorylation of extracellularly regulated kinases (ERK) 1 and 2 and reduced degranulation of perforin and granzyme B, which are the killing small molecules secreted from NK cells [82, 83].

3.2. Cytotoxic T lymphocytes

Other types of cytokilling immune cells, CTLs, are also involved and have their functional and cellular properties altered by asbestos exposure. From in vitro analyses using peripheral CTLs in a mixed lymphocyte reaction (MLR), it was found that differentiation and proliferation of CD8+ naïve T cells were disturbed by the presence of cocultured asbestos fibers with decreased expression of killing small molecules, such as granzyme B and interferon γ (IFNγ) [84]. Moreover, alteration of killing molecules, as well as the phenotype of CD8+ cells, was manifested by CD45RA as the marker of effector/memory T cells. Freshly isolated CD8+ cells derived from asbestos-exposed patients, such as PP and MM, showed a higher predominance of CD45RA-negative cells compared with HD [85]. However, the cytokilling activity differed between isolated and in vitro-stimulated CD8+ cells. CD8+ cells from PP and MM revealed an increase in the number of perforin-positive cells; however, after in vitro stimulation, only CD8+ cells from MM showed a decrease in the perforin-positive cell population when subtracted from the unstimulated base line [85].

These findings indicated that asbestos exposure caused dysfunction of CTLs, while specific cell functions differed depending on disease status, for example, PP patients do not carry any malignant tumors, whereas MM patients suffer from mesothelioma. However, the impact of asbestos fibers on CTLs is considered to involve a reduction of tumor immunity, as we showed in NK cells mentioned above [84, 85].

3.3. Th 1 cells

Asbestos fibers are also known to modify Th1 cells. We developed continuously exposed sublines using a cell line model. The cDNA microarray data were examined of the original cell line, which has had no contact with asbestos fibers, and six independently established sublines, which were continuously exposed to asbestos fibers for more than 8 months using an asbestos concentration that did not induce apoptosis in more than half of the cells by transient exposure. The microarray showed a decrease in IFNγ and related molecules, such as IFN regulatory factor 9 (IRF9) and IFN-stimulating gene factor-3 (ISGF3), in addition to a decrease in CXC chemokine receptor 3 (CXCR3), which is regulated by IRF9 [86].

CXCR3 is important in antitumor immunity to summon IFNγ-positive tumor antigen recognizing Th1 cells to the tumor. Thus, the asbestos-induced reduction of CXCR3 and IFNγ seems to cause a reduction of antitumor immunity in asbestos-exposed patients. As we assumed, examination of freshly isolated CD4+ cells from HD stimulated in vitro and cocultured with

asbestos fibers as well as peripheral CD4+ cells from PP and MM revealed a decrease in the cell surface expression of CXCR3 in addition to a decrease in the number of intracellular IFNγ-positive cells [87].

Taken together, one of the immunological risks resulting from asbestos exposure concerns a reduction of Th1-type T cell–derived antitumor immunity.

3.4. Treg cells

Treg cells are important in antitumor immunity. If the function and number of Treg cells are enhanced, immune cells responding to tumor antigen show suppressed function, which causes a reduction of antitumor immunity [75, 76].

Our cell line model continuously exposed to asbestos fibers using MT-2, a human T-lymphotropic virus type 1, which causes adult T cell leukemia/lymphoma, showed excess production of transforming growth factor (TGF) β and IL-10, typical soluble factors examined to reveal the function of Treg cells [88, 89]. Overproduction of IL-10 is regulated by the Src-family receptor and is used by the IL-10 receptor via autocrine mechanisms, which then causes activation of the signal transducer and activator of transcription 3 (STAT 3) and upregulation of antiapoptotic molecule Bcl-2 located downstream of STAT3 [88]. Continuously exposed sublines acquire resistance to apoptosis induced via transient exposure to asbestos [88]. Furthermore, overproduction of TGFβ induces resistance to TGFβ-induced growth inhibition in continuously exposed sublines with phosphorylation of p38, one of the signaling molecules in the mitogen-activated protein kinase (MAPK) signaling pathway, as well as phosphorylation of SMAD3 [SMAD; vertebrate homologues of *Sma* and *Mad* [Drosophila protein, mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA (from the sma gene for small body size)] [89].

In addition to the two aforementioned typical soluble factors, continuous exposure of MT-2 sublines to asbestos resulted in markedly higher suppressive activity when mixed with cultures of CD4+ responder cells activated with anti-CD3 antibody and autologous peripheral blood monocyte-derived dendritic cells compared with the original MT-2 cell line, which has had no contact with asbestos [90].

Taken together, exposure to asbestos results in enhanced Treg function, which is manifested by a reduction of antitumor immunity [11, 12, 15, 16, 80, 81].

3.5. Risks of asbestos on antitumor immunity

As mentioned above and shown in **Table 2**, all of the examined effects of asbestos on NK cells, CTLs, Th1, and Treg cells indicate that asbestos exposure can cause a reduction of antitumor immunity. These findings are considerable and the risks associated with asbestos exposure may be used as early detection markers for the occurrence of asbestos-induced malignancies. Additionally, the ability to mitigate the observed reduction of antitumor immunity through the use of chemopreventive substances derived from foods or plants may be an important strategy in the treatment of high-risk groups exposed to asbestos, such as residents who have

a history of living near factories handling asbestos and workers in the building demolition and rubble processing fields.

4. Conclusion

Risks associated with exposure to fibers, such as asbestos, and particulates, such as silica, were discussed based on our experimental findings and analyzed using cell lines, freshly isolated peripheral immune cells from HD, as well as patients exposed to silica particles, exposed to asbestos fibers, and patients with silicosis, PP, and MM. The immunological risks manifested in different directions, in that silica caused dysregulation of autoimmunity, whereas asbestos induced a reduction of antitumor immunity. Both cellular and molecular alterations contributed to the complications of silica exposure, the occurrence of autoimmune diseases and asbestos exposure, and the development of malignant tumors.

These risks may be detected using findings described in this chapter, and early detection of these risks may assist workers, as well as other exposed populations, in avoiding further exposure and therefore prevent the onset of various pathological states caused by exposure to fibrous and particulate substances. Recently, although exposure to silica and asbestos has been reduced through the improvement of work-related environments as well as banning the use of asbestos, new substances, such as nanomaterials, which are widely used in the industrial fields, are now feared to cause health risks. It should be reiterated that risks, and particularly immunological ones which hitherto have not received a great deal of attention, caused by classical types of particulate and fibrous substances, such as silica and asbestos, require continued and greater consideration in an effort to further prevent the health impairment caused by environmental substances.

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Chapter 9

Environmental Factors in Causation of Diabetes Mellitus

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Additional information is available at the end of the chapter

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Abstract

Environmental factors play a role in etiopathogenesis of diabetes. Environmental factors include polluted water, soil, unhealthy diet, stress, lack of physical activity, vitamin D deficiency, exposure to enteroviruses, and damage to immune cells.

Keywords: diabetes, virus, diet, stress, pollutants, prenatal factors, cow's milk, vitamin D deficiency

1. Introduction

India is currently experiencing an epidemic of diabetes mellitus (DM). According to the World Health Oganization, India has the unique distinction of being the country with the largest number of diabetic patients in the world. Type-2 DM accounts for more than 90% of all patients with diabetes worldwide. The prevalence of diabetes in adults is showing an upward trend worldwide [4% in 1995 to 5.4% in 2025 (projected)]. The majority of this increase will however occur in developing countries. Asians, particularly from the Indian subcontinent, will be mostly affected. Reports from different parts of India show a rising trend in the prevalence of diabetes. In 1995, prevalence of diabetes was 19.4 million and is projected to increase to nearly 80 million in 2030. Migrants have a higher risk of developing type-2 diabetes. The long-term impact of obesity and change in diet are two main causes of increased prevalence of diabetes in migrant Indians. The migrants are shifting from the high-fiber staple diet to polished and milled rice.

Diabetes is a heterogeneous syndrome where it is impossible to formulate a unified etiopathogenesis. In our country (India), diabetes of the young is often seen. Type-2 diabetes occurs a decade earlier and type-1 occurs a decade later. In type-2, strong genetic basis exists. But the

time of onset depends on the environmental factors. It is triggered by obesity, increase in age, and diet indiscretion.

In type-1 diabetes, in a susceptible individual (HLA B8), interaction between environmental factors triggers autoimmune response.

Environmental factors play a role in the etiopathogenesis of diabetes. They include polluted air, soil, water, unhealthy diet, stress, lack of physical activity, vitamin-D deficiency, exposure to enteroviruses, and damage to immune cells.

2. Diet

Unhealthy food rich in saturated fatty acids, refined carbohydrates, and sweets cause obesity and DM. Three major dietary toxins that trigger diabesity are cereal grains (especially refined flour), omega-6 industrial seed oils (corn, cottonseed, safflower, and soybean), and fructose (high-fructose in corn syrup). These nutrients in excessive quantities increase the risk of obesity and diabetes, especially if there is genetic risk. These dietary factors increase inflammation and act as potential risk factors for diabetes. A recent study showed that a diet with an omega 6:3 ratio of 28 (meaning 28 times more omega-6 than omega-3 fats) increased obesity in mice in experimental studies [1, 2].

Fructose is found primarily in fruits, vegetables, and sweeteners. Fructose is shunted directly to the liver where it is converted to fat. Excess fructose consumption causes a condition called nonalcoholic fatty liver disease, which is directly linked to both diabetes and obesity. Shifting 25% of dietary calories from glucose to fructose caused a fourfold increase in abdominal fat. Abdominal fat is an independent predictor of insulin sensitivity, impaired glucose tolerance, high blood pressure, high cholesterol and triglyceride. Fructose reacts with polyunsaturated fat and protein to form advanced glycation end-products (AGEs). A donut is the perfect diabesity food. It has refined flour, trans-fatty acids, and plenty of high-fructose corn syrup.

3. Food practices and diabetes

Diet and related factors that promote diabetogenesis:

- Calorie (energy) intake in excess of physical needs leading to obesity
  - Preferential and excess consumption of high-glycemic index carbohydrate rich-food
  - Refined flour, pasta, raw rice
  - Overcooked products of the above by baking or roasting
  - Aerated soft drinks
  - Sweets and free sugar
- High-fat diet with greater content of saturated fats, such as dairy and/or meat fat

http://dx.doi.org/10.5772/62543

- Coconut or palm kernel oil
- Vanaspathi—Hydrogenated oil
- High omega-6 fatty acid intake, particularly corn oil, sunflower oil, and saffola

- Energy-dense fat- and sugar-rich snacks and meals at fast-food outlets
- Reduced consumption of legume, fresh fruits, and vegetables

4. Secondary causes

Low protein diet impairs beta-cell function. This phenomenon is shown in rats and rhesus monkeys. Such beta-cell dysfunction in case of malnutrition in utero as well as in infancy and early childhood has been projected as the basic mechanism in the pathogenesis of malnutrition-modulated DM seen among the poor in some tropical areas [3].

Stress hormones like adrenalin and cortisol can increase blood sugar. Stress causes the liver to release more glucose. In individuals with family history of diabetes, stress can definitely precipitate DM.

4.1. Infant nutrition and type-1 DM

Autoimmune damage to islet cells responsible for type-1 diabetes may have a nutritional basis. Clinical presentation of type-1 DM peaks around puberty or sometime later, but islet cell antibodies are seen by the age of 5 years. The implication is that the involvement of type-1 islet is early in life but may burn faster in some than in others. An association between bottle-feeding and type-1 DM has been found in Finland. It is hypothesized that molecular mimicry between islets antigen P69 and bovine serum albumin in cow's milk may occur.

4.2. Fibrocalculous pancreatic diabetes (FCPD)

Chemicals	Altered beta-cell function
a) Rodenticide	a) Calcific fibrosis
b) Streptozotocin	b) Cassava
c) Nitrosamine	c) Protein malnutrition

Table 1. Beta cell damage caused by:

Malnutrition = Cassava intake.

Cassava (tapioca) contains linamarim, a cyanogenic glycoside, which on hydrolysis releases hydrocyanic acid. This hydrocyanic acid is normally inactivated by conjugation with sulf-hydryl radicals derived from aminoacids—methionine and cysteine, and combines with hydrocyanic acid to form thiocyanate, which is excreted in urine. In protein-calorie malnutri-

tion, there is a deficiency of these aminoacids, and accumulation of hydrocyanic acid occurs which may damage the pancreas.

4.2.1. Rich food sources of cyanide

Cassava (Tapioca), sorghum, yam, millet, some varieties of beans are some of the rich sources of cyanide.

4.3. Prenatal infections and diabetes

Child whose mother is type-1 is less likely to develop DM than when the father is affected. Risk is increased with higher birth weight in elderly parents.

4.4. Role of demographic factors in diabetes etiology

There is an increased incidence of incidence of type 1 DM in spring and autumn season; probably related to viral infection. Viral infections through autoimmune mechanisms or directly damaging beta cell may precipitate diabetes. Most of the viral infections are aymptomatic and may precipitate diabetes even after several years of diabetes. Prenatal infections of viruses such as congenital Rubella, cytomegalovirus, and enterovirus are known to be diabetogenic.

4.4.1. Virus and beta-cell damage

- Virus persists in host, leading to protracted beta-cell dysfunction
- Virus directs acute beta-cell destruction
- Virus activates beta-cell defense mechanism, leading to inflammatory cytokines
- Virus produces immune-mediated beta-cell destruction [4]

4.5. Insulitis

Activated T-lymphocytes infiltrate the pancreatic islets prior to or spontaneous with the development of diabetes. Islet inflammation leading to beta-cell failure is a factor in pathogenesis. Interleukin (IL)-1 is central to this insulitis. Islet infiltrating macrophages are a major source of IL-1 and other cytokines in response to elevated levels of glucose and saturated fatty acids. IL-17A exacerbates proinflammatory chemokine expression and secretion by human islets exposed to cytokines. This suggests that IL-17A contributes to the pathogenesis of type-1 diabetes by two mechanisms, namely the exacerbation of beta-cell apoptosis and increased local production of chemokines, thus potentially aggravating insulitis.

- Exposure to cow's milk in infancy may precipitate DM. Coeliac disease and type-1 DM can overlap with the haplotypes [5–8].

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- Vitamin D: Type-1 DM patients have low vitamin D levels, and lack of sunlight correlates well with increased incidence of type-1 DM at higher altitudes [9–14]. Young people who have higher vitamin D levels have lesser chances of developing T2DM later in life, compared to people who have lower vitamin D levels. People with newly diagnosed type-2 diabetes often have low vitamin D levels than people without diabetes. Vitamin D may have a role in pancreatic beta-cell function, insulin action, and inflammation. There are some specific receptors in pancreatic beta-cell that start turning on if they get enough vitamin D. There is an association between low vitamin D and decreased insulin sensitivity. Vitamin D plays an important part in the regulation of calcium. Calcium helps to control the release of insulin; so, alterations in calcium can have a negative effect on beta-cell function. People with higher vitamin D in blood, that is, over 25 ng/ml had a decreased chance of getting type-2 DM later in life compared to those with lowest levels, that is, below 14 ng/ml. A person with highest vitamin D level in blood had a 19% decreased chance of developing type-2 DM. For every 4 ng/ml increase in vitamin D, there was 4% lower risk of developing type-2 DM. In one study, it was shown that vitamin D supplementation improved pancreatic beta-cell function [15–18].
- High vitamin E levels are associated with diabetes, and beta-carotene, the precursor of vitamin A, is a protective factor in diabetes prevention.
- Overweight and inactivity results in insulin resistance and diabetes.
- Age: with increasing age, risk of diabetes increases. Increased triglyceride increases the risk of type-2 diabetes.

4.6. Drugs

Drugs like thiazide diuretics, diphenylhydantoin, pentamidine, and cyclosporin may cause diabetes. Experimental chemical agents that induce diabetes are afloxan and streptozotocin.

4.6.1. Advanced glycation and lipoxidation end-products (AGEs and ALEs) as dysmetabolic risk factors

AGEs are not only endogenously produced but also have an exogenous origin. Thermally processed food products contain high amounts of heat-accelerated protein and lipid-associated pro-oxidant. Foods rich in exogenous AGEs and ALEs are foods cooked at high temperature, roasted foods, fried foods, and dairy products processed with sugar [19].

AGEs and ALEs add to the systemic toxic pool of these powerful pro-oxidants. The glycoxidation product may be considered an important risk factor for insulin resistance, diabetes, and other chronic illness. Increased concentrations of reactive carbonyl compounds have been observed in patients with diabetes, leading to the formation of various AGEs in the body. Both AGER1 (advanced glycation receptor-1) and SIRT1 (survival factor sirtuin 1) were independently found to be suppressed in chronic oxidative stress conditions like diabetes and ageing. AGEs may contribute to insulin resistance. It seems plausible that excess intra-adipose AGEs could impair normal lipolysis via suppression of SIRT1, a factor implicated in fatty acid mobilization. AGEs deplete host defenses, raise basal oxidative stress and inflammation, and

increase susceptibility to dysmetabolic insulin resistance. Pyridoxamine was observed to decrease AGEs accumulation. AGEs are increasingly considered in the pathogenesis of cardiomyopathy, retinopathy, and nephropathic complications of diabetes. AGE levels correlate with these complications.

Restriction of AGEs would result in increased expression of SIRT1 and AGER1 and reduction in insulin resistance . AGE inhibition and Sirtuin activation will be the targets of treatment in future for DM.

Urbanization has been recognized as a potent environmental predisposing factor for the development of type-2 DM. Urban rural differences in the prevalence of diabetes in the same community have been highlighted in most of the epidemiological studies.

S.No.	Protecting factors	Precipitating factors
1	Breast-feeding	Cows' milk allergy
2	Nicotinamide	Infant feeding through bottle-milk
3	Zinc	Weight gain in infancy
4	Vitamin C, D, and E	Enterovirus infection during pregnancy
5	–	Preeclampsia
6	-	Excessive weight gain in pregnant women
Environmental agent		Diabetogenic effect
Viruses		Increase
• Rubella		Increase
• Enteroviruses (Coxsackie B, Echoviruses)		Increase
• Cytomegalovirus, mumps, retroviruses, rotaviruses		
Diet		Increase
• Cow's milk		Increase
• Early cereal supplements		Increase
• N-nitroso compounds		Increase
• Vitamin D deficiency		
Childhood vaccination		No effect
Older maternal age		Increase
Increase birth weight (large babies) (more than 4 kg / 8lb 13 ounce)		Increase

Table 2. Nutritional risk factors in type-1 DM.

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4.7. Gut microbes and type-2 diabetes

Gut microbiota–host interactions control energy homeostasis, glucose metabolism, and lipid metabolism. In addition to genetic and environmental factors, gut microbes may play an important role in the modulation of metabolic diseases. It was found in type-2 diabetes by a moderate degree of gut microbial dysbiosis, decrease in the abundance of some universal butyrate-producing bacteria and increase in various opportunistic pathogens, conferring sulfate reduction and oxidative stress resistance in a Chinese study. Obese people with insulin resistance have in their gut elevated firmicutes/bacteroidetes ratio when compared with healthy people. Change in gut microbes in obese people modulates intestinal permeability and increases metabolic endotoxin secretion that leads to chronic low-level inflammation, leading to insulin resistance and type-2 diabetes. Probiotic strains modulate immune system, down-regulate inflammatory interferon (IFN)-α and IL-2 or IL-1β, and enhance anti-inflammatory IL-10 production [20].

It was found that *Saccharomyces boulardii*-treated mice exhibited reduced fat mass, reduced hepatic steatosis, and reduced inflammatory reaction, thereby concluding this probiotic will prove beneficial to the treatment of obesity and type-2 diabetes.

Nutrition during fetal life and infancy may be crucial to the development of DM; infants who are small for dates (a marker for poor intrauterine nutrition) have fewer beta-cells. Impaired glucose tolerance and type-2 DM may both result from poor nutrition early in life interacting adversely with abundant nutrition later on. Obesity in late years leads to insulin resistance.

4.7.1. Night-shift workers and diabetes

Men who work on shifts face a massively increased risk of developing diabetes. Shift-working men face a 37% increased risk of developing diabetes compared to general population with an overall increased risk of 9%. Shift workers tend to put on weight and suffer from increased appetite. Rotating shifts make it harder for people to adjust to a regular sleep–wake cycle. Lack of sleep or poor quality of sleep may worsen insulin resistance. Night-shift workers have higher risk of developing obesity, DM, and sleep problems [21].

While multiple genetic loci may operate in combination with environmental factors to produce diabetes, other determining factors are age of onset, severity, and rate of progress. Developments in diabetogenesis occur slowly to manifest characteristic abnormalities like IFG, IGT, frank diabetes, and lipid abnormalities. These changes take 7–8 years to progress into diabetes.

The most important promoters of metabolic syndrome and diabetes identified among environmental factors are lifestyle changes and dietary practice. Principal changes in diet consists of greater consumption of calorie-dense foods, particularly fast-foods with greater fat and refined carbohydrate contents, and decreased preference for natural fiber-containing whole grain products, fruits, and vegetables with low glycemic index. These dietary changes lead to obesity and dyslipidemia and glucose intolerance, which are characteristic of type-2 diabetes.

4.7.3. Physical inactivity

Sedentary life style with constant use of automobiles even for short distances, sitting long hours at office/study table, and more so in watching television and using computers have transformed the lives of most urbanites, starting from childhood throughout the rest of their life. These practices have increased the incidence of diabetes and heart disease. Increased physical activity by regular walking, gardening, and doing both endurance and resistance types of exercises have been found to prevent the onset of diabetes.

4.7.4. Obesity and BMI

Risk of diabetes is estimated to increase by 9% for every increase in 1 kg body weight. Obesity alone accounts for nearly 64% of diabetes in men and 74% in women. Independent of BMI, android distribution of fat and waist circumference predict diabetes risk. Obesity predisposes to insulin resistance and beta-cell failure.

4.7.5. Exposure to chemicals

Chemicals from water pollution, plastic packaging, cleaning and beauty care products have all been found to increase insulin resistance and diabetes.

Persistent organic pollutants (POPs) are pollutants that accumulate in the environment due to extensive use of pesticides and industrial chemical products. Pollutants in the environment, consumer products, and cosmetics may all increase risk of diabetes. Endocrine-disrupting chemicals are the substances present in the environment, food, and consumer products, which have the ability to affect hormone production and metabolism among other functions. Type-1 DM is seen more in people who consume fish that have higher amounts of heavy metal pollutants like cadimum and lead present in them. Plastic bottles used for water-storing are known to give out Endocrine-Disrupting Chemical (EDC) [22–25]. Europe has moved away from plastic to glass. There is a connection between pollution and obesity. They are implicated in immune dysfunction and type-2 DM. The organic pollutants are present in animal fats, which when consumed can cause diabetes by producing insulin resistance. Obesity per se may not cause DM, but the presence of POPs in obese people make them susceptible for the development of diabetes. The best preventive measure against POP-induced diabetes will be to minimise the use of pesticides and saturated fats [26–31].

POPs include hundreds of different chemical compounds with common properties, such as long-term persistence, widespread diffusion in the environment, and bioaccumulation through the food chain. These POPs which are cytochrome P450 enzyme inducers are found in the environment, either in the air, soil, or water. They accumulate in the body as white adipose tissue (WAT), which acts as a reservoir of lipophilic environmental pollutants. They are resistant to biological and chemical degradation, leading to their accumulation. They are detectable in virtually all of the general population, most of who experience only background exposure through food consumption, but their persistence has led to multiple health effects, including diabetes. A total of 12 POPs, all chlorine-containing organic compounds, have been

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chosen as priority pollutants by the United Nations Environment Program (UNEP) for their impact on human health and environment. These POPs have been divided as follows:

- Pesticides: Aldrin, dieldrin, endrin, chlordane, DDT, heptachlor, mirex, toxaphene and hexachlorobenzene (HCB) [32–38]
- **Industrial chemical products**: Polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB)
- **Unwanted by-products**: Polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (**PCDFs**), **polychlorinate**d biphenyls (PCBs), hexachlorobenzene (HCB)

4.8. Adverse effects on human health

High-dose acute exposure results from accidental fires or explosions of electrical capacitors or other PCB-containing equipment, or food contamination.

Mid-level chronic exposure is predominantly due to the occupational exposure or increased consumption of POP-contaminated dietary source, such as fish or other marine animals.

Chronic, **low-dose exposure** is seen worldwide in most of the population in different quantities, depending on the variations of diet, geography, and the level of the industrial pollution.

Short-term exposure to high concentrations of certain POPs has been shown to result in illness and death. In 1990, Rice farmers and mango sprayers of Phillippines suffered acute endosulfan poisoning.

Chronic exposure to sublethal concentrations of POPs over prolonged exposure can cause the following:

- Immune dysfunction
- Dermatological effects like chloracne
- Neurodevelopmental and neurobehavioral effects
- Reproductive anomalies
- Endocrine problems
- Rheumatological disorders like arthritis
- Carcinogenesis

Swedish investigations have reported that dietary intake of PCBs (polychlorinated biphenyls), dioxins, and furans may be linked to important reductions in the population of natural killer cells (lymphocytes). Studies of high-level exposure also indicate that some biochemical changes, for example, in enzyme and hormone levels may be induced.

Very high levels of dioxin (2, 3, 7, 8-TCDD), hundreds of times greater than natural levels, are known to cause a reversible skin condition known as chloracne (acne-like eruption of blackheads, cysts, and pustules). Lifetime presence of chloracne, abnormal nails, hyperkeratosis,

skin allergy, goiter, headache, gum pigmentation, and broken teeth were observed more frequently in the men and women exposed to PCB/PCDF. The exposed women reported anemia 2.3 times more than controls. The exposed men reported arthritis and herniated intervertebral disks 4.1 and 2.9 times, respectively, more than controls. The developing fetuses and neonates are particularly vulnerable to POPs' exposure due to transplacental and lactational transfer of maternal POP, some of which can potentially affect neurodevelopment.

4.9. POPs and diabetes

Dae-Hee Lee and colleagues were the first to analyze serum concentrations of POPs which showed the prevalence of DM more than five times higher in groups with higher concentrations of PCB153, oxychlordane, or trans-Nonachlor than in those with lower concentrations. In a study, they found a strong dose–response relationship between diabetes and the six POPs (one PCB (hexachlorobiphenyl), two dioxins (heptadioxin and OCD dioxin), two pesticides (oxychlordane and trans-Nonachlor), and a pesticide metabolite (DDE, a metabolite of DDT)) [5]. In general, older people had higher levels of individual contaminants than younger people. Men tended to have lower concentrations compared to women. For all but one contaminant (PCB153), Hispanics tended to have higher levels as did poorer people [36–38].

The various mechanisms implicated by these POPs in causing diabetes are as follows:

- Insulin resistance
- Obesity
- Decreased insulin production
- Disrupted glucose homeostasis

4.9.1. Insulin resistance

Serum concentrations of organochlorine (OC) pesticides were found to be strongly and positively associated with insulin resistance among nondiabetic subjects. Results suggest that the background environmental exposure to some POPs, especially OC pesticides, may be critically involved in the pathogenesis of diabetes through a pathway involving insulin resistance. Among several OC pesticides, both oxychlordane (metabolites of chlordane) and trans-Nonachlor (impurity of chlordane) were most strongly associated with insulin resistance. Individuals with high levels of POPs (DDT, dioxins, PCBs, and chlordane, among others) in their body were found up to 38 times more likely to be insulin-resistant than individuals with low levels of these pollutants, though a cause and effect relationship could not be established.

4.9.2. Obesity

Because POPs are lipophilic, people with higher body mass index may be more likely to store higher levels of these organic pollutants than people with lower body mass index with equivalent exposure. A study by the National Health and Nutrition Examination Survey on

http://dx.doi.org/10.5772/62543

population found that obesity and diabetes were associated only among participants with detectable levels of POPs [5]. The diabetogenic effect due to dioxin exposure has also been shown to be stronger among obese compared with lean individuals. Another possibility is that insulin resistance causes increased accumulation of POPs. Obesity was associated with diabetes only in people who tested high for these pollutants. Lee et al. found no association between obesity and diabetes in individuals with nondetectable levels of POPs. Obesity was a risk factor for diabetes only if people had blood concentrations of these pollutants above a certain level. Thus, this indicates that obesity is only a vehicle for the accumulation of POPs in the body which eventually results in diabetes.

4.9.3. Decreased insulin production

Pancreatic β-cells contain muscarinic acetylcholine receptors, which are involved in the glucose-dependent production of insulin. Organophosphate insecticides are known inhibitors of acetylcholinesterase, the enzyme responsible for the degradation of acetylcholine. Thus, exposure to sufficiently high levels of organophosphate insecticides would be expected to result in increased accumulation of acetylcholine, potentially leading to overstimulation and eventual downregulation of its receptors and reduction in insulin production.

4.9.4. Disrupted glucose homeostasis

Dioxins are known to be a frequent contaminant of herbicides and have been used in the past as a contaminant by the name of Agent Orange (a code name used by the military) [16, 17]. It involves an estrogen-dependent peroxisome proliferator-activated receptor (PPAR) pathway and thereby upregulates insulin-like growth factor binding protein-1 (IGFBP-1) in MCF-7 cells. Studies have suggested that exposure to these contaminated herbicides increased the risk of diabetes by disrupting the glucose and insulin homeostasis.

4.10. Prevention and control

International efforts to minimize exposure to these compounds include the banning of their use. With the exception of DDT, few, if any, of these compounds have been authorized for use. PCBs, which were widely used in capacitors, transformers, and lubricating oils, have not been manufactured for several decades, but linger in the environment. Chlorinated dibenzodioxins and dibenzofurans are by-products of products made from chlorophenols.

4.10.1. At the individual level

Since fetuses and infants are especially sensitive to the effects of toxic compounds, efforts should be made to reduce girls' and women's exposure to dioxins in foods during the years well before childbearing, so that lesser amounts of these compounds accumulate in their bodies and are passed on through the placenta and breast-milk. Fats in meat, poultry, fatty fish, whole milk, and full-fat dairy products are the principal sources of most people's exposure, and their ingestion should be minimized. Choosing a balanced diet that is low in saturated and total fats

helps minimize any potential exposure to dioxin from food, because dioxins are found mostly in animal fats (due to the fat-soluble properties of the dioxin compound).

- Choose leaner cuts of beef, pork, and poultry; trim the fat and remove skin from chicken before cooking.
- Choose nonfat and low-fat milk and milk products.
- Choose a diet high in fruits, vegetables, whole grains, and lean proteins.

These strategies help lower the intake of saturated fats and total fats and therefore help reduce the risk of exposure to dioxins.

4.10.2. At the industry level

The industries should make an effort to produce alternatives to the banned pesticides which are biodegradable. Research and development for new products should be undertaken rigorously by industries. Training of farmers in terms of efficient and judicious use of pesticides should be undertaken.

Research in the following direction may be useful:

- Serum concentration of POPs in genders, obese and nonobese diabetics, type-1 and gestational diabetics should be studied to find the relationship between POPs and DM.
- Relationship between fatty foods, oil, and water levels of POPs in humans should be studied.

4.10.3. At the government level

The government plays an important role in the prevention of POPs. They should improve the surveillance system to monitor any manufacturing, import, or export of banned pesticides. Carrying out changes according to the POP elimination programs should be their main goal.

5. Conclusion

Environmental factors have a major role both in type-1 and type-2 DM. Even when there is genetic proneness, environmental factors precipitate diabetes. These environmental factors are reviewed in detail. The accumulation of POPs in the environment and their various effects on humans in general have been given in short. Their implications in the causation of type-2 DM, with a special reference to their mechanism, including preventive measures, have been discussed. Further study is needed regarding urban and rural concentrations of POPs as well as study of POPs in type-1 DM, type-2 DM, obese DM, and gestational DM.

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Chapter 10

Amoxicillin in the Aquatic Environment, Its Fate and Environmental Risk

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Additional information is available at the end of the chapter

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Abstract

Amoxicillin is a broad-spectrum antibiotic widely used for treating both human and animal diseases, and it belongs to a group that are excreted unchanged within urine and faeces; therefore, it is possible to find traces of this drug or its degradation products in environmental water bodies. In water, it is rapidly degraded by biotic and abiotic factors, yielding different intermediate products; these are suspected of being more resistant to degradation, and potentially more toxic, than the parent compound. In the water bodies, these compounds may produce toxic effects on the aquatic organisms from different trophic levels and produce an ecological imbalance. Amoxicillin may bioaccumulate in fish muscle tissues, with the possibility of the occurrence of these drugs in food, leading to a passive consumption of this antibiotic resulting in undesirable effects on consumer health such as immunoallergic responses. However, the main problem related with the presence of this antimicrobial compounds in fish tissues is the possibility of inducing bacterial resistance genes. At present, the available scientific knowledge is less than what is needed to fully assess the risks that amoxicillin pose to the environment, and it is still necessary to conduct large amount of research works before a thorough understanding of this severe environmental issue.

Keywords: Amoxicillin, risk, toxicology, occurrence, analytical methods

1. Introduction

In the recent decades, the term "emerging pollutants" have been used widely to refer to a variety of chemical compounds without regulatory status in the environment and its impact on the environment and human health are poorly understood. Within the category of emerging pollutants, the antibiotics are one of the most significant groups.

Antibiotics are among the most successful drugs used for human therapy; however, these drugs are also recognized by its use and benefits in many different activities such as agriculture, aquaculture, bee keeping and livestock as growth promoters. Wise in 2002, estimated that the consumption in the market of antibiotics at worldwide level, was between 100,000 and 200,000 tonnes annually; however, the World Health Organization states that the amount of antibiotics is not precisely known because only a few countries have national statistics of the use, amounts and patterns of antibiotics [1–9].

Due to the extensive use in human and veterinary medicine, it is a reality that antibiotic compounds may be found in different environmental compartments. Indeed, in the last few years, antibiotics have been detected and reported by several researchers around the world in the μg/L range in municipal sewage, hospital effluents, sewage treatment plants effluents, surface water, ground water, marine water and drinking water [10–23]. The excretion of incompletely metabolized antibiotics by human and animals is the primary source of antibiotics in the environment, and other sources include the disposal of unused antibiotics and waste from pharmaceutical manufacturing process; residential (private residences, dormitories, hotels and residential care facilities) and commercial facilities (including hospitals) are also known as contributors of antibiotics to municipal wastewater. Other potential contributors of antibiotics to surface and groundwater are effluents from wastewater treatment plants and industrial facilities, and surface runoff from concentrated animal feeding operations [22, 24].

The occurrences of these compounds in different water bodies represent a potential threat to the environment, since these drugs are designed to have a pharmacological effect and to be persistent; moreover, the greatest concern about the presences of antibiotics in the environment is the emergence and dissemination of antibiotic resistance genes [1, 4, 12, 13, 16, 25–28].

In addition, antibiotics are of concern due to their high toxicity to algae and bacteria at low concentrations, potential genotoxic effects, disruption of aquatic ecology, promotion of antibiotic resistance and possibly even increased human health risks [20, 22, 29].

As a result, Sanderson et al. in 2004 classifies the antibiotics as pollutants of high priority for measuring environmental risk due to the probability of potential effects on human and environmental health, even more than sex hormones, cardiovascular drugs and antineoplastics [30].

Risk analysis is the scientific methodology used internationally to assess toxic effects on the environment [31–33]. In 2002, Jones et al. reported that the risk quotient (RQ) for amoxicillin in the United Kingdom exceeded the 1.00 unit, as well as, Park and Choi in 2008 reported that

http://dx.doi.org/10.5772/62049

amoxicillin has a risk quotient (RQ) in Korea of 1.62, suggesting the need for further research to this antibiotic as an ongoing and future environmental monitoring toxicological investigations [34, 35].

Likewise in 2004, Boxall et al. scored the amoxicillin as "high priority" in environmental monitoring and prioritization of toxicological studies [34–36].

2. Amoxicillin

Originally introduced in the early 1970´s for oral use in United Kingdom, this drug has found gradually a regular place as a broad-spectrum antibiotic. In 1981, SmithKline Beecham patented amoxicillin/clavulanate potassium tablets, and first sold the antibiotic in 1998 under the trade names of amoxicillin, amoxil and timox [37–39].

The amoxicillin is a semi-synthetic drug, which belongs to a class of antibiotics called the penicillins (β-lactam antibiotics). This drug has been shown to be effective against a wide range of infections caused by gram-positive and gram-negative bacteria and is used for the treatment and prevention of respiratory, gastrointestinal, urinary and skin bacterial infections due to its pharmacological and pharmacokinetics properties [37, 40, 41]. Besides its use in human medicine, amoxicillin is also used for treating and preventing animal diseases as well as it is used as growth promoters for many domestic and food animals, including dogs, cats, pigeons, horses, broiler chickens, pigs, goats, sheep, pre-ruminating calves, cattle and fishes [42, 43]. It is well absorbed from gastrointestinal tract. The apparent volume of distribution of amoxicillin is approximately 0.26-0.31L/Kg and widely distributed to many tissues, including liver, lungs, prostate muscle, bile, ascetic, pleural and synovial fluids, and ocular fluids, accumulates in the amniotic fluid and crosses the placenta but penetrates poorly into the central nervous system unless inflammation is present. It is approximately 17–20% bound to human plasma proteins, primarily to albumin [44, 45].

Amoxicillin is very closely related to ampicillin with the same spectrum of activity and potency but is much better absorbed when given orally, achieving blood concentrations approximately twice as high as those obtained with ampicillin [37].

It acts binding to penicillin binding protein (PBP-1A) located inside the bacterial cell wall, the amoxicillin acylate the penicillin-sensitive transpeptidase C-terminal domain by opening the lactam ring causing inactivation of the enzyme, prevents the formation of a cross-link of two linear peptidoglycan strands, inhibiting the third and last stage of bacterial cell wall synthesis, which is necessary for cell division and cell shape and other essential processes, producing as a consequence the lisis of the bacteria cells [37, 46–49]. The two major metabolites of amoxicillin are amoxicilloic acid and piperazine-2, 5-dione (diketopiperazine). These metabolites have lost antibacterial activity of the parent component, but the amoxicilloic acid could have potential allergic properties [50–52].

The drug´s terminal half-life of elimination is 1 to 1.5 hours. Excretion of amoxicillin is predominantly renal, more than 80% of the original drug is recovered unchanged in the urine, leading to very high urinary concentrations and is also secreted in milk [53–55].

3. Amoxicillin sales and occurrence in water bodies

Amoxicillin acts against a broad spectrum of gram-positive and gram-negative microorganisms; therefore, it is used as first-line antibiotic both in human medicine and in veterinary medicine in many different countries [56–59]. Table 1 is listed numbers of data reported of sales of amoxicillin in different countries around the globe.

Country	Amount (kg)	Reference
Italy	209, 500 in 2001	[60]
UK	71, 467	[34]
Korea	106, 673	[61]
Australia	959,000	[62]
Australia	4 million scripts filled for amoxicillin which equates to approximately 40, 000kg of this antibiotic	[63]
UK	170, 432	[64]
Brazil	In Brazil, it is the most commonly prescribed antibiotic and have been sold 6 billions units of amoxicillin between 2007–2011	[65]
China	In China, Amoxicillin and Penicillin are the two most prescribed antibiotics	[18]
Nederland	Reported that amoxicillin and ampicillin are the two most consumed antimicrobial drugs in 71 countries between 2000–2010	[25]

Table 1. Sales of amoxicillin in different countries around the world.

Besides to the numbers of sales and use of this drug that have been mentioned above, amoxicillin belongs to a group of drugs that are excreted unchanged in urine and faeces at high rates; therefore, it is likely to find amoxicillin concentrations traces in the environment. Table 2 is listed the occurrence and quantity of amoxicillin that have been found in different water bodies of different countries around the world.

Country	Water body	Concentrations ($ng\ L^{-1}$)	Reference
Italy (Cagliari)	Effluents of sewage treatment plants	7.40	[66]
Italy (Cosenza)		1.80	
Italy (Palermo)		120.35	
Italy (Roma)		15.20	
Italy (Napoli)		1.80	

Country	Water body	Concentrations (ng L^{-1})	Reference
Italy (Torino)		4.74	
Italy (Varese Olona)		4.68	
Italy (Varese Lago)		4.68	
Italy (Palermo)	Effluents of sewage treatment plants	120	[67]
Italy (Latina)		15	
Italy (Varese Olona)		25	
UK (Merthyr Tydfil)	Surface waters	39–49	[68]
UK (Trefforest Estate)		198–245	
UK (Cardiff)		56–60	
Australia	Raw sewage	280	[69]
Australia (Brisbane)	Effluent of conventional treatment plant	30	
Australia	Hospital effluent	900	[20]
	Wastewater treatment Plant influent	6940	
	Wastewater treatment Plant effluent	50	
	Surface waters	200	
Italy	Wastewater of sewage treatment plants	622	[26]
Spain	Surface water	200	[70]
	Hospital effluent	900	
	Urban effluent	1670	

Table 2. Occurrence of amoxicillin in different water bodies around the world.

Although amoxicillin is an antibiotic drug highly consumed for human and veterinary medicine, and therefore is expected to be found at a relatively high concentration in wastewater and surface water, it is worth noting that there is no much information available in the literature regarding with its occurrence in environmental compartments. This can be explained by the clear fact that the chemical structure of this compound has an unstable β-lactam ring causing it readily undergo hydrolysis shortly after excretion; moreover, this might be the consequence of the incapability of the analysis to assess the presence of all antibiotic compounds presents in the different samples or even that its concentration in the aquatic media is lower than the limits of detection and quantification of the analytical methods.

3.1. Methods of analysis for amoxicillin

British, India and US Pharmacopoeia recommended liquid chromatography and potentiometric methods for the analysis of pure amoxicillin in pharmaceutical dosage forms (tablet, capsule, oral suspension and injection) [71–73].

However, the extensive literature survey showed that there are several methods, which can be used for assaying amoxicillin in drug substances, formulation products, biological fluids and environmental water samples, that is ultraviolet spectroscopy methods, colorimetric

methods, bienzimetic UV-spectrophotometric methods, polarography methods, spectrofluorometric methods, microbiological assays, enzyme linked immunosorbent assays (ELISA), and high-performance liquid chromatography methods (HPLC) [37].

The literature are reported several high-performance liquid chromatography methods with fluorescence, UV or mass spectrometry detection for the determination of amoxicillin residues in edible tissues of cattle, pig, sheep and goat as well as for cow and sheep milk [75–78].

Table 3 is summarized methods to measure amoxicillin residues in animal tissues that are reported in the literature.

Tissue	Method	Limit of detection and limit of quantification	Reference
Catfish and salmon tissues	HPLC	LOQ: 1.2ng/g	[79]
Pig, cattle and chicken muscle	HPLC Fluorescence	LOQ: 5 μg/Kg	[80]
Pig liver, kidney, muscle and fat	LC-MS/MS	LOD: 2.3–12 μg/Kg LOQ: 25 μg/Kg	[81]
Chicken muscle, kidney, liver, fat.	LC-MS/MS	CC α: 51.6–57.0 μg/Kg CC β: 72.4 μg/Kg	[82]
Pig kidney, liver, fat and muscle	LC-ESI-MS/MS	LOD: 1.5–3.5 ng/g LOQ: 25ng/g	[83]
Sheep serum and tissue cage fluid	HPLC Fluorescence	LOD: 0.06–0.10μg/mL LOQ: 0.10–0.20 μg/mL	[84]
Bovine milk	UHPLC-MS/MS	LOD: 1.0ng/mL LOQ: 5ng/mL	[85]
Bovine muscle	LC-MS/MS	CC α: 61.2 μg/Kg CC β: 72.4 μg/Kg	[86]
Chicken muscle, kidney and liver	HRMS	LOD: 10 μg/Kg LOQ: 15–25μg/Kg	[87]
Eggs chicken	LC-MS/MS	LOD: 0.6 μg/Kg LOQ: 1.8 μg/Kg	[88]

Notes: CC α: Decision limit; CC β: Detection capability

Table 3. Methods to measure amoxicillin residues in food animal tissues.

The literature are also reported a few high-performance liquid chromatography methods with fluorescence, UV or mass spectrometry detection for the determination of amoxicillin in effluents and environmental water samples [60, 67]. Table 4 summarises some methods to measure amoxicillin and its degradation products in water samples.

The chromatographic methods for amoxicillin analysis in animal tissues and environmental water samples play a significant role in the regulation of the use of this drug in livestock as

Molecule	Method	Limit of detection or quantification	Reference
Amoxicillin	HPLC-MS-MS	LOQ: 6ng/L	[60]
Amoxicillin	HPLC-MS/MS	LOQ: 2.08ng/L	[67]
Amoxicillin	UPLC-MS/MS	LOD: 2.5 μg/L LOQ: 10 μg/L	[68]
Amoxicillin	LC-MS/MS	LOD: 14ng/mL	[69]
Amoxicillin	HPLC-MS/MS	LOD: 20ng/mL	[20]
Amoxicillin, Penicilloic acid, Penilloic acid, AMX diketopiperazine AMX-S-oxide	LC-MS	No reported	[89]
Amoxicillin	LC-MS/MS	LOD: 3.54 μg/L LOQ: 10 μg/L	[90]

Table 4. Methods to measure amoxicillin and metabolites in water samples.

well as in the creation of future regulations and monitoring programs for this drug in effluents from wastewater treatment plants, hospitals and households. Regulatory agencies rely on data generated from these methods to establish regulatory actions. Therefore, it is necessary to develop sensitive, accurate and reliable methods to support regulatory programs.

4. Environmental degradation of amoxicillin

Several processes can affect the fate and transport of organic compounds in the environment including 1) sorption, 2) biotic transformation and 3) abiotic transformation. The knowledge of the chemical properties and structures of compounds can allow preliminary estimation of their fate and persistence in the environment [91].

Despite that it is well known that β-lactam drugs contain a lactam ring, which is unstable and easily opened by β-lactamases (a widespread enzyme in bacteria) [24] as well as have shown thermal degradation with the hydrolytic cleavage and ultimate mineralisation to CO_2 and water [20], there was no detailed information regarding to the transformation of this antimicrobial compound in the environment until recent years.

In 2013, Gozlan et al., suggested a full degradation pathway of amoxicillin in aqueous medium. This starts with the opening of the four-membered β-lactam ring by hydrolysis to yields the intermediate AMX-penicilloic acid, which contains an extra free carboxylic acid group. Also it was reported that various metal ions such as mercury, zinc, cadmium, cobalt and copper might catalyse the degradation of the β-lactam ring. Subsequently depending of the pH of the medium, this intermediate compound could yield two different more stable compounds [89, 92, 93].

At high pH, the lone pair electrons on the amine group of the AMX-penicilloic acid are available for nucleophilic attack on the carbonyl group to yield a six membered stable diketopiperazine ring and the AMX diketopiperazine degradation product [89]. In the other hand, at low pH, the AMX-penicilloic acid suffers a decarboxylation process yielding the AMX penicilloic acid degradation product [89].

Another metabolite of amoxicillin is yield under sunlight irradiation merely as an indirect photolysis process enhanced by the presence of natural photo-sensitisers like humic acids, which activate the oxygen dissolved in water to oxidise the amoxicillin, forming the AMX-S-oxide degradation product; also this product is obtained under ozonation process. It is worth noting that the presence of this compound in aquatic environments is of great concern, because the AMX-S-oxide β-lactam ring is still active and may lead to the development of resistant bacteria and even cause other possible health hazards to human and wild and domestic animals [93].

To sum up, it is important to remark that these degradation products are suspected of being more resistant to degradation, and potentially more toxic, than the parent compound [36, 66, 70, 93].

5. Bioaccumulation and bacteria resistance genes

Antibiotics like amoxicillin are used not only for treating human health issues; they are also wide used in livestock farming and fish farming for treating diseases. The improper administration of theses pharmaceutical compounds may lead to the occurrence of these drugs in food supplies [4, 78, 94]. Over the last decades, the occurrence of antibiotics in tissues of aquatic organisms has raised the interest of different organisations around the globe, particularly the FDA in USA. It has been reported that continuous exposure to these pharmaceutical compounds may result in accumulation of the parent compound, their metabolites or both in tissues of aquatic organisms [95].

Due to the risk associated with direct and indirect effects on human health due to both passive and active consumption of antibiotics has lead to set regulations on the use of some antibiotics and the establishment of maximum residue limits. The US official limit tolerance for amoxicillin residues is 0.01ppm (10 ppb o 10ng/g) in milk and uncooked cattle tissue; however, no tolerance has been established for amoxicillin residues in fish tissues at the present time [78, 95, 96].

It is important to remark that the presence of these drugs in animal tissues can have undesirable effects on consumer health such as allergies; however, the main problem related with the presence of this antimicrobial compounds in fish tissues is the non-controlled ingestion of antibiotics, possibly inducing resistance in bacterial strains [4, 66, 94, 95].

It has been recently shown an exchange of genes for resistance to antibiotics between bacteria in the aquaculture environment and bacteria in the terrestrial environment, including bacteria of animals and human pathogens [4, 97–99]. Therefore, the presence of antibiotics in the aquatic

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environment can result in the appearances of resistance among human pathogens forming part of its microbiota [4, 100–102].

To sum up the unrestricted use of antibiotics like amoxicillin for any purpose in any country has the potential to affect human and animal health on a global scale; hence, this problem should be dealt through unified local and global preventive approaches [4, 103, 104].

6. Immuno-allergic potential

In the literature, it is reported that antibacterial drugs like amoxicillin are not considered as important toxics substances, even at high concentrations; however, an frequent use or exposure to this drugs may produce allergic reactions. Currently, the widely prescribed β-lactam antibiotics are among the drugs most frequently eliciting allergic reactions in human populations [105–108].

Protein haptenation plays a key role in immunological reactions to amoxicillin. The haptenation process occurs through the nucleophilic opening of the β-lactam ring by the attack of free amino groups in proteins, particularly modelling molecular studies found that the most reactive residues towards to amoxicillin is the Lysine, favouring the amoxicilloyl-protein adduct formation, which is able to elicit an immune response [109–112].

Clinically, drug allergy is characterized by a spectrum of immune reactions ranging from mild skins rashes to angio-oedema or life-threatening anaphylaxis, meaning a serious and potentially life-threatening problem, causing injury to tissues throughout the body. Moreover, diagnosis is complicated and requires a careful medical history, laboratory studies and in many cases oral food challenge to confirm a diagnosis. Particularly, some people have hypersensitive immune systems that overreact to these types of drugs, and without immediate medical treatment, allergic reactions may be fatal [113, 114]. Residues of many antibiotics and antibacterial agents, or haptenised macromolecules, for example penicilloylated proteins in meat and other dietary products from food animals and farmed fish might be responsible for hypersensitivity reactions in human population. Up to 7–10% of the general population in the world has true allergic sensitivity to penicillin and their related compounds due to prior medical treatment. However, in the literature, only a very small number of cases of hypersensitivity reactions related to food containing residues of penicillin have been reported [115–117]. Even though these cases are very rare, they remark the continuing need to control antibiotic residues vigilantly [117].

7. Toxicological risk of amoxicillin

In aquatic environments, few studies have reported the effects of amoxicillin in fish, probably because it is not described as a toxic substance important in the scientific literature reporting that the LC_{50} at 96 hours for *Oryzias latipes* was 1000 mg / L [35].

However, Andreozzi et al., in 2004, reported a high toxicity of amoxicillin in the blue green algae *S. leopoliensis* (NOEC = 0.78μg / L; LOECs = 1.56μg / L; EC*50* = 2.22μg / L) at 96 hours in concentrations between of 50 ng/L and 50 mg/L [66].

Furthermore, in 2013 at the University of Aveiro Portugal, Oliveira et al., measured the effects of amoxicillin in embryos and adults of *Danio rerio*; the effects found in embryos exposed with amoxicillin were premature hatching, malformations such as edema and deformities in the tail as well as abnormal development of fish. In the other hand, in adults exposed to amoxicillin, they found that amoxicillin produce the inhibition on the activity of the enzyme catalase (CAT) in certain tissues such as gills and brain, as well as produce an induction of the activity of the enzyme glutathione transferase (GTS) in tissues such as muscle, gills and brain, indicating that this antibiotic is capable of modifying the normal enzyme activity in fish [118].

In addition, Liu et al., in 2015, reported that the exposure of the photosynthetic cyanobacteria *Microcystis aeruginosa* to different concentrations of amoxicillin produced a significant increase in reactive oxygen species (ROS) and an increase in the activity of superoxide dismutase (SOD), peroxidase (POD) and glutathione-S-transferase (GST) as well as an increase in the content of glutathione (GSH) and malondialdehyde (MDA) [119].

Similarly, Li, et al., in 2007 reported that amoxicillin has the potential to produce genomic injuries in human deoxyribonucleic acid (DNA), possibly by intracellular induction of reactive oxygen species (ROS) [120].

Although the full extent and consequences of antibiotics in the aquatic environment are still largely unknown, their biological activity and toxicology is of concern [70]. However, despite being an issue of global concern, research regarding toxicological testing and biological activity is still insufficient and necessary.

9. Conclusion

Due to the lack of information regarding with environmental occurrence, ecotoxicity tests and it unstable structure, β-lactam drugs like amoxicillin are not generally thought could be of concern as environmental pollutants, however, amoxicillin is one of the most consumed antibiotics in the world, hence this pharmaceutical compound could be present at concentrations of concern by continual infusion in to the environment.

In the literature, there have been found traces of this antimicrobial compound in different water bodies; furthermore, some toxicological tests reported toxic effects in algae and fishes as well as the calculation of the risk quotient (RQ) in UK and Korea suggest that amoxicillin should be consider as a pollutant of high priority in the environment.

However, at present, the available scientific knowledge is less than what is needed to fully assess the risks that amoxicillin pose to the environment; therefore, future works will need to focus on more detailed ecotoxicity testing, using a wide range of aquatic organisms, in order to fully understand the environmental toxicity of this antimicrobial product and how may

affect both the aquatic and terrestrial environments and indicate possible remediation strategies; future works are needed in the development of new analytical methods that are more sensitive, accurate and reliable in order to assess the occurrence of the amoxicillin and its metabolites in different water bodies.

A large amount of research work is necessary before a thorough understanding of this severe environmental issue.

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This book, Environmental Health Risk - Hazardous Factors to Living Species, is intended to provide a set of practical discussions and relevant tools for making risky decisions that require actions to reduce environmental health risk against environmental factors that may adversely impact human health or ecological balances. We aimed to compile information from diverse sources into a single volume to give some real examples extending concepts of those hazardous factors to living species that may stimulate new research ideas and trends in the relevant fields.

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