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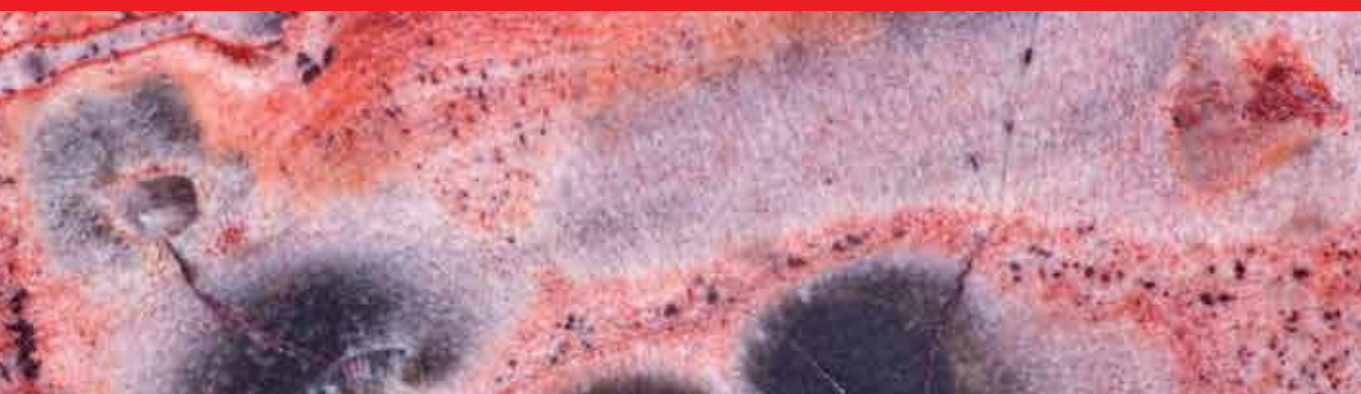
Edited by Cristiana Filip and Elena Albu



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Edited by **Cristiana Filip** and **Elena Albu**

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Contributors

Wiliam M. Chilian, Lindsey Rinker, Roma Patel, Joanna Peng, Giulia Guerriero, Gerardino D'Errico, Gaetano Ciarcia, Anna De Maio, Anna Rita Bianchi, Oladokun Sulaiman Olanrewaju, Abdurrahim Koçyiğit, Eray Metin Guler, Murat Dikilitas, Nina Filip, Elena Cojocaru, Alexandru Filip, Bogdan Veliceasa, Ovidiu Alexa, Kazutaka Hirakawa, Chanchai Boonla, Patricia Siques, Julio Brito, Eduardo Pena, Yun-Wen Zheng, Hui Xu, Qi Liu, Yu-Mei Li, Zhou Hu-Chen, Feng-Lin Luo, Hiroko Isoda, Nobuhiro Ohkohchi, Li-Ping Liu, Teppei Takeshima, Shinnosuke Kuroda, Yasushi Yumura, Filip Cristiana

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Meet the editors

Dr. Cristiana Filip is an Associated Professor at the Department of Biochemistry, Faculty of Medicine, "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania, since 2008. She became a PhD in Chemistry in 2001 with the thesis entitled "Analytical methods for assessing the bioavailability of some drugs bound to a polymer support" at the Faculty of Chemistry, "Alexandru Ioan Cuza" University, Iasi, Romania. She is a senior chemist since 2010, in the Public Health Network, in the field of Medical Biochemistry. Her scientific activity for the last 10 years consists of 4 books (national/international) and 8 papers (international). She participates as a team member in seven research grants (national/international).
She has experience in pharmacokinetic, clinical laboratory of biochemistry, reactive species, and homocysteine metabolism. Her fields of research are reactive species, homocysteine metabolism, and endothelial dysfunction in cardiovascular diseases.

Elena Albu is an Associate Professor of Pharmacology at the Faculty of Medicine "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania, since 2008.
She became a PhD in Pharmacology by accomplishing in 2002 the thesis entitled "Pharmacology research of mastocyte and mast cell release substances." She had obtained the clinical specializations in hematology, internal medicine, and clinical pharmacology. Currently, she practices clinical hematology. Her scientific activity for the last 10 years consists of 8 books (national/international) and 16 papers (international). She participates as a team member in three research grants (national/international).
Her scientific interests are in the field of clinical and preclinical research in pain, oxidative stress, hemato-oncology, and cardiovascular pathology.

Contents

Preface

The book aims to bring together the newest scientific data in the field of reactive species biology.

The chapters contain scientific works from scientists of different leading researcher groups in the field of reactive species.

The main subject of the book refers to the "metabolism" of the reactive species and cellular signaling. The book focuses on cellular signaling as a vital process in adapting the body to the stress factors. The book also highlights the boundary between beneficial and negative effects of reactive species as being the key in the elucidation of their role.

The book presents ROS involvement in many types of pathologies such as renal lithiasis and bladder cancer, hepatocellular carcinoma, male infertility, bone fragility, skin diseases such as psoriasis and vitiligo, and the cardiovascular system functioning in hypoxia at high altitude.

The presentation of very different but widespread pathologies, in which reactive species are involved, may be very useful to physicians in their therapeutic approach. The book provides information on new techniques for producing reactive species in situ for therapeutic purposes in cancer therapy. It also contains useful information for treatment of skin diseases, male infertility, kidney stone and bladder cancer, as well as bone fragility. The readers will find well-documented information that advises on the conditions and precautions to be taken when using phytochemicals in the treatment of cancer.

By linking the fundamental notions regarding reactive species to altered mechanisms of various diseases, the authors emphasize the clinical aspects of the book.

The research in the field of reactive species will certainly continue, as the most recent data indicate that they have a major role in epigenetics. Last but not least, the book contains extremely useful information about high-sensitivity ROS detection techniques and new devices designed for ROS quantification.

The book addresses mainly to those who have knowledge of chemistry, biochemistry, pharmacology, physiology, and physiopathology. It presents information to be of benefit for medical students, practicing physicians, biologists, biochemists and researchers as well.

I would like to express my gratitude to all the scientists who had chosen to join this project by submitting their work.

I would also like to thank Ms. Maja Bozicevic, Publishing Process Manager at IntechOpen, for her help and guidance along this work.

Cristiana Filip and Elena Albu
"Grigore T. Popa" University of Medicine and Pharmacy
Iasi, Romania

Main Concepts

Chapter 1

Introductory Chapter: The Biology of Reactive Species

Filip Cristiana

Additional information is available at the end of the chapter

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1. Main concepts

Reactive species are a relatively old topic in the field of biomedical research, and yet many aspects of their role have remained unclear.

The initial theory, still valid, considers reactive species to be involved in the phenomenon of cellular aging by assigning them an exclusively harmful role.

Current data indicate the involvement of reactive species in cell signaling, assigning them an additional role in the physiological process of adapting the body to stress factors.

Much more scientific evidence indicates that, similar to many other biological molecules in the body, the reactive species trigger different cellular responses depending on their concentration. It is now accepted that in physiological concentrations, reactive species exert a beneficial role by modulating a large number of processes, but both diminishing and accumulating their concentrations trigger pathological processes. That is why although the notion of reactive species is maintained today, there is much more talk about the biology of reactive species.

Until now, the mechanisms by which reactive species act in antibacterial defense and in some signaling processes such as nitric oxide (NO) activity in vascular relaxation have been fully elucidated.

Now, the most current researches pursue several directions such as:

- identifying the reactive species that could trigger the activation of specific proteins and the inclusion/exclusion of these species in the category of second messengers;
- identifying the cascade reactions that can cause cellular response under the action of reactive species and the mechanism of its regulation;

- identifying the links between reactive species and the inflammatory and carcinogenic processes;
- identifying the mechanism by which reactive species influence epigenetics.

From the enumeration of these themes, it is obvious that research of reactive species is far from being elucidated and exhausted.

Survival of aerobic organisms depends on the presence of oxygen. The main use of oxygen is its participation in the energy generation process. In all processes using oxygen, reactive oxygen species (ROS) are constantly formed as secondary products. ROS are a group of compounds with increased reactivity including anion superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), singlet oxygen ($1O_2$), and hydroxyl radical ($OH^{\bullet}$), which can be formed in living organisms. In addition to ROS, cellular metabolites, generated by exogenous/endogenous nitrogen, can form reactive nitrogen species (RNS) that include nitric oxide, peroxynitrite, and nitrite/nitrate. The two types of reactive species, ROS and RNS, can act together generating the so-called nitrosoative stress ROS/RNS.

Thus, living organisms are continuously assaulted by reactive species from external to internal sources. The main problem is the concentration of reactive species and the time that their action lasts so that these two factors set the boundary between beneficial and negative effects of ROS. This boundary probably is the key to elucidating the mechanism of action of reactive species.

As for the role of reactive species, NO is the only reactive species that have been identified so far as a second messenger.

Regarding ROS, the debate continues, but from all ROS, only hydrogen peroxide appears to act as a second messenger.

Superoxide anion is a radical; however, it cannot diffuse remotely because of its limited lipid solubility and high reactivity. Hydroxyl radical ($OH^{\bullet}$) indiscriminately reacts with any structure in its path thus being devoid of specificity. Hydrogen peroxide, on the other hand, is lipid-soluble, diffuses through the lipid membranes [1], has a longer life span, and appears to be more selective in its reactions to biological molecules [2].

To meet the second messenger criteria, a structure must have a certain reactivity and specificity [2]. Hydrogen peroxide is less reactive than superoxide or hydroxyl anion, which allows it to have better diffusivity. This higher diffusivity makes hydrogen peroxide capable of reaching certain target proteins. Scientific data show that H_2O_2 has some specificity to oxidize cysteine residues belonging to specific proteins called protein-tyrosine phosphatases [3, 4]. Through this mechanism, H_2O_2 interferes with the known MAP kinase pathway that functions in cell signaling [5].

The possibility of reversing the oxidation of protein-tyrosine phosphatases under certain conditions makes this process suitable for regulation. But in the case of an intense oxidative process, the oxidation of protein-tyrosine phosphatases becomes irreversible, resulting in blocking the signaling process at a certain phase and thus triggering the pathological processes [3].

2. Reactive species in pathology

A subject of particular interest is inflammation. Inflammation is a complex process involving both a stage of destruction of damaged tissue and a repair stage to the initial structure. The complexity of this process is not only due to the large number of molecules involved but also to the mechanisms that must be perfectly correlated and synchronized. Shortening the global process leads to an inefficient repair or an irreversible damage to the affected area [6]. The prolongation of inflammation causes a large number of pathologies, some of which are moderate, chronic, and some lead to cancer. Scientific works demonstrate the links between inflammatory mediators and the emergence of tumor phenomena in various pathologies. This leads to the idea of a common mechanism that current research studies seek to elucidate, but the involvement of reactive species in cancer is far from being elucidated.

Recent data show that reactive species play a double role in cancer. On the one hand, ROS facilitate cell proliferation and adaptation to hypoxia; on the other hand, it can trigger the death of tumor cells by initiating autophagy [7]. Furthermore, tumor cells can themselves generate reactive species [8] and, at the same time, can increase their antioxidant activity to ensure their survival in the oxidation medium so formed [9].

Disrupting the balance of any of these mechanisms leads to various pathologies such as kidney disease, bladder cancer, hepatocellular carcinoma, male infertility, bone fragility, skin diseases, and cardiovascular system dysfunctions.

In an effort to limit the damage of reactive species, the administration of natural antioxidants is used in therapeutics. On the other hand, there are current approaches that envisage the use of reactive species to kill cancer cells. Therefore, there is a constant and high interest in the quantification of reactive species.

3. Perspectives in reactive species' research

At present, there is an increased interest in studying the action of reactive species in epigenetics. Recent data demonstrate that oxidative stress induces changes in chromatin by initiating histone methylation/demethylation processes. As these changes occur in physiological and pathological processes, reactive species open a new branch in biomedical research.

Author details

Filip Cristiana

Address all correspondence to: cfilip2000@yahoo.com

Department of Biochemistry, Faculty of Medicine, Grigore T. Popa University of Medicine and Pharmacy, Iasi, Romania

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Chapter 2

Reactive Oxygen Species: The Good and the Bad

Roma Patel, Lindsey Rinker, Joanna Peng and
William M. Chilian

Additional information is available at the end of the chapter

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Abstract

This chapter summarizes recent research on the biology of reactive oxygen species (ROS). The chapter is focused on the bimodal actions of ROS, which can be summarized as both beneficial and negative. The beneficial aspects of ROS are related to their effects on the redox state of cells and the important role that some ROS play in signaling cascade. The detrimental effects of ROS are related excess amounts of these chemical moieties, which are caused by excessive production and/or insufficient actions of endogenous antioxidants. The generation of these species is also discussed.

Keywords: reactive oxygen species, oxidative stress, superoxide

1. Introduction

Reactive oxygen species (ROS) are defined as chemically reactive oxygen radicals as well as non-radical derivatives of oxygen [1]. The varying range of reactivity each reactive oxygen species exhibits is crucial to its impact at the molecular level. Their significance in the development of many cardiovascular diseases is well known, but they also have beneficial roles in cells. Developing a balance between the overproduction of ROS and its utilization is important in maintaining healthy redox processes within the cells.

2. Generation of reactive oxygen species

The main types of reactive oxygen species discussed in this paper are superoxide and hydrogen peroxide, both of which play a large role in cardiovascular diseases. Additionally, the production of hydroxyl radicals and singlet oxygen will be mentioned, as these are the most

reactive, and subsequently dangerous, of the ROS. Lastly the generation of peroxynitrite will be considered. See **Table 1** for an overview of the mechanism of generation for each.

Superoxide is produced by the one-electron reduction of molecular oxygen. Superoxide is then converted to hydrogen peroxide via the mitochondrial enzyme superoxide dismutase (MnSOD), or into diatomic oxygen [2]. Hydrogen peroxide itself is fairly unreactive, but plays a role in the Fenton reaction to generate hydroxyl radicals that can be damaging to cellular structures and molecules. Haber and Weiss demonstrated in 1934 that a superoxide molecule and a hydrogen peroxide molecule could interact with each other to produce these reactive hydroxyl radicals in the following net reaction: $\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$ [3]. Hydroxyl radicals can also be formed from reactions with hypochlorous acid, which is produced by the enzyme myeloperoxidase [4]. The production of hydroxyl radicals in tissue is significant for its contribution to a variety of pathologies, but also for the fact that it cannot be removed enzymatically in the same way that superoxide is converted back to oxygen via SOD.

There are many enzymatic pathways by which ROS can be generated in the cell. Most pathways involve the initial production of superoxide, which, as previously indicated, lead to the production of even more reactive compounds. Although some enzymatic systems "intentionally" generate superoxide, e.g., NADPH oxidases, this ROS is also a consequence of metabolism. Specifically, in aerobic cellular respiration, superoxide is a byproduct of oxygen utilization. The electron transport chain (ETC) of the mitochondria is a major source its generation. The ETC is made up of three complexes and an ATP synthase enzyme; it functions by transferring electrons through a series of electron carriers. The transfer of electrons is coupled with the release of protons into the intermembrane space of the mitochondria, creating an electrochemical potential, Δp, across the inner membrane, which drives the production of ATP [5]. However, when electrons are leaked from the complexes, instead of being transferred, these leaked species are those that reduce oxygen to form superoxide.

The first complex in the ETC is composed of a flavin mononucleotide group and is a significant site of production of superoxides. The donation of electrons is initially provided to the chain by NADH to the FMN, which subsequently passes them along a chain of FeS to the reduction site CoQ [6]. Superoxide is produced when FMN is fully reduced; its degree of reduction has been shown to be dependent on the ratio of NADH/NAD+, with the proportion

Reactive oxygen species	Mechanism of generation
Superoxide (O^-_2)	Reduction of molecular oxygen in the electron transport chain of mitochondria [4, 6], and other enzymatic routes: monooxygenase, NADPH oxidase, xanthine oxidase [8, 10]
Hydrogen peroxide (H_2O_2)	Converted from O^-_2 by enzyme superoxide dismutase (SOD) [18]
Hydroxyl radical ($^\bullet OH$)	Produced in Haber-Weiss reaction from O^-_2 and H_2O_2 [2]
Singlet oxygen ($^1O_2$)	Produced in reaction of hypochlorous acid (HOCl) and H_2O_2 [3]
Peroxynitrite ($ONOO^-$)	Produced in reaction of nitric oxide (NO) and (O^-_2) [15]

Table 1. Production of reactive oxygen species.

of FMNs reduced correlating to a higher ratio [7]. This has been confirmed with the experimental addition of rotenone, a complex I inhibitor; its function is to limit the transfer of electrons away from complex I, creating a condition where they are "backed up" onto the NADH and are available for superoxide generation [7].

The third complex in the ETC is also a significant site of superoxide generation. Complex III is where electrons are transferred from CoQ to the cytochrome *c*. Changes in the Δp or in the reduction state of CoQ are contributors to the production of superoxide at Complex III, as well as the addition of the inhibitor antimycin [5]. Compared to complex I, the superoxide generation at this site is less significant.

Outside of the ETC, ROS can be generated in the mitochondria by different means. In the event that complex I is inhibited, and 2-oxoglutarate is added as a substrate, there is still a high level of superoxide generated by the enzyme α-ketoglutarate dehydrogenase, which also contains a flavin subunit and utilizes the reduced NADH pool of electrons that complex I is unable to use [8]. Cytochrome P450 is another enzyme within the mitochondria that has been implicated in ROS production. Its regular function involves complex reactions converting cholesterols and other steps in steroid biosynthesis; it catalyzes monooxygenase reactions that require electrons from NADPH, which can "leak" and interact with diatomic oxygen to produce superoxides [9]. These are just two examples of a variety of mitochondrial reactions involving the utilization of electrons that produces ROS at this organelle.

Mitochondrial respiration is not the only source of ROS generation. Other sources of ROS production include the processes of the enzymes nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), xanthine oxidoreductase, and myeloperoxidase [10].

NADPH oxidase functions as a multi-subunit enzyme which, via electrons donated by NADPH, can reduce oxygen to superoxide. NADPH oxidase is well known in leukocytes, but also exists in other tissues in different forms, such as in vascular smooth muscle. The leukocyte NADPH oxidase is primarily found in polymorph-nuclear neutrophils, or PMNs, and its function is the generation and subsequent release of superoxide as a mechanism for combating bacterial infection. The vascular NADPH oxidase is mainly activated by angiotensin II, but also thrombin, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-alpha), and other mechanical stimuli such as shear stress and strain [11]. Its function in these cells correlates to the development of cardiovascular disease, but also the dismuatation of superoxide results in the production of the vasodilator, H_2O_2 [12].

Xanthine oxidoreductase has two interconvertible forms as an enzyme, both of which are essential to purine catabolism by oxidizing hypoxanthine to xanthine to uric acid. Its dehydrogenase oxidizes NADH to NAD, while in its oxidase form, the enzyme is capable of producing both superoxide and hydrogen peroxide from diatomic oxygen [13].

Myeloperoxidase is highly significant not for the production of superoxides but because hypochlorous acid is its main product [14]. HOCl is a key intermediate for the generation of many different ROS and is, in itself, highly reactive—it can react with superoxides to produce hydroxyl radicals, or with hydrogen peroxide to produce a singlet oxygen [4]. Singlet

oxygens are extremely reactive species. Hypochlorous acid itself is implicated in various cellular reactions as well, such as initiating lipid peroxidation, or oxidizing protein sulfhydryl and thioether groups on proteins [4, 15, 16].

Nitric oxide, which is in the family reactive nitrogen species, plays a significant role in vascular tone in the circulatory system. While its generation is not discussed here, it is important to mention because it greatly increases in toxicity when it reacts with superoxide to generate peroxynitrite (ONOO─), a powerful oxidant [17]. Peroxynitrite itself reacts slowly, giving it selective reactivity within the cell, giving it wide implications for cellular pathology [18]. Despite its high reactivity, it is highly stable with a negative charge delocalized over the whole molecule, providing the ability to be a highly influential oxidant. **Table 1** summarizes the generation of reactive oxygen species.

3. The "good side" of ROS

Reactive oxygen species (ROS) have been given a considerable amount of scrutiny due to the disease states that they have been linked to, such as aging, cancer, and atherosclerosis [19]. However, ROS is imperative for redox homeostasis, as well as proper function in the cardiovascular system, and immune system. The body requires a balance in its ROS levels for homeostasis. If the level of ROS exceeds that which the body can handle, then oxidative stress occurs [20]. On the other hand, if the level is too low, reductive stresses occur and can also cause pathologies ranging from cancer to cardiomyopathy [21].

Redox regulation is imperative for the body to maintain proper signaling processes. These redox reactions usually entail ROS interacting with the amino acid cysteine on proteins. ROS modulates cell proliferation and apoptotic pathways to ensure proper regulation of the cell cycle and programmed cell death. There are multiple kinases in these pathways that interact with ROS. The mitogen- activated protein kinase (MAPK) has a MAPKKK upstream called apoptosis signal regulated kinase 1 (ASK1). ASK1 regulates transcription factors JNK and p38, which can trigger apoptosis by phosphorylating MAPKK4,3, and cGMP dependent protein kinase (PKG) and protein kinase A (PKA) are both activated by ROS as well and are involved in the MAPK signaling process. ROS can also inhibit protein phosphatases through cysteine oxidation that prevents the inhibitory actions of the phosphatase on MAPK signaling. Consequently, transcription factors such as p38 can be regulated this way as well. Protein tyrosine phosphatase (PTP) is oxidized and inhibited by ROS and helps maintain appropriate levels of growth factor signals. Tyrosine phosphatases are affected by ROS in a manner consistent with our concept of the redox window. Physiological levels of H_2O_2 will activate tyrosine kinases through cysteine oxidation to sulfenic acid; however, high levels of ROS oxidize cysteine into sulfinic and sulfonic acids which lead to complete inactivation of the phosphatase through irreversible modification of the catalytic cysteine [22]. Another major signaling pathway, phosphoinositide 3-kinase (PI3K), is regulated by ROS through oxidation reactions. The body maintains a homeostatic level of ROS because ROS products activate antioxidant genes through mechanisms such as PI3K-NFE2-like2 (Nrf2)-antioxidant response element

(ARE) [23]. Ref-1, also known as redox factor-1, is an endonuclease that is regulated through transcription factors such as activator protein 1 (AP-1), p53, nuclear factor kappa B (NFkB) and hypoxia inducible factor 1 (HIF-alpha). When cytoplasmic Ref-1 is subjected to oxidative stress, by exposure to ROS, it moves to the nucleus and helps the redox factor interact with transcription factors so an antioxidant defense system can be initiated [23].

Many of these redox regulatory pathways are evident when examining the impact of ROS on collateral blood vessel growth, which is a major area of interest in all forms of vascular disease, e.g., peripheral artery disease, ischemic heart disease. A conundrum about the role of ROS in coronary collateral growth pertains to observations that too much ROS, and the concurrent oxidative stress, inhibits collateral growth. On the opposite side of the spectrum, too little ROS and the consequential reductive stress, also inhibits coronary collateral growth. This optimal "level" of ROS has been dubbed "redox window" [24], which is the level of the redox state that is optimal for growth factor signaling. p53 is thought to be the connection between redox dependent and growth factor dependent signaling. Angiogenesis is mediated through a transcription factors, such as nuclear factor kappaB, and ROS such as H_2O_2, NO and other oxidants$_7$. H_2O_2 is a major mediator of HIF-1a [25], a major transcription factor required for vascularization in ischemic settings [26]. Vascular endothelial growth factor (VEGF) activates NADPH oxidase to produce ROS. ROS produced through this process works in conjunction with VEGF to trigger endothelial cell migration and proliferation. Vascular NADPH oxidase, a generator of ROS, is also triggered by angiotensin II (Ang II), which is a key component of angiogenesis. The necessity of ROS to appropriately activate angiogenesis is yet another exemplification of the beneficial use of ROS in the body [27]. The thyroid hormone can activate angiogenesis by triggering transcription factors such as VEGF and HIF-1a [28]. Both of which were described above as producers of ROS that further propagate the angiogenesis process.

Vascular smooth muscle cells require ROS for appropriate cell growth [29]. PDGF and thrombin are both agonists to help cell proliferation, and both agonists require ROS in their mechanisms to amplify and further their signal for greater cell growth [30]. ROS also plays an important role in the expression of transcription nuclear factor-kB, which helps the body's inflammatory process by activating the monocyte chemotactic protein-1 (MCP-1) and interleukin-6 [31]. Many reactive oxygen species, such as H_2O_2, play a big part in vasomotor tone such as vasorelaxation in the pulmonary, coronary and mesenteric systems [32].

Reactive oxygen species has an important role in the immune system. A lack of ROS in the immune system can cause disease states that impair an individual's ability to fight against foreign invasion. The innate immunity that utilizes macrophages, neutrophils, and dendritic cells are key. These cells use toll like receptors to determine a cell that is foreign to the body, such as a bacterium. As a part of the innate immune system, macrophages, neutrophils and dendritic cells can phagocytose foreign material and then express it to the acquired immune system. The phagocytosis process is made possible by the use of reactive oxygen species. As previously mentioned, the ROS used in this process is made on the endosomes of the phagocytosing cells using NADPH oxidase. The immune system ensures the production of ROS when a foreign substance is detected due to the toll like receptor-4 binding to NADPH

oxidase. Such makes certain that when a foreign substance is detected in the body and it binds to the toll like receptor-4, the NADPH oxidase is consequently triggered to make sure there is ROS production to breakdown the foreign entity [31].

Nitric oxide a reactive nitrogen species that easily diffuses across most tissues, but has a difficult time being carried through blood because oxyhemoglobin breaks it down. However, due to its rapid diffusion rates, it reacts with superoxide with diffusion limited kinetics, resulting in the formation of the potent oxidant, peroxynitrite. Although by convention peroxynitrite is viewed as deleterious [17], it is noteworthy to add that this species is used by the immune system destroy bacteria. Macrophages produce peroxynitrite, and this mechanism was found to kill amounts of *Escherichia coli* in proportion to the amount of peroxynitrite produced in the macrophage [33].

When ROS production is not appropriate, many disease states can occur. Chronic granulomatous disease (CGD) is a rare hereditary disease where there is a defect in the NADPH oxidase. As a result, infections such as pneumonia and osteomyelitis can occur. Since the body cannot fight the infection, it creates granulomas around the infections. The treatment for the disease is designed to help the immune system through antibacterial, antifungal and immunomodulatory therapy. Stem cell transplants and gene therapy are both definitive treatments used as the first clinical interventions [34].

All some reactive oxygen species such as superoxide and hydrogen peroxide have beneficial effects (at physiological levels), some reactive oxygen species, such as the hydroxyl radical, react and form bonds with almost all organic molecules in the body. As a result, this particular species is exclusively deleterious when produced within a cell. When the ROS levels deviate away from the "redox widow," imbalance in these systems occurs and detrimental consequences can be triggered [24].

However, while redox homeostasis of ROS is imperative for normal bodily function ranging from an effective immune system to angiogenesis, an imbalance in said homeostasis is not always terrible. When the body experiences acute trauma such as a hemorrhage, the renin-angiotensin system comes into play [35]. Angiotensin II helps constrict the blood vessels to increase the blood pressure that considerably drops due to the loss of blood [36]. Angiotensin II activates NADPH oxidase in smooth muscle cells causing a production of superoxides [37]. The superoxides partake in the angiotensin II mechanisms. However simultaneously there is a decrease in the nitric oxide present in the blood because NO scavengers, such as NOX, actively eliminate them [38]. By increasing the levels of superoxides and decreasing the levels of NO, vasodilation is minimized and platelet coagulation is much more effective [39]. A hemorrhage is a wonderful example of how the body naturally handles crises by tipping the redox homeostatic scale toward a greater production of superoxides.

4. The "bad side" of ROS

The production of mitochondrial reactive oxygen species (ROS) is found in both physiological and pathological conditions. When ROS production increases above basal level, however,

the excessive amounts of ROS can lead to pathologies ranging from autoimmune diseases to cardiomyopathies. As mentioned previously, there are numerous sources of ROS in a cell that may occur in cytosolic, extracellular, and mitochondrial domains. The relative amounts of mitochondrial ROS produced are indicative of the metabolic needs of the cell by acting as a mode of cell signaling [40]. At lower levels of production, the presence of ROS may be beneficially used as a metabolic response to hypoxia by regulating the stability of HIF-1a. Medium levels of ROS production are more indicative of an inflammatory response by activating mitogen-activated protein kinase (MAPK) and proinflammatory cytokines. Excessive levels of ROS production, however, become pathological, and may lead to mitochondrial and cell apoptosis through activation of the apoptosome protein complex. Interaction of apoptosis activating factor (APAF-1) with mitochondrial cytochrome c plays in integral role in activation of the apoptosome, which will then lead to the activation of a chain of apoptotic caspases. The decision of whether the cell enters a state of inflammation or apoptosis, dictated by relative amount of mitochondrial ROS found within the cell, highlights the importance of ROS in choosing which cell signaling pathway will proceed. Overproduction of ROS is observed to be the cause of inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, and atherosclerosis by over-activating MAPKs [40].

This state of overproducing ROS may be stimulated by a multitude of enzymes. An example includes myeloperoxidase (MPO), a subfamily of peroxidases, due to its role in producing hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2) during an immune response. MPO's, unique to neutrophils and monocytes, are active in respiratory burst, a cytotoxic mechanism to kill pathogens and bacteria. Excessive production of HOCl, however, may cause oxidative damage, apoptosis and inflammatory disease. The clinical significance of excessive ROS production through MPO can be seen in its role in the formation of nitrotyrosine in endothelial regions of inflammation, impairment of NO-dependent relaxation of blood vessels, and inactivation of select neutrophil granule contents during inflammation, which may then to a prolonged respiratory burst. These detriments are apparent in pathologies associated with MPO defects, such as atherosclerosis and plaque formation, multiple sclerosis and Alzheimer's disease [4].

When produced above basal levels necessary for cell signaling and transduction, the cell requires specific mechanism to eradicate ROS in order to return to physiological conditions. The toxic effects of excessive mitochondrial ROS production necessitate that the cell has developed antioxidant mechanisms to scavenge them after generation. These mechanisms to counteract ROS production include the use of the Superoxide Dismutase (SOD) family, which catalyze the initial reaction of O_2—to hydrogen peroxide (H_2O_2), a product that will eventually reduce to water through glutathione peroxidase and catalase. The SOD family, found in three isoforms, can be found within the cell cytoplasm, mitochondria and nucleus. The isoform SOD2 exemplifies the detrimental effects of overproduction of ROS: the removal of mitochondrial SOD2 in species such as yeast, flies and mice is associated with cardiomyopathy, aging and early death, and atherosclerosis. The SOD2 gene codes for the protein Manganese SOD (MnSOD), and is found within the inner mitochondrial membrane and dismutates superoxide anions, produced by the mitochondrial electron transport chain, into H_2O_2. Overproduction or incomplete metabolism of these superoxide anions can cause

oxidative damage. The importance of MnSOD was reinforced in a study where knockout mice for SOD2 displayed increased mitochondrial oxidative damage and cell apoptosis. The mice deficient of MnSOD died within 1 week due to either dilated cardiomyopathy or neurodegeneration [11]. Deficiencies in other members of the SOD family did not demonstrate the same severity in oxidative damage as SOD2. Additionally, MnSOD and SOD2 play imperative roles in the intrinsic pathway for apoptosis, through its involvement in mitochondrial permeability transition. Mitochondrial permeability transition utilizes mitochondrial cytochrome c, APAF-1 and caspase 9 to ensure cell death, an outcome possible from excessive production of ROS [41].

In physiological conditions, the presence of the ROS superoxide can be quickly eradicated by the presence of these SODs; however, when synthesized in close proximity to NO, the toxic radical peroxynitrite (ONOO-) may be spontaneously formed. The reaction, which does not require an enzyme, may outcompete the scavenging capabilities of SOD [18]. Peroxynitrite, once formed, can cross cell membranes through both anion channels or passive diffusion and reacts selectively throughout the cell by nitrating tyrosine residues on proteins [17]. The presence of nitrotyrosines will alter the conformation and function of proteins such as neurofilaments and actin, leading to pathologies such as atherosclerosis, myocardial ischemia and irritable bowel syndrome [17]. Additionally, peroxynitrites can oxidize the heme groups of various proteins, including hemoglobin, myoglobin and cytochrome c [18]. By reacting in the same manner with inducible NOS, peroxynitrite can alter negative feedback of itself in inflammatory conditions. Peroxynitrite may also damage DNA through oxidation of bases and the DNA backbone, and contribute to apoptosis. During reperfusion or states of inflammation, the mitochondrion produces higher levels of NO. NO has the effect of inhibiting complex IV of the electron transport chain, increasing electron leakage, and consequently, the formation of superoxide. Therefore, the subsequent increase in peroxynitrite from NO and superoxide, can cause mitochondrial oxidative damage and increase the amount of free radicals present [18]. These elevated concentrations of peroxynitrite and superoxide are found in endothelium due to the uncoupling of endothelial NOS and vascular NADPH oxidase. In pathological conditions, due to excessive ROS production, NO is altered to become ONOO─, preventing endothelial-dependent relaxation, and causing endothelial dysfunction. The initial adverse event due to the decrease in NO bioavailability is impaired endothelium-dependent vasodilation, which may spiral into long-term cardiovascular complications due to the decreased vasorelaxation. This dysfunction in vascular endothelium is then associated with pathologies ranging from hypertension, preeclampsia, and atherosclerosis to coronary artery disease [42].

The premise that inadequate scavenging of excessive ROS is detrimental to normal cellular function is reinforced by the existence of multiple antioxidant mechanisms, such as glutathione peroxidase (GPx). Decreased efficiency of GPx, which catalyzes the reduction of H_2O_2 to water using NADPH as a substrate, may lead to pathologies such as atherosclerosis and vascular inflammation [2]. Additionally, deficiencies in antioxidant enzymes such as peroxiredoxin and mitochondrial thioredoxin 2 (Trx2) lead to mitochondrial apoptosis and vascular pathologies and myocardial infarction. Knocking out these imperative enzymes within mice models demonstrate that insufficient removal of ROS due to inefficient antioxidant mechanisms will also lead to excessive ROS amounts and its damaging effects.

Other proteins involved with the cellular response to stress and in physiological conditions include the family of heat shock proteins. Heat shock proteins (HSP's), such as HSP70 and HSP27, are observed as stress-response proteins induced by ROS through the JAK-STAT pathway [43]. In normal, physiological conditions, HSP's serve as molecular chaperones required to prevent improper folding of proteins found within a cell. Their synthesis, however, is increased in response to environmental stresses in an attempt to prevent protein aggregation. Additionally, HSP's are observed to be directly involved in the signaling pathways that lead a cell to undergo apoptosis in response to stress. For example, the overproduction of the reactive oxygen species H_2O_2 activates the JAK-STAT pathway that leads to HSP70 production. The beneficial expression of HSP70 in response to ROS-induced stress was exemplified in the myocardium of transgenic mice [44]. Following a period of ischemia, the correction of metabolic acidosis and re-establishment of correct phosphate stores due to the presence of HSP70 exemplify its protective effects against cardiomyopathy. Likewise, the reduction of expression of heat shock protein 27 inhibits the regulation ROS-induced apoptosis in cardiomyocytes [45]. This observation is exemplified by the overproduction of Hsp27 in the rat cardiac cell line H92c in comparison to a control, and the consequent suppression of H_2O_2-induced injury and apoptosis and the protective increase in phosphatidylinositol 3-kinase (PI3K)—protein kinase B(Akt) pathway activation when plentiful amounts of Hsp27 are present [45]. Both Hsp70 and Hsp27 serve to demonstrate the integral role of heat shock proteins in both physiological conditions and stressful conditions such as ROS-induced oxidative stress.

The impact of the presence of excessive ROS may also be found in its role in protein post-translational modification, in both irreversible or reversible protein oxidative modifications. The interaction between ROS, reactive nitrogen species (RNS), and amino acid residues has been observed to lead to aging and protein dysfunction. Commonly, these post-translational modifications occur most readily on the thiol (—SH) functional group found on cysteine residues: the electron rich sulfur atom within the thiol group allows for the oxidization a cysteine to sulfenic, sulfinic or sulfonic acid in addition to other oxidative posttranslational modifications (Ox-PTM) such as nitrosylation, sulfhydration, glutathionylation, and sulfenylation [46]. When ROS/RNS react with the thiol through nitrosylation, studies have found the reaction serves a function for cardioprotection [47]. More specifically, nitrosylation of cysteine residues acts as a barrier during periods of oxidative stress against further modification and oxidative damage, and may therefore lead to a faster recovery time [47]. ROS/RNS reactions with the thiol through glutathionylation as a reversible Ox-PTM have been previously linked with neurodegenerative and cardiovascular disease. ROS also have the ability to form thiyl radicals (RS.) to react with thiolates and form disulfide bonds, causing static protein conformations. The formation of disulfide bonds can alter the geography of the protein, and change the conformation and therefore function of the protein itself. Sulfenylation, a highly reactive and unstable form of modification has been associated with irreversible oxidative damage and apoptosis of a cell. Other modifications can include carbonylation, and phosphorylation [48]. These modifications may alter the polarity of the amino acid, ultimately modulating cell signal transduction and its downstream effects. In addition, the modifications may alter metal cofactor interaction and may inadvertently affect inhibitor reactions and impact physiological and drug reactions. A common effect of protein carbonylation, specifically, may be protein inactivation; the inactivation of membrane

transporters such as glucose (GLUT) transporters and Na+-K+ ATPases may lead to a multitude of neurodegenerative disorders. Additionally, oxidation of residues such as methionine to a sulfoxide may occur as well, which may serve to decrease cell signaling, and cause phosphorylation [49]. The inactivation of important antioxidant mechanisms such as glutathione peroxidase and thioredoxin due to protein modification may occur and act to aggravate oxidative stress within a cell. Other modifications may induce the phosphorylation of HSP27 in an attempt to prevent the unfolding and degradation of imperative proteins necessary for cell metabolism [45]. The fluctuations in ROS production, which distinguishes physiological metabolism from pathological metabolism, is revealed through the relative amount of Ox-PTM of critical cysteine thiols due to its role in regulation of oxidative stimuli.

The amount of mitochondrial ROS ranging from lower, basal physiological levels to excessive pathological levels highlights the importance of ROS and its maintenance. Excessive ROS production or inadequate scavenging by the cell's antioxidant mechanisms may cause a multitude of complications within a cell, leading to mitochondrial oxidative damage or cell apoptosis. The overproduction of ROS by MPO demonstrates how excessive amounts of HOCl may lead to pathologies such as atherosclerosis, while deficiencies in SOD2/MnSOD, GPx, peroredoxin, and Trx2 demonstrate how inadequate scavenging may lead to vascular and inflammatory complications as well. The role of HSP's to prevent protein aggregation caused by ROS accumulation underscores the importance of cell signal transduction pathways in response to excessive ROS. The detrimental outcomes of high levels of ROS can be seen through the multitude of effects due to oxidative protein post-translational modifications. ROS, both good and bad, can widely affect the intricate network of a variety of distinct proteins found within a single cell.

5. Conclusions and gaps

We hope the readers understand the there are two sides to reactive oxygen species—a "good side" and a "bad side." We opine that if one reads the literature, ROS are equated with pathology, and adverse consequences. Our goal is this chapter was to reinforce the concept that these species have many important physiological actions. The largest gap we see in our understanding of ROS and their actions pertains to defining the boundaries of the redox window. This will be important to study and understanding since the boundary marks the transition of ROS from being beneficial to being detrimental.

Author details

Roma Patel, Lindsey Rinker, Joanna Peng and William M. Chilian*

*Address all correspondence to: wchilian@neomed.edu

Department of Integrative Medical Sciences, Northeast Ohio Medical University, USA

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Chapter 3

Role of Antioxidant Phytochemicals in Prevention, Formation and Treatment of Cancer

Abdurrahim Kocyigit, Eray Metin Guler and Murat Dikilitas

Additional information is available at the end of the chapter

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Abstract

Reactive oxygen species (ROS) played an important role in cancer. Although low levels of ROS can be beneficial in normal physiological functions, chronic exposure to ROS is associated with increased risk of cancers. Increased ROS levels can also induce apoptosis and cell death in various types of cancer. Taken together, the role of ROS in cancer prevention, formation and therapy is extremely complex and very challenging to study. Although the antioxidant activity of phytochemicals is well recognized and generally used to prevent cancer, they can have pro-oxidant and ROS generating activities under certain conditions, especially at high doses or in the presence of metal ions. The basal redox levels of cancer cells are also different from those of normal cells. Therefore, higher levels of free form of metal ions and higher levels of endogenous ROS production in cancer cells sensitizes them to phytochemicals mediated pro-oxidant cytotoxicity. In conclusion, people tend to intake of antioxidant phytochemicals for the detrimental effects of ROS. However, excessive intake of phytochemicals could have cancer development or therapeutic potential by generating ROS. In this section, the role of phytochemicals in the prevention, development and removal of cancer has been discussed.

Keywords: phytochemicals, cancer, pro-oxidant, reactive oxidant species

1. Introduction

Cancer is already a major health problem and is the second leading cause of death in the world. With a 1% increase every year, when it comes to 2030, there will be 26.4 million new cases of cancer and about 17 million cancer deaths per year. For this reason, it is necessary to develop effective chemopreventive strategies when human life expectancy and environmental

conditions are taken into consideration [1]. In addition to genetic factors such as hereditary mutations, hormones and immune conditions, environmental factors such as tobacco, diet, radiation and infectious organisms are the major cause of cancer formation. These factors modulate important cellular elements, including genes such as proto-oncogenes, tumor suppressor genes and DNA repair genes, through cellular intermediates [2].

Oxidative stress is a key component of environmental toxicity during the cancer process and reactive oxygen species (ROS) are generated in response to both endogenous and exogenous stimuli [3]. ROS such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) singlet oxygen ($^1O_2$) and hydroxyl radical ($HO^{\cdot}$), are well known to be cytotoxic and have been implicated in the etiology of a wide array of human diseases, including cancer [4]. Various carcinogens show their effect by forming ROS during their metabolism [5]. Oxidative DNA damage can cause mutations and can, therefore, play an important role in the initiation and progression of carcinogenesis [6]. For this reason, the antioxidant balancing function against elevated ROS levels is important for many diseases, including various cancers [7]. Researchers have noticed that in recent years, the role of ROS depends on their level. While a moderate amount of ROS is required for tumor formation, excess ROS serves to kill tumor cells [8].

The relationship between dietary antioxidants and non-communicable diseases (cancer, cardiovascular diseases, and cataracts) is largely based on epidemiological studies. These studies have shown that there is potential for cancer prevention in plant foods and phytochemicals. Hence, in recent years, studies on phytochemicals promoting healthy and disease-preventing potential have increased [9]. The interest in plants and phytochemicals in recent years has increased not only for cancer but also for the prevention of chronic diseases such as cardiovascular diseases. The vast majority of the studies are investigations of the antioxidant properties of phytochemicals [10]. However, some of these phytochemicals act as antioxidants, as well as act as pro-oxidants and ROS-producing agents that cause oxidative stress in high doses or metal ions, especially in the presence of iron and copper [11–13]. In this regard, polyphenols known as antioxidants such as quercetin, epicatechins, and epigallocatechin-3-gallate (EGCG) and gallic acid have also been shown to produce ROS by pro-oxidant activity in cell models [14–16].

While lower levels of ROS are required for signal transduction and cell proliferation, moderate exposure to chronic ROS has been shown to degrade the antioxidant defense system in favor of oxidants, leading to oxidative modification of DNA bases and carcinogenesis [17]. It has been commonly accepted that oxidative damage by DNA is one of the most important causes of cancer [18]. For example, green tea is advised as a healthy drink due to possessing of chemicals which inhibit cancer development [19]. However, when it is consumed very frequently (>1 l/d), it has been associated with increased incidence of esophageal cancer in some countries such as northern Iran or India, even though this has been proposed to be due to consumption of hot tea [20, 21]. It has been shown that green tea can produce H_2O_2 in the mouth cavity [22]. It has also been shown that people who took 20 mg/kg β-carotene or 30 mg/day β-carotene and 25,000 IU retinyl palmitate supplementation alone developed the incidence of lung cancer in smokers [23, 24].

Various methods are used in cancer treatment including chemotherapy, radiotherapy and/or surgery, and chemotherapy is one of the basic modalities in the treatment of cancer patients [25].

Most of the chemotherapeutic and radio therapeutic agents kill cancer cells by increasing ROS [3], and induce either necrosis or apoptosis of tumor cells [26, 27]. However, they have a number of side-effects that can limit their efficacy [28, 29]. Many of the anticancer agents are also carcinogenic themselves and the patients may suffer secondary cancers following primary remission from the initial tumor [30]. For this reason, studies focused on plant-derived compounds or their active ingredients with low toxicity and high selectivity for killing cancer cells kill plant-derived compounds or cancer cells. In the United States, about 50–60% of cancer patients are treated with chemotherapy and/or radiation therapy concurrently or alone with phototherapeutic agents that have been confirmed for their anticancer activities [31]. There are sufficient evidences to support phytochemical-mediated production of ROS [3], a pro-oxidant action that is responsible for their ability to induce apoptosis in cancer cells [32, 33]. It has been demonstrated that curcumin and ascorbic acid have cytotoxic, genotoxic and apoptotic effects on various cancer cells by preclinical and clinical studies [34, 35].

In this chapter, we have tried to explain how phytochemicals derived from plants behave like double-edged swords acting as antioxidants or prooxidants according to their dose and environment and how they play a role in cancer prevention, formation and treatment.

2. Role of reactive oxygen species in cancer

2.1. Molecular basis of reactive oxygen species

Comprehensively, ROS can be divided into free radicals and non-radical molecules. Although free radicals contain one or more unpaired electrons in the outer orbitals of the molecules, non-radical ROS do not contain mismatched electrons, but they are chemically active and readily convert to free radicals. Superoxide, H_2O_2 and hydroxyl radicals are the most common ROS and studied in cancer. ROS sources are both exogenous and endogenous [36].

Sources of exogenous ROS are food, tobacco, smoke, drugs, xenobiotic, radiation, and other mediators. Ionized radiation causes ROS production through interaction with water. Upon interaction, an electron is lost and, in turn, a hydroxyl radical ($HO^{\cdot}$), hydrogen peroxide (H_2O_2), a superoxide radical ($O_2^{-\cdot}$) and eventually oxygen (O_2) [37]. ROS are also produced endogenously in the cell through multiple mechanisms, including mitochondria, peroxisomes, endoplasmic reticulum and NADPH oxidase (NOX) complex in cell membranes [38, 39]. Mitochondria contain the electron transport chain that transfers electrons from succinate to NADPH during respiratory ATP synthesis. During ATP synthesis, the leakage of electrons from the electron transport chain causes the molecular oxygen to be reduced to the superoxide [40]. The superoxide produced from the mitochondria passes from the mitochondrium to the cytoplasm, exiting through the pores in the outer mitochondrial membrane [41]. Superoxide is converted into H_2O_2 both in mitochondrial matrix (Mn-SOD) and cytosol (with Cu-ZnSOD) [41]. Peroxisomes are also crucially important organelles as mitochondria for the production of superoxide and H_2O_2 with the action of various enzymes such as catalase and xanthine oxidase [42]. H_2O_2 is then converted into water by catalase or it can be converted to highly reactive hydroxyl radicals in the presence of transition metals [43]. Superoxide is also able to react with reactive nitric

oxide (NO) forming peroxynitrite (ONOO–) [44]. Another source of ROS is NOX localized in various parts of cellular membranes [45].ROS are also produced during the process of protein folding and disulfide bond formation in the endoplasmic reticulum. Glycoprotein endoplasmic reticulum oxidoreductin 1, protein disulfide isomerase and NOX4 are the main sources of ROS in the endoplasmic reticulum [46]. Under normal physiological conditions, the cells try to stabilize by eliminating the ROS production with the cleaning system [47]. Detoxification of ROS is facilitated by non-enzymatic molecules (e.g., Glutathione, flavonoids and vitamins A, C and E) or antioxidant enzymes that metabolize different ROS products.

SOD is a metalloenzyme that catalyzes the conversion of superoxide anion to H_2O_2. SOD uses metal ions such as copper (Cu^{+2}), zinc (Zn^{+2}), manganese (Mn^{+2}) or iron (Fe^{+2}) as cofactors. "Different SOD enzymes are found in different compartments of the cell and are highly specific in regulating bound biologically bound processes" [48]. Catalase is an enzyme that facilitates the decomposition of hydrogen peroxide into water and the free oxygen molecule. The major localization of catalase in most eukaryotes is cytosol and peroxisomes [49]. Peroxiredoxins are thioredoxin peroxidases, which catalyze the reduction of hydrogen peroxide, organic hydroperoxides and peroxynitrite [50]. Glutathione has a significant role in cellular signaling and antioxidant defense system. It reacts directly with ROS and reactive nitrogen species (RNS) and is responsible for the detoxification of free radicals, membrane protection, metabolic regulation, modulation and signal transduction. The glutathione system involves reduced (GSH) and oxidized (GSSG) forms of glutathione. The enzymes required for the system includes glutathione reductase (GR), glutathione peroxidase (GPX), glutathione S-transferase (GST) [51]. Glutathione protects the cells against oxidative stress by reducing the disulfide bonds of cytoplasmic proteins to the cysteines. It is mostly synthesized in the cytosol of the cells and prevalent in most of the cells. It is then oxidized to glutathione disulfide. However, the oxidized form, GSSG, is predominant in endoplasmic reticulum. Glutathione peroxidase (GPx) is an antioxidant enzyme that effectively reduces H_2O_2 and lipid peroxides to water and lipid alcohols [52]. Glutathione reductase converts GSSG to GSH [53]. Under physiological conditions, almost all of the glutathione are in reduced form because of a constitutive activity of glutathione reductase in cells, and the glutathione S-transferases (GSTs) are detoxifying enzymes that catalyze the ligation of various exogenous and endogenous electrophilic compounds [54]. GSTs are overexpressed in a variety of tumors to regulate MAPK pathways and also play a role in the development of resistance to chemotherapeutics [55].

Normally, the human body naturally tries to compensate by producing endogenous or exogenously produced antioxidants against endogenously produced or exogenously taken oxidants. Endogenous and exogenous antioxidants act as "free radical scavengers" by preventing and repairing damages caused by ROS [7]. However, under oxidative stress conditions, excessive ROS can damage cellular proteins, lipids and DNA and cause deadly lesions in the cells, which contribute to many human diseases, including cancer [56].

2.2. Role of reactive oxygen species in tumor formation

Cancer is a major health problem in almost all parts of the world. It could be resulted from both internal and environmental factors. These factors such as inherited mutations,

inefficient immune system, smoking, bad diet,, infectious organisms, etc., are able to modulate our genes, especially tumor suppressor genes and DNA repair genes [8]. Cellular intermediates, along with unstable structures, affect cellular signaling pathways through transcription factors. These are nuclear factor-kappa B (NF-κB), signal transduction and transcription activator (STAT)-3, hypoxia inducible factor (HIF)-1α, kinases, various growth factors, cytokines and other proteins [56]. Extensive research showed that ROS has crucially important roles in modulating genes. Although low levels of ROS can be beneficial to cell, however, excessive accumulation of ROS could modify cell signaling pathways through the transcription factors [57]. Although ROS is balanced via endogenous antioxidant defense system, sometimes exogenous antioxidant needs to be supplied to counterbalance ROS-mediated injury. However, when oxidative status arises due to inefficient antioxidant system, chronic or cumulative oxidative stress eventually causes deleterious modifications in macromolecules such as protein, lipid and DNA [3]. ROS can also react with other cellular components such as phospholipids and proteins and result in the generation of secondary reactive intermediates and causes irreversible DNA bases by forming DNA adducts [58]. Formation of DNA adducts is the main step of carcinogenic process because, if such adducts cannot be repaired, they may lead to DNA damage and eventually to mutations [59]. Oxidative lesions play an important role in the etiology of cancer and (8-oxo-dG) lesions can be used as a critical biomarker for oxidative DNA damage [60, 61]. ROS can cause 8-OHdG in DNA and cause GC → TA transversions [62]. Therefore, 8-OHdG is widely used as biological markers of oxidative stress in studies of antioxidants and diseases associated with ROS [63]. Excess ROS levels cause DNA mutations such as GC → TA transversion mutations [64], single strand breaks and instability [65]. In human tumors, transversion from G to T is the most common mutation of the p53 repressor gene [66]. Excessive ROS levels can also increase carcinogenesis by inducing and sustaining oncogenic phenotypes of cancer cells [67, 68]. ROS are associated with three stages of carcinogenesis, initiation, promotion and progression. In cancer formation, ROS contributes to the initiation of nuclear or mitochondrial DNA mutations, including point mutations, deletions, chromosomal translocations, and others [69, 70]. The initiation stage transforms normal cells into cancer cells. Following the initiation stage, the initiated cells are expanded into colonies in the promotion stage, accompanied by cell proliferation and/or inhibition of apoptosis in this stage [7]. ROS promote the expansion of malignant cells by regulating cell proliferation/apoptosis-related genes and transcription factors such as nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1), nuclear factor erythroid 2-related factor 2 (Nrf2), and hypoxia-inducible factor (HIF) [67, 71, 72]. Compared to normal cells, ROS levels have increased as a consequence of their metabolism in cancer cells, and they have a modified redox status to preserve malignant phenotypes [73].

Compared to normal cells, cancer cells are loaded with more ROS. Therefore, they are more vulnerable to further ROS attack produced by exogenous ROS-generating factors (prooxidants) [74]. The role of ROS in cancer cells is described as "live by the sword, die by the sword" by Schumacher [75] or "a breath of life and death" by Fruehauf and Meyskens [76]. Therefore, prooxidant strategy should well be exploited to develop anticancer agents [77]. Although this strategy has not commonly been followed in conventional medicinal chemistry and is opposite to antioxidant therapy, however, it has promising sites that several ROS producing natural

compounds such as phenethyl isothiocyanate [78], piperlongumine [79], curcumin [80] and parthenolide [81] are able to kill cancer cells efficiently. Recognizing the importance of ROS in cancer therapy, Jim Watson wrote: "The vast majority of all agents used kill ROS, directly or indirectly, cancer cells and produce ROS that inhibits important steps in the cell cycle" [74].

3. Phytochemicals

3.1. Phytochemicals and their presence in foods

Phototherapy is the use of plant-based materials to prevent and treat diseases or to promote healthy life [82]. The word "phyto" comes from the Greek word for plant meaning, so phytochemicals mean plant chemicals. Phytochemicals are the bioactive non-nutrient plant chemicals found in fruits, vegetables, grains and other plant foods that have health-related effects. Vegetables and fruits consumed fresh or processed are the most important sources of phytochemicals necessary for human nutrition. Up to now, about 200,000 phytochemicals have been identified and 20,000 of them are derived from fruits, vegetables and grains [83].

Phytochemicals can be classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds and organosulphur compounds [84].These compounds are secondary metabolites with a variety of identifiable structures and are common features of the benzene ring and one or more hydroxyl groups. Generally, they are classified as flavonoids (anthocyanins, flavan-3-ols, flavonols, proanthocyanidins or flavones, non-hydrolyzable tannins, isoflavones and flavanones) and non-flavonoids (hydroxycinnamic, hydroxybenzoic acid, hydrolyzable tannins, benzoic acids and stilbenes) [85]. Phytochemicals are essential for the growth and reproduction of plants, and are produced as a response for defending plants against pathogens and stress in general [86]. In the last decade, the results of many researches have shown that phytochemicals have also a positive effect on human health. In general, phytochemicals, which are secondary metabolites found in plant foods such as alkaloids, phenolic compounds (flavonoids, isoflavonoids and anthocyanins) and terpenoids, have gained importance due to antioxidant, antiviral, antibacterial and anticancer effects [87]. Preclinical and clinical studies demonstrated that especially phenolic compounds have antimicrobial, anti-inflammatory, antioxidant, antiviral, anti-allergic, anticancer, anti-ulcer, antidiabetic, anti-plasmodia, antihypertensive and anticonvulsant effects [83]. In addition, food scientists and nutritionists think that consuming phytochemicals as part of a normal human diet is important for a healthy lifestyle [88].

3.2. Phytochemicals and its antioxidant/prooxidant action

A number phytochemicals, especially phenols and flavonoids, are found naturally in food and can behave like antioxidants [89]. Most plant foods contain phenols and flavonoids. Green leafy vegetables, fruits and yellow vegetables are especially rich in carotenoids, flavonoids and vitamin C vitamins. Vitamin C and vitamin E prevent the formation of carcinogenic nitrosamine [90]. Turmeric (*Curcuma domestica*), widely used in Indian food, contains a curcumin active ingredient, which is a strong antioxidant and a yellow coloring feature that can provide

protection against cancer [91]. There have been several recent epidemiological studies that implicate dietary antioxidant phytochemicals such as carotenoids [92], phenolic compounds [93] and flavonoids [94] as protective agents against cancer and cardiovascular disease. Many studies have been conducted on how phytochemicals such as vitamin C and E, carotenoids, flavonoids and phenolic acids show anticarcinogenic effects [95–97].

It is known that the concentration of antioxidant micronutrients such as vitamin C, vitamin E and carotenoids changes between high micromolar and low millimolar levels in human plasma and organs, while polyphenol concentrations are at high nanomolar to low micromolar levels [14]. However, despite their low levels, polyphenols have been reported to be more effective against oxidative stress than vitamin C [14]. In this respect, it has been suggested that phenolics are among the most active substances from natural sources, displaying a variety of health-promoting properties such as cytoprotective, antibacterial, antiviral, antiaging and anti-inflammatory effects [98, 99]. Some phytochemicals such as catechin and quercetin may show antioxidant effects not only due to molecular structures, but also by activating signaling pathways such as Nrf-2 [100].

Although the antioxidant activity of phytochemicals is well recognized [98, 99, 101], they can also display prooxidant activities under certain conditions, such as at high doses or in the presence of metal ions [14, 102, 103]. Prooxidant or antioxidant activity has been shown to be dependent on the concentrations of phytochemicals and, in this context, studies using cell models have emphasized the prooxidative activities of polyphenols known as antioxidants such as quercetin, epicatechin and catechins containing epigallocatechin-3-gallate (EGCG) [14, 15, 104, 105]. For example, at high doses, quercetin (50 μM) has been shown to enhance the production of superoxide radical (O_2^-) in isolated mitochondria and cell culture medium [104]. In another study, quercetin has been shown to reduce cell survival and viability, thiol content, total antioxidant capacity and SOD, CAT and glutathione transferase activity at higher concentrations (> 50 μM), while antioxidant activity of quercetin is observed only at low doses (0.1–20 μM) [15]. It has also been shown that flavonoids present in high concentrations can produce ROS with autoxidation (e.g., miksetin and quercetin) and redox cycling (e.g., quercetin) [106, 107].

In addition to the antioxidant concentration, prooxidant activity has been reported to be directly proportional to the total number of hydroxyl groups in a flavonoid molecule, and the presence of metal ions plays an important role [108]. Phytochemicals containing mono- and dihydroxy-flavonoids showed no significant prooxidant activity, while compounds containing multiple hydroxyl groups, particularly in group B, have been shown to significantly increase hydroxyl radical production by the Fenton reaction [109, 110]. Galati et al. [16] found that EGCG was isolated in the presence of transition metals and caused oxidative damage to cellular DNA. In the presence of metal ions owing to their reducing capacity and forming chelates, antioxidants act directly on free radicals (−R·) by a scavenging process characterized by the donation of hydrogen atoms (resulting in the formation of –RH) or electrons (resulting formation of –R–) [111]. However, strong reducing power of antioxidants may have potential to affect metal ions such as Fe^{+3} and Cu^{+2}, because they increase their ability to form highly reactive HO^{-}· radicals, originating from peroxides via Fenton's reaction [101, 112].

$$\text{Antioxidant (AH)} + Fe^{3+} \text{ (or } Cu^{2+}) \rightarrow A\cdot + Fe^{2+} \text{ (or } Cu^{+}) + H^{+} \quad (1)$$

$$H_2O_2 + Fe^{2+} \text{ (or Cu+)} \rightarrow HO\cdot + HO^{-} + Fe^{3+} \text{ (or } Cu^{2+}) \quad (2)$$

$$Fe^{2+} + O_2 + H_2O \rightarrow Fe^{3+} + HO_2\cdot + HO^{-} \quad (3)$$

$$Fe^{2+} + HO_2\cdot + H_2O \rightarrow Fe^{3+}\,HO^{-} + H_2O_2 \quad (4)$$

The antioxidant phenolic compounds, when scavenging the free radicals, can form less reactive phenoxyl radicals and are stabilized by delocalization of unpaired electrons around the aromatic ring [113]. However, even though these radicals are relatively stable, they may also show prooxidant activities [16]. However, it should be emphasized that natural compounds may have harmful effects as well as beneficial effects (independent of their anti-oxidative properties); for example, inflammation processes, activation of certain cellular pathways such as nitrogen and dicarbonyl metabolisms [16, 114]. It has been demonstrated that β-carotene at low doses exhibited antioxidant [115] and anti-inflammatory [116] properties in human HL-60 cells. However, at high doses, it exhibited prooxidant activity [115] and pro-inflammatory effects [116].

4. Chemopreventive role of phytochemicals in cancer

4.1. The role of phytochemicals in cancer prevention

Epidemiological studies have shown that the consumption of fruits and vegetables regularly reduces the risk of developing chronic diseases such as cancer and cardiovascular diseases [117]. The data suggest that people fed on an antioxidant-rich diet have a higher risk of chronic diseases and mortality than those who consume less fruits and vegetables. In a cohort study, Serafini [118] suggest that high intake of antioxidant-rich fresh fruits, root vegetables and vegetables is associated with a reduction in mortality and antioxidant-rich nutraceuticals have a protective effect on cancer development.

While the biological functions of polyphenols and/or metabolism in the human body are not completely known, there is consensus that antioxidant activity of flavonoids may be a combination of metal chelating and free radical scavenging properties [118]. Therefore, the structure of polyphenols enables them having free radical scavenging activity. Degree of methoxylation and the number of hydroxyl groups are important factors enabling them to have antioxidant properties. As for phenolic acids, inhibition of oxidation is associated with the cleavage of alkoxyl and peroxyl radicals, the cleavage of metal ions by the orthodihydroxy phenolic structure, and the production of α-tocopherol by reduction of tocopheryl radical [119]. Recently, we have shown that the naringenin-oxime compound, having one or more hydroxyl groups than naringenin, had more antioxidant and anti-genotoxic potentials than the naringenin in

the human mononuclear leukocyte cells [120]. Oxidases such as lipoxygenase (LO), myeloperoxidase (MPO), NADPH oxidase and xanthine oxidase (XO) are considered to be one of the important mechanisms by which phytochemicals inhibits the formation of high amounts of ROS [119]. Phytochemicals also inhibit enzymes indirectly involved in the oxidative process, such as phospholipase A2 (FLA2), by stimulating known antioxidant enzyme activities such as catalase and superoxide dismutase (SOD) [121]. For this reason, flavonoids can be considered as phytochemicals that can interfere directly or indirectly with the formation of free radicals [122].

4.2. The role of phytochemicals in cancer formation

It appears that antioxidants are found in the body at sufficient concentrations to prevent accumulation of prooxidants (oxidative stress state) and that exogenous antioxidants play an important role in maintaining healthy biological systems and establishing redox hemostasis at physiological (nutritional) doses [123]. However, exogenous antioxidants, particularly phenolic compounds, can participate in redox reactions that can function as antioxidants (electron donors) or prooxidants (electron acceptors), depending on their environment [14, 103]. The antioxidant or prooxidant activity also depends on their concentration [98]. It is now assumed that exogenous antioxidants, including polyphenols, act as "double edged swords" according to their cellular redox status [123]. Yordi and Pérez [124] recently published a list of such compounds and their dietary sources. Some of the most abundant flavonoids and phenolic acids found in plants were reported to act as prooxidants, besides antioxidant activities: quercetin, curcumin, mycetin, kaempferol and caffeic, chlorogenic, ferulic acids and phenolic acids were also demonstrated as prooxidants [125–129]. Several studies have shown that oxidative stress is either mediated through the formation of ROS or inhibition of antioxidant systems from prooxidant agents [130]. For this reason, the type, phylogenetic, and matrix of phytochemicals may be determining factors affecting the balance between beneficial or deleterious effects of these natural compounds [123]. It is known that the development of many chronic diseases may be due to an oxidative stress, which antioxidant/pro-oxidant balance cannot provide and may lead to a pathological process [131]. The prooxidants catalyze the oxidative reactions of biomolecules, which may lead to cellular dysfunction [132]. It was demonstrated that increased prooxidant activity had damaged to biomolecules such as DNA, proteins and lipids that were able to lead to a variety of diseases such as cancer and cellular death [98, 132]. It was firstly demonstrated that resveratrol can induce oxidative DNA damage in the presence of copper ions [133]. Although it is associated with consumption of hot tea, it has been shown that too much tea consumption (>1 l/d) is associated with an increase in the incidence of esophageal cancer in some countries such as northern Iran or India [20]. At the same time, green tea has been shown to produce H_2O_2 in the mouth cavity [132]. Because of this, taking plants with high dose phytochemicals is not always effective or safe, sometimes may have toxic effects.

In the use of phytochemicals, it is necessary to distinguish pharmacological doses from physiological (nutritional) dosages. Clinically, physiological doses are usually used to optimize or maintain optimal health. The pharmacological dose generally requires a doctor's prescription

to treat the specific disease, because in the intake of antioxidant micronutrients, pharmacological doses are not equal to physiological doses and the intake of antioxidant micronutrients may be toxic and can generate cancer.

4.3. Role of phytochemicals in cancer therapy

One of the main features of cancer cells is the survival ability. For this reason, the main goal of cancer therapy is to kill cancer cells by selecting them without harming normal cells. There are various therapeutic methods to treat cancer including chemotherapy, radiotherapy, and/or surgery. Chemotherapy is one of the basic modalities in the treatment of cancer patients [110]. Although chemotherapy is aimed at removing the desired primary target tumor cells, normal cells are also affected and produce many side-effects in multiple organ systems [26, 134, 135]. For this reason, efforts are being made to develop alternative and effective treatment methods. The studies have focused on the active components of plants with low toxicity and high selectivity for killing cancer cells.

The primary mechanism of many chemotherapeutic drugs against cancer cells is the formation of ROS or free radicals [26, 27]. Indeed, there is a realistic approach to treatments aimed at strikingly increasing intracellular ROS to kill cancer cells by reducing antioxidant capacity [136]. This can be achieved by using compounds that inhibit antioxidant systems or by inhibiting specific signaling pathways that upregulate antioxidants in cancer cells. The resulting increase in ROS can stimulate tumor cell death through harmful functions of ROS, or through apoptosis-specific induction of death signaling pathways. Chemotherapeutics that makeup ROS include alkylating agents (melfalan, cyclophosphamide), anthracyclines (doxorubicin, epirubicin), podophyllin derivatives (etoposide), platinum coordination complexes (cisplatin, carboplatin) and camptothecin (topokan, irinotecan) [137]. Because, high ROS levels results in acute damage to cellular components such as DNA, proteins and lipids. ROS can attack DNA due to its strong reactivity and can cause DNA base oxidation, DNA lesion and damage to the DNA helix [138]. One of the first drugs developed based on ROS production characteristics was the procarbazine [139]. It hydrolyzes in aqueous solutions and the cytotoxic effects of the drug are the result of H_2O_2 production [140]. Similar to chemotherapy, radiotherapy also kills cancer cells by producing ROS [141]. Cancer cells can be killed by three pathways: apoptosis, necrosis, and autophagy [142–144]. Apoptosis is a tightly regulated form of cell death and can be initiated by death receptors (extrinsic pathways) or mitochondria (internal pathways), and both extrinsic and intrinsic pathways of apoptosis are associated with ROS [145]. ROS is also required for Fas phosphorylation at the tyrosine residue, a signal for Fas-associated death pathway and caspase-8 and for apoptosis induction [146]. Although it is not known that ROS induces apoptosis in excessive amounts, high levels may cause necrotic cell death. In some cases, ROS can trigger both apoptosis and necrosis in cancer cells. For example, it has been determined that low H_2O_2 concentrations in Jurkat T lymphocytes cause apoptosis by caspase activation in cells, while higher concentrations cause cell death by inducing necrosis [147]. Studies have also shown the role of ROS as a signaling molecule in the stimulation of autophagic cell death in cancer cells [148]. Besides the ability of cells to kill, ROS is also necessary for the survival of cancer cells. In fact, the ability of cancer cells to differentiate ROS as a

survival or apoptotic signal is related to the dose, duration, type and location of ROS production. In short, while moderate levels of ROS are required for cancer cells to survive, extreme levels kill them [149, 150].

Although phytochemicals of plant origin are known to have preventive effects on cancer and they are widely used in developed countries [151], numerous studies have revealed that many of these agents can kill cancer cells [56]. Some drugs, nowadays, used in chemotherapy are natural plant-derived products such as (e.g., paclitaxel, vincristine, vinblastine, bleomycin, mitomycin, doxorubicin, idarubicin, aclarubicin and actinomycin D). Other chemicals such as curcumin, epigallocatechin-3-gallate, genistein, resveratrol, camptothecin, perillyl alcohol, lycopene, phenylethyl isothiocyanate, sulforaphane, aplidin, eicosapentaenoic acid, linoleic acid, ursodeoxycholic acid, and vitamin C are in the clinical test stage for the treatment of cancer [1].

The potentials of some phytochemicals to treat cancer were evidenced by both *in vitro* cell culture systems and *in vivo* mice models [152, 153]. However, their therapeutic effect on cancer cells has not been elucidated yet. There are several mechanisms offered for the cytotoxicity of phytochemicals including the inhibition of topoisomerases, kinases and prooxidant actions [16]. Although many phytochemicals known as antioxidants can protect the cell from the oxidative stress and neutralize the damaging effect of ROS, however, they can, on the other hand, be cytotoxic at high concentrations. However, the mechanism of dual protective-destructive behavior of flavonoids is not exactly known. It is highly possible that the prooxidant effect is responsible for the selective antiproliferative activity of these compounds, and ROS are key signaling molecules to modulate cell death [154]. Because, many phytochemical agents exhibit prooxidant action, particularly in the presence of transition metal ions such as copper [13, 155]. The prooxidant activity of individual dietary polyphenols and their ability to induce mitochondrial dysfunction and consequently apoptosis has been suggested a possible anticancer mechanism [136]. There seems to be enough evidence to support phytochemicals-mediated production of ROS, a prooxidant action that is responsible for their ability to induce apoptosis in cancer cells. It was observed that the accumulation of H_2O_2 is crucial for paclitaxel-induced cancer cell death both *in vitro* and *in vivo* [156, 157]. Being well known that H_2O_2 can induce selective killing of cancer cells, it seems possible that paclitaxel induced H_2O_2 production plays a role in the selective anticancer effects of this natural product [158]. Several *in vitro* and *in vivo* studies have also shown that phytochemicals such as catechins, phisapubesin B, daucosterol and hesperetin can induce apoptosis induction in various cancer cells and animal models [33, 159–162]. Recently, we also demonstrated cytotoxic, apoptotic, ROS generating and DNA damaging effects of naringenin on cancer and normal cells *in vitro* in cell culture medium [163]. In the same line, the apoptosis inducing effect of EGCG has been shown to be due to an increase in caspase-3, -9 and -8 [76–79] expression [164]. Similarly, the intrinsic pathway (FAS-independent, caspase 8-independent) by down regulation of ellagic acid Bcl-xL and release of cytochrome C, and colon cancer induced apoptosis in Caco-2 cells [165]. Interestingly, ellagic acid, quercetin and curcumin were found to induce ROS formation, DNA damage and apoptosis synergistically in cancer cell lines [132, 166, 167]. Recently, a combination of chemotherapeutic drug imatinib and curcumin has been used in a cancer patient and has been shown to increase efficacy in cancer treatment [168]. In addition,

pharmacological doses of vitamin C and curcumin have been reported to be used in the treatment of different types of cancer, and successful results have been reported [169–174].

Studies have shown that many phytochemicals show more cytotoxic effects in various cancer cells than in normal cells [175–177]. Some studies have shown that polyphenols such as EGCG and genistein kill cancer cells more at the same dose with apoptosis than normal cells [178, 179]. We also showed that an herbal medicine named ankaferd, derived from different plant extracts, killed cancer cells more than normal cells at the same doses [180]. However, it is not clear how this differential effect occurs. This may be due to the difference in metabolism between cancer cells and normal cells. Since the metabolism of cancer cells is higher than normal cells, endogenous ROS production levels are much higher than normal cells [181]. The use of prooxidant phytochemicals emerges as an exciting strategy to target tumor cells selectively, due to further increase in ROS levels in cancer cells. Indeed, our findings show that the production of ROS in cancer cells to which phytochemicals are applied is significantly higher than normal cells and that there is a positive correlation between ROS level and cell death [163, 180]. The advantage of such a strategy is that it is not significantly affected by the fact that basal ROS levels of normal cells are lower than cancer cells and therefore less dependent on antioxidants.

Although many *in vitro* cell culture studies have been carried out on the use of phytochemicals in the treatment of cancer, the number of experimental animals and clinical trials *in vivo* is low. An important problem in *in vivo* studies is that phytochemicals are digestion, absorption and bioavailability. Bioavailability is very low due to low absorption rate of many phytochemicals [182]. Therefore, enteral administration is preferred for cancer treatment [168, 171].

5. Conclusion

In conclusion, extensive researches over the past half a century have indicated that oxygen ROS play an important role in cancer metabolism. ROS are one of the main components of cell signaling pathways and have been shown to take roles in regulating cell transformation, survival, proliferation, invasion, angiogenesis, and metastasis [73, 168]. On the other hand, ROS can also suppress tumor progression. However, most chemotherapeutic and radiotherapeutic agents are designated to reduce the impact of ROS by augmenting ROS stress in cancer cells [183]. Due to these dual roles of ROS, both prooxidant-based and antioxidant-based anticancer agents have been developed [184]. It is clear that numerous chemotherapeutics mediate their effects by inducing ROS generation. However, unwarranted side effects of synthetic anticancer drugs should be minimized in healthy cells. For this reason, researchers continue to look at the nature and explore the potential for cancer treatment. A great number of phytochemicals including some of the vitamins, flavonoids, terpenoids, carotenoids, phenolics, phytoestrogens, minerals and antioxidants in plant materials are used for chemoprevention of cancer. However, various *in vitro* and *in vivo* experiments have shown that phytochemicals have carcinogenic potential as well as protective and curative effects against cancer. Many studies have shown that these three different effects of phytochemicals on cancer are related to the molecular structure of phytochemicals, bioavailability, dose and the oxidative status of the administered organism. For this reason, the molecular structure, bioavailability and

knowledge of the oxidative status of the applied organism are vital to the phytochemical agent used in the prevention or treatment of cancer. Otherwise, treatment with phytochemicals may result in an opposite result to the desired effect.

Author details

Abdurrahim Kocyigit[1]*, Eray Metin Guler[1] and Murat Dikilitas[2]

*Address all correspondence to: abdurrahimkocyigit@yahoo.com

1 Department of Medical Biochemistry, Faculty of Medicine, Bezmialem Vakif University, Istanbul, Turkey

2 Department of Plant Protection, Faculty of Agriculture, Harran University, Sanliurfa, Turkey

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Section 2

Reactive Species Involvement in Pathology

Chapter 4

Reactive Oxygen Species and Bone Fragility

Nina Filip, Elena Cojocaru, Alexandru Filip,
Bogdan Veliceasa and Ovidiu Alexa

Additional information is available at the end of the chapter

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Abstract

Reactive oxygen species (ROS) are key signaling molecules that play an important role in the progression of inflammatory disorders. In the last decade, studies have indicated that ROS, including superoxide and hydrogen peroxide, are crucial components that regulate the differentiation process of osteoclasts. Osteoclasts (OCs), cells specialized for bone resorption, utilize ROS as second messengers during receptor activator of NF-κB ligand (RANKL)-induced differentiation and activation. The purpose of this chapter is to explore the current understanding of reactive oxygen species involvement in bone pathophysiology.

Keywords: fragility fractures, free radicals, bone, osteoporosis, oxidative stress

1. Introduction

Reactive oxygen species (ROS) are known to determine oxide-reducing balance alteration, oxidative stress, and carcinogenicity. Many diseases, including cancer and other pathologies associated, like arteriosclerosis and cataracts, are related to mitochondrial dysfunctions provoked by reactive oxygen species [1, 2]. Free radicals (O^{2-}, $HO^{\bullet}$, H_2O_2) react easily and cause damage to the DNA and cell membranes, generating strong oxidant agents inside cells [3]. Reactive oxygen species (ROS) play a role in a number of degenerative conditions including osteoporosis [4].

Bone is an important organ performing three essential physiological functions: mechanical support, mineral homeostasis, and support of hematopoiesis. Bone diseases in the elderly are associated with increased morbidity and mortality. Osteoporosis is one of the most important diseases that affects the quality of life for the elderly [5]. Although the bones stop growing after adolescence, the bone is a very dynamic tissue. Bone tissue is continuously reabsorbed and regenerated by constantly changing its structure.

One of the most striking features of the bones is their ability to reshape. This remodeling process occurs during growth and continues throughout its life. During bone formation, the bone is formed and deposited, according to a particular pattern, by a process called ossification. The remodeling is continuous, leading to the organization of the bone structure in regular units, allowing the bone mass to gain maximum resistance to the mechanical forces acting on it. The older bone is removed by osteoclasts (bone-resorbing cells), and osteoblasts (bone-forming cells) lead to the deposition of new bone tissue. Osteoclasts secrete enzymes that break down the bone matrix as effectively as acids and convert calcium salts resulting in a soluble form (that can be absorbed into the bloodstream).

Osteoclast activity occurs behind the epiphyseal growth zone to reduce bone margins enlarged at the width of the bone stem that undergoes an elongation process.

The remodeling process is especially important for long bones supporting the limbs. These bones are widest at the ends and narrower in the middle, which gives added strength to the joints.

As osteoclasts destroy the old bone at the epithelial ends of the bone, the osteoblasts within the growth zone create a new epiphysis.

In each of the tubular spaces released by osteoclasts within the bone, the osteoblasts come in to deposit a new bone layer.

A bone that is poorly used, such as a lower immobilized member after a trauma, will be prone to reabsorption because bone destruction goes beyond bone formation.

Bones subjected to increased stress are permanently remodeled. The femur, for example, is completely replaced every 6 months.

Bone remodeling gives the different form of long bones. They are wider at each end and thinner in the middle. Bone remodeling not only changes the bone structure but also helps regulate the level of ionic calcium in the blood. Calcium is necessary for normal nervous transmission, for cell membrane formation, and for blood clotting. The resorption and bone formation sequence is shown in **Figure 1**.

Osteoblasts (bone-forming cells), osteoclasts (bone-resorbing cells), and osteocytes (load-sensing cells) are the cell types participating in bone remodeling. During remodeling, these cell types are spatially and temporally organized in functional structures called basic multicellular units (BMUs). The remodeling sequence operated by a BMU follows well-defined phases [5]. The bone remodeling sequence is influenced by a number of regulatory factors produced by hormonal glands, bone cells involved in sensing the mechanical environment, lymphocytes, and even tumor cells [6].

Osteoporosis is characterized by the parallel reduction of bone minerals and bone matrix so that the bone is in a low amount but with a normal percentage composition.

Despite rapid advances in our understanding over the last few years, the morbidity and mortality of patients resulting from this disease are still too high [4], and there is an urgent need for a proper assessment of the underlying mechanisms and the development of new treatment strategies to address this pathophysiological issue.

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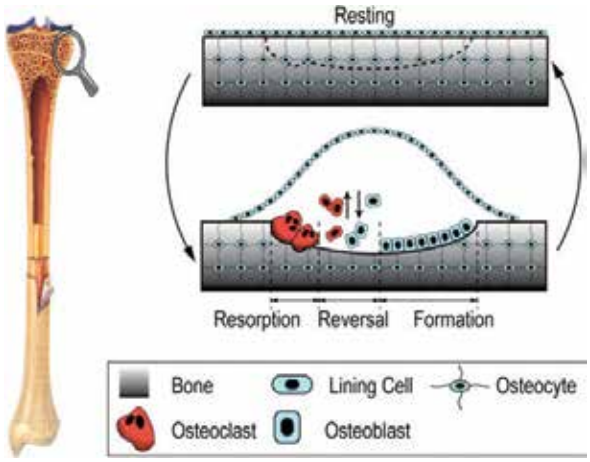

Figure 1. Bone remodeling sequence executed by different bone cell types within basic multicellular units (BMUs) (figure from Ref. [5], reproduced with permission).

2. Reactive oxygen species

Oxygen (O_2), nitrogen oxide (NO), and iron (Fe^{2+}) are present in erythrocytes. These elements may in some conditions form reactive species, called radicals that affect red blood cells and vascular endothelium. A chemical species that presents an unpaired electron in outer orbitals and which can exist independently is called free radical. Radicals are very reactive trying to complete their own orbits by taking an electron from a neighboring molecule. Oxygen and nitrogen are the two chemical elements that in the body give rise to reactive species called the reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively.

2.1. ROS: general definitions

Reactive species can be defined as atoms or molecules that contain in their structure an odd electron that causes an atom/molecule energy instability, which is reflected in their increased reactivity.

Reactive species are produced in the body for physiological purposes in defense, vaso-relaxation, cellular or accidental exposure to toxic, or radiation exposure. When the amount of reactive species exceeds nontoxic physiological levels, the so-called oxidative stress occurs. Oxidative stress can be defined as the status of reactive oxygen or oxygen radicals in a biological system. It results from the imbalance between oxidants and antioxidants, in favor of oxidants, with destructive and pathogenic potential [7, 8].

The reactive species of oxygen are superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$). Free oxygen radicals (ROS) produce harmful effects on the body called oxidative stress (OxS). The reactive oxygen species are constantly formed as reaction products in all cells by aerobic processes such as oxidative phosphorylation.

The base state of oxygen is triplet. This means that it has two unpaired electrons that are positioned on two different molecular orbitals.

Because electrons have parallel spin, oxygen can only rotate in the same orbit. Since molecular oxygen is paramagnetic, it is concluded that it has the structure represented in the right-hand side of **Figure 2**. Molecular oxygen can react rapidly with single unpaired electrons of transitory metals (e.g., Fe, Cu, Mn). One mole of properly chelated copper could catalyze consumption of the oxygen in an average room within 1 s in [9]. Depending on the conditions, O_2 can be both kinetically stable (not reactive) and very reactive.

For proteins and enzymes that have a metal in their structure, the oxidative attack of O_2 is slow, and in conclusion the superoxide radical will slowly form.

Superoxide (O_2): this radical constitutes the first step of oxygen activation by way of univalent reduction, forming in most of the autoxidation reactions by the capture of an electron that is placed on one of the antiknocking orbital n (**Figure 3**).

After an electron has been acquired, the following electrons are added more easily, and the reactions take place faster, leading to the generation of reactive oxygen species [10].

Reducing the hydroxyl radical to water takes place in the last stage of electron acceptance (**Figure 4**).

Superoxide radical (O_2) can react in the presence of ferric iron with hydrogen peroxide generating the most potent hydroxyl radical through a nonenzymatic reaction named Haber-Weiss reaction.

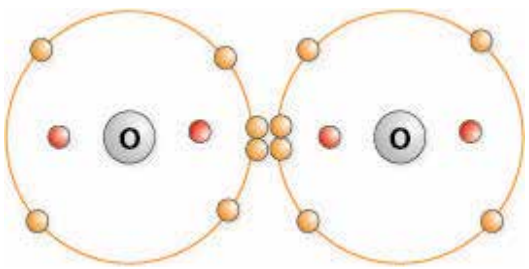

Figure 2. The structure of oxygen molecule.

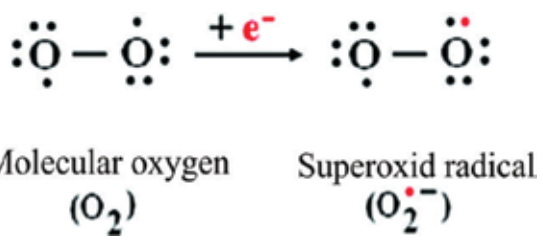

Figure 3. Superoxide radical generation.

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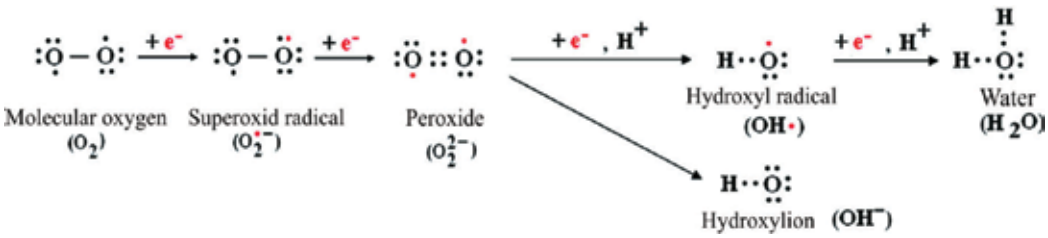

Figure 4. Reactive oxygen species generation.

The reaction involves two steps:

$$O_2 + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet \quad (2)$$

The nonenzymatic reaction occurring in living cells is:

$$O_2 + H_2O_2 \rightarrow OH^\bullet + OH^- + O_2 \quad (3)$$

In the presence of ferric iron, superoxide radical is a source of hydroxyl radical.

Most of the superoxide radicals are formed by electrons derived from the coenzyme Q cycle in complex III and by NADH flavoprotein dehydrogenase (complex I). The superoxide radical, which has a relatively low degree of toxicity compared to other reactive oxygen species, is kept undetected by enzymes called superoxide dismutase (SOD) that catalyzes the dismutation reaction.

Hydrogen peroxide (H_2O_2) is not a free radical due to the fact that it has no unpaired electron in the structure, which gives it reactive stability, being the most easily measurable reactive species. H_2O_2 is a weak oxidizing agent that forms as a result of the O_2-dismutation reactions or oxidase-catalyzed reactions. This process takes place at the level of peroxisomes, cellular organisms with rich enzyme equipment. Concentration of hydrogen peroxide is controlled by catalase (present in peroxisomes) and by glutathione peroxidase (localized to the cytosol).

Hydroxyl radical ($OH^\bullet$) is a highly reactive free radical which diffuses over small distances until it reacts with any organic molecule encountered such as carbohydrates, amino acids, lipids, nucleic acids, and organic acids. The toxicity of this molecule comes from the fact that it can initiate chain reaction.

Singlet oxygen ($^1O_2$) can result from oxidoreduction reactions. Single oxygen is more reactive than the hydroxyl radical by reacting with many organic compounds such as polyunsaturated fatty acids, cholesterol-forming hydroperoxides.

Singlet oxygen can be formed from superoxide radical in [11]:

$$2O_2 + 2H^+ \rightarrow H_2O_2 + {}^1O_2 \quad (4)$$

Although it is not a radical, singlet oxygen is more reactive than the hydroxyl radical. The most reactive radical is hydroxyl ($OH^\bullet$) which takes electrons from other molecules around it, whereas superoxide (O_2) and hydrogen peroxide (H_2O_2) are more selective in their reactions with various molecules [12].

The processes so far presented above take place in all human cells. The molecular oxygen that is transported to the tissues is found for a short period of time unbound. It is assumed that in this state, it generates the radicals previously presented. Oxygen binds to hemoglobin molecule at the ferrous iron (Fe^{2+}). Some of Fe^{2+} is transformed by O_2 to ferric form (Fe^{3+}) in resulting methemoglobin. Methemoglobin reductase is an enzymatic system which restores Fe^{3+} to Fe^{2+} and reduces methemoglobin back to hemoglobin. Oxygenated hem has some of the electronic characteristics of a $Fe^{3+}OO$ peroxide anion [10]. Like in this study [13], the author found that the $Fe^{3+}O^{2-}$ complex is able to generate superoxide radical during the normal molecular oxygen transport to tissues through the hemoglobin auto-oxidation.

Some researchers propose that hemoglobin may have oxidative reaction in the oxygen-releasing process.

Like in this study from 2006 [14], the author demonstrates that at intermediate oxygen pressure, where hemoglobin partially releases molecular oxygen, the superoxide radical production increases. They show that superoxide radical is released in the hydrophobic hem pocket.

Through the Haber-Weiss reaction, H_2O_2, even if it is not a radical, can form short and very active hydroxyl radicals [15]. The hydroxyl radical ($OH^\bullet$), which is highly reactive, reacts with any biomolecules it encounters. There are studies according to which the high reactivity of the hydroxyl radical decreases its ability to diffuse [16]. Since the hydroxyl radical may generate an autocatalytic reaction in the chain, we can say that it is particularly dangerous.

There are some researchers who believe that the hydroxyl radical is responsible for cellular damage and others that ferric ion promotes initiation of the chain reaction [17–19]. In conclusion, both oxidative species can form in living cells [20].

Like in **Figure 4**, ROS are produced accidentally and physiologically in various enzyme-catalyzed reactions.

2.2. Effects and signaling of ROS

Reactive oxygen species can interact with any biological molecule causing damage to lipids, proteins, and DNA. Prospects for effective therapeutic intervention may fail if the initial target of oxidative stress is unknown. For example, it is known that DNA is the primary target of lesions produced by the addition of H_2O_2 to mammalian cell cultures, so that DNA strand breaks occur before lipid peroxides or "detectable" oxidized proteins. The method of detecting lesions produced on target molecules may give incomplete information. The evidence of protein damage by detecting carbonyl radicals in the initial stages of lesions may be negative, but the determination of SH oxidation, which occurs earlier, is positive. During peroxidation

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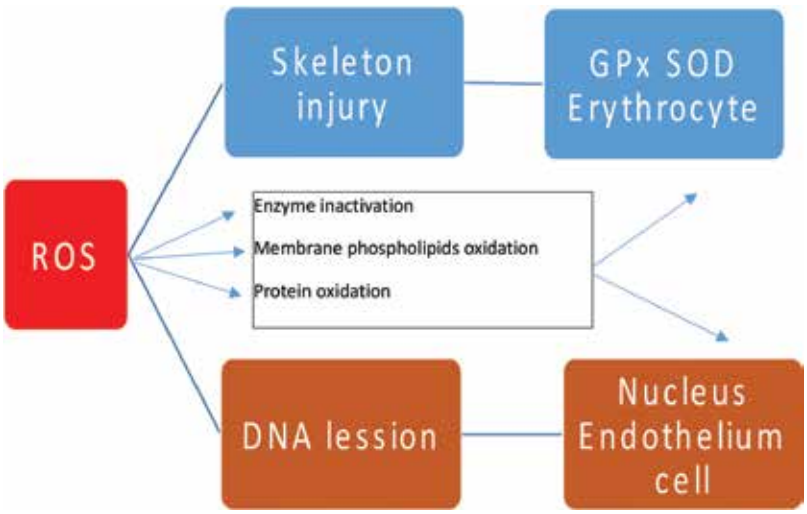

Figure 5. ROS damaging activity.

of lipids, the peroxyl radicals are formed by chain reactions. These intermediates that are formed can amplify the lesion. The attack of ROS affects both erythrocytes and endothelial cells. DNA damage may occur in nucleus containing endothelium cell besides lipid and protein oxidation. Interaction between free radicals and DNA can lead to strand breaks or structural changes such as adduct formation (**Figure 5**) [21, 22].

In general, lipid peroxidation occurs in late stages of aggression, so therapies directed against lipoperoxidation may be less beneficial. This is another reason that can lead to erroneous conclusions about the importance of oxidative stress and antioxidant therapies.

As long as they do not accumulate in excess, ROS have positive effects in the body, being involved in various cellular mechanisms. For example, biosynthesis of thyroid hormones involves the formation of hydrogen peroxide in order to assemble thyroglobulin iodine to synthesize thyroxine. On the other hand, in the immune response, in neutrophils and macrophages, NADPH oxidase catalyzes the formation of superoxide anion by internalizing

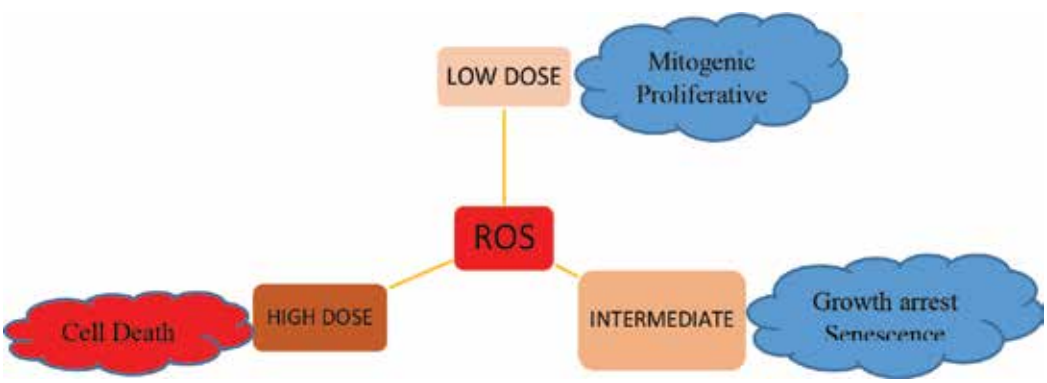

Figure 6. Cells response under ROS attack [23].

bacterial antigenic structures. The process is completed by the action of SOD, which will produce hydrogen peroxide that will induce bacterial lysis. It has been shown that ROS acts as a signaling molecule [21, 22]. In response to the intensity of attack of the ROS, cellular responses are triggered, which prepare the cells to survive or to die (**Figure 6**).

Oxidative stress can trigger cellular response through different signaling modes. ROS modulates different types of enzymes, the activity of transcription factors, and the ionic channel. Both kinases and phosphatases can be modulated by ROS.

Mitogen-activated protein kinase (MAPK), which has three subfamilies, namely, N-terminal c-Jun kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK), is part of the kinase class [24]. These MAPK pathways are structurally the same. The difference between them is found at a functional level. ROS activates these three MAPK pathways [25] and may inhibit tyrosine phosphatase activity.

In gene and protein expression, ROS are involved by activating transcription factors.

The plasma membrane Ca^{2+} and K^{+} channels are stimulated by ROS. The increase in calcium ion concentration ROS mediated leads to the oxidative stress-mediated activation of PKC and to the transcriptional induction of the AP-1 proteins c-Fos and c-Jun [26].

Cells can be protected from attack by reactive species through various mechanisms. Antioxidants can be divided into two groups: enzymatic like superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase and nonenzymatic like vitamins E and C, provitamin A (b-carotene), and glutathione.

SOD is found in all aerobic cells as well as in aerobic optional bacteria. Initially, it was interpreted as a copper storage form, and later three types of SODs were described, based on the type of metal in the catalyst center.

The role of superoxide dismutase is to catalyze the dismutation reaction of the superoxide radical anion according to the equation (**Figure 7**):

$$2\,O_2^{\bullet -} + 2\,H^{+} \xrightarrow{\text{Superoxide dismutase}} O_2 + H_2O_2$$

Figure 7. Superoxide dismutase activity.

The enzyme may also inhibit the production of singlet oxygen and indirectly the peroxidation of polyunsaturated fatty acids. In addition to the role of the oxidative marker, SOD is tested as a potential therapeutic agent under pathological conditions in which the oxidative stress has a clear role, such as ischemia-reperfusion syndromes, hepatic disorders and acute and chronic inflammation, cataracts, rheumatic arthritis, diabetes, and neoplasia. One of the enzyme-catalyzed reactions leads to the appearance of another harmful compound—hydrogen peroxide [27].

GPx is part of a family of enzymes catalyzing the degradation of H_2O_2 resulting from normal metabolic processes and providing the protection of proteins, lipids, and nucleic acids from the action of oxidizing molecules, using glutathione as an electron donor or, in some cases, thioredoxin or glutaredoxin (**Figure 8**).

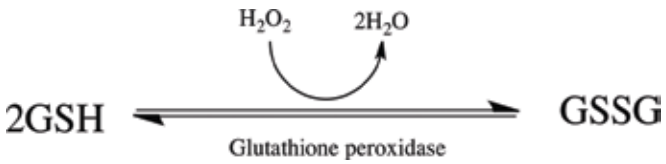

Figure 8. Glutathione peroxidase activity.

GPx is a dependent selenium enzyme, found in cytosol (70%) and in mitochondria (30%). It is irreplaceable in the antioxidant arsenal, especially in mitochondria, as they do not contain catalase for peroxide metabolism. GPx also provides protection against organic H_2O_2 and helps to regenerate the reduced vitamin C form. Imbalances in the GPx level have been observed with aging and a variety of disorders such as cancer, cardiovascular disease, and diabetes [28].

GPx is an enzyme that has a potentially greater antioxidant than SOD and catalase due to the wide-specific substrate. Other roles of GPx are regulating prostaglandin biosynthesis by inhibiting lipoxygenase and, in conjunction with glutathione reductase, contributing to the restoration of reduced glutathione.

3. Oxidative stress in bone remodeling

Bone tissue undergoes, throughout life, a continuous renewal through a process called bone remodeling, which is controlled by the activity of osteoclasts mediating bone resorption and parallel activity of osteoblasts which mediate bone formation [29]. A bone remodeling cycle involves three stages: (1) initiation, during which osteoclasts are formed and resorb damaged bone; (2) reversal, the transition of osteoclast to osteoblast activity; and (3) formation, when osteoblasts replace the portion of the bone that was resorbed [30]. The hormonal imbalance or the aging process can lead to disruption of the balanced formation process and bone resorption. This may result in decreased bone mass and osteoporosis, which increases the risk of fractures. A very important factor underlying the pathogenesis of osteoporosis is the receptor activator of NF-κB ligand (RANKL). It plays an important role in osteoclast differentiation and activation [31]. For this reason, inhibition of RANKL represents an innovative therapeutic target for controlling osteoclastogenesis [32].

In the bone remodeling process, the alternative NF-κB pathway, which mediates the activation of the p52/RelB NF-κB complex, is involved. The difference of the alternative mechanism is represented by p100 processing of a NF-κB2 precursor protein. Both NF-κB-inducing kinase (NIK) and IKKα, a downstream kinase, promote induction of phosphorylation-dependent ubiquitination for p100. NF-κB-inducing kinase is processed by a tumor necrosis factor (TNF) receptor-associated factor-3 (TRAF3)-dependent E3 ubiquitin ligase. NIK activates the alternative path of NF-kB. This occurs after signals mediated by a subset of TNF receptor superfamily members [33]. The inhibitory role of p100, in both basal and stimulated osteoclastogenesis in bone formation and resorption, has been clearly demonstrated [34]. In the alternative NF-κB pathway p52 derived from p100 through NIK, binding of p52 and RelB induces effects on osteoclast biology [34]. However, to date, the precise physiologic importance of

alternative NF-κB in bone biology is not completely elucidated. Furthermore, the currently known intracellular signaling pathways activated after receptor binding of RANKL include the nuclear factor of activated T cells [35], mitogen-activated protein kinases (MAPKs), TRAFs, c-Jun N-terminal kinases (JNKs), and ROS [36, 37]. In addition, NF-κB is a transcription factor, which pleiotropically regulates osteoclast formation, function, and survival [35].

The reactive oxygen species are involved in the regulation of RANKL-dependent osteoclast differentiation. They act as intracellular signaling compounds. ROS have cytotoxic effects, including lipid peroxidation and DNA damage.

Since the relationship between Nrf2 and osteoclastogenesis is known, stimulation of precursors of osteoclasts with RANKL will lead to the upregulation of Keap1 and downregulation of cytoprotective enzymes. Osteoclast precursors are primary peritoneal macrophages and RAW 264.7 cells. Keap 1 is a negative regulator of Nrf2. However, overexpression of Nrf2 leads to the regulation of the expression of heme oxygenase-1 and gamma-glutamylcysteine synthetase enzymes associated with decreased reactive oxygen species and slowing of bone destruction [38]. Consistent with this line of evidence, overexpression of Keap1 or RNAi-induced knockdown of Nrf2 resulted in effects opposite to those obtained by stimulation of Nrf2-dependent DNA binding activity [38]. We still do not know the exact mechanisms by which RANKL stimulation reduces Nrf2. It is known that Keap1 has in its structure thiol groups. These groups are highly reactive, and oxidation at their level produces conformational changes to Keap1. Ultimately these will lead to dissociation from Nrf2 and promotion of nuclear Nrf2-dependent DNA binding activity [38].

In addition, Nrf2 autoregulates its own expression [39–41]. Taken together, this evidence implies that an increase in ROS levels induced by stimulation with RANKL may upregulate Nrf2. It has also been reported that Nrf2 regulates Keap1 by controlling its transcription [39–41].

Decreasing translation through miRNA can modulate downregulation of Nrf2. The downregulation of Nrf2 may be modulated by decreasing translation by miARN or by modifying the mRNA stability Nrf2. Bach1 is an inhibitor of Nrf2 binding to ARE. This inhibitor is implicated in the mechanism indicated by the inhibited osteoclastogenesis discovered in Bach1 knockout mice [38]. In conclusion, the Keap1/Nrf2 axis regulates RANKL-dependent osteoclastogenesis both by modulating intracellular ROS signals and by expressing cytoprotective enzymes. Elucidation of the precise mechanism linking Nrf2 to stimulation with RANKL may be a therapeutic treatment for destructive bone disorders through the Keap1-Nrf2 axis [42].

4. Oxidative stress in the establishment of bone disease

4.1. Osteoporosis

The human aging implies a failure in bone formation and a loss of bone mass, but the specific molecular mechanisms arbitrating these effects are still unclear. Numerous studies in experimental animals have offered arguments for a damaging effect of oxidative stress in bone tissue, supporting the idea that an increase in reactive oxygen species (ROS) with advancing age characterizes a pathophysiological mechanism essential in age-related bone damage. The disproportion between

bone resorption and formation with age is related to various factors. A reduced wall width represents the most common histological feature in aged human bone due to a reduced deposition of bone matrix related to an insufficient number of osteoblasts involved in bone remodeling [43, 46]. It was found that the low number of osteoblasts in the aging bone is correlated with a decrease in the number of mesenchymal stem cells and improper proliferation and differentiation of progenitor cells [45]. An additional histologic feature of aged human bone tissue is a decrease in osteocyte density in the structure of bone lamellae accompanied by a mineralization of osteocyte lacunae. The precise mechanism of this phenomenon—named micropetrosis—is not very well known, but it seems to contribute to the decrease in osteocyte density with age [44–46].

The histopathological examination reveals thinned bone trabeculae that lose continuity being separated from each other by enlarged areolae with adipose degeneration of the marrow. The reduction of the trabecular connectivity is related to osteoporosis stages. The decrease of the medullar cellularity together with its enhancement in fat cells has negative outcomes on the bone. The areolar spaces are stretched, bordered by incomplete bone septa. This fact could be explained by the decrease of the connectivity of the bone trabeculae that will lead to the connection of the areolae by the osteolysis of certain walls (**Figures 9** and **10**).

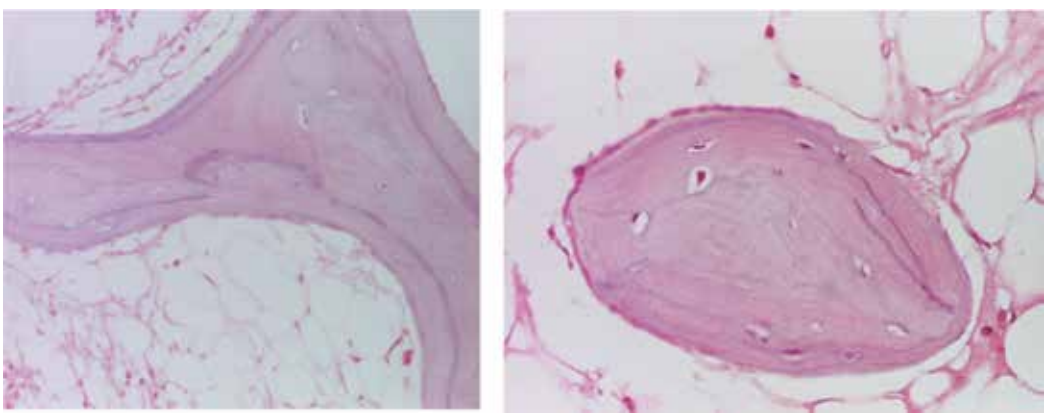

Figure 9. Osteoporotic bone—hematoxylin-eosin (HE) stain.

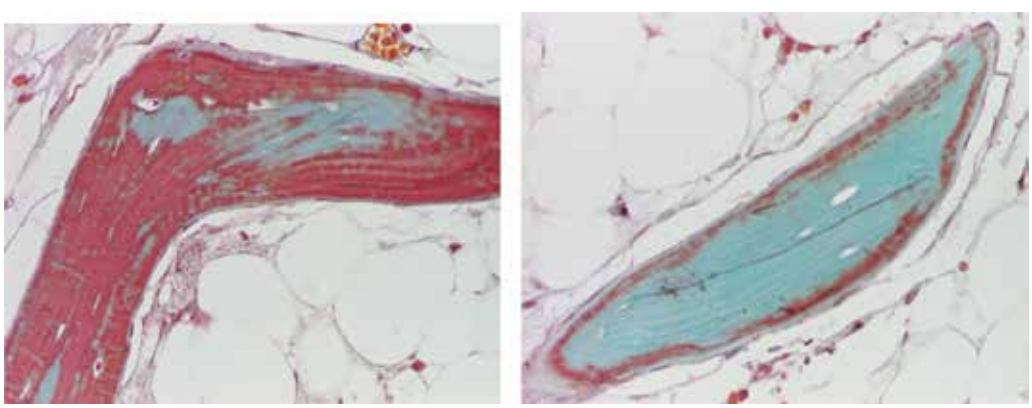

Figure 10. Osteoporotic bone—trichromic Goldner-Szekely stain.

4.2. Bone tumor development

Healthy people have a balance between bone formation and bone resorption. In various bone diseases, even in malignancy, loss of this balance leads to damage to the normal structural integrity of the skeleton [47, 48]. Tumor cells act by excessive stimulation of both osteoclasts and osteoblasts.

Oxidative stress is one of the most important events that gives rise to the conditions leading to tumor onset and progression [49]. Reactive oxygen species (ROS) is one of the most important species of free radicals. ROS controls many cellular processes, including cell proliferation, and thus stimulates the uncontrolled cell growth which may lead to tumor development [50]. In the case of chronic inflammation, the secretion of ROS may lead to the amplification of dysregulated processes and eventually to the development of a preneoplastic state.

When the endogenous antioxidant response is exceeded by the amount of ROS produced, oxidative damage can occur in the lipids, proteins, and DNA. These lesions can lead to genetic alterations and ultimately to dysregulation of suppressor genes. Since oxidative stress and chronic inflammation processes are linked, failure to block these processes can lead to the initiation of carcinogenesis through genetic alterations [51]. In patients who have cancer, low total antioxidant capacity can be detected even before oncological treatment is started [52].

4.3. Diabetes-associated bone complications

Diabetic patients have been shown to increase lipid peroxidation and decrease the activity of erythrocyte antioxidant enzymes. Hyperglycemia is due to impaired insulin secretion in T1DM and insulin resistance in T2DM.

In hyperglycemic conditions, oxidative stress occurs as a result of the increased activity of the polyol pathway [53], the hexosamine pathway [54–56], and the promotion of the activator of protein kinase C [57]. Hyperglycemia also leads to a higher activation of the nuclear-kappa B (NF-κB), by protein kinase C in vitro. The nuclear factor-kappa B is a central transcription factor involved in the regulation of many proinflammatory genes, including cytokines (TNF-tumor necrosis factor, interleukin) and hematopoietic growth factors [58]. Under hyperglycemic conditions, the NADH/NAD + cytosolic ratio increases because sorbitol is oxidized by NAD +. In this situation, the activation of the glyceraldehyde-3-phosphate dehydrogenase enzyme is inhibited. Although there is still little research, type 1 diabetes has been associated with osteoporosis. Therefore, we can say that type 1 diabetes may increase the risk of fractures [59]. Type 1 diabetes affects both bone density and bone quality [60, 61]. In T2DM, BMD is equal or increased according a meta-analysis, but the fracture risk is increased despite this increase in BMD [62, 63].

Osteocalcin (OST) is the second structural protein after collagen, a component of bone tissue, mainly involved in the process of bone mineralization and calcium homeostasis. It is synthesized by osteoblasts, under the action of vitamins K and D3, from a pre-pro-osteocalcin precursor made up of 98 amino acids. Clinical research has shown that serum osteocalcin levels

are significantly lower in patients with type 2 diabetes, becoming normal after improved glycemic control. Osteocalcin is an independent factor associated with glucose and glycated hemoglobin for menopausal women.

It has been revealed that diabetes causes a reduction in the number of osteoblasts [64]. Diabetes can affect bone-forming cells through increased apoptosis. Like in this study from 2007, authors found that AGEs induced apoptosis of osteoblasts through the MAP kinase pathway [65]. Another mechanism by which diabetes is associated with bone formation is the reduction in the expression of transcription factors involved in the regulation of osteoblast differentiation [66]. Studies in rats with diabetes showed a decrease in both alkaline phosphatase activity and the formation of the mineralized matrix [67, 68].

RAGE, the AGE receptor, is expressed at higher osteoblast levels in rats with diabetes, thus rendering the animals more susceptible to AGEs effects [69].

Some enzymes called serine kinases can be induced by oxidative stress. Their induction affects the ability of insulin to activate protein kinase B and glucose transport. When insulin action is impaired, diabetic complications may occur. In this context, the NF-κB, p38 MAPK, and JNK/SAPK pathways are sensitive to oxidative stress [70].

A considerable amount of evidence has accumulated indicating that metabolic and endocrine alterations caused by diabetes affect bone quantity and quality over the last decades of life [71].

Hyperglycemia-induced oxidative stress is a major contributor in the development of long-term complications of diabetes mellitus [72–75].

5. Conclusion

In this chapter we discussed about the role of oxidative stress in bone pathophysiology and the possibility of ROS production being a relevant therapeutic target under certain conditions.

Bone remodeling is a process of continuous formation and resorption occurring in specific areas of the matrix. Novel therapeutic strategies have been developed that focused on the inhibition of excessive bone resorption and promotion of bone formation process. As in this study from 2017 [47], the author proposed as osteoblast inhibitors in dexamethasone (Dex)-induced apoptosis the synthetic derivatives of benzo[1,2,5]selenadiazole (SeDs). These compounds may have a protective effect. Dex treatment leads to overproduction of ROS in the cells, DNA fragmentation, caspase-3/caspase-9 activation, p53 phosphorylation, and MAPK-pathway activation. When cells were pretreated with SeDs, these modifications were blocked, suggesting to the authors that SeDs may present a therapeutic application to antagonize osteoporosis induced by glucocorticoids [47]. Accordingly, basic research can contribute to the identification of specific pathways that can be effectively targeted by novel compounds able to treat and possibly reverse osteoporosis, particularly which occur in already chronically severed patients, such as in neurodegenerative disorders. In conclusion, we have shown that osteoclast differentiation and bone resorption are associated with the generation of ROS and oxidative stress. These data also indicate that by reducing bone resorption, antioxidants have the potential to treat osteoporosis.

Author details

Nina Filip[1]*, Elena Cojocaru[2], Alexandru Filip[3], Bogdan Veliceasa[3] and Ovidiu Alexa[3]

*Address all correspondence to: zamosteanu_nina@yahoo.com

1 Department of Biochemistry, University of Medicine and Pharmacy "Grigore T. Popa", Iasi, Romania

2 Department of Morphopathology, University of Medicine and Pharmacy "Grigore T. Popa", Iasi, Romania

3 Department of Orthopaedic and Traumatology, University of Medicine and Pharmacy "Grigore T. Popa", Iasi, Romania

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Chapter 5

Reactive Oxygen Species in Skin Repair, Regeneration, Aging, and Inflammation

Hui Xu, Yun-Wen Zheng, Qi Liu, Li-Ping Liu, Feng-Lin Luo, Hu-Chen Zhou, Hiroko Isoda, Nobuhiro Ohkohchi and Yu-Mei Li

Additional information is available at the end of the chapter

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Abstract

As the most important and largest surface barrier, the skin provides a necessary protection to the organism from the external factors, including chemical, biological, and physical irritation, injury, and others. External environmental irritants or their metabolites are inherent oxidants and/or directly or indirectly drive the production of various reactive oxidants, reactive oxygen species (ROSs), owing to the redox imbalances. ROSs, the most common free oxygen radicals, participate in a series of physiological and pathological skin processes. Here, we discussed the role of oxidative events in injury, repair, photoaging, and cutaneous disease development. Intrinsic and extrinsic factors lead to the skin barrier damage, which leads to the disequilibrium in oxidant and antioxidant balance and induces excessive ROS production. The underlying mechanisms include DNA damage, MAPK/AP-1, NF-κB, and JAK/STAT-signaling pathways, apoptosis and autophagy, and autoimmune reaction of melanocytes and keratinocytes. The skin employs a number of antioxidant agents to protect the oxidative balance, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), ascorbic acid, and tocopherols. The results presented here indicate that antioxidant treatments may be effective when applied in the therapy of cutaneous diseases where oxidative stress plays a prominent pathogenic role.

Keywords: reactive oxygen species, antioxidant, ultraviolet radiation, apoptosis, photoaging, vitiligo, psoriasis, autophagy

1. Introduction

1.1. Reactive oxygen species (ROS) definition and endogenous and exogenous antioxidants

Oxidative stress represents the imbalance between oxidative and antioxidative events, which induces oxidative reactions; it is involved in free radical production, and it is a factor responsible

for skin aging and disease development. Reactive oxygen species (ROSs) represent the major agents of oxidative stress, which may be both beneficial and deleterious to the skin, and this group includes singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), H_2O_2, hydroxyl radical (•OH), and others. The gradual reduction of $^1O_2$ leads to the production of $O_2^{\cdot -}$, H_2O_2, and •OH [1]. Free-radical-induced reactions are usually respiratory chain reactions. Electron acceptors, such as molecular oxygen, react readily with free radicals, which lead to the generation of free oxygen radicals. An additional source of oxygen radicals in the skin and other organs is the infiltration of activated leukocytes that possess systems capable of generating these species, such as $O_2^{\bullet -}$ and hypochlorite. The generation of H_2O_2, one of the most stable forms of ROS, in skin may be induced by both exogenous and endogenous factors. Exogenous factors include pathogens, chemicals, ultraviolet (UV) light, and others. Endogenous factors are represented by different acute and chronic inflammations [2], which include hyperglycemia and antioxidant enzyme products.

Skin is the heaviest, largest, and most complex organ, functioning as a physical barrier to protect the internal milieu from water loss and external harmful agents such as pathogens, chemicals, physical agents, and UV light [3]. The fundamental purpose of the generation of high ROS levels during skin inflammation is the removal and destruction of the invading microorganisms and/or degradation of the damaged tissue structures. The ROS system is ubiquitous in aging, photoaging, inflammation, wound healing, tumorigenesis, and other processes in the skin. The imprecise ROS targeting can induce oxidative stress in adjacent normal cells, leading to the aggravation of pathologic processes. Two types of antioxidant systems exist, which include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX); for example, SOD catalyzes the dismutation of $^{\bullet}O_2^-$ into O_2 and H_2O_2, while CAT catalyzes H_2O_2 into O_2 and H_2O. The non-enzymatic antioxidant system includes vitamins C and E, glutathione (GSH), carotenoids, melatonin, A-lipoic acid, Zn(II)-glycine, and polyphenols, and some of these molecules are exogenous antioxidants. The antioxidative systems in human skin are interdependent, but they collaborate. The treatment with known antioxidants such as ascorbic acid, tocopherols, and polyphenols increases the resistance of organism to ROS and prevents skin aging and inflammation [4].

1.2. Physiological ROS roles

ROSs are ubiquitous in organisms and are continuously formed at low levels in skin cells. For example, oxidized lipids and proteins induce alterations at the skin surface, while UV irradiation-generated ROSs stimulate sebaceous gland function, by increasing oxidized lipid and triglyceride hydroperoxide levels, in order to maintain the emollience of the skin and prevent the development of fungal infection [5]. Furthermore, ROSs show a paradoxical effect on melanocytes, both inducing depigmentation and increasing the skin pigmentation. The skin of patients with vitiligo vulgaris, characterized by circumscribed depigmented macules, contains high levels of SOD and low levels of CAT [6]. Impaired nuclear factor erythroid 2-like 2 (NRF2) signaling and decreased antioxidative enzyme levels, including heme oxygenase-1 (HMOX1), have been reported in patients with vitiligo. These antioxidative mechanisms are essential for the protection of melanocytes against H_2O_2-induced damage [7].

In contrast to this, ROSs can accelerate skin pigmentation as well. Keratinocytes adjacent to melanocytes were shown to contribute to UV-induced skin pigmentation, and keratinocyte-derived NO induces melanogenesis by increasing the levels of melanogenic factors, tyrosinase and tyrosinase-related protein 1 (TYRP1) [8]. ROSs, including NO, induce skin erythema through prostaglandin E2 synthesis. The expression of prostaglandin-endoperoxide synthase (PTGS), an enzyme crucial for prostaglandin E2 synthesis, is upregulated by ROS, which stimulates the inflammation [9]. Low levels of ROS are required for cellular signaling during the wound-healing process, primarily for angiogenesis maintenance, which indicates that these physiological low levels of ROS are necessary for the maintenance of skin functions and metabolism [10].

1.3. Increased ROS levels during injury, skin repair, and inflammatory diseases

ROSs have important roles in the wound healing, inflammatory, apoptotic, and other processes.

Excessive ROS production or impaired detoxification of the aggressive molecules can induce oxidative stress, which has been identified as an important feature in the pathogenesis of chronic, non-healing wounds. Excessive ROS levels lead to the oxidative modifications and biomolecular damage, altering lipid/protein/DNA structure and functions, inducing the irreversible oxidation of reactive protein thiol groups, which is a hallmark of oxidative stress, and the dysregulation of cell-signaling pathways, triggering downstream signaling cascades leading to altered cytokine release and exacerbation of inflammatory skin diseases. Malondialdehyde (MDA) levels were shown to be higher in the chronic ulcers than those in the acute wounds, and this molecule is an excessive ROS-induced lipid peroxidation product in skin wounds [11]. The significant increase in the allantoin to uric acid ratio was observed in wound fluids from chronic leg ulcers than those obtained from acute surgical wounds, representing a feature of oxidative stress [12].

ROSs were suggested to act as the secondary messengers in the induction of biological processes, such as the activation of MAPK/AP1, NF-κB, and JAK/STAT-signaling pathways during the pathogenesis of psoriasis or acne [13], and *in vitro* and *in vivo* investigations indicated the pathogenetic roles of ROS in vitiligo development [14, 15]. ROSs lead to a decrease in the activity of cellular proteins, such as TYRP1 (DHICA oxidase) in patients with vitiligo [16], and it may be involved in the pathogenesis of allergic skin reactions, photodermatosis, and drug eruption [17].

2. ROS roles in skin injury and repair

2.1. Skin injury and repair: inflammation, new tissue formation, and matrix remodeling

The wound-healing process consists of three partially overlapping stages: inflammation, new tissue formation, and tissue remodeling [18, 19]. Following the injury, vascular damage can cause platelet blockage and blood clot formation, resulting in the temporary closure of the wound and the invasion of various immune cells [20]. Neutrophils are recruited first, followed by the mononuclear cells, differentiated into mature tissue macrophages. These innate immune system cells secrete proteolytic enzymes and proinflammatory cytokines. They also produce

and secrete increased amounts of ROS, required to protect the organism from bacteria and other microorganisms. After the decrease in the immune cell numbers and proinflammatory cytokine levels, keratinocytes, fibroblasts, and endothelial cells localize to the wound and begin to proliferate.

The second stage is the new tissue formation stage. One to 2 days after an injury, the keratinocytes that have migrated to the injured dermis initiate the epithelialization, repairing the wounded skin. The new tissue, originally replacing the lost dermal tissue, is the granulation tissue, comprising fibroblasts, endothelial cells, and inflammatory cells. In this tissue, fibroblasts differentiate into myofibroblasts, responsible for wound contraction and the formation of collagen and other extracellular matrix proteins. New lymphatic vessels are generated to restore the lymphatic vessel system.

A long reconstruction stage follows. The resaturation of the original thickness of epidermis by keratinizing cells leads to the recovery of the epidermal barrier. In the granulation tissue, endothelial cells, myofibroblasts, and inflammatory cells undergo apoptosis, resulting in a significant reduction in cell numbers. Reconstruction of the extracellular matrix also occurs. Granulation tissue is characterized by collagen type III, and collagen type I is involved in the enhanced intermolecular crosslinking [19].

2.2. ROS roles in skin defense and repair of skin injury

The wound-healing process is regulated by a variety of different growth factors, cytokines, and hormones [19, 21]. Additionally, several recent studies revealed that nitric oxide and ROS represent the crucial regulators of this process [22, 23]. ROSs are required for the defense against invading pathogens [24], and at low concentrations, they are the crucial mediators of intracellular signaling [25]. A previous study showed that low H_2O_2 levels are important for the efficient neoangiogenesis in wounds [26].

ROS is produced by all cells during normal metabolic processes such as respiration. Additionally, NADPH oxidase, expressed by inflammatory cells in injured and inflamed tissues, is responsible for the generation of these molecules at high concentrations [27, 28]. Following the NADPH oxidase activation, the cells produce highly active superoxide radicals, which are rapidly decomposed into peroxides and water, a process mediated by SOD, leading to severe cellular damage [29, 30].

Due to the short half-life of ROS, their *in vivo* concentrations are difficult to determine. However, the level of H_2O_2 can be determined by using real-time electrochemical measurements, and using these techniques, low concentrations of H_2O_2 at the wounded site were detected. By contrast, higher levels were determined at the early stage of inflammation (at day 2 after the injury). At the later stages, together with the tissue remodeling (at day 5 after the injury), in addition to H_2O_2, superoxide was detected at the wound edges, and ROS levels were indirectly determined in these studies [26, 30].

The main product of lipid peroxidation that can be detected is 4-hydroxy-2-nonenal (4-HNE), and it was shown, using mouse models, that its expression is associated with the wound-healing process [31]. This suggests that low concentrations of ROS, produced during the process of wound healing, are important for the repair process. An additional lipid

peroxidation product is MDA; however, no differences in MDA levels were detected in wound fluids from the acute and chronic human wounds in this study [32]. Essential fatty acids are produced by propofol, a prostaglandin, and a significant increase in the concentration of 8-isopropionic acid was found in the liquid obtained from chronic venous ulcers, showing the oxidative stress conditions in chronic ulcers, which may be due to the persistence of strong inflammatory processes [31].

2.3. Excessive ROS production in skin wounds

Excessive ROS levels are harmful and can cause severe cellular damage. In human and animal cells in the presence of nitric oxide, calcium, and pathogens, the balance between oxidant and antioxidant systems is affected, promoting the generation and accumulation of ROS in cells, eventually inducing oxidative stress. Oxidative stress can result in DNA damage, mutations, and double-strand aberrations. Additionally, X-ray, UV light, alkylating agents, and intercalating agents that lead to the DNA damage *in vivo* and *in vitro* induce oxidative stress as well [33].

Melanocytes generate more ROS than other skin cells, such as keratinocytes, despite the ROS-scavenging activities of melanin. These cells are constantly under oxidative stress conditions due to melanogenesis, known as melanogenesis stress. Melanocytes were recently found to have a significantly lower repair capacity for both oxidative DNA damage and bulky photoproducts, making them more vulnerable to mutagenesis and tumorigenesis, and it may explain why melanocytes in the regions that have never been exposed to sunlight may still form mucosal melanomas. DNA damage in these melanocytes may be due to the oxidative stress and the generation of metabolites that interact with DNA [34].

3. ROS, skin cell apoptosis, and autophagy

3.1. Relationships between apoptosis, autophagy, and skin regeneration

Autophagy is considered one of the programmed cell death types, in addition to apoptosis, and it can be activated in all cells in response to stress or nutrient deprivation [35]. Induction of autophagy not only facilitates the degradation of damaged cellular components but provides the cell with molecular building blocks and energy as well [36]. Apoptosis is a unidirectional programmed cell death, activated to remove the aging and abnormal cells, and apoptotic defects can result in tumor development. A number of direct molecular connections between autophagy and apoptosis were identified, showing a potential causal link between the two processes [37]. Autophagy is involved in skin regeneration, cell differentiation, and tissue reconstruction as well, and this process may play a role in the protection or damage of tissue, at different healing stages and different wound severity. With the aging of the skin, autophagy levels decrease. Caloric restriction may induce autophagy, while food metabolism may induce ROS generation. Leptin is an obesity hormone, secreted by the adipose tissue that has a variety of systemic biological effects following the binding to its receptors, and it was shown to regulate autophagy in various cellular types [38]. Autophagy can restrain aging through anti-ROS effects, and leptin affects the respiratory chain, inhibits protein expression, decreases ROS

production, and regulates autophagy in the skin, slowing down the process of aging and improving skin regeneration by fibroblasts [39].

3.2. Relationships between ROS generation, apoptosis, autophagy, and skin regeneration

ROS and reactive nitrogen species (RNS)-induced stress affects many physiological processes, including cell survival and death. Although high ROS/RNS concentrations primarily lead to cell death, low free radical levels can directly modulate the activity of transcriptional factors, such as NF-κB, p53, and NRF2, and regulate numerous protein kinase cascades that participate in the regulation of the crosstalk between autophagy, apoptosis, and regeneration [40].

ROSs show molecular aggregation, which can not only affect intracellular proteins, lipids, DNA, and sugars but also induce other structural and functional damages. Additionally, these molecules may represent signaling molecules, regulating the initiation and transduction of apoptotic and autophagy signaling. Therefore, ROS play important roles in skin apoptotic processes, including those occurring in burn and other wounds and in vitiligo. Autophagy exists in eukaryotic cells, and the degradation of various molecules and organelles resulting from the process of autophagy generates a range of degradation products that can be used as raw materials in cells.

The pathogenesis of vitiligo was shown to be associated with ROS, apoptosis, autophagy, and the regeneration of melanocytes and keratinocytes. UV irradiation and chemical irritation can alter cellular membrane lipids, leading to the production of excessive ROS levels, which further induces an increase in the levels of 7-tetrahydrobiopterin ($7BH_4$), finally resulting in

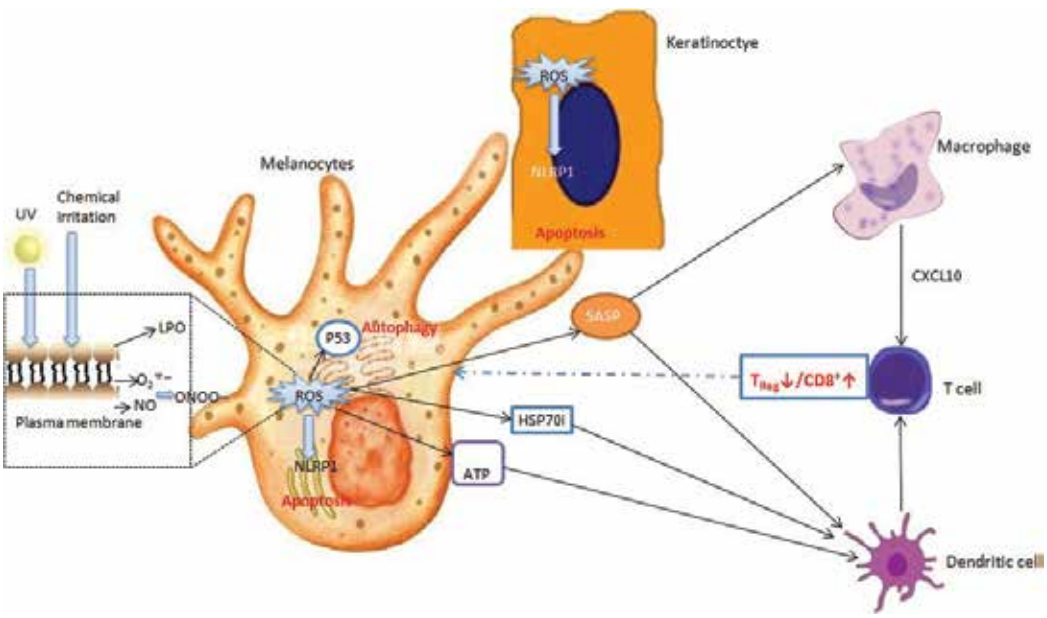

Figure 1. Reactive oxygen species, apoptosis, autophagy, and autoimmune reaction of melanocytes and keratinocytes in vitiligo patients. UV and chemical irritation alter membrane lipids and produce excessive ROS; ROS-induced autophagy and apoptosis by 7BH4, ATP, HSP70i, and NLRP1; ATP, SASP, and HSP70i induce an imbalance between T_{Reg} and $CD8^+$ T-cells, which lead to melanocyte death through an autoimmune reaction.

the autophagy of melanocytes. In the following step, ATP is released, together with the increase in inducible heat shock protein 70 (HSP70i) levels and the induction of senescence-associated secretory phenotype (SASP) [41]. Furthermore, ROSs generated in keratinocytes and melanocytes induce the expression of NLR family pyrin domain containing 1 (NLRP1) protein, which may be associated with the apoptosis of keratinocytes and melanocytes. Following the release of ATP and HSP70i and the induction of SASP, macrophages and dendritic cells in the proximity are activated, leading to the disturbance in T_{Reg} and $CD8^+$ T-cell balance with the recruitment of excessive melanocyte-specific $CD8^+$ T-cells that recognize melanocyte antigens. Moreover, the regeneration of melanocytes was shown to decrease in an environment containing ROS [42]. Taken together, these results show that ROS production is associated with the apoptosis, autophagy, and autoimmune reaction in vitiligo patients (**Figure 1**).

4. ROS, aging, and photoaging

4.1. UV irradiation and skin

The skin aging can be due to endogenous, natural aging, and exogenous aging, mainly photoaging. With age, the skin becomes thinner, dries, wrinkles, develops uneven pigmenting or liver spots (solar lentigines), and wound-healing processes are delayed. This may affect the quality of life and interfere with social or occupational functions. Therefore, the prevention of skin aging and improvement of wrinkling and pigmentation with minimal adverse effects represents the main goals of skin care and treatments [43, 44]. Chronic exposure to UV radiation is a major cause of skin aging, leading to the development of wrinkles, skin relaxation, and other photoaging characteristics. Skin structure is altered due to photoaging, including the epidermal stratum corneum integrity, hydration and lipidation, skin thickness, color, and light-absorbing properties. Similar to the physiological skin aging, photoaging is characterized by the development of wrinkles, roughness, and pigmentation, while histopathological analyses show a decrease in collagen levels and an increased expression and activity of matrix metalloproteinases (MMPs) in the dermis [45].

4.2. ROS and inflammation in the photoaging

UV exposure can lead to the excessive generation of ROS in the skin, and mitochondria are particularly susceptible to oxidative stress. Additionally, ROS can activate skin aging-related signaling pathways: MMP1-mediated aging, MAPK/AP-1/NF-κB/tumor necrosis factor (TNF)-α/IL-6-mediated inflammation-induced aging, and p53/BAX/cleaved caspase-3/cytochrome c-mediated apoptosis-induced aging [46].

UVB-induced ROS generation activates MAPK pathway, resulting in the expression of MMPs in the skin. MMPs, especially MMP1, are responsible for the extracellular matrix degradation in the skin [47], which may lead to the formation of wrinkles. Cytokines, such as PTGS2, tumor necrosis factor (TNF)-α, and IL-1b recruit neutrophils and induce the production of MMPs in dermis [48].

Inflammation-mediated skin aging is stimulated by the UVB-induced oxidative stress. Inflammatory factors, such as iNOS, PTGS2, cytokines, as well as IL-6, IL-1β, and TNF-α, are produced

by the immune cells. Following the exposure of skin to UV radiation, these cells release inflammatory cytokines, leading to the development of chronic inflammation and inflammatory aging [49]. Excessive ROS levels activate MAPK-signaling pathway and induce AP-1- and NF-κB-mediated expression of inflammatory proteins. AP-1, activated by MAPK signaling, can induce the production of inflammatory proteins, which further boosts inflammation and skin aging and may even lead to cancer development.

Most aging-associated diseases share some common inflammation-related characteristics, such as the activation of transcription factors including NF-κB and sirtuins, which directly or indirectly promote inflammation-induced signaling and are involved in cellular oxidative stress aggravation by increasing ROS production and inducing skin cell apoptosis [50]. NF-κB is a kappa light-chain synthesis promoter in B cells that is involved in the development of diverse skin diseases, such as psoriasis vulgaris, allergic dermatitis, and skin cancer, and which was shown to induce the expression of MMP1 [51]. The functions of NF-κB are associated with cellular longevity, as it regulates the expression of telomerase genes, inflammation, angiogenic, and anti-apoptotic factors, cellular proliferation, and other processes.

Acute dermal overexposure to UV radiation causes sunburn and induces an inflammatory response with increased prostaglandin and proinflammatory cytokine production, causing erythema, vasodilation, and leukocyte infiltration. After UV exposure, keratinocytes and other skin cells upregulate proinflammatory cytokine production, including that of IL-1, IL-6, and TNF-α, and induce the expression of vascular adhesion molecules. TNF-α is thought to be a central mediator of UV-induced inflammation. An effective topical antioxidant may reduce UV-induced skin cancer development and prevent or delay skin photoaging through the reduction of UVA-induced ROS generation. Cycloheterophyllin was shown to inhibit UVA- and ROS-induced phosphorylation of the member of MAPK pathway [52]. Furthermore, JP4-039, a gramicidin S-conjugated nitroxide, was shown to be a potent electron-scavenging agent that can provide the protection of mitochondrial membrane from ROS-induced damage [53]. Therefore, the inhibition of several signaling and cytokine pathways may represent a beneficial anti-photoaging approach, and antioxidant supplements may improve skin health.

5. ROS, inflammation, and skin disease development

5.1. Relationship between ROS production and inflammation in the development of skin diseases

ROSs have roles in the skin injury, repair, regeneration, aging, and inflammatory processes, and skin is heavily affected by the oxidative stress. Although harmful effects of ROSs are attenuated by endogenous antioxidants, increased or prolonged presence of free radicals can prevent the effectiveness of ROS defense mechanisms and lead to the activation of cellular responses that result in the development of various skin disorders, including photosensitivity-associated diseases and skin malignancies. Skin prevents the exposure to the ionizing and UV light irradiation, chemicals, such as redox-active quinones, or their metabolites, and pathogens, which may induce the excessive generation of ROS that can further prevent the efficacy of antioxidant systems and other oxidant-degrading pathways [54].

ROS can directly affect the activity of kinases, phosphatases, and transcription factors, or modulate cysteine-rich redox-sensitive proteins. In human keratinocytes, ROSs enhance EGFR phosphorylation and activate ERKs and JNKs. MAPK family includes p38, ERK, and JNK, which interact [55]. However, the ERK pathway primarily mediates cellular responses to growth factors, whereas the JNK and p38 pathways primarily mediate cellular responses to cytokines and physical stress. A recent study demonstrated that the peroxisome proliferator activated receptors, whose natural ligands are polyunsaturated fatty acids and their oxidation products, may be involved in the pathogenesis of psoriasis or acne [56]. Furthermore, oxidative stress compromises the function of cellular proteins, such as tyrosine-related protein 1 (TRP1), in patients with vitiligo [57]. ROSs have been observed to induce the apoptosis of keratinocytes, which could result in melanocyte detachment at the borders of vitiligo lesions. This finding may explain the role of ROS in the pathogenesis of vitiligo [58].

5.2. ROS and vitiligo pathogenesis

Vitiligo is an acquired chronic depigmenting disease that affects 0.5–2% of the world population. Vitiligo develops due to progressive and gradual disappearance of epidermal melanocytes, which is associated with polymorphisms in genes involved in the immune response and melanogenesis, and as the result of a complex interplay between biochemical, environmental, and immunological events. Recently, ROSs were shown to play important roles in the development and progression of vitiligo. In active vitiligo, ROS and H_2O_2 were found in excess, and these molecules can affect biological processes. Additionally, excess H_2O_2 levels impair tyrosinase activity through the oxidation of methionine residues in this key melanogenic enzyme [59]. Processes that are involved in oxidative damage repair can be damaged by H_2O_2 as well [60], and numerous proteins and peptides, in addition to tyrosinase, are affected during oxidative stress. Therefore, increased ROS levels lead to melanocyte destruction [61]. *In vitro* and *in vivo* experiments showed an increased susceptibility of melanocytes in vitiligo patients to the increased ROS levels. In these cells, p53 is overexpressed, and some of its target genes induce SASP, which is characterized by the production of IL-6, MMP3, PTGS2, insulin-like growth factor-binding protein 3 (IGFBP3), and IGFBP7 [62, 63]. The overexpression of p53 further induced autophagic processes and ATP releases, leading to the initiation of degenerative process. The released ATP and SASP activate dendritic cells, which induce the imbalance between T_{Reg} and $CD8^+$ T-cells through CXCL10 activity [64]. Taken together, these results indicate the correlation between ROS generation and immune responses during skin depigmentation.

5.3. ROS and psoriasis pathogenesis

Psoriasis is a frequent recurrent chronic immune-mediated hyperproliferative inflammatory skin disease which affects about 2% of the world population [13]. Several proinflammatory cytokines, such as ILs, TNF, and interferon-γ (IFN-γ), were shown to be overexpressed in psoriatic lesions [65]. These findings demonstrate that ROS-mediated oxidative stress is involved in many biological responses leading to DNA modification, lipid peroxidation, and inflammatory cytokine production [66] and confirm that ROSs play a role in the pathogenesis of psoriasis. Treatment strategies including the application of antioxidants were shown to be effective in the treatment of psoriasis patients.

Several signaling transduction pathways are involved in the pathogenesis of psoriasis, such as MAPK/AP1, NF-κB, and JAK/STAT, which act by upregulating the expression of proinflammatory cytokines and chemokines [67]. ROSs were confirmed to act as secondary messengers by modulating these transduction cascades and inducing psoriasis development. Recent studies identified ROS-mediated activation of the MAPK/AP1-signaling pathway and the activation of RAS, MEKK1, ASK1, and MLK3 receptors, subsequently leading to the expression of their target genes. Furthermore, JNK/p38 MAPK pathway activation induces the expression of inflammatory cytokines [68]. Additionally, ROS modulates the expression of PKCζ, a signal transduction molecule downstream of TNF that is involved in the overexpression of CD1d, an HLA-class-I-like molecule, which is potentially involved in keratinocyte-natural killer T-cell interactions in psoriatic lesions [69]. These findings demonstrate that ROSs are involved in the pathogenesis of psoriasis and that the application of antioxidants may be useful for the treatment of psoriasis and other inflammatory diseases with considerable ROS involvement.

6. The application of antioxidants in dermatology

6.1. Main antioxidants: ascorbic acid, tocopherols (vitamin E), carotenoids, Zn(II)-glycine, and polyphenols

Under stress conditions, the overproduction of ROS in plants is common. Ascorbic acid (vitamin C, AsA) is one of the universal non-enzymatic antioxidants with ROS-scavenging potential and affecting many functions in plants under both stress and physiological conditions [70]. This molecule is an antioxidant and a key substrate during the removal of ROS. Furthermore, it plays diverse physiological roles in humans, while in the skin, it represents a cofactor required for the enzymatic activity of prolyl hydroxylase, which hydroxylates prolyl residues in procollagen and elastin [71]. Ascorbic acid can be applied as a depigmentation agent due to its tyrosinase-inhibition effects [72].

Vitamin E belongs to a group of fat-soluble antioxidants, which includes tocotrienols and tocopherols, and it was shown to lead to a decrease in the levels of PKC, an important cellular signaling molecule. Vitamin E can regulate the inflammatory arachidonic acid cascade by increasing cytosolic phospholipase A2 and PTGS2 activities [73]. Four tocopherols are absorbed through food, but only R7BH4 RRR-α-tocopherol represents a vitamin [74]. The antioxidative mechanism of tocopherols is partially due to the hydroxyl group in the chromanol ring donating a hydrogen atom to reduce free radical levels [72]. Tocopherol has preventive effects in various oxidative stress conditions. A detailed study of the ROS-scavenging activity indicated that γ-tocopherol is superior to α-tocopherol in NO scavenging [75]. Therefore, tocopherol may suppress melanogenesis. Several clinical studies showed that the nutraceutical formula combining omega-3 and omega-6 fatty acids with vitamins (PLP10), including vitamins A, C, and E, may provide beneficial antioxidant effects. In one small clinical study (8–12 participants per treatment arm), a mixture of several PUFAs, monosaturated fatty acids, and saturated fatty acids, together with vitamin E and vitamin A, considerably reduced multiple sclerosis relapse rate (10%) compared with that in the control (58%) [76]. The authors showed that vitamin E is required in this combination, but the limited number of patients enrolled in the study prevented a definitive conclusion. Population studies showed that vitamin intake levels did not correlate with increased

multiple sclerosis risk or disease progression when adjusted for age, time, latitude of birthplace, smoking, and total energy levels [77, 78].

Carotenoids are organic pigments that are naturally produced by plants, algae, some fungi, and bacteria, and this group includes β-carotene, astaxanthin, and lycopene. Carotenoids can quench $^1O_2$ and are used to prevent UV-induced damage. Lycopene concentration in the skin was shown to be related with skin roughness, suggesting that higher antioxidant levels in the skin correlate with lower skin roughness, which represents an early stage of wrinkle formation [78].

Zn(II)-glycine, a coordinated Zn^{2+} and glycine compound, is a cell-membrane-permeable inducer of metallothionein expression, which prevents UVB-induced cell damage and suppresses IL-1a secretion and prostaglandin E2 synthesis in human keratinocytes [79]. Additionally, this molecule leads to the reduction in pro-MMP1 production and MMP1 levels in dermal fibroblasts [4].

Polyphenols are the most abundant dietary antioxidants, found in fruits, vegetables, and cereals, and characterized by the presence of phenol units. They were shown to have antioxidant and scavenging activities [73]. Epigallocatechin gallate (EGCG) is a representative polyphenol, and the oral administration of EGCG for 8 weeks was demonstrated to significantly increase the minimal UV-induced erythema dose (MED) and to protect against the disruption of the epidermal barrier function. These findings indicate that EGCG enhances the skin tolerance to the UV-induced stress [80].

6.2. Dimethyl formamide (DMF), simvastatin, *Ginkgo biloba*

Since 1959, a drug containing DMF has been used as the oral treatment of moderate to severe psoriasis, showing a high level of efficacy [81]. *In vitro* and *vivo* studies indicate that DMF induces the upregulation of GPX and NAD(P)H: quinone oxidoreductase 1 (NQO1), two antioxidative pathways [82].

The effects of atorvastatin and simvastatin on oxidative stress markers in rats with hyperhomocysteinemia (Hhcy) were analyzed, and simvastatin was shown to have a superior antioxidant activity compared with that of the atorvastatin, independent of its effects on the lipid profile, but dependent on the homocysteine concentration. Simvastatin was shown to protect human melanocytes from H_2O_2-induced oxidative stress by activating NRF2, which indicates that this compound may be used for vitiligo treatment [83]. Sufficiently powered prospective clinical and intervention studies are required to determine the antioxidant effectiveness of simvastatin [84].

G. biloba extract is obtained from *G. biloba* tree leaves, and the commercially available products contain mixtures of biologically active compounds [85]. The percentage of each compound in different *G. biloba* extract components varies between suppliers, but the most common research formulation is EGb-761, which contains approximately 24% flavone glycosides, 7% proanthocyanidins, and 6% terpene lactones. *G. biloba* was reported to have beneficial effects as a monotherapy for the slowly spreading vitiligo [86]. No severe side effects related to *G. biloba* have been reported [73].

Coenzyme Q10 (CoQ10) is an intracellular antioxidant that can reduce UVA-induced DNA damage levels in human keratinocytes *in vitro*. CoQ10 suppresses MMP1 production in dermal

fibroblasts due to the downregulation of IL-6 in UVB-irradiated keratinocytes [87]. Moreover, CoQ10 accelerates the production of basement membrane components, such as laminin 332 and type IV and VII collagens in keratinocytes and fibroblasts, respectively. However, no effects on type I collagen production in fibroblasts were reported. CoQ10 was shown to have anti-aging effects, by accelerating the production of epidermal basement membrane components [88].

Grapes are one of the most widely grown fruits, and grape seeds are rich in proanthocyanidins, which have been shown to scavenge free radicals. Grape seeds contain 40% fiber, 16% oil, 11% proteins, and 7% complex phenols such as tannins, and they represent flavonoid source, including monomers, dimers, trimers, oligomers, and polymers. The monomeric compounds contain (+)-catechins, (−)-epicatechin, and (−)-epicatechin-3-O-gallate. Grape seeds exhibit a broad spectrum of antioxidative properties and may have potential health benefits including anti-diabetic, anti-cholesterol, anti-platelet (anticoagulant), and oxidative damage-protective functions [89].

7. Summary and future perspectives

As the heaviest, largest organ, with the most complex functions, the skin is very vulnerable to a variety of redox reactions, and the balance between oxidants and antioxidants must be maintained. ROSs at low concentrations exert their physiological activity, but the increased levels of these molecules are involved in the pathological processes, including injuries, repair, tissue regeneration, aging, autophagy, apoptosis, and inflammation. UV exposure and aging induce ROS generation, and antioxidants such as EGCG and resveratrol may represent effective treatments for the prevention of UV-induced skin aging. Additionally, ROSs play significant roles in the pathogenesis of several inflammatory cutaneous diseases, including psoriasis and vitiligo. ROSs are involved as the secondary messengers in the MAPK/AP1, NF-κB, and JAK/STAT-signaling pathways, which are activated early during the development of inflammatory disorders such as psoriasis. Numerous studies demonstrated that ROSs represent the trigger factors that can induce autophagy, apoptosis, and autoimmune responses in melanocytes during the pathogenesis of vitiligo. The molecular mechanisms underlying the regulation of ROS-mediated signaling pathways remain unclear. Many issues should be further investigated, concerning the physiological and pathological effects of ROS as well as the molecular mechanisms underlying these processes. ROS involved in the skin injury, repair, regeneration, aging, autophagy, apoptosis, and inflammatory process should be identified as well. Some organic compounds that can induce antioxidative responses were shown to be effective therapeutics for the treatment of skin diseases where the oxidative stress plays a prominent pathogenic role. With the development of phytoextraction and medicinal chemistry technology, an increasing number of antioxidant agents may be applied in the treatment of skin diseases and to decelerate aging.

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Author details

Hui Xu[1,2], Yun-Wen Zheng[1,3,5]*, Qi Liu[1,2], Li-Ping Liu[1,2,3], Feng-Lin Luo[1,2], Hu-Chen Zhou[1,2], Hiroko Isoda[4], Nobuhiro Ohkohchi[3] and Yu-Mei Li[1,2]*

*Address all correspondence to: ywzheng@md.tsukuba.ac.jp; l.yumei@aliyun.com

1 Research Center of Stem Cells and Regenerative Medicine, Jiangsu University, Zhenjiang, China

2 Department of Dermatology, Affiliated Hospital of Jiangsu University, Zhenjiang, China

3 Faculty of Medicine, University of Tsukuba, Tsukuba, Japan

4 Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan

5 Department of Regenerative Medicine, School of Medicine, Yokohama City University, Yokohama, Japan

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Chapter 6

Reactive Oxygen Species and Sperm Cells

Teppei Takeshima, Shinnosuke Kuroda and Yasushi Yumura

Additional information is available at the end of the chapter

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Abstract

Many cases of male factor infertility are idiopathic, but 30–40% of cases may have excessive levels of reactive oxygen species (ROS) in their semen. The origins of endogenous ROS are leukocytes and immature spermatozoa, and external causes are various. On the contrary, seminal plasma contains various antioxidants. Low levels of ROS are essential for the fertilization process, but excessive levels of ROS lead to oxidative stress and can have harmful effects such as lipid peroxidation of a membrane, sperm deoxyribonucleic acid fragmentation, and apoptosis on the fertile capacity. In order to evaluate oxidative stress appropriately, ROS is measured by the chemiluminescence method with neat semen and quantification of 8-OH-2′-deoxyguanosine and malondialdehyde in seminal plasma. Antioxidant potential is often measured using total antioxidant capacity (TAC) assay. The oxidation-reduction potential measured by a MiOXSYS analyzer is a novel, easier, quicker, and less expensive technology to measure oxidative stress. In order to minimize oxidative stress and improve clinical outcomes, sperm-sorting methods, lifestyle modifications, shortening the ejaculatory abstinence, and treatments such as oral antioxidants, varicocelectomy, and testicular sperm extraction are taken into account. As a future prospect, proteomics, metabolomics, and genomics are still developing areas that have the potential to discover new findings and highly sensitive biomarkers.

Keywords: reactive oxygen species, oxidative stress, lipid peroxidation, sperm DNA fragmentation, antioxidants, male infertility

1. Introduction

Infertility is defined as the inability to achieve pregnancy despite 1 year of regular, unprotected, and well-timed intercourse [1], and it is a global problem faced by 15% of couples. Approximately 50% of all cases presenting at infertility clinics are due to male factors [2]. However, the etiology of male factors is multifactorial and many cases are idiopathic [2]. Various studies have been

conducted to elucidate the pathologies of idiopathic male infertility. Since Aitken and Clarkson first detected reactive oxygen species (ROS) in processed human ejaculate using the chemiluminescence method in 1987 [3], there have been many reports on the influence of oxidative stress on male fertile capacity. A low level of ROS is essential for the process of fertilization, such as capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion [4, 5]. On the contrary, a high level of ROS results in lipid peroxidation (LPO), deoxyribonucleic acid (DNA) damage, and induction of apoptosis [6], which has been reported to negatively affect sperm concentration [7, 8], motility [9–11], morphology [12, 13], and male fertile capacity [14]. Clinically, several studies reported that oxidative stress resulted in significantly lower fertilization rates, implantation failure, impaired embryonic development, recurrent pregnancy loss, lower live-birth rates, and poor assisted reproductive treatment (ART) outcomes [15–17]. It is considered that an understanding of the etiologies and influences of ROS on sperm, measuring oxidative stress appropriately, and treating based on the etiologies play an important role in improving the outcomes of male infertility.

2. Etiology of ROS in human semen

Oxidative stress in human semen develops as a result of an imbalance between ROS production and antioxidant capacity [18]. ROS are categorized as (1) free radicals, such as superoxide anion ($\cdot O_2^-$), hydroxyl radicals ($\cdot OH$), and peroxyl radicals ($\cdot RO_2$) and (2) nonradical species, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO) [19]. Free radicals contain at least one unpaired valence electron that are highly reactive but have a short life span. Therefore, they become paired by depriving an unpaired electron from other compounds, which causes oxidation. Nonradical species, such as hydrogen peroxide itself, is not very reactive, but it generates hydroxyl radicals in the presence of metal ions in vivo [20].

However, it has become apparent in the past two decades that ROS at low levels function as signaling molecules to regulate biological and physiological processes [21]. ROS induce cyclic adenosine monophosphate in spermatozoa and elevate the level of tyrosine phosphorylation. Localization of tyrosine phosphorylation to the flagellum also leads to hyperactivation in the female genital tract. Moreover, tyrosine phosphorylation leads to binding the spermatozoon to the zona pellucida and is necessary for the acrosome reaction [4, 5]. On the contrary, oxidative stress caused by high levels of ROS has various adverse effects in a living body, such as cranial nerve disease (e.g., Alzheimer's disease and Parkinson's disease), arteriosclerosis, diabetes, and inflammatory bowel disease (e.g., ulcerative colitis and Crohn's disease) [22–25]. Similarly, ROS in semen function as an essential second messenger in the fertilization process at low levels, including capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion [4, 5]. Conversely, high levels of ROS in semen have been reported to be present in 25–40% of infertile men [9, 15, 26, 27]. Oxidative stress in semen is well known to cause LPO of the sperm membrane, which leads to a loss of fluidity, sperm DNA fragmentation, and apoptosis.

The principal sources of endogenous ROS in semen are seminal leukocytes [3, 28] and immature spermatozoa with an abnormal head morphology and cytoplasmic retention [29, 30]. When inflammation and infection occur in the male genital tract, chemotaxis and activation of leukocytes are stimulated, and they destroy pathogens by activating the myeloperoxidase system

[26], which produces ROS. On the contrary, in morphologically normal spermatozoa, cytoplasm deposits in the midpiece are extruded to allow cell elongation and condensation to occur during spermiogenesis, and the cytoplasm deposits contain large amounts of the glucose-6-phosphate dehydrogenase enzyme that produces nicotinamide adenine dinucleotide phosphate (NADPH). As a result, ROS is generated from NADPH via an intramembrane-located NADPH oxidase (NOX) [31]. So, immature spermatozoa are characterized by large amounts of cytoplasm that are expected to produce higher levels of ROS. It is important to determine the source of ROS in semen because the clinical implications of infiltrating leukocytes are quite different from those of pathological conditions in which the immature spermatozoa themselves are the source of ROS [32].

Myeloperoxidase staining (the Endz test) is useful to differentiate leukocytes (neutrophils and macrophages) from germinal cells [33]. Peroxidase-positive leukocytes in semen are identified for their capacity to generate high levels of ROS, contributed largely by the prostate and seminal vesicles [34]. These activated leukocytes can produce 100-fold higher amounts of ROS than nonactivated leukocytes because they increase NADPH production via the hexose monophosphate shunt [35].

Nitroblue tetrazolium (NBT) staining is used to detect ROS generation by immature spermatozoa [36]. NBT is a yellow water-soluble nitro-substituted aromatic tetrazolium compound that reacts with cellular superoxide ions to form a formazan derivative that can be monitored spectrophotometrically. Oxidation in the cytoplasm helps transfer electrons from NADPH to NBT and reduces NBT into formazan. The principle of this test is based on the conversion of NBT into blue-pigmented diformazan after interacting with superoxide. The concentration of diformazan is correlated with intracellular ROS concentration [37]. This NBT staining is a cost-effective and easy-to-use method that can predict ROS levels and simultaneously detect the source of ROS generation.

3. External causes of potential oxidative stress

External causes of ROS are shown in **Table 1**. They are classified roughly into lifestyle, environment, infection, autoimmune, testicular, idiopathic, iatrogenic, and chronic disease factors. Especially, tobacco contains more than 400 kinds of constituents, including nicotine, tar, carbonic monoxide, polycyclic aromatic hydrocarbons, and heavy metals, so the toxicological mechanism of smoking is complicated. Especially, nicotine is oxidative and can induce double-strand DNA breaks in sperm DNA in vivo [38]. It is reported that smokers had a 48% increase in seminal leukocyte levels and a 107% increase in seminal ROS levels compared with nonsmokers [39]. The sperm DNA fragmentation index (DFI) is also increased in infertile smokers compared with infertile nonsmokers (37.66% vs. 14.51%, $P < 0.001$) [40]. Furthermore, natural antioxidants such as vitamins C and E in seminal plasma were decreased in smokers, which indicates a reduced protection against oxidative stress [41].

Scrotal. hyperthermia may result from wearing close-fitting underwear, sauna use, longtime bathing, and cycling [42–45]. The position of the scrotum acts to maintain the temperature of the testes (34–35°C) lower than that of the body (36–37°C) [46]. An elevated scrotum temperature may negatively but reversibly affect spermatogenesis and oxidative stress. Rao et al. concluded that intermittent heat exposure could more seriously damage spermatogenesis than consecutive heat exposure [45]. Oxidative stress may participate in the suppression of spermatogenesis.

Lifestyle	Smoking
	Obesity
	Alcohol abuse
	Aging
Environmental	Pollution
	Heavy metals
	Heat
	Mobile phone radiation
	Phthalate
Infection	Genitourinary tract
Testicular	Varicocele
	Testicular torsion
Iatrogenic	Centrifugation
	Cryopreservation
	Drug
Others	

Table 1. External cause of oxidative stress.

Obesity also provokes oxidative stress because proinflammatory cytokines are released from adipose tissues, which leads to an increase in leukocytes [47]. Moreover, in numerous studies, a positive correlation between body mass index and DFI has been reported [48].

A clinical varicocele is almost exclusively left-sided and is a pampiniform plexus of the spermatic cord that forms a tangle of distended blood vessels in the scrotum. It has an incidence of approximately 15% in the general male population and 30–40% in men with primary infertility and 75% with secondary infertility [49]. A varicocele is considered to be the most common surgically correctable cause of male infertility [50]. Oxidative stress is considered to be the main factor contributing to infertility in men with a varicocele, to which the testis responds by way of high scrotal temperature, testicular hypoxia, adrenal metabolite backflow, or production of vasodilators such as nitric oxide [51]. The dominant mechanism is that impairment of protamination and chromatin compaction in sperm increases the susceptibility of affected cells to oxidative stress causing defective spermiogenesis [52]. Most studies reported that the level of seminal ROS in men with clinical varicoceles was higher than control subjects [53]. Moreover, the antioxidant level in both seminal plasma and blood was reduced in patients with a varicocele [54].

4. Lipid peroxidation

Elevated ROS production causes peroxidation of polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid, which has six double bonds per molecule in the sperm cell

membrane [55]. This LPO of the sperm cell membrane causes a loss of membrane fluidity and integrity that are required for sperm-oocyte fusion [56]. LPO has two phases: the first phase is "initiation," which is the abstraction of a hydrogen atom from an unsaturated fatty acid, and the second phase is "propagation," which is the formation of a lipid alkyl radical followed by its rapid reaction with oxygen to form a lipid peroxyl radical [57]. A peroxyl radical can remove a hydrogen atom from an unsaturated fatty acid to produce a lipid radical and lipid hydroperoxide [58].

The products of LPO are malondialdehyde (MDA), conjugated dienes, and secondary peroxidation products such as saturated and unsaturated aldehydes, ketones, oxo- and hydroxyl acids, and saturated and unsaturated hydrocarbons (e.g., ethane and pentane). The methods used to detect and measure LPO include the spectrophotometric thiobarbituric acid (TBA) test, a fatty acid analysis by high-performance liquid chromatography, and an oxygen electrode. TBA test has been frequently used as an indicator of the peroxidation of PUFAs [55, 59]. Several studies have shown that LPO has detrimental effects on sperm concentration, motility, and morphology and is associated with poor sperm quality [60].

5. Sperm DNA fragmentation

Excessive amounts of ROS can damage sperm DNA directly or indirectly through the activation of sperm caspases and endonucleases. DNA fragmentation occurs after spermiation during comigration of mature and immature sperms from the seminiferous tubules to the cauda epididymis by ROS exposure, resulting in the formation of 8-OH-guanine and 8-OH-2′-deoxyguanosine (8-OHdG) [61].

DNA fragmentation can occur in single-strand (ss-) DNA and double-strand (ds-) DNA. The ss-DNA damage can be repaired by the human oocyte and embryo, although the repair ability decreases with advanced maternal age [62]. However, spermatozoa with ds-DNA damage fall into apoptosis [63]. The presence of unrepaired DNA damage above the critical threshold in an embryo has an adverse effect on embryo development and implantation, which has been characterized as the "late paternal effect." [64] On day 2–3 of human embryo development (between the four-cell and eight-cell stage), as embryonic genome activation begins, embryo development switches from being dependent on the maternal factor to being dependent on the embryo's own genomes [65]. Therefore, sperm with DNA damage has a detrimental effect on the rate of blastulation, implantation, and pregnancy. Several studies have investigated the relationship between ART outcome and high DNA damage in sperms. The meta-analysis of Zhao et al. showed that sperm DNA damage was significantly associated with pregnancy [combined relative risk (RR): 0.81; 95% confidence interval (CI): 0.70–0.95; $P = 0.008$] and miscarriage (combined RR: 2.28; 95% CI: 1.55–3.35; $P < 0.0001$) [66].

6. Antioxidants in human semen

All human ejaculate contains intra- and extracellular antioxidants in the seminal plasma. They are categorized as enzymatic and nonenzymatic antioxidants (**Table 2**) [67]. Enzymatic

Enzymatic antioxidants	Superoxide dismutase
	Glutathione peroxidase
	Catalase
Nonenzymatic antioxidants	Ascorbic acid (vitamin C)
	Alpha-tocopherol (vitamin E)
	Urate
	Coenzyme Q10
	L-Carnitine
	Melatonin
	Myo-inositol
	Lactoferrin
	Astaxanthin

Table 2. Antioxidants in human semen.

antioxidants contain superoxide dismutase (SOD), catalase, and glutathione peroxidase. Nonenzymatic antioxidants contain ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), urate, melatonin, transferrin, carnitine, and lactoferrin [67–69]. These antioxidants function as scavengers of ROS by self-protection mechanisms. A total antioxidant capacity (TAC) score has often been used to measure the total nonenzymatic antioxidant capacity in seminal plasma [69]. Seminal TAC can be measured as the total available antioxidants in the seminal plasma, while measuring by specific antioxidant assays is expensive, cumbersome to perform, and provides limited information about the assessed antioxidants [70]. The principle of this assay is based on the ability of all antioxidants in the seminal plasma specimen to inhibit the oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) to $ABTS^{+}$, resulting in a change of absorbance at 750 nm to a degree that is proportional to their concentration. The capacity of the antioxidants present in the sample to prevent ABTS oxidation was compared with that of standard Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analog. The results are reported as micromoles of a Trolox equivalent. The total antioxidant concentration of each sample was calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values from each sample into the equation [71]:

$$\text{antioxidant } (\mu M) = \left[(\text{unknown average absorbance} - Y - \text{intercept})/\text{slope}\right] \times \text{dilution} \times 1000. \quad (1)$$

Past studies showed that low levels of seminal TAC were related to male infertility [72]. Mahfouz et al. reported the best cutoff value of seminal plasma TAC level was 1420 μM with high sensitivity and specificity [73]. However, TAC levels are not routinely measured as a standard infertility evaluation because TAC assay is cumbersome and employs costly equipment and skills. To measure more precise oxidative stress, the ROS-TAC score described below is more useful than TAC alone [12].

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7. Measurement of ROS in human semen

ROS measurements can be determined by direct or indirect assays (**Table 3**). Direct assays measure the oxidation levels of the sperm cell membrane. Indirect assays measure the detrimental effects of oxidative stress, such as sperm DNA damage or LPO levels [74]. The chemiluminescence method is a direct assay that is commonly used to measure seminal ROS. Takeshima et al. [9] and Yumura et al. [11] reported that chemiluminescence was recorded using a computer-driven luminometer after the addition of 40 μL of 100 mmol/L 5-amino-2,3-dihydro 1,4-phtalazinedione (luminol) to 500 μL of unwashed semen. The Luminometer 1251™ (LKB Wallac, Turku, Finland) was used to measure the ROS level of unprocessed semen according to the previously reported method. When the peak level was ≥0.1 mV/s, ROS formation was considered positive. The integral level of ROS production in the present study was calculated by subtraction of the area under the baseline from total chemiluminescence values between 0 and 30 min after the addition of luminol to unwashed semen and expressed as mV/s/30 min/10^6 spermatozoa (**Figure 1**) [9]. The Monolight 3010™ Luminometer (BD Biosciences Pharmingen, Ltd., San Diego, CA, USA) was similarly used to measure ROS levels. The subtraction of the integrated chemiluminescence between 0 and 200 s before and after the addition of luminor to the sample was measured. The calculated chemiluminescence value was expressed as relative light units (RLU)/200 s/10^6 spermatozoa. The chemiluminescence ROS level threshold was defined as 4332.4 RLU/200 s [11]. A variety of luminometers are available. Single- and double-tube luminometers are sensitive and inexpensive but can only measure one or two specimens at one time, so it is not suitable for a center where many specimens are handled [75].

As described above, NBT assay is a cost-effective and user-friendly direct assay. The advantage of this assay is that it is can evaluate the ROS level and its potential source (such as spermatozoa or leukocytes) using a light microscope. The concentration of diformazan is correlated with the intracellular ROS concentration [36, 37].

MDA is an end product of LPO, and it represents the level of LPO. In a TBA assay, sperm MDA concentration is measured using spectrophotometry [76]. Sperm MDA levels are positively correlated with seminal ROS in infertile men [76].

8-Hydroxy-2-deoxyguanosine (8-OHdG) is a product of oxidative DNA damage following specific enzymatic cleavage after 8-hydroxylation of the guanine base and is also used as a sensitive marker of oxidative DNA damage caused by ROS in human sperm [77]. 8-OHdG in seminal plasma can be measured by the enzyme-linked immunosorbent assay, and 8-OHdG

Direct assays	Indirect assays
Chemiluminescence	Myeloperoxidase (Endz) test
Nitroblue tetrazolium test	Lipid oxidation level
Flow cytometry	Oxidation reduction potential
Electron spin resonance	Total antioxidant assay

Table 3. Direct and indirect semen assays of ROS.

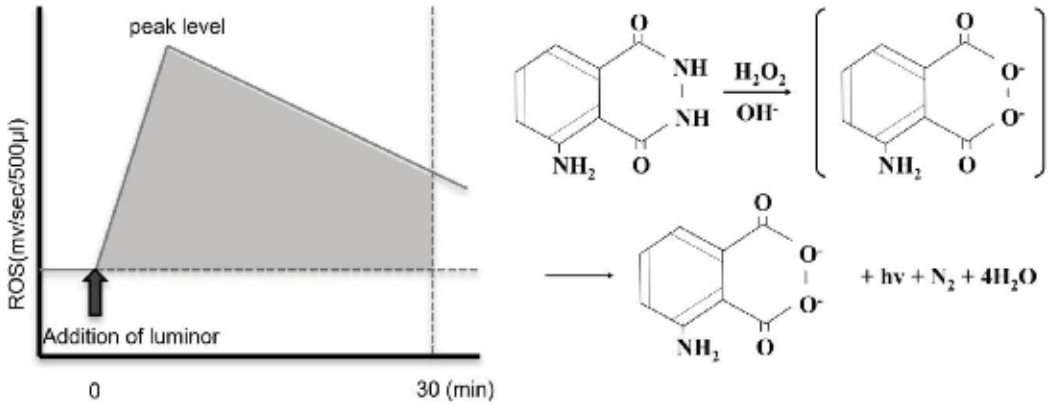

Figure 1. ROS measurement by chemiluminescence assay.

in testicular tissue can be quantified by immunohistochemical staining using an anti-8-OHdG monoclonal antibody as a primary antibody [78].

As described above, seminal TAC can be measured as the total available antioxidants in the seminal plasma [71]. But instead of ROS or the TAC score alone, an ROS-TAC score is thought to be a better predictor of oxidative stress [79]. The ROS-TAC score is a parameter derived from the ratio of ROS concentrations in washed sperm suspensions and TAC in seminal plasma using a principal component analysis to obtain a standard index of oxidative stress. A cutoff value of 30 is determined as the lower limit of a normal range ROS-TAC score. Patients with scores <30 are thought to be at risk of infertility [80]. However, a TAC assay is not carried out routinely because it is cumbersome and requires the use of costly equipment and technical skills [12].

Instead, the oxidation-reduction potential (ORP) is a direct measurement of oxidative stress by the MiOXSYS system, which is a novel, easier, quicker, and less expensive technology to measure the transfer of electrons from reductants to oxidants in human semen [81, 82]. Past studies have successfully evaluated ORP in the blood of patients with cerebral vascular disease, heart disease, and metabolic syndrome. In recent years, ORP measurements in semen have been carried out [81, 82]. Using a MiOXSYS Analyzer (Aytu BioScience Inc., Englewood, CO), ORP values were calculated and expressed as mV/10^6 spermatozoa/mL. A higher ORP level indicated an imbalance in the activity of all available oxidants relative to all available antioxidants in the ejaculate and indicates a state of oxidative stress. Agarwal et al. confirmed the cutoff level as 1.36 mV/10^6 spermatozoa/mL [83]. This MiOXSYS system has shown promise as a diagnostic tool in the evaluation of male infertility.

8. Prevention and treatment

There are several types of sperm-sorting methods, lifestyle modifications, and treatment strategies that can be used to minimize the detrimental effects of oxidative stress on reproductive function.

8.1. Sperm-sorting methods

Approaches of sperm selection that overcome production of oxidative stress and remove sperm with DNA damage include density gradient centrifugation (DGC) [84], electrophoretic separation [85], intracytoplasmic morphologically selected sperm injection (IMSI) [86], hyaluronic acid binding assay [87], and annexin-V magnetic activated cell separation (MACS) [88]. Especially, DGC can separate motile spermatozoa from immotile spermatozoa, leukocytes, cell debris, and toxic ROS prior to ART. Takeshima et al. reported that DGC could newly generate ROS, but ROS were pooled in the upper layer. Therefore, DGC can select mature spermatozoa without enhancing oxidative stress [84].

8.2. Lifestyle modification

External causes of ROS generation are shown in **Table 1**. To minimize exogenous ROS generation, cessation of smoking [89], weight loss through diet education and moderate exercise [90], and decreasing the opportunity of exposure to phthalate [91] are useful preventive measures. It is also well established that alcohol abuse [92], an elevated temperature around the scrotum [44, 45], and exposure to toxins such as heavy metals and organic solvents [93] lead to an increase in oxidative stress and can have harmful effects on one's fertile capacity. Avoiding cycling with tight pants, avoiding taking long hot water baths and saunas, and avoiding using a laptop on closed legs will also help to minimize oxidative stress [42–45]. Further, using protective equipment at work places that reduces the exposure to chemicals and vapors leading to oxidative stress is also an effective preventive way to minimize oxidative stress. Because mobile phone radiation increases ROS production and decreases antioxidant activities [94], storing a mobile phone somewhere other than a trouser pocket is a useful measure for minimizing oxidative stress [95].

8.3. Shorter period of ejaculatory abstinence

As mentioned above, sperm cells in the cauda of the epididymis and vas deferens may be subject to a harmful seminal microenvironment of oxidative stress before or after ejaculation. Therefore, increasing the ejaculation frequency may reduce spermatozoal exposure to toxic ROS, thereby improving sperm motile function. Several studies showed that a shorter period of ejaculatory abstinence was associated with a higher seminal TAC and a lower sperm DFI. But, there was no significant difference in the degree of sperm membrane LPO when comparing the period of ejaculatory abstinence [96]. A shorter period of ejaculatory abstinence may improve sperm quality and DNA integrity by protecting sperm from ROS damage.

8.4. Oral antioxidant therapy

An oral antioxidant treatment has been reported to reduce sperm damage and improve intracytoplasmic sperm injection (ICSI) outcomes in patients with oxidative stress and sperm DNA damage. According to systematic reviews in the Cochrane database [97], 48 randomized controlled trials (RCTs) that compared single and combined antioxidants with a placebo

in a population of 4179 infertile men were reviewed. The results showed that antioxidants might have increased clinical pregnancy rates [odds ratios (OR): 3.43, $P < 0.0001$, 7 RCTs, 522 men] and live birth rates (OR: 4.21, $P < 0.0001$, 4 RCTs, 277 men), but these evidences were graded low. Representative oral antioxidant therapies are vitamin C alone (400–1000 mg/day) [98] and a combination of vitamins C and E [99, 100]. Vitamins C and E work synergistically, and many studies on the positive effect of combined antioxidants on reducing DNA fragmentation and increasing the clinical pregnancy rate have been reported. Zinc is an element essential for spermatogenesis and sperm DNA synthesis. It also prevents LPO and works as a component of SOD [101, 102]. Selenium is also an essential component of the glutathione peroxidase selenoproteins [103]. Several studies reported that the mixed antioxidants including zinc and selenium could decrease sperm DNA fragmentation and increase clinical pregnancy rates [104]. L-Carnitine and coenzyme Q10 are strong antioxidants that prevent LPO and sperm DNA fragmentation. Meta-analysis indicated that both elements improved the conventional sperm parameters [105, 106]. And as to L-Carnitine, erectile function was also improved [105].

8.5. Varicocele repair

As described above, current evidences suggest that oxidative stress and elevated levels of sperm DNA fragmentation are the main factors that contribute to infertility in men with a varicocele [52]. Moreover, current evidences also suggest that varicocele repair in men who have a clinically palpable varicocele with documented infertility significantly improves the male fertile capacity [50, 107]. Surgical options for varicocele repair include the traditional high retroperitoneal (Palomo) and inguinal (Ivanissevich) approach, laparoscopic high ligation, and microsurgical low ligation via an inguinal or subinguinal incision. Microsurgical low ligation of the spermatic vein by the subinguinal approach is considered the gold-standard technique for varicocele repair because of lower postoperative recurrence and complication rates (e.g., hydrocele, testicular atrophy, and wound pain) compared to other techniques [108]. Several studies indicate that a varicocele repair reduces oxidative stress in seminal plasma and ameliorates sperm DNA damage. Moreover, varicocele repair significantly increases antioxidant levels indirectly because of a decrease in exhaustion due to less ROS formation after the surgery [109].

8.6. Testicular sperm extraction

As mentioned above, spermatozoa in the ejaculate are affected by ROS in the process of ejaculation. The testes have substantial antioxidant systems, but once spermatozoa are released from the Sertoli cells and migrate from the seminiferous tubules to the epididymis, they become susceptible to oxidative stress [110]. DNA damage in testicular spermatozoa is threefold lower compared with ejaculated spermatozoa [111]. Testicular sperm extraction (TESE) is a method of surgically retrieving sperm from the testis in patients with azoospermia or cryptozoospermia. ICSI using testicular sperm has a higher implantation rate and pregnancy rate than that using ejaculated sperm [112, 113]. However, testicular sperm has a significantly higher aneuploidy rate than ejaculated sperm [111]. Therefore, this method should be carried out with limited indication of recurrent ART failure and severe oligozoospermia cases.

9. Future prospects

In recent years, with the development of proteomics technology, identification of proteins that can be used as biomarkers of diseases has been carried out [114, 115]. Identifying the proteins expressed in spermatozoa and seminal plasma in semen with high ROS levels can lead to the discovery of new biomarkers of idiopathic male infertility. The proteomic analysis uses one- or two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to quantify the protein in addition to a shotgun analysis by mass spectrometry to qualify the protein expressed in sperm or seminal plasma. The current proven overexpressed proteins in the ROS-positive group include glutamine synthetase (GLUL), heat shock 70 kDa protein 5 (HSPA5), histone cluster 1, H2ba (HIST1H2BA), and sperm acrosomal membrane protein 14 [115].

Metabolomics, the study of cellular metabolic products and genomics, the study of identifying genetic abnormalities are current progressive research areas [116]. These studies have a great potential for identifying highly sensitive biomarkers.

Conflict of interest

The authors report no declarations of interest.

Author details

Teppei Takeshima*, Shinnosuke Kuroda and Yasushi Yumura

*Address all correspondence to: teppeitalia@gmail.com

Department of Urology, Reproduction Center, Yokohama City University Medical Center, Japan

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Chapter 7

Reactive Oxygen Species at High Altitude (Hypobaric Hypoxia) on the Cardiovascular System

Patricia Siques, Julio Brito and Eduardo Pena

Additional information is available at the end of the chapter

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Abstract

Reactive oxygen species (ROSs) play important physiological and physiopathological roles in the cardiovascular system. An imbalance between ROS and antioxidants, termed oxidative stress, can contribute to endothelial dysfunction and cardiovascular remodeling. ROSs have been demonstrated to be increased and to regulate the following main pulmonary vasculature changes that occur at high altitude (hypobaric hypoxia): hypoxic pulmonary vasoconstriction (HPV), pulmonary hypertension, right ventricular hypertrophy (RVH), and ultimately, cardiac failure. Thus, ROS increases are a public health concern for the increasing number of people living or working at high altitudes. ROSs trigger the activation of different metabolic signaling pathways that alter the activity of redox-sensitive transcription factors and translational signals. Consequently, we provide a comprehensive review of the literature on the main factors, sources, and mechanisms of action of ROS and their effects on the cardiovascular system under hypobaric hypoxic conditions. Although ROS generation is a normal physiological activity, under hypobaric hypoxia (high altitude) conditions, ROS levels are elevated. The principal sources of ROS are nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-4 (NOX4) in the vascular system and NOX2 in cardiac tissue. Thus, the information presented in this review provides a broad view of the relationship between ROS and hypoxia.

Keywords: reactive oxygen species, altitude, hypoxia, cardiovascular system, pulmonary hypertension, right ventricle hypertrophy

1. Introduction

In the cardiovascular system, reactive oxygen species (ROSs) and reactive nitrogen species (RNSs) play important physiological roles in the control of endothelial functions, vascular tone, and cardiac functions, as well as a pathophysiological role in inflammation, hypertrophy, fibrosis,

angiogenesis, cell proliferation, apoptosis, and migration. The regulation of this biological activity is the result of a balance between oxidants and the buffering action of antioxidants, such that an imbalance between ROS or RNS and antioxidants (called "oxidative stress"), wherein ROS or RNS is increased, contributes to cellular signaling that leads to endothelial dysfunction and cardiovascular remodeling. On the one hand, ROS triggers the activation of different cellular pathways by activating specific proteins (e.g., Akt1/2: serine/threonine protein kinase; PKC: protein kinase C; PDK: 3-phosphoinositide-dependent kinase; Erk1/2: extracellular signal-regulated kinase; JNK: c-Jun N-terminal kinase; PI3K: phosphatidylinositol-3-kinase; and JAK: Janus kinase) in different tissues. On the other hand, ROSs alter the activity of redox-sensitive transcription factors (i.e., AP-1: activator protein 1; NF-κB: nuclear factor-κB; HIF-1α: hypoxia-inducible factor-1α; and STAT: signal transducer and activator of transcription) to induce direct effects on enzymes, receptors, or ion channels and different cellular responses [1]. Oxidative stress is generated by external factors, such as a decrease in the partial pressure of oxygen (PO_2) in hypobaric hypoxia due to high-altitude exposure. Over 100 million people live in hypoxic conditions worldwide [2, 3], the number of people exposed to hypoxic conditions is higher if we include people traveling to high altitudes for either leisure or work. Human beings, except Tibetans, are not naturally adapted or genetically equipped to live at high altitudes. Therefore, depending upon its degree and duration or the altitude, hypoxia generates several physiological or pathological effects on the human body [4].

The main effects of exposure to hypobaric hypoxia are excessive erythrocytosis and high-altitude pulmonary hypertension (HAPH). Features of the latter include high pulmonary artery pressure, vascular remodeling of pulmonary arteries, right ventricle hypertrophy (RVH), and cardiac failure. In addition to the mechanical explanation usually considered for this phenomenon, new data suggest other, mechanical-independent mechanisms. We attempt to provide a comprehensive review of the principal factors, sources, and mechanism of action of ROS in the development of cardiovascular diseases under hypobaric hypoxia and/or similar stressors, with a specific focus on the cardiovascular system.

2. High altitude (hypobaric hypoxia) and oxidative stress considerations

2.1. ROS and RNS

ROSs are small molecules that derive from O_2 and include the superoxide anion ($O_2^{\bullet -}$), hydroxyl ion (OH), peroxyl (RO_2), and alkoxy agents ($RO^{\bullet}$), as well as certain nonradicals that are either oxidizing or easily converted into radicals, such as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). There are other types of molecules that are oxidizing agents but contain nitrogen; these radicals are called RNS. One example is peroxynitrite (NOO^-), which is derived from nitric oxide (NO) when oxidized by $O_2^{\bullet -}$ [5]. These molecules are highly reactive due to the presence of an unpaired valence electron layer [6, 7], and through this electronic condition, ROSs avidly interact with a large number of molecules, including the plasma membrane and organic macromolecules such as proteins, lipids, carbohydrates, and

nucleic acids, to achieve electron stability. Through such interactions, ROS can irreversibly alter or destroy the function of specific molecules in the cell; for this reason, ROSs are recognized as important players in many cellular signaling and physiological processes [8].

Based on the above, ROSs are considered harmful molecules that promote cellular aging in biological organisms. However, to date, at least one beneficial function has been described: ROSs produced by leukocytes, neutrophils, and macrophages were found to play a major role in the defense against host molecules or foreign agents [9]. Additionally, ROSs were recently proposed to participate not only in cellular damage and the destruction of pathogens but also in several reversible regulatory processes in all cells and tissues [9]. In other words, in a healthy organism, the cell normally produces low levels of ROS, which activates specific signaling pathways that contribute to normal responses to various stimuli [10]; however, the inability to adequately compensate for an increase in ROS by the antioxidant system of the tissue or organism (known as "oxidative stress") can result in the development of several pathologies [11].

Therefore, under oxidative stress, high levels of ROS produce changes in the cell through the following mechanisms: (1) activating redox-sensitive protein kinases, such as JAKs, PKC, PI3K, and PDK; (2) activating mitogen-activated protein kinase (MAPK) family members, such as Akt, JNKs, Erk1/2, and p38, which are involved in angiogenesis and cell proliferation, differentiation, migration, growth, motility, survival, and apoptosis; (3) altering the activity of redox-sensitive transcription factors, such as AP-1, NF-κB, HIF-1α, and STAT; (4) inhibiting protein tyrosine phosphatase (PTP), which produces high levels of phosphorylated proteins; (5) producing an increase in the concentration of intracellular calcium $[Ca^{2+}]_i$; (6) producing direct effects on cellular structures, such as enzymes, receptors, and ion channels, or generating indirect effects on these structures through polyunsaturated fatty acids (PUFAs), which are highly susceptible to ROS, such that the oxidative breakdown of n-3 PUFAs may compromise membrane lipid matrix dynamics and, hence, the structure and function of membrane-associated proteins, such as enzymes, receptors, and transporters; and (7) stimulating the activity and expression of pro-inflammatory molecules and pro-oncogenes [1, 7, 8, 12–14].

For these reasons, regulating ROS production modulates the activity of various intracellular molecules and various cell signaling pathways, thereby inducing specific acute and chronic changes in the phenotype and function of a cell (commonly referred to as "redox signaling"). Thus, with a specific focus on the cardiovascular system, ROSs play an important physiological role in the control of endothelial functions, vascular tone, and cardiac functions, as well as a pathophysiological role in inflammation, hypertrophy, fibrosis, angiogenesis, cell proliferation, apoptosis, and migration, whereby all these processes synergistically contribute to endothelial dysfunction and cardiovascular remodeling [7], as we demonstrate later in this chapter.

2.2. High-altitude exposure: types of exposure and principal cardiovascular responses

As a result of decreased barometric pressure and oxygen partial pressure (PaO_2), exposure to high altitudes generates an important effect on the cardiovascular system known as *hypobaric hypoxia*, where reduced uptake of oxygen leads to a decrease in O_2 transported by the blood

to all the cells in the organism [15, 16]. The important physiological effects in living beings are derived from acclimatization or adaptability to high altitude, and these effects fundamentally depend on the level of altitude and the duration of exposure [3].

Acute hypoxia (AH) occurs when a person (e.g., a tourist or alpinist) is exposed to high altitudes for short periods of time (days or hours), whereas *chronic hypoxia* (CH) occurs when a person is permanently exposed to hypoxic conditions (i.e., living at high altitude). A new and distinct form of exposure has recently been shown to be different from all types of hypobaric hypoxia described to date and is related to mining exploitation, thus termed "Chilean mining model of chronic intermittent exposure to high altitude" [17]. This type of hypobaric hypoxia involves working over 3000 m above sea level in shifts (days of work at high altitude and days of rest at sea level) and maintaining this condition for years. It has been estimated that over 200,000 people work under these conditions [18]. This biological condition is classified as chronic intermittent hypobaric hypoxia (CIHH).

There are many effects of high altitude that could ultimately lead to pathologies. However, the principal effects are an increase in hematocrit levels by accumulative red cell production or excessive erythrocytosis (chronic mountain sickness) and the development of acute mountain sickness (AMS), which can begin as mild to severe (as cerebral edema or lung edema). Another effect is the development of hypoxic pulmonary vasoconstriction (HPV), which leads to HAPH, with a prevalence of up to 15% in individuals exposed to high altitude [4].

The latter is of utmost interest, since its consequences are the clinical development of pulmonary hypertension and RVH or cor pulmonale [19–21]. Nevertheless, it must be noted that these effects appear to be less severe in CIHH exposure than in chronic exposure (CH) [16, 22].

3. ROS, hypoxia, and the cardiovascular system

3.1. The cardiovascular system and hypoxia-induced ROS

Previously, it was suggested that exposure to high altitude limits O_2 supplementation in the organism in general and thus reduces the generation of free radicals (ROS), which are derived from this important gas [23]. However, this concept was later disputed with data suggesting that exposure to high altitude (>3000 m) leads to an increase in ROS production in many cell lines, thus generating an O_2 supplementation paradox [24–26]. Finally, the high-altitude-induced increase in ROS products was confirmed by human studies, in which the concentrations of specific biomarkers of oxidative stress (plasmatic lipid peroxidation and iso-8-prostaglandin F-2α level in urine) were found to be increased after acute or chronic exposure to high altitude (4300 m) and without exercise [6]. Therefore, these findings suggest that exposure to hypoxia produces oxidative stress, thus causing all the aforementioned effects on both physiological and pathological cell signaling responses [9, 27].

Studies have evaluated the main sources of ROS in several cell lines under hypoxic conditions and concluded that the predominant source of ROS in the cardiovascular system is the enzymatic

complex *nicotinamide adenine dinucleotide phosphate* (NADPH) *oxidase* (NOX), which prevails over other ROS-generating systems, such as mitochondria and xanthine oxidase [5, 28]. NADPH oxidases comprise a complex multicomponent family of transmembrane and cytosolic proteins that use NADPH as an electron donor to reduce molecular oxygen to the superoxide anion and hydrogen peroxide. The prototype NADPH oxidase was formerly known as gp91phox and was first described in leukocytes [1]. However, it is important to highlight that subsequent studies characterized seven members of the NOX family (NOX1 to 5 and dual oxidases 1 and 2) with diverse distributions among specific tissues and organs [10]; these NOX family members have since been described in nonphagocytic cells, including neurons, skeletal muscle, myocytes, hepatocytes, endothelial cells, hematopoietic cells, stem cells, and cardiomyocytes [28]. For example, previous studies found that stimulating rat cardiomyocytes with angiotensin II (Ang II) directly activated the NADPH oxidase complex, specifically the NOX2 isoform. This NOX2 complex can be activated in healthy organisms by several factors, including a G-protein receptor agonist (Ang II) and endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), and mechanical shear stress from blood flow. However, the pathological activation of NOX2 (e.g., cytokines such as tumor necrosis factor-alpha) can result in the generation of much higher concentrations of ROS that appear to contribute to pathological states, including endothelial dysfunction, myocardial hypertrophy, fibrosis, heart failure, inflammation, atherosclerosis, coronary artery disease, stroke, and renal and pulmonary fibrosis [10].

Studies of the vascular system have shown that the predominant isoform of the NADPH complex is NOX4 [27, 29], and previous investigations revealed that NOX4 is involved in oxygen sensing, vasomotor control, angiogenesis, fibrosis, cell proliferation, differentiation, migration, apoptosis, and senescence. Elevated expression of NOX4 has been reported in a number of cardiovascular diseases, including atherosclerosis, pulmonary fibrosis, cardiac failure, and ischemic stroke [30].

Notably, previous studies have demonstrated that a single mutation in NOX4 disrupts $O_2^{\bullet-}$ production; these studies showed that although $O_2^{\bullet-}$ production was undetectable in NOX4-transfected cells, there was robust production of H_2O_2, in contrast to the mixture of $O_2^{\bullet-}$ and H_2O_2 production following transfection with NOX1-NOX3 and NOX5 [31].

This effect of NOX4 was found due to the mutation of a highly conserved histidine residue in the E-loop of the NOX4 structure that promotes the rapid dismutation of $O_2^{\bullet-}$ before it leaves the enzyme [32], highlighting that higher concentrations of NOX4-produced H_2O_2 also elicit multiple effects. These effects are smooth muscle cell hypertrophy, activation of metalloproteases, and a low concentration of NOX4, which has been proposed as a cardiac protector [33]. Preliminary data from DNA microarray screens indicate that H_2O_2 causes a more than two-fold induction in the expression of nearly 100 genes, with a more than two-fold reduction in the expression of many more. Further, many transcription factors have been shown to be activated by H_2O_2. For example, as mentioned above, nuclear factor-κB (NF-κB) usually resides in the cytoplasm in association with an inhibitor protein (IκB) but is dissociated from IκB in the presence of H_2O_2. This process generates the nuclear translocation of NF-κB, and other transcription factors directly affected by exogenous H_2O_2, such as activator protein 1 (AP-1) (a complex composed of the *jun* and *fos* gene products) [1].

3.2. HAPH and NOX4-produced ROS

As mentioned previously, HAPH is one of the principal pathologies involved in hypoxic exposure and arises from the narrowing of pulmonary arteries, which elevates pulmonary vascular resistance and, consequently, pulmonary artery pressure. HAPH is characterized by excessive proliferation and hypertrophy of pulmonary arterial medial smooth muscle and adventitial remodeling. ROS may serve as important regulators of pulmonary vascular remodeling, and some evidence supports a prominent role of NOX4 in the pathogenesis of HAPH [27]. For example, NOX4 is the major NADPH oxidase homolog expressed in human pulmonary artery smooth muscle cells, and its expression at both the mRNA and protein levels is significantly increased in lungs from patients with idiopathic pulmonary arterial hypertension (IPAH) compared to that in healthy lungs [34], which may suggest a strong correlation between NOX4 and the onset of HAPH.

In addition, NOX4 expression was found to be increased in a CH-induced pulmonary artery hypertension (PAH) experimental mouse model. Therefore, NOX4 may also mediate hypoxia-induced growth of human pulmonary smooth muscle cells [35]. Indeed, this was corroborated in studies that silenced NOX4 expression by RNA interference; the results demonstrated a decrease in the growth of human pulmonary arterial smooth muscle cells and fibroblast proliferation [30].

Furthermore, if we focus on the HPV response to high altitude (hypobaric hypoxia), this effect is explained by smooth muscle cell contraction. Studies have shown that one of the main pathways involves an increase in intracellular calcium $[Ca^{2+}]_I$ from the extracellular space and intracellular stores through voltage-activated potassium channels (KV) and nonspecific cation channels (NSCC) [36]. Nevertheless, further studies in lung cells found an increase in hypoxia-induced ROS that produced the activation of a calcium sensor (SMIT1) in the endoplasmic reticulum (ER), where this protein activates CRAC channels that contribute to the increase in intracellular Ca^{2+} [37].

In the nitric oxide (NO) pathway, studies have reported that intermittent hypobaric hypoxia exposure reduces the bioavailability of NO in lung parenchyma and vasculature [27, 38]. NO is an endogenous vasodilator that activates cyclic GMP, which in turn activates protein kinase G (PKG) and ultimately causes reuptake of Ca^{2+} and the opening of calcium-activated potassium channels, leading to the relaxation of vascular smooth muscle cells (VSMCs). The decrease in NO bioavailability observed following exposure to intermittent hypobaric hypoxia may be due to the destruction of NO by hypobaric hypoxia-induced ROS, such as superoxide anion (O^{2-}), which is produced by the enzymatic complex NAPH oxidase, specifically the NOX4 subunit [27, 29]. In agreement with these findings, studies silencing NOX4 and p22phox, another subunit involved in the activation of NADPH oxidase-NOX4, showed attenuation of ROS formation and proliferation in human and rat pulmonary artery smooth muscle cells (PASMCs) [39]. Therefore, all these ROS-activated cellular mechanisms may contribute to pulmonary artery remodeling, pulmonary hypertension, and finally, cardiac failure due to RVH.

Recently, other studies noted that NOX4 does not contribute to the development of hypoxia-induced pathologies, such as HPV or pulmonary hypertension. However, these studies found increases in superoxide anion ($O_2^{\bullet -}$) levels in SMCs of NOX2- and NOX1-overexpressing mice and that NOX4 overexpression increased H_2O_2 levels. Therefore, NOX4 may be incapable of

destroying NO; this contrasts with NOX1- and NOX2-derived $O_2^{\bullet-}$, which destroys NO and contributes to the formation of $ONOO^-$, thus leading to vascular dysfunction [40].

NOX4 has received considerable attention because it differs from NOX1 and NOX2 in several aspects: (1) NOX4 mRNA expression is higher than that of the other NOX homologs (>1000-fold higher copy number than NOX1 and NOX2) and different from NOX1 and NOX2, which are induced by Ang II in VSMCs. This is supported by studies in cultured cells showing the expression of NOX4 mRNA at copy numbers greater than 10- to 100-fold that of NOX2 and greater than 100-fold that of NOX1 [41]. Therefore, NOX4 is the most abundant NOX isoform in the vasculature. However, one must be mindful that mRNA levels may not accurately reflect protein expression levels of the various NOX isoforms [42]. (2) NOX4 expression increases over the course of differentiation and is required for the maintenance of the differentiated phenotype in cultured cells. (3) NOX4, unlike NOX1 and NOX2, is independent of cytosolic activator subunits and thus is potentially constitutively active. This is supported by overexpression studies conducted mostly in HEK293 cells, which have suggested that NOX4-dependent ROS production is controlled by the abundance of the enzyme. This aspect does not exclude the possibility that other interacting proteins, such as Poldip2 or protein disulfide isomerase, alter the activity of NOX4. Conversely, NOX4 predominantly releases H_2O_2, which cannot alter NO. NOX4 overexpression in the presence of NO does not lead to $ONOO^-$ formation, which strongly argues against significant $O_2^{\bullet-}$ formation by the enzyme [33].

Therefore, eNOS uncoupling is an important mechanism that leads to endothelial dysfunction. It is becoming progressively clear that the presence of low concentrations of H_2O_2 not only acts as a vasodilator by activating kinase G Iα^3 but also may activate and induce eNOS by several mechanisms [33]. Thus, NOX4 might have an antagonistic function to NOX1 and NOX2, since it differs from these NADPH oxidases. NOX4 is a special NOX because it is highly constitutively active and is highly expressed in many cardiovascular cells. However, studies using both anti-NOX4 antibodies and *in situ* hybridization showed that NOX4 is primarily expressed in the middle layer of pulmonary blood vessels in both mice and humans [34].

Numerous studies have shown that NOX4 is robustly upregulated in response to transforming growth factor-beta (TGF-β) stimulation in various cell types, including aortic and pulmonary smooth muscle cells, pulmonary and cardiac fibroblasts, and endothelial and embryonic kidney cells [43]. However, tumor necrosis factor-alpha is less specific and can increase NOX1, NOX2, and NOX4 activity and/or the expression of these oxidases in various vascular cells. Other stimuli that induce NOX4 expression are ER stress, shear stress, hypoxia, and ischemia, as well as the activation of PKCα, NF-κB, HIF-1α, and Nrf2. These pathways are also likely dependent on the stimulus and cell type [44], and as mentioned above, Ang II has been shown to potently activate NOX1 and NOX2, but its effect on NOX4 expression is much less pronounced [30].

3.3. Cardiac myocytes and HIF-1α

HIF-1α is a heterodimeric subunit of the transcription factor HIF-1, which regulates the transcription of genes involved in adaptive responses to hypoxia. Therefore, HIF-1 induces and promotes the expression of several genes containing hypoxia-responsive elements (HREs) in their

regulatory region, such as proangiogenic factors (VEGF) or stromal-cell derived factor-1α (SDF-1α, CXCL12), vasoconstrictors (endothelin-1), and inflammation-associated genes (iNOS—inducible nitric oxide synthase and COX2—cyclooxygenase). Many of these factors promote angiogenesis and wound healing and are thus critical for the response to local hypoxia and injury. This HIF-1 system is also used to measure the systemic oxygen supply and to control the formation of red blood cells [12] through the glycoprotein erythropoietin (EPO). EPO has strong organ-protective effects in the heart, brain, and kidney, promotes re-endothelialization, and induces the mobilization of endothelial progenitor cells (EPCs), where ROS-NOX2 production is fundamental for EPO-induced mobilization of EPCs and vascular repair in hypoxic conditions [12].

However, the role of HIF-1α in the development of cardiac hypertrophy has been sparsely documented [45]. More interestingly, carvedilol, a β-receptor blocker, has emerged as a beneficial treatment for cardiac hypertrophy, as it inhibits the overexpression of HIF-1α during pressure overload in the rat heart [46]. Subsequent studies in cardiomyocytes under mild hypoxic conditions showed that HIF-1α controls the process of cardiac hypertrophy through the activation of transient receptor potential canonical 3 (TRPC3) and 6 (TRPC6), producing an increase in the levels of $[Ca^{2+}]_i$ and calcineurin [47].

TRPC channels are nonselective cation channels that mediate Ca^{2+} influx into several cell types, including cardiac myocytes [48]. TRPC expression in cardiac hypertrophy has been studied by several laboratories, with somewhat variable results. For example, previous studies have shown that TRPC3 promotes cardiomyocyte hypertrophy in several animal models, including abdominal aortic banding (AAB) rats and spontaneous hypertensive heart failure rats [47]. Other studies have demonstrated that TRPC6 sequentially initiates a calcineurin signaling circuit during pathological cardiac hypertrophy. However, Ohba et al. [49] demonstrated that TRPC1, TRPC3, TRPC5, and TRPC6 are constitutively expressed, but only TRPC1 expression is significantly increased in hypertrophic hearts from AAB rats. However, these studies regarding the role of HIF-1α in cardiac hypertrophy were based on pathological situations, and their conclusions were controversial. Therefore, the potential role of HIF-1α in adaptive cardiac hypertrophy needs to be clarified.

Further, previous studies showed that the HIF-1 pathway is involved in hypoxia-induced autophagy in cardiomyocytes and that HIF-1-induced autophagy may, therefore, help cardiomyocytes to overcome hypoxic injury and increase survival [50]. In other words, HIF-1α upregulation can increase autophagy and ameliorate the hypoxia-induced reduction in cell viability. Regarding survival and cardiac viability in hypoxic conditions, cardiac muscle cell survival plays a critical role in maintaining the correct function of the heart and, possibly, in cardiac embryogenic development. In contrast, adult cardiomyocytes are thought to be terminally differentiated and therefore have lost their proliferative capacity. One of the mechanisms that cardiomyocytes employ to protect themselves from deleterious stimuli is the release of survival cytokines capable of promoting cytoprotection in an autocrine/paracrine manner [51, 52]. One of these cytokines is cardiotrophin-1 (CT-1). CT-1 is a member of the interleukin-6 family with hypertrophic properties in neonatal and adult cardiomyocytes [53]. In adult cardiomyocytes, CT-1 exerts a protective function in response to death stimuli (apoptosis and necrosis), such as Ang II, H_2O_2, and ischemia-reperfusion. The cardioprotective properties of CT-1 under stress conditions suggest that it may be upregulated during cardiac diseases that are characterized by

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an environment of reduced oxygen availability, inflammation, and oxidative stress. Indeed, circulatory levels of CT-1 are elevated in pathological conditions associated with ischemia, including unstable angina pectoris, acute myocardial infarction, hypertensive heart disease, and heart failure. Importantly, studies have shown that hypoxia increased CT-1 in cardiac cells (*in vitro* and *in vivo*) through direct regulation of the *CTF1* promoter by HIF-1α, and this CT-1 activation may protect cells from apoptosis, thus supporting a protective role of CT-1 as a survival factor for cardiomyocytes [52].

3.4. Myocardium, myocytes, and hypoxia-induced ROS

HAPH-induced RVH or end-stage cor pulmonale [19–21] is primarily explained as a compensatory effect of right ventricular afterload. However, numerous investigations have established new avenues for the development of cardiac hypertrophy that highlight oxidative stress as the main mediator [5, 54].

To support the involvement of oxidative stress, a study evaluating both smooth muscle cells and endothelial cells in the development of pulmonary artery remodeling in CH was conducted. This study found that such arterial remodeling occurs via a mitochondrial factor, which requires the Rieske iron-sulfur protein (RISP), a mitochondrial complex III protein required for ROS generation. RISP depletion in endothelial cells and smooth muscle cells prevented CH-induced pulmonary hypertension, but it did not prevent RVH, suggesting that right ventricle remodeling in CH occurs through a mechanism independent of the increase in pulmonary artery pressure [55]. Thus, RVH could be directly produced by hypoxia-induced ROS, such that some *in vitro* experiments showed increased ROS levels in chicken cardiomyocytes and Hep3B cells cultured under AH [56].

Therefore, acute and chronic hypoxic exposure could generate oxidative stress [6] and may activate a large variety of protein kinases, such as MAPK, tyrosine kinases, and Rho kinases, and transcription factors (NF-κB, AP-1, and HIF-1α) that are derived from cellular hypertrophy [57] may also inactivate PTP. Both combined and separate effects induce an increase in the phosphorylation cascade or produce an increase in the concentration of intracellular calcium $[Ca^{2+}]_I$ and stimulate the activity and expression of pro-inflammatory genes and proto-oncogenes [7].

Regarding cardiomyocytes, NOX2-mediated $O_2^{\bullet-}$ formation has been found to activate the protein kinase B or serine/threonine kinase (Akt) signaling pathways through PI3K, JNK, ERK1/2, and p38-MAPK. Thus, activation of these signaling pathways may play a central role in Ang II-stimulated cardiomyocyte hypertrophy [5, 58, 59]. Consequently, oxidative stress could play a fundamental role in cardiac hypertrophy, specifically RVH (possibly independent of the mechanical explanation) as a result of exposure to hypoxia. This is congruent with other studies demonstrated that NOX2 knockout attenuated Ang II and myocardial infarct-induced myocardial fibrosis and cardiomyocyte hypertrophy in mice. Hence, it could be surmised that NOX2 may play an important role in the development of cardiac hypertrophy in either hypoxic or other conditions, and this role may be independent of changes in blood pressure [60].

In addition to NOX2, several studies have reported a relative abundance of NOX4 expression in human and mouse cardiac myocytes [61, 62] and in pulmonary arteries under hypoxia [27].

NOX4 is induced in experimental models of heart failure and in humans [61]. Recent studies using cardiac-specific NOX4 knockout mice revealed decreased levels of ROS and improved performance along with reduced hypertrophy, fibrosis, and apoptosis. Conversely, an experiment using a transgenic cardiac-specific NOX4-overexpressing mouse showed deleterious effects, such as promoting dysfunction, fibrosis, and apoptosis, in response to pressure overload [62]. While these results suggest that NOX4 is a major source of oxidative stress involved in the failing heart, there are reports showing opposite effects using a global NOX4 knockout and cardiac-specific NOX4 transgenic model [63]. These contradictory findings could be explained by differences in the methodology used to induce heart failure.

Supporting a more active role of NOX4, studies have revealed that NOX4 induces positive endothelial effects by producing H_2O_2, which in turn activates protein kinase G Iα by thiol oxidation and subsequent dimerization. Moreover, H_2O_2 also activates endothelial NOS (eNOS). Therefore, it is necessary to determine how NOX4 may mediate such contradictory roles [30].

Another important source of ROS in cardiomyocytes is the mitochondrial complex (electron transport chain). Previous studies have found that mitochondria in cardiomyocytes increase their generation of ROS during hypoxia (1–5% O_2), with the increased ROS generation originating from the proximal region of the electron transport chain, most likely complex III. These observations suggest that ROS generated by mitochondria may trigger p38 phosphorylation (activation) during hypoxia and thus highlight that the role of p38 phosphorylation in cardiomyocytes is highly dependent on PO_2. Moreover, this ROS-induced p38 activation has been shown through another source independent of the electron transport chain, cobalt chloride [64]. However, hemoglobin (Hb), which is increased in hypobaric hypoxia exposure, depending on the type and duration of exposure, has intrinsic heme-oxidase activity that leads to the production of superoxide and thus contributes to oxidative stress. Therefore, the release of superoxide by Hb is favored in the T structure. Thus, sustained or excessive desaturation of Hb (T structure) may increase ROS production [65], and the phosphorylation of p38 MAPK during hypoxia may involve several ROS sources.

Although ROS generation is a normal physiological process, its counterbalance seems to be impaired under hypobaric hypoxia. The resulting imbalance leads to changes with potential pathological consequences for the cardiovascular system.

4. Conclusion

Under hypobaric hypoxia, ROS levels are elevated, resulting in a subsequent unbalanced oxidative status. The principal sources of hypobaric hypoxia are NOX4 in the vascular system and NOX2 in cardiac tissue. The main effects of this oxidative increase include cellular damage, impaired NO pathway signaling, and the activation of calcium channels, transcription factors, pro-inflammatory molecules, and kinase proteins, all of which have deleterious effects on the cardiovascular system.

Therefore, this exaggerated or unbalanced ROS activity is closely related to the development of specific changes in the cardiovascular system under hypoxia, such as HPV, altitude pulmonary

hypertension, pulmonary artery remodeling, and RVH. Notably, although most of the sources in this review described results from nonhypobaric hypoxia conditions, the information gathered reveals a broad view of the relationship between ROS and hypoxia. However, it is still necessary to further elucidate the undefined aspects of this association and the controversies concerning the poor characterization of hypobaric hypoxia.

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Author details

Patricia Siques*, Julio Brito and Eduardo Pena

*Address all correspondence to: psiques@tie.cl

Institute of Health Studies, Arturo Prat University, Iquique, Chile

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Section 3

Applications and Perspectives

Chapter 8

Oxidative Stress in Urolithiasis

Chanchai Boonla

Additional information is available at the end of the chapter

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Abstract

Oxygen is absolutely essential for the survival of our life. However, metabolic consumption of oxygen inevitably yields reactive oxygen species (ROS). Imbalance of ROS production and antioxidant capacity causes oxidative stress that potentially damages biomolecules leading to cell injury and death. In fact, ROS have two-faceted functions. Under physiologic condition, ROS function as signaling molecules and participate in maintaining redox balance. In pathology, ROS induce oxidative stress that critically involves in the development of several diseases including urolithiasis (UL). UL or urinary stone disease is a common urologic condition in all countries with progressively increasing prevalence. Most of UL are multifactorial with polygenic susceptibility and highly recurrent nature. Formation of urinary stones is driven by supersaturation of urinary lithogenic ions, and calcium oxalate (CaOx) is the most prevalent stone type. Oxidative stress clearly plays an active role in UL development. In vitro, lithogenic crystals induce ROS generation in renal tubular cells leading to oxidative stress, cell injury and release of inflammatory mediators. In nephrolithic rats, oxidative stress and CaOx deposit are gradually increased in the rats' kidneys. Intervention with antioxidants efficiently reduces oxidative damage and crystal deposits. Human studies show that patients with UL have increased oxidative stress and renal tubular injury relative to the non–stone-forming individuals. Increased oxidative lesions and inflammation are observed in the stone-containing kidneys of the patients. Furthermore, renal fibrosis mediated through tubular epithelial-mesenchymal transition is observed in kidneys of stone patients. Increased renal fibrosis is significantly associated with decreased kidney function. From therapeutic point of view, nutraceutical regimens that are able to reduce oxidative stress may be clinically useful alternatives for preventing stone formation and recurrence. This chapter has an intention to provide a basic knowledge of ROS generation and oxidative stress and up-to-date research findings of oxidative stress in UL based on the published articles as well as the author's studies.

Keywords: oxidative stress, reactive oxygen species, urolithiasis, kidney stone, treatment

1. Introduction

Aerobic living organisms require oxygen for their metabolism mainly to generate adenosine triphosphate (ATP) through the electron transport chain (ETC). In the aerobic metabolism, molecular oxygen (O_2) is sequentially reduced to water (H_2O), and reactive oxygen species (ROS) are generated (**Figure 1**) [1, 2]. Therefore, it is no doubt that oxygen is absolutely essential for aerobic life, but it can be very harmful under the condition that ROS are excessively generated. These good and evil faces of oxygen are called "oxygen paradox" [3–5]. A complete reduction of O_2 to H_2O requires stepwise addition of four electrons, and three ROS, viz., superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) are respectively produced (**Figure 1**). In addition to ETC, another significant endogenous source of ROS is from oxidase and oxygenase enzymes [6]. Oxidases use oxygen as electron acceptor [7]. They catalyze the transfer of two electrons from electron donor to oxygen, and H_2O_2 is usually a byproduct. In case of oxygenases (monooxygenases and dioxygenases), they catalyze an incorporation of oxygen into substrate [7]. Dioxygenases incorporate both atoms of oxygen into substrate, while monooxygenases add one oxygen atom to substrate to yield hydroxyl substrate and water. Exogenous source of ROS includes UV/ionizing radiation, toxins, environmental pollutants, heavy metals, drugs, xenobiotics, pathogens and inflammatory cytokines [8–10]. Exposure to these substances causes increased production of ROS that further involves in the initiation of disease development. Besides ROS, reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite [11–13], and reactive chloride species (RCS), such as hypochlorous acid [14, 15], also play important physiological and pathological roles in human. Fundamentally, RNS and RCS are produced by reacting with ROS, for example, peroxynitrite is formed from reaction of superoxide anion and nitric oxide. Overproduction of ROS in cells creates a tense condition called oxidative stress.

Oxidative stress is defined as an imbalance condition between amount of generated oxidants (mainly ROS) and antioxidant contents in the body, which further causes oxidative damage and injury (**Figure 2**). Oxidative stress is associated with a number of human diseases such as neurodegenerative diseases, cardiovascular diseases and cancers, and it has been

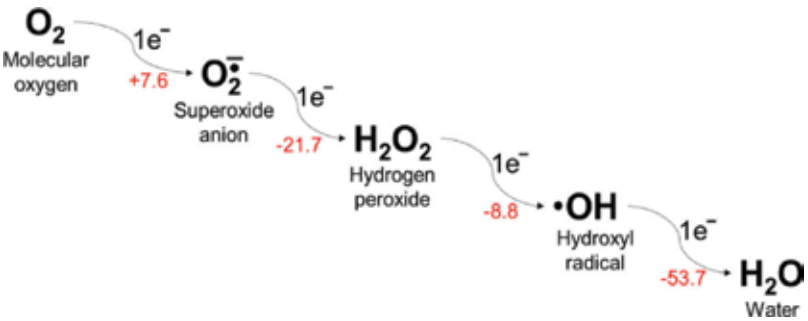

Figure 1. Stepwise reduction of O_2 to H_2O (sequential addition of four electrons). Numbers indicate the difference in Gibb's free energy (Kcal/mol) for each reaction.

experimentally proved to mediate the disease development [16–18]. The term oxidative stress is first described by Helmut Sies in 1985 [19]. ROS are primarily generated in an aerobic metabolism, and they have a powerful oxidizing capability to damage all kinds of biomolecules in the cells [20]. Therefore, some say oxidative stress may be viewed as the price that we have to pay for the use of oxygen in our metabolism [21]. However, our body has an antioxidant defensive system to scavenge ROS, deter oxidative damage and remove oxidized lesions in order to prevent the development of oxidative stress-mediated diseases (**Figure 3**). Both enzymatic and non-enzymatic scavenging antioxidants are the first line defense to combat ROS and inhibit the formation of oxidative lesions [22]. Once lesions formed, the second line of defensive system is to fix the lesions (mostly oxidized DNA) via repairing mechanisms [23–26] or to degrade them (mostly oxidized proteins) through proteasome and turnover mechanism [27–29]. The signaling pathway that regulates cytoprotective response to ROS is the Nrf2 (nuclear factor erythroid 2 [NF-E2]-related factor 2)-Keap1 (Kelch-like ECH-associated protein 1) pathway [30–32]. In response to ROS, transcription factor Nrf2 is activated and moves to the nucleus to bind to antioxidant responsive element (ARE) in the regulatory region of target genes to initiate transcription of antioxidative genes involved in the maintenance of cellular redox homeostasis (called redox biology) [33–35]. In pathological conditions, ROS are overwhelmingly generated, and antioxidant defense systems are not sufficient to counteract resulting in oxidative injury and disease progression. Therefore, activation of Nrf2 pathway and intervention with antioxidants have been considered to be a clinically useful alternative to ameliorate oxidative stress, delay aging and reduce risk of oxidative stress-related diseases [36]. However, clinical evidences of antioxidant supplement for disease prevention are still controversial and not conclusive [37, 38]. Intake of natural antioxidants through diets, rather than commercial supplements, is believed to be a better effective way to naturally boost up antioxidative capacity in the body. In summary, ROS are a part of normal human metabolism. When ROS are chronically produced and antioxidant systems are overwhelmed, excessive ROS directly attack cellular biomolecules, cause tissue injury and eventually lead to pathology [22, 39, 40]. However, oxidative stress may not solely act as the only causative factor for disease development. It rather acts in concert or interacting with other cellular processes in order to initiate and promote the pathogenesis of diseases.

Figure 2. Oxidative stress is firstly conceptualized in 1995 by Helmut Sies as a disturbance in the oxidant-antioxidant balance in favor of oxidant, potentially leading to oxidative damage [19, 41–45].

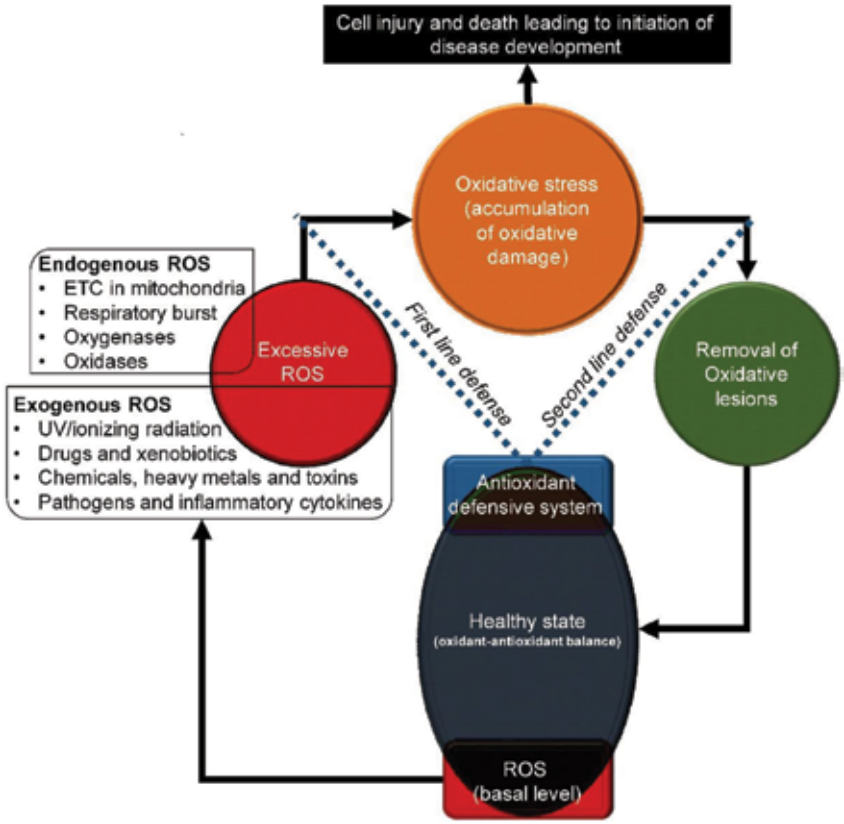

Figure 3. ROS generation, antioxidant defensive system, oxidative stress and consequences. Increased ROS production leads to oxidative damage. Antioxidant defensive system is activated to prevent oxidative injury. The first line defense is enzymatic and dietary antioxidants that directly scavenge the generated ROS. The second line defense is pathways to repair the oxidized lesions (repairing process) or degrade the oxidatively modified biomolecules (turnover process). Failure of these defensive mechanisms causes accumulation of oxidative lesions and increased degree of oxidative stress leading to cell injury, apoptosis and eventually initiation of disease development.

2. Oxidative stress

Oxygen is essential for our metabolism, but at the same time harmful ROS are produced during the reduction of oxygen to water (**Figure 1**). This two-faceted effect of oxygen is called "oxygen paradox" that is firstly conceptualized through an observation of a massive deleterious effect of reoxygenation in myocardium [46, 47]. The well-known oxygen paradox in clinical setting is a reperfusion injury [48]. In hypoxic tissues, xanthine oxidase appears to be a major source of superoxide anion after reoxygenation, and this superoxide is further reduced to form other ROS and cause an oxidative injury (**Figure 4**) [49, 50].

Excessive production of ROS and inadequacy of antioxidants cause an imbalance of oxidant-antioxidant system and result in oxidative stress. The term oxidative stress has gained more recognition and used in several research fields even in the public outside scientific community.

Usage of this term is sometimes overstressing or misusing. Therefore, the refined definition of oxidative stress is suggested in 2007 as follows: an imbalance between oxidants and antioxidants in favor of the oxidants, leading to the disruption of redox signaling and control and/or molecular damage [44, 51].

Increase in ROS production is the most common cause of oxidative stress in human body. There are at least six conditions that cause overproduction of ROS (**Table 1**). First, consumption of energy-rich diets directly increases aerobic metabolism and oxidative phosphorylation leading to increase in mitochondrial ROS production [52]. Mitochondrial superoxide anion is usually formed from an electron leakage in complexes I and III of ETC [53–55]. Second, high rate of oxygen use by strenuous work, competitive sport and exhaustive exercise is known to increase ROS generation through metabolic reactions [56–58]. Third, during reperfusion in surgery and organ transplantation, ROS are excessively generated, and ischemia or reperfusion injury is an unavoidable consequence (**Figure 4**) [59]. Forth, exposure to radiation such as UV (non-ionizing) and X-rays (ionizing) directly initiates ROS formation and causes damages to cellular biomolecules [60]. Fifth, excessive activation of phagocytic cells through respiratory burst consumes large amount of oxygen to generate superoxide anion, hydrogen peroxide and hypochlorous acid (HOCl) [61]. Sixth, exposure to toxicants activates cytochrome P450 monooxygenase (CYP450) in phase I xenobiotic biotransformation. Many reactive metabolites are unavoidably formed to cause oxidative damage [62].

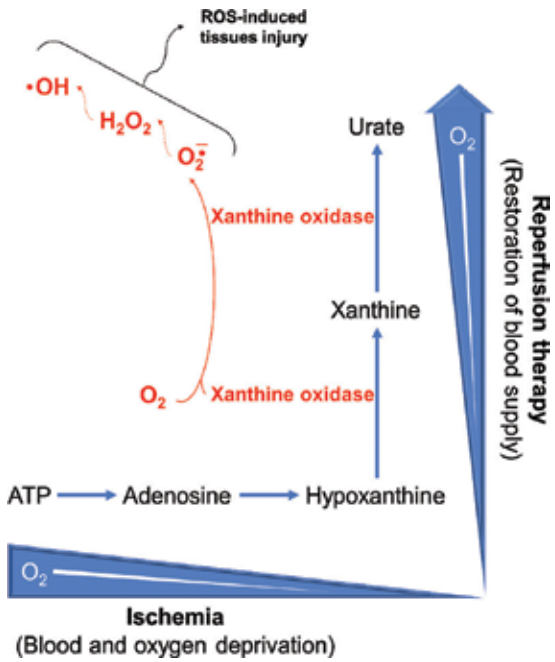

Figure 4. Proposed mechanism of excessive ROS production during reoxygenation leading to a reperfusion injury. Reduction of blood flow causes decreased oxidative phosphorylation and ATP production. Subsequently, purine precursor used in ATP synthesis is degraded to hypoxanthine. Restoration of blood supply and oxygen triggers conversion of the accumulated hypoxanthine into xanthine and uric acid by xanthine oxidase. This reaction generates superoxide anions and other ROS that further initiate oxidative tissue injury. Modified from Refs. [49, 50].

Increased ROS production	Decreased antioxidant capability
High intake of energy-rich foods	Inadequate intake of dietary antioxidants
High rate of oxygen consumption	Genetic mutation of antioxidant enzymes
Ischemic reperfusion	Depletion of glutathione as a consequence of increased rate of xenobiotic detoxification (via glutathione conjugation)
Exposure to radiation	
Excessive activation of phagocytic cells	
Exposure to xenobiotics activates CYP450 monooxygenase to produce reactive metabolites	

Table 1. Conditions contributed to disturbance between oxidants and antioxidants leading to oxidative stress in human body.

On the other side, decrease in antioxidant content in the body is attributed by at least three ways as follows: inadequate intake of dietary antioxidants, mutations in antioxidant genes and depletion of cellular glutathione as a consequence of detoxification of a large amount of xenobiotics (**Table 1**). Increased production of ROS beyond capability to cope by antioxidants leads to progressive augmentation of oxidized lesions that further promote pathogenic process such as in aging [63], atherosclerosis [64], neurodegenerative diseases [18, 65, 66] and cancers [67–71].

2.1. Generation of ROS

Reactive species include free radicals and other molecules that are themselves capable of converting to free radicals or have a powerful oxidizing property. By definition, free radicals are atoms or molecules having an unpaired valence electron. These molecules are chemically unstable and highly reactive towards other molecules. In fact, oxygen (O_2) in the air has two unpaired electrons, thus it is a biradical or diradical. Parallel spin of the unpaired electrons in oxygen molecule makes it chemically stable and inactive. This state of oxygen is a ground state or triplet oxygen ($^3O_2$). However, if triplet oxygen is activated by sufficient energy to create an antiparallel spin of unpaired electrons, a highly reactive non-radical species, called singlet oxygen ($^1O_2$), is formed. UVA exposure and phagocytosing neutrophils appear to be main sources of singlet oxygen formation in the human body [72]. Like other ROS, singlet oxygen is deleterious and capable of oxidizing lipids, proteins and nucleic acids leading to tissue damage and inflammation [73]. Common ROS and RNS found in the biological system are shown in **Figure 5**.

Superoxide anion is the first ROS generated in the stepwise reduction of oxygen, and it is a free radical precursor of hydrogen peroxide and hydroxyl radical (**Figure 1**). Superoxide anion is principally produced in mitochondrial ETC through complex I (NADH:ubiquinone oxidoreductase) and complex III (ubiquinol:cytochrome c oxidoreductase) [74]. The other clinically significant source of superoxide anion is from oxidases, particularly NADPH oxidase in the respiratory burst and xanthine oxidase in the reperfusion therapy [61].

NADPH oxidase is usually found in plasma membrane and phagosomes of phagocytic cells. Xanthine oxidase is primarily expressed in liver and small intestine located on outer surface of plasma membrane and in cytoplasm. Once activated, these oxidases produce large amount of superoxide anion. Superoxide anion itself is not highly reactive, and it has a relatively short half-life. Moreover, superoxide anion is negatively charged that is unable to cross the lipid membrane. Thereby, attack of superoxide anion to cellular biomolecules is confined at the site of origin. However, superoxide anion is capable of converting into a more diffusible reactive species, hydrogen peroxide. In addition, superoxide anion is able to react with other reactive species to produce more powerful oxidants, for instance, its interaction with nitric oxide ($NO^{\bullet}$) generates peroxynitrite ($ONOO^{-}$) [55]. Peroxynitrite is a very powerful non-radical oxidant that is injurious to cells and has crucial roles in pathogenesis of many diseases [75]. Reactive species that are derived from nitric oxide is collectively termed RNS (**Figure 5**), and a cellular stress that is caused by RNS with elevated level of nitrosylation marker (e.g., nitrotyrosine) is called "nitrosative stress" [75]. As it is beyond the scope, RNS, nitrosative stress and their contribution to disease development are not elaborated in this chapter.

Hydrogen peroxide (H_2O_2) is an uncharged non-radical species with reactive potential. Dismutation of superoxide anion produces hydrogen peroxide, and this reaction can be spontaneously occurred or catalyzed by superoxide dismutase (SOD) enzyme. SOD is first observed in 1969 [76]. The rate of hydrogen peroxide formation in SOD-catalyzed dismutation is much greater than the spontaneous one. In human, SOD has three distinct forms with a comparable reaction rate constant, i.e., SOD1 or Cu/Zn-SOD in cytosol, SOD2 or Mn-SOD in

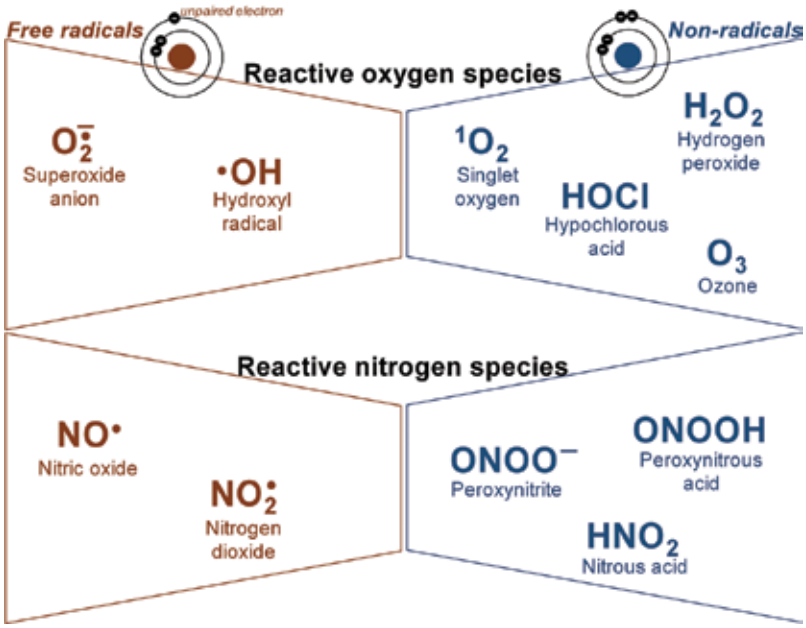

Figure 5. Common radical and non-radical species of ROS and RNS generated in the body.

mitochondria and SOD3 or extracellular SOD (Cu/Zn-ecSOD). To detoxify H_2O_2, catalase and glutathione (GSH) peroxidase are employed to convert H_2O_2 into water. These two enzymes have been shown to have an equal contribution of H_2O_2 disposal in human red blood cells [77]. In addition to its direct toxic effect, H_2O_2 can be converted into two main ROS, including hypochlorous acid (HOCl) by myeloperoxidase (MPO) in phagocytes and hydroxyl radical ($^{\bullet}OH$) by reacting with transition metals. In the actual fact, H_2O_2 per se is poorly reactive under a condition without transition metals [78].

Hydroxyl radical ($^{\bullet}OH$) is the most destructive ROS with the strongest oxidizing capacity to attack biomolecules in cells. The well-known reaction for the production of hydroxyl radical is Fenton reaction [79, 80]. Ferrous ion (Fe^{2+}) reacts with hydrogen peroxide to give ferric ion (Fe^{3+}) and hydroxyl radical. The Fenton chemistry was first delineated by H.J.H Fenton an over century ago, based on an observation of tartaric acid oxidation by H_2O_2 in the presence of Fe^{2+} [81, 82]. In addition to Fe^{2+}, other transition metals such as Cu^{2+} can catalyze the Fenton reaction [22]. The other reaction that is closely related to the Fenton reaction is Haber-Weiss reaction [83, 84]. It was firstly described (in German) by Haber and Willstätter in 1931 [85], secondly demonstrated its kinetics by Baxendale et al. in 1946 [86] and experimentally verified that hydroxyl radical is produced from an interaction between hydrogen peroxide and superoxide anion by Weiss in 1949 [87]. This reaction indeed enlightens the toxicity of superoxide anion to generate a detrimental ROS, hydroxyl radical. In biological system with a presence of iron, generation of hydroxyl radical is mainly mediated through the iron-catalyzed Haber-Weiss/Fenton reaction (**Figure 6**) [22, 83, 87]. Therefore, in conditions with iron overload, ROS are increasingly generated via this iron-catalyzed Haber-Weiss/Fenton reaction causing accumulation of oxidative damage that further promotes disease progression [88–90].

2.2. Roles of ROS in physiology and pathology

ROS are poisonous and pathogenic at the uncontrollable high concentration, but they are our good friend exerting many beneficial functions at the nontoxic physiological level. These two paradoxical functions of ROS, depended on how well our body can regulate and control their production, can be viewed as the Janus faces (**Figure 7**). ROS under normal circumstance exert critical actions in cells such as signal transduction, gene transcription and immune response [91]. Superoxide anion produced by NADPH oxidase is vitally important in killing invaded pathogens in phagocytic cells (macrophages, monocytes, neutrophils, eosinophils) through respiratory burst or oxidative burst (**Figure 8**). SOD converts superoxide anion into H_2O_2 to be used by myeloperoxidase (MPO) to form hypochlorous acid (HOCl). In the presence of iron, $^{\bullet}OH$ can be produced from H_2O_2. These generated ROS are believed to be responsible for destroying the engulfed pathogens in the phagolysosome. Mutation in genes encoding for NADPH oxidase complexes resulting in insufficient ROS production that is a direct cause of chronic granulomatous disease (CGD) [92]. CGD is a rare inherited immune disorder caused from the inability of phagocytes to kill the ingested microbes, and its typical manifestation is frequently recurrent subcutaneous abscess formation together with hyperinflammation. There is still an argument that ROS and MPO-mediated halogenation are not the main killing system for the invaded microorganisms [93, 94].

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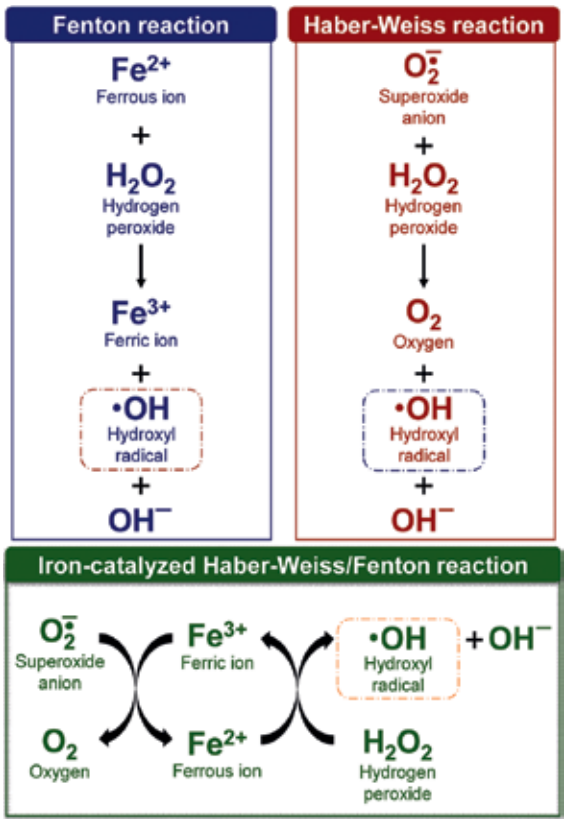

Figure 6. Fenton reaction and Haber-Weiss reaction for generation of hydroxyl radical. In the body with availability of iron ions, hydroxyl radical is principally produced via the iron-catalyzed Haber-Weiss/Fenton reaction.

ROS, particularly H_2O_2, are known to activate a stress-regulated transcription factor NF-*k*B to withstand the physiological stress, and that activated NF-*k*B induces transcription of a number of genes requiring for survival, apoptosis resistance and inflammatory response [95–97]. Some ROS act as substrate for enzymes, for instance, H_2O_2 is a substrate for heme-peroxidases involved in iodination of thyroid hormone [98]. H_2O_2 has been considered as a key cellular redox sensor and signaling molecule. At physiological levels (1–10 nM), it regulates redox signaling to maintain physiological stress (called oxidative eustress), and at higher level, it activates Nrf2/Keap1/ARE signaling pathway to initiate cytoprotective response and NF-*k*B activation to promote cell survival. At the extremely high or supraphysiological concentrations (>100 nM), H_2O_2 damages cellular biomolecules, disrupts redox signaling and causes oxidative distress leading to pathological development [99].

Nitric oxide, originally discovered as endothelium-derived relaxing factor, is the best-known free radical with signaling characteristic. It participates in several cellular and organ functions such as relaxation and proliferation of vascular muscle cells, leukocyte adhesion, platelet aggregation and angiogenesis. Nitric oxide is synthesized from L-arginine and oxygen by nitric oxide synthase (eNOS in endothelial cells, nNOS in neurons and iNOS in many cell

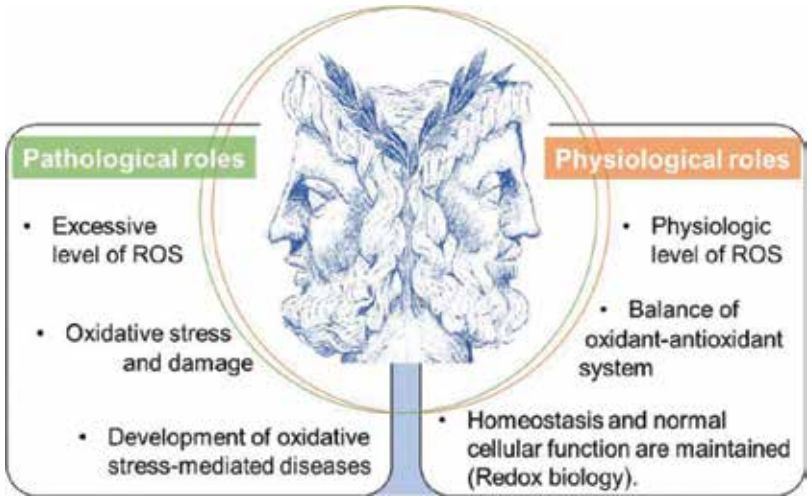

Figure 7. Janus faces of ROS in the human body regarding their physiologic and pathogenic functions. The process that ROS function as signaling molecules to maintain the physiological function is called "redox biology" [101], and the maintenance of intracellular redox homeostasis requires cooperative action and network of various antioxidants such as glutathione, peroxiredoxin, thioredoxin and antioxidant enzymes [102].

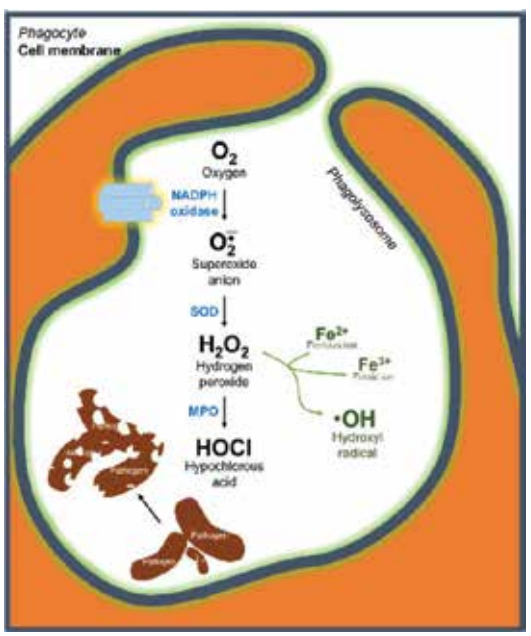

Figure 8. Respiratory burst for killing pathogens in phagocytes. In the presence of iron ions, hydroxyl radical can be formed through Fenton reaction and/or iron-catalyzed Haber-Weiss/Fenton reaction.

types following induction) using NADPH as electron donor. Sildenafil (VIAGRA®), a well-known drug for treating erectile dysfunction, is developed to interfere the nitric oxide signaling cascade in vascular smooth muscle cells [100]. Nitric oxide synthesized from endothelial

cells activates soluble guanylyl cyclase to convert GTP into cGMP leading to relaxation of vascular smooth muscle cells and vasodilation. The drug inhibits the cGMP degrading enzyme (phosphodiesterase-5), and this inhibition in turn causes a persistent increase in cGMP to stimulate vascular relaxation.

2.3. Oxidative stress in diseases

Oxidative stress is critically involved in the pathogenesis of almost all diseases ranging from infection to chronic diseases including cancers. Many infectious agents are well recognized to trigger the production of ROS and RNS [103]. *Helicobacter pylori*, a well-known bacterial agent implicated in the development of gastritis, peptic ulcer and gastric carcinoma, is shown to induce ROS generation, oxidative stress and apoptosis in human gastric epithelial cell lines [104]. Oxidative stress induced by influenza virus is clearly demonstrated in many studies, and antioxidant intervention is an alternative therapeutic strategy to combat the virus [66]. In hepatocellular carcinoma (HCC), oxidative stress induced by hepatitis B and C viruses is a well-established mechanism to drive malignant transformation of hepatocytes [105]. Toxicity and carcinogenicity induced by heavy metals (e.g., Hg, Cd, Ni, As) are demonstrated to mediate through ROS formation (mainly via Fenton reaction) yielding oxidatively modified products with highly carcinogenic and mutagenic potential [106, 107]. Undoubtedly, pathogenesis and complication of diabetes [108, 109], atherosclerosis [110, 111] and Parkinson's disease [112] is critically involved ROS generation and oxidative damage. ROS directly cause oxidized lesions on DNA. Increased formation of oxidized lesions together with failure of DNA repair introduces a bunch of genetic mutations. Cancer is a disease of accumulated genetic mutations, and ROS production in cancer cells is markedly higher than normal cells. It is well established that ROS and oxidative stress have both direct and indirect contributions to carcinogenesis and progression of cancers [69, 113, 114]. The question is that how do cancer cells survive under the highly oxidative microenvironment. It turns out that cancer cells cope with the oxidative stress by reprograming their metabolism and empowering the antioxidative capability through Nrf2/Keap1/ARE pathway [115–119]. In this chapter, it is focused only on oxidative stress in urinary stone disease.

3. Urolithiasis

UL or urinary stone disease is a condition with mineral masses in the urinary system. It is indeed an ancient condition. The oldest urinary stone was found in the pre-historic Egyptian tomb by Professor G. Elliot Smith in 1901. He observed the calculus lying among the pelvic bones of a 16-year-old boy mummy. This bladder stone composed of several types of minerals including uric acid (UA), calcium oxalate (CaOx), calcium phosphate (CaP) and magnesium ammonium phosphate (MAP). It was dated before 4500 B.C., meaning that the first evidenced urinary stone occurred over 7000 years ago [120, 121]. Even though UL is a long-standing disease staying with us since the origin of human history, the mechanism of urinary stone formation is still not fully understood. Moreover, UL cannot be cured completely. Surgical treatment of stones removes only symptoms, not causes. The challenging issue of stone disease management is how to prevent the stone recurrence.

According to the location of stones in the urinary tract, stone disease is classified into three main forms as follows: kidney or renal stone (nephrolithiasis), ureteric stone (ureterolithiasis) and bladder stone (vesical calculi). Kidney stone is the most prevalent one. Bladder stone is accounted approximately 5% of all stones. It is prevalent in children in the developing countries, and diet is the main risk factor [122]. Stone lodged in the ureters (ureteric stone) is found about 20% of all stones [123]. It is believed that ureteric stone has a kidney origin. Kidney stone moves downwards to ureter due to the flush of urine flow and the size of stone (normal ureter diameter: 3–4 mm). Size does matter for spontaneous passage of ureteric stones, as ureteric stones about 4 mm in width have a spontaneous passage rate over 80% [124].

Prevalence of UL is progressively increasing in all countries across the world [125–127], especially in the tropical regions [128]. The lifetime risk of stone formation in the USA is over 12 and 6% in men and women, respectively [129]. In Japan, the lifetime prevalence is of 15.1% in men and 6.8% in women [130, 131]. Overall kidney stone prevalence in Europe is ranged between 5% and 10% [132]. In Germany, data in 2001 show stone prevalence of 4.7% in men and 4.0% in women [133]. The highest lifetime prevalence of 20% is reported in Saudi Arabia, a country with desert climate [130]. In Thailand, UL is endemic in the northeastern region, and the disease rate examined by abdominal ultrasound in 1997 is of 16.9% [134]. Our preliminary unpublished data of a community survey in 2017 for detecting asymptomatic urinary stones in villagers who reside in the northeastern region using computed tomography scan reveal the prevalence of asymptomatic stones at 12%, which is relatively high. Additionally, pattern of stone onset greatly varies among regions, for instance, the ureteric stone is much more common in the southern region of Thailand compared to the other regions [135]. In sum, the data of stone prevalence clearly indicate that stone formation varies across countries, depending greatly on climate. Stone prevalence is lower in colder countries, but higher in warmer countries.

Change in lifestyle and dietary habit is believed to have major contribution to an increasing stone prevalence [136]. Stone disease is a disease of urine concentration. Fluid intake and fluid loss due to hot climate are greatly contribute to urinary stone formation. An impact of global warming on rising in stone prevalence was first reported in 2008 in the USA [137]. Later, the adverse effect of climatic change on increase in UL onset is confirmed by studies [138] from various countries, viz., Korea [139, 140], Iran [141] and USA [142, 143]. In our ongoing research of climate change and UL onset, a trend of global warming in Thailand is observed. Increased temperature is positively associated with increased UL onset. Contrary, rainfall has a negative association with onset of urinary stone. Our finding confirms a significant contribution of global warming to UL development.

Stones are built from lithogenic crystals formed in the supersaturated urine. Type of urinary stones is, therefore, classified according to primary mineral components into four main types, namely CaOx, CaP, MAP and UA stones. Miscellaneous stones, including cystine and xanthine stones, are usually caused by genetic mutations of certain genes, and they are found mainly in children. CaOx is the highest prevalent stone type that is found up to 80% of all stones, and it is frequently mixed with CaP or hydroxyapatite. MAP or struvite stone is

formed in the alkali urine and associated with urinary tract infection of urea-splitting microorganisms such as *Proteus*, *Klebsiella*, *Serratia*, *Pseudomonas*, *Staphylococcus* and *Mycoplasma*. Nowadays, prevalence of struvite stone is decreasing, perhaps due to a widespread use of antibiotics. UA stone is the second most common urinary stones found up to 40%, and its formation is associated with acidic urine [144]. Precipitation of UA depends chiefly upon urine pH. UA has a pKa of 5.75 [145]. In urine pH over 5.8, it exists as urate and readily solubilizes in water, whereas in urine pH below 5.8 it predominantly presents in the form of insoluble UA. Our hospital-based data from four main regions of Thailand (Northeast, North, Central and South) revealed that CaOx, CaP, UA and MAP stones were found at 74, 5, 16 and 5%, respectively [146]. This is consistent with the global picture of urinary stone types as CaOx is the most common one followed by the UA stone.

Urinary stone has multifactorial etiology with polygenic susceptibility. It mainly affects adults. Monogenic stone condition, such as cystinuria (found approximately at 1% of all stones and 7% of stones in children) and primary hyperoxaluria, is a relatively rare condition that is often found in children, so-called childhood UL [147, 148]. Cystinuria is an autosomal recessive trait caused by mutations in *SLC3A1* or *SLC7A9* gene resulting in an inborn error in transport of urinary cystine, ornithine, lysine and arginine (commonly known as COLA) that subsequently initiate cystine stone formation. Stone formation without any identifiable clinical causes is labeled "idiopathic," which is commonly observed in the CaOx formers [149]. However, it has been suggested that genetic screening should be performed in the previously classified idiopathic calcium UL in order to certainly rule out an underlying genetic susceptibility [148].

Stone formation is more prevalent in males than females. Male-to-female ratio varies from 3.13:1 (in Germany) to 1.15:1 (in Iran) [126, 150]. In Thailand, we found a much lower of male-to-female ratio at 1.1–1.2:1 implying that Thai men and women have a comparable chance to develop urinary stones [146, 151]. For age, a peak of prevalence is found between 40 and 50 years old for CaOx stone, but for UA stone the age peak is shifted to 60–70 years old [126, 146, 150, 151]. Increased body mass index and diabetes are associated with increased risk of UL, particularly UA stone formation [152–154]. Certain anatomical abnormality of kidneys also increases the risk of stone formation [155]. UL is known as the most frequent complication of horseshoe kidneys, which can be found up to 60% [156]. Likewise, up to 50% of patients with calyceal diverticula are inflicted with stones [157].

Family history is another factor known to increase a risk of stone formation. The data from study in the USA show that men with positive family history have 2.57 times higher risk of incident stone formation than those without [158]. Familial aggregation of kidney stone disease is more prominent in the northeastern region of Thailand with a relative risk of 3.18 among members of the affected families [159]. Based on our data, a positive family history is accounted for 32–35% implying that contribution of genetic susceptibility to drive stone formation is observed only in one-third of cases [146, 151]. Stone formation in the majority of cases (two-third) is chiefly influenced by environmental and behavioral factors, especially diets. Inadequate fluid intake (recommended at 2 liters per day), increased consumption of food rich in animal protein and lithogenic substances (such as oxalate and purines) and low

intake of food containing antilithogenic substances (especially citrate) markedly contribute to stone development [136]. However, argument is raised from a prospective study of the large cohorts in the USA that demonstrates that dietary oxalate (as well as spinach intake) is not a major risk factor for incident nephrolithiasis [160]. For protective factor, dietary calcium and intake of fruits and vegetables reduce a risk of stone formation [161, 162]. In sum, dietary factor has a large contribution to stone formation; therefore, development of UL is possibly preventable.

Exposure to risk factors mentioned above causes changes in concentrations of urinary substances, including lithogenic substances (called stone promoters) and antilithogenic substances (called stone inhibitors). Disproportion of urinary stone promoters and inhibitors predisposing to crystallization and stone formation is defined as metabolic risk factor or metabolic abnormality. Metabolic abnormality includes an increase in urinary stone promoters, e.g., hypercalciuria, hyperoxaluria and hyperuricosuria, and a decrease in urinary stone inhibitors, e.g., hypocitraturia, hypokaliuria and hypomagnesiuria. Although hypercalciuria is found in UL patients more frequent than hyperoxaluria, evidence suggests that mild degree of hyperoxaluria has much more influence on CaOx stone formation than hypercalciuria [163]. Citrate is the most potent stone inhibitor in urine, and low urinary citrate excretion is a common manifestation found in UL patients. Hypocitraturia in UL is reported between 20% and 60% in western studies [164]. In endemic area, hypocitraturia is much more prevailing, plausibly due to difference in lifestyle and dietary habit [165, 166]. Our data demonstrate that hypocitraturia (80–100%) and hypokaliuria are the most common metabolic risk factors found in Thai stone patients. In addition, we show that individuals with hypocitraturia have about 10 times higher risk for kidney stone development than those without [167, 168].

Mechanism of kidney stone formation has been proposed, although it is not entirely understood (**Figure 9**). Building blocks of stones are lithogenic crystals, such as CaOx, CaP and UA crystals, formed in the urine. Chemically, CaOx crystals have three forms, i.e., calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD) and calcium oxalate trihydrate (COT), but the most deleterious form with highest lithogenic potential is COM. A process of crystallization from solution has two phases. The first phase is birth of new crystals (called nucleation), and the second phase is growth of crystals to get larger size (called crystal growth). Supersaturation of urine, caused by increase in concentration of lithogenic ions and/or decrease in urine volume and stone inhibitors, triggers nucleation in renal tubules. The crystals grow and aggregate to reach sufficient sizes and retain in the kidney. Surplus crystals are toxic and injurious to renal tubular cells resulting in renal tubular injury. Injured tubule is a suitable site for crystal attachment and retention. Nidus (site of stone origin) is then formed, grown and finally become stone. There are pathological changes occurred during the lithogenic process. Excessive crystals induce ROS production in the exposed renal tubular cells leading to oxidative stress and renal tubular damage. Crystals also induce production and release of inflammatory mediators to activate inflammatory response that further enhances tubular injury. Both oxidative stress and inflammation cause release of various proteins and sloughing of cell debris into urine creating a vicious cycle to enhance crystal formation, aggregation, retention and finally stone formation (**Figure 9**).

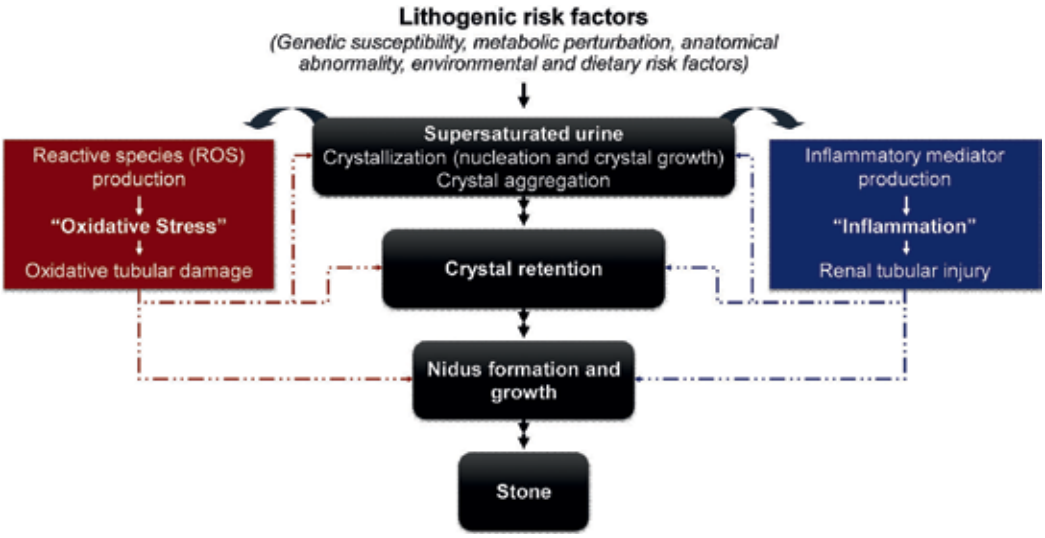

Figure 9. Key mechanistic steps in the process of kidney stone formation (see text for detail).

Although it is known that urinary crystals are building blocks for building urinary stone, it is not precisely known how the microscopic crystals transform to be a macroscopic stone. Crystal aggregation is one of the critical steps in lithogenic process. Urinary crystals have to adhere to each other to form a bigger mass. If urinary crystals do not adhere or bind to each other, stone cannot be formed—similar to granulated sugar that each minute granule stays separately without clumping. Studies show that stacking of urinary crystals to form stone requires biological glue to adhere crystals together, and that glue is called stone matrix [169]. Stone matrix contributes about 5% of the stone mass composing of cellular biomolecules, cell debris and whole cells. We investigated lipid and protein constituents in stone and urine samples of nephrolithiasis patients. We found that glycolipids and phospholipids released into urine are actively incorporated into stone matrix [170]. Majority of proteins in stone matrix and nephrolithiasis urine are inflammatory and fibrotic proteins [171]. We also demonstrate that S100A8 is an abundant inflammatory protein found in urine and stone matrix of the patients, and it could be a marker to indicate an extent of intrarenal inflammation in nephrolithiasis patients.

4. Oxidative stress in urolithiasis

ROS are experimentally proved to have a critical role in the pathogenesis of kidney stone [172]. Oxidative stress and inflammation are clearly demonstrated to mediate lithogenic process [173, 174]. Exposure of renal tubular cells to oxalate, COM, CaP and UA crystals causes increases in ROS production and oxidative stress leading to cell injury [175, 176] as well as release of monocyte chemoattractant protein-1 (MCP-1) [177–179] and interleukin-6 (IL-6) [180]. Our human data show an elevated urinary excretion of oxidative DNA lesion, 8-hydroxydeoxyguanosine

(8-OHdG), along with rise in renal tubular injury in patients with nephrolithiasis [181]. We also show an increased expression of 8-OHdG lesion in stone-containing renal tissues [182]. MCP-1 and IL-6 mRNA expression in stone-containing kidney tissues are increased, and their increment is related to declined creatinine clearance [183]. Our findings indicate that patients with nephrolithiasis persistently have increased oxidative stress and intrarenal inflammation, and these pathological changes contribute to renal impairment. An inevitable consequence of chronic inflammation is fibrosis. We show an evidence of renal fibrosis in the kidneys of nephrolithiasis patients, and the renal fibrogenesis at least in part mediates through transforming growth factor-beta 1 (TGF-ß1)-induced epithelial-mesenchymal transition (EMT) [184]. In **Figure 10**, we propose the putative cellular mechanism of crystal-induced inflammation leading to interstitial fibrosis in nephrolithiasis patients. Lithogenic crystals are readily formed in the supersaturated urine. In healthy individuals, crystals are flushed out by the urine flow without any harm. In contrast, crystals grow, aggregate and adhere to renal tubular cells in stone-forming patients. Crystals are internalized to be dissolved in lysosomes, and the remnants are exocytosed into renal interstitium. Alternatively, lithogenic ions such as calcium, phosphate and oxalate ions may diffuse through tubular lining towards the renal interstitium to form interstitial crystals. Crystals as well as oxalate ions induce oxidative damage to renal tubular cells via increased ROS generation. MCP-1, osteopontin (OPN), IL-6 and TGF-ß1 are

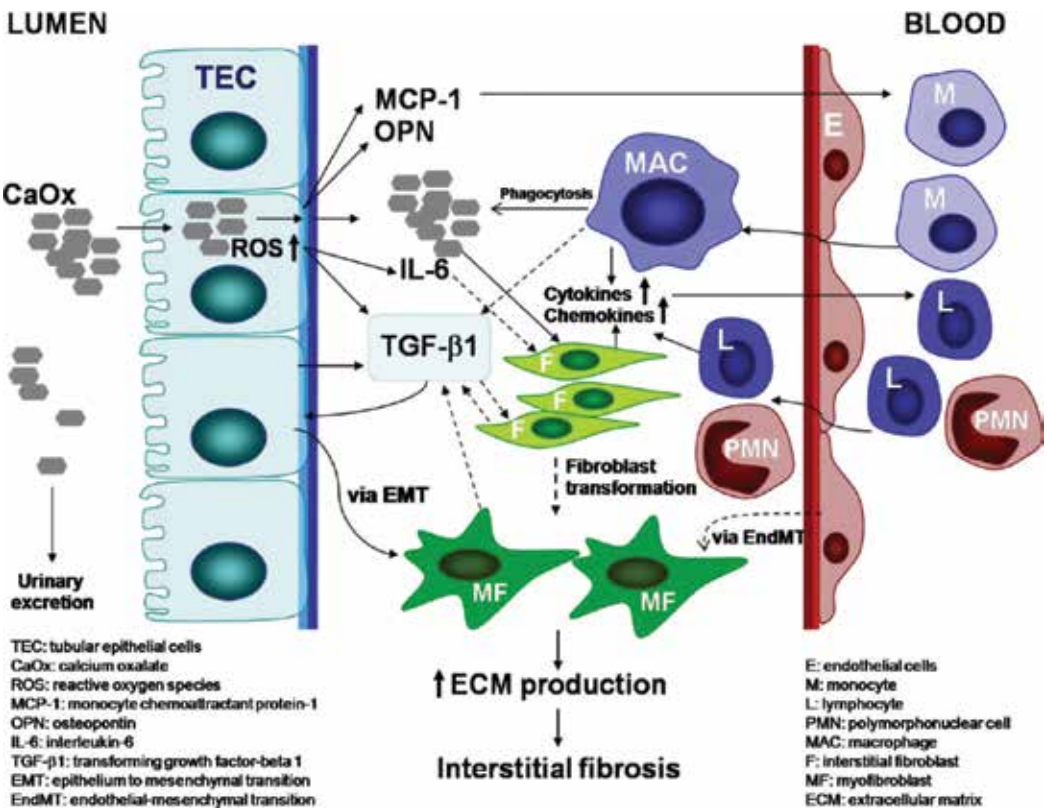

Figure 10. Putative cellular mechanism of crystal-induced inflammation leading to interstitial fibrosis in nephrolithiasis patients. Solid lines indicate demonstrated pathways and dash lines represent hypothesized pathways.

upregulated in the crystals/oxalate-exposed renal tubular cells. MCP-1 and OPN exert chemotactic activity to recruit monocytes and macrophages into the renal interstitium and initiate inflammatory response. The infiltrated immune cells phagocytose the interstitial crystals and release a variety of cytokines, chemokines and growth factors, leading to further recruitment of leukocytes and inflammatory amplification. Excessive and chronic inflammatory reaction causes renal damage and activates wound healing process. IL-6 might stimulate the proliferation of renal tubular cells and interstitial fibroblasts in order to replace the severely injured and dead renal cells. TGF-ß1 produced by tubular cells in stone-forming kidneys activates the transformation of interstitial fibroblasts into α-smooth muscle actin (αSMA)-expressing/extracellular matrix (ECM)-producing myofibroblasts and induces the transdifferentiation of renal tubular cells via EMT leading to overproduction of myofibroblasts and ECM. TGF-ß1 is also capable of inducing endothelial-mesenchymal transition to generate myofibroblasts from endothelial cells. TGF-ß1 is in turn overproduced by monocytes/macrophages, fibroblasts and myofibroblasts. Chronic inflammation is further amplified. Excessive repairing process causes excessive deposition of ECM proteins leading to scar formation. Thereby, lithogenic crystals that actively and chronically form in the nephron of nephrolithiasis patients cause a sustained inflammatory injury and excessive repair, which eventually lead to renal fibrosis. Urinary obstruction by large stone mass also initiates the renal fibrogenic cascade through TGF-ß1.

The goal of UL therapy is to remove stones and prevent stone recurrence. Removal of stones requires surgical approaches while prevention of stone relapse requires medical management. The current drug of choice for stone therapy is potassium citrate [185, 186]. The drug delivers citraturic and urine alkalinizing effects to elevate urinary citrate and increase urine pH. According to the stone management guideline, treatment with potassium citrate requires at least 6 months to effectively reduce the likelihood of recurrent stone formation [187]. Since citrate is a key therapeutic ingredient for inhibiting stone formation, citrus fruits (such as orange, lemon, lime and grapefruit) and non-citrus fruit (such as melon) with high content of citrate have been considered as alternatives for stone treatment [188]. Intervention with antioxidants is shown to effectively inhibit CaOx crystal deposits in experimental nephrolithic rats [189, 190]. Regarding this, herbs and medicinal plants with high antioxidant property that have been traditionally used for treating stone disease in various countries are suggested to be alternative nutraceuticals or complementary therapeutic options for stone disease [191]. Banana stem (*Musa sapienta* L.), which is an Ayurveda remedy to treat kidney stones, had been shown to significantly reduce urinary stone risk in hyperoxaluric rats [192]. We have been investigated the clinical efficacy of lime juice and banana stem beverage as alternatives for UL treatment. We show that our inhouse limeade-based regimen, designated lime powder regimen (LPR), efficiently delivers citraturic, alkalinizing and antioxidative actions in nephrolithiasis patients [193]. Our preclinical and phase 1 clinical trial reveals that LPR inhibits COM crystal growth and attenuates oxidative stress in vitro and is capable of increasing urine citrate, pH and antioxidant capacity in healthy individuals [194]. Importantly, LPR is well tolerated and safe for daily intake.

Based on our research experience over 10 years three major etiological factors are identified in UL patients including (1) an inadequate intake of water, (2) low urinary excretion of citrate and (3) increased oxidative stress. We have developed an innovative beverage-based regimen for preventing urinary stone formation, named HydroZitLa (patent pending). Our inhouse HydroZitLa beverage contains therapeutic dose of citrate and naturally antioxidants

derived from banana stem, *Clitoria ternatea L.* and *Caesalpinia sappan*. In vitro, HydroZitLa efficiently inhibits COM crystal aggregation and exerts antioxidative action to reduce oxidative damage in COM-treated HK-2 cells as well as H_2O_2-treated bladder cancer cells. In vivo experiment reveals an antilithigenic efficacy of HydroZitLa in inhibiting CaOx deposits in kidneys of ethylene glycol-induced nephrolithic rats. The antilithogenic effect of HydroZitLa is comparable to that of potassium citrate drug (Uralyt-U). Our findings indicate a promising clinical potential of LPR and HydroZitLa as alternative nutraceuticals for UL treatment.

5. Conclusion

ROS are both friend and enemy. At physiological level, they are required for metabolic reactions and play a vital role in redox biology. At the uncontrollable high level, they are very destructive. High rate of oxygen consumption (through ETC and oxidases/oxygenases) and presence of transition metals are the main factors to generate ROS in an excessive amount. Cells combat ROS through activation of the cytoprotective Keap1-Nrf2-ARE signaling pathway. Chronically excessive production of ROS causes oxidative stress that disrupts redox signaling and control resulting in damage to biomolecules and cell injury. Oxidative stress mediates pathogenesis of a number of diseases ranging from infection to cancer. Evidences from in vitro, animal and human studies strongly support the active involvement of oxidative stress in urinary stone formation. Lithogenic crystals formed in urine directly induce ROS generation in renal tubular cells causing oxidative damage and release of inflammatory mediators. Sustained tubular injury, oxidative stress and inflammation in turn accelerate crystal formation, growth and aggregation and ultimately stone formation. Renal fibrosis is also found in the stone-containing kidneys of the patients and believed to be a main contributing factor to kidney dysfunction in the stone patients. Stone disease is greatly contributed by environmental and behavioral factors, and the disease is frequently recurrent. Based on our research experiences, major risk factors of stone formation (in particular CaOx stone) include inadequate daily intake of fluid, low urinary excretion of citrate (hypocitraturia) and low antioxidative capability (high oxidative stress). Therefore, regimens or approaches to recuperate these depleted conditions are promising to be a new therapy for UL. Citrate is a potent stone inhibitor, and potassium citrate is a current drug used for preventing the stone recurrence. We recently developed a novel herb-based antilithic drink (called HydroZitLa, patent pending) containing a therapeutic dose of citrate and high amount of natural polyphenol antioxidants. Our in vitro and animal studies show a great promise of HydroZitLa to be an alternative for preventing urinary stone formation. Clinical trials are now planning to be conducted to observe the side effect and to test the clinical efficacy of HydroZitLa in reducing the risk of stone formation in the real clinical setting.

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Conflict of interest

C. Boonla is one of the inventors of HydroZitLa (Patent pending).

Author details

Chanchai Boonla

Address all correspondence to: chanchai.b@chula.ac.th

Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

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Chapter 9

Biomolecules Oxidation by Hydrogen Peroxide and Singlet Oxygen

Kazutaka Hirakawa

Additional information is available at the end of the chapter

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Abstract

Hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are important reactive oxygen species (ROS) for biological and medicinal fields. Oxidation processes of chemical materials by molecular oxygen are important H_2O_2 source, whereas photochemical reaction is important for $^1O_2$ production. Reactivity and biomolecule damage by these ROS depend on the surrounding conditions and targeting molecules. In this chapter, production mechanisms of H_2O_2 and $^1O_2$, biomolecule oxidation by these ROS, their detection methods, and production control of $^1O_2$ are briefly reviewed.

Keywords: hydrogen peroxide, singlet oxygen, DNA damage, protein damage, photooxidation

1. Introduction

Biomolecule damage, for example, oxidation of DNA and/or protein, by reactive oxygen species (ROS) is closely related to carcinogenicity [1–3] and/or toxicity [4–6]. Furthermore, oxidative damage to unwanted tissue can be applied to the treatment of disease including cancer treatment [7–9], and similar reaction is applied to sterilization [10–14]. Hydrogen peroxide (H_2O_2) is a relatively long-lived ROS compared with a short-lived ROS such as superoxide anion radicals ($O_2^{\bullet-}$) [15]. One of the most important producing mechanisms of H_2O_2 is a dismutation of $O_2^{\bullet-}$, which is easily formed though oxidation of various materials by dioxygen molecule (O_2). Various carcinogenic chemical compounds produce H_2O_2 through their oxidation processes. Relationship among molecular oxygen and ROS is shown in **Figure 1**. Oxygen molecules are easily reduced by surrounding materials, and various ROS and the intermediates are formed (**Figure 1A**). In the case of photosensitized reaction, excited states of oxygen molecules are produced (**Figure 1B**). Singlet oxygen ($^1O_2$), which is also an important ROS, can

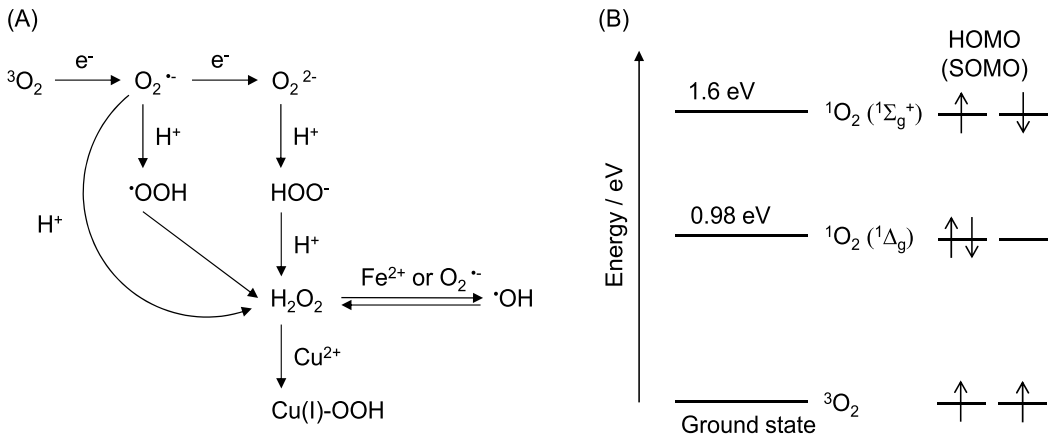

Figure 1. Relationship among ground-state oxygen molecule ($^3O_2$) and ROS (A) and the energy levels of oxygen molecule (B). HOMO and SOMO are the abbreviations of highest occupied molecular orbital and semi-occupied molecular orbital, respectively. The "arrows" in (B) indicate the electron spin.

be easily generated via photosensitized reaction [16–18]. The $^1\sum_g^+$ state ($^1O_2(^1\sum_g^+)$) is mainly produced through the excitation energy transfer from the excited state, in general triplet excited (T_1) state, of photosensitizer [16–18]. The $^1O_2(^1\sum_g^+)$ has higher energy, 1.6 eV, corresponding to the ground state of oxygen molecule ($^3O_2$). The lifetime of $^1O_2(^1\sum_g^+)$ is several picoseconds, and $^1O_2(^1\sum_g^+)$ is rapidly converted to the $^1\Delta_g$ state ($^1O_2(^1\Delta_g)$) [16–18]. Because the lifetime of $^1O_2(^1\Delta_g)$ (several microseconds) is markedly longer than that of $^1O_2(^1\sum_g^+)$, $^1O_2(^1\Delta_g)$ is a more important ROS. After that, $^1O_2$ indicates $^1O_2(^1\Delta_g)$ without explanation in this chapter. Visible light, other than ultraviolet radiation, has sufficient energy to produce $^1O_2$ from the ground state of oxygen molecule. Therefore, $^1O_2$ production is an important mechanism of phototoxicity and/or photo-carcinogenicity under strong light illumination with phototoxic materials. The purpose of this chapter is a review of the ROS-mediated biomolecule damage and the related topics.

2. Hydrogen peroxide

Hydrogen peroxide itself is not strongly ROS. However, other ROS including hydroxyl radicals (•OH) are produced from H_2O_2. In general, H_2O_2 is produced from the dismutation of $O_2^{\bullet-}$, and, in vivo, production of H_2O_2 and $O_2^{\bullet-}$ occurs in mitochondria [19]. In this section, H_2O_2 formation from compounds, specifically artificial materials, is introduced.

2.1. Hydrogen peroxide formation through oxidation of chemical compounds

One of the most important processes of H_2O_2 production is a dismutation of $O_2^{\bullet-}$. Various chemical compounds or metals can be oxidized by oxygen molecules. In the case of a simple electron transfer-mediated oxidation, $O_2^{\bullet-}$ is produced by the electron extraction from chemical compounds or metals. The lifetime of $O_2^{\bullet-}$ in aqueous solution is about several milliseconds [15]. The produced $O_2^{\bullet-}$ in aqueous media is converted to H_2O_2 through the dismutation by proton (H^+) as follows:

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

For example, hydroquinone, which is one of the metabolites of benzene, can produce H_2O_2 through the autoxidation process (**Figure 2**) [20]. This process is markedly enhanced by the presence of metal ions, specifically Cu^{2+} ions [20]. In the presence of sacrificial reductants, for example, nicotinamide adenine dinucleotide (NADH), the oxidized form of hydroquinone, *p*-benzoquinone, is reduced to the parent hydroquinone. Consequently, the redox cycle is formed, leading to the production of H_2O_2 abundantly. It has been also reported that hydrazine analogues produce H_2O_2 through their autoxidation processes (**Figure 3**) [21–23].

2.2. Hydrogen peroxide production through photochemical processes

Photochemical processes also contribute to the formation of H_2O_2. Because the reorganization energy of the reduction of small molecule, such as O_2 molecules, through electron transfer becomes large due to the Marcus theory [24, 25], the $O_2^{\bullet-}$ production through photoinduced electron transfer is energetically difficult [26, 27]. However, ultraviolet radiation to reductive photosensitizer, such as NADH (**Figure 4**), can produce $O_2^{\bullet-}$ as follows [28]:

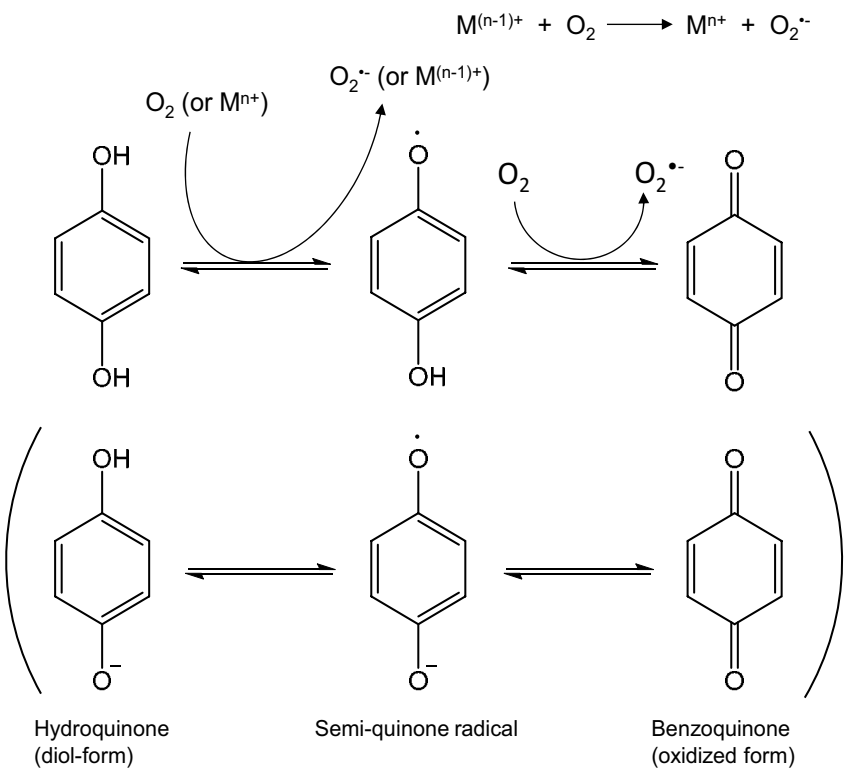

Figure 2. Autoxidation process of hydroquinone and ROS production.

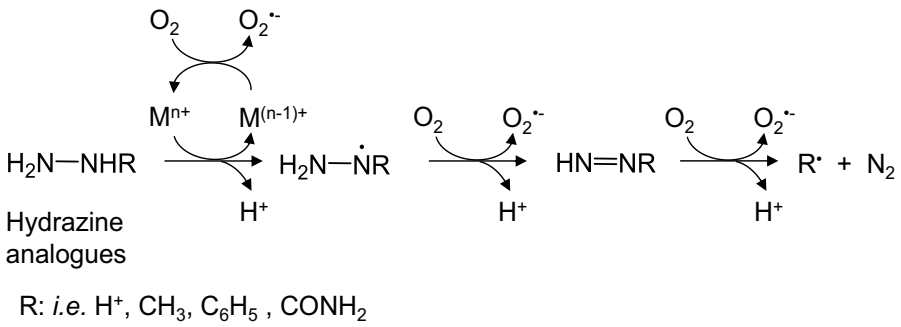

Figure 3. Autoxidation process of hydrazine analogues and ROS production.

$$NADH + h\nu \rightarrow NADH^{*} \tag{2}$$

$$NADH^{*} + O_2 \rightarrow NAD^{\bullet} + H^{+} + O_2^{\bullet -} \tag{3}$$

where NADH* is the photoexcited state of NADH and $NAD^{\bullet}$ is the radical form. $NAD^{\bullet}$ undergoes further oxidation by oxygen molecules to NAD^{+}, the final oxidized product. The formed $O_2^{\bullet -}$ is also converted to H_2O_2 through the dismutation process of Eq. (1).

Photocatalytic reaction can also produce H_2O_2 [29–34]. For example, the surface of titanium dioxide (TiO_2) can reduce relatively oxidative molecules under ultraviolet A (UVA; wavelength, 315–400 nm) irradiation [29–32]. Two crystalline forms of TiO_2, anatase and rutile with band gap energies of 3.26 and 3.06 eV, respectively, are well-known semiconducting photocatalyst [29–32]. The adsorbed oxygen molecules on the TiO_2 surface is reduced to $O_2^{\bullet -}$ by the electron of conduction band, which is excited from the valence band by UVA energy (**Figure 5**).

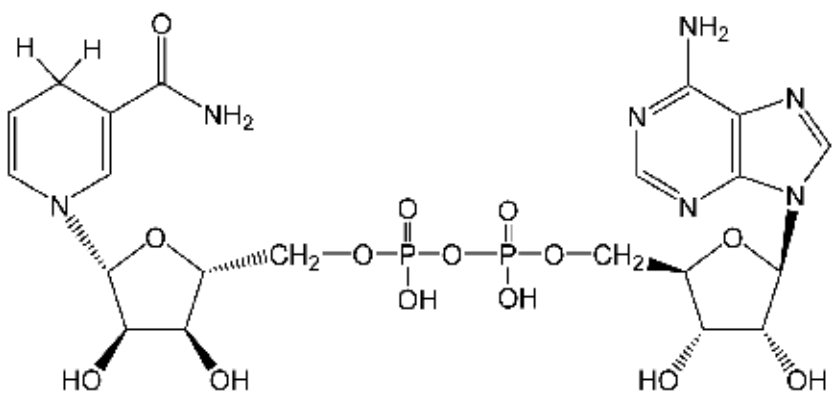

Figure 4. Structure of NADH.

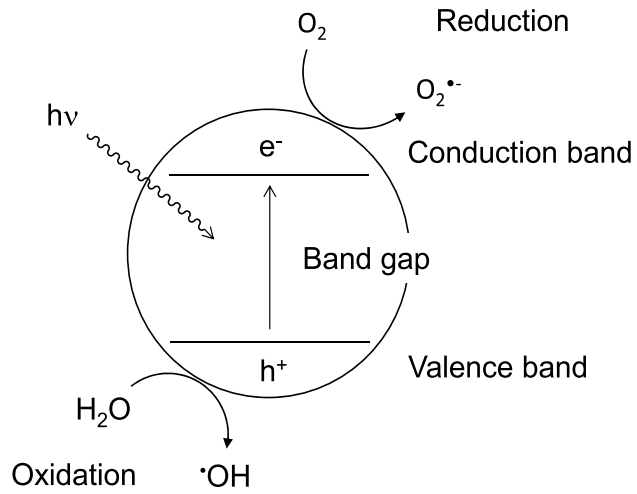

Figure 5. Photocatalytic production of ROS by TiO_2.

Similarly to the abovementioned reaction, $O_2^{\bullet-}$ is also converted to H_2O_2 through the dismutation process of Eq. (1). In addition, oxidation reaction of TiO_2 photocatalyst also produces H_2O_2. The formed hole (h^+) in the valence band by UVA irradiation oxidizes water molecules on the surface of TiO_2 to $^{\bullet}OH$. The reaction of two $^{\bullet}OH$ species can produce H_2O_2 as follows:

$$2^{\bullet}OH \rightarrow H_2O_2 \tag{4}$$

Although TiO_2 particles are barely incorporated into cell nucleus [35], cellular DNA damage was reported [36–39]. Because H_2O_2 has a transparency for nuclear membrane, the cellular DNA damage can be explained by H_2O_2-mediated mechanism [32]. The activation of H_2O_2 and DNA damage by H_2O_2 are described later.

2.3. Secondary formation of hydrogen peroxide through photocatalytic reaction

Photocatalytic reaction can produce oxidized intermediates other than final oxidized products of chemical compounds. For example, photooxidized amino acids [40] and sugars [41] by TiO_2 photocatalyst produce H_2O_2 through secondary oxidation reaction in the presence of metal ions (**Figure 6**). Titanium dioxide can photocatalyze the production of $^{\bullet}OH$, a strong oxidant, through the decomposition of H_2O. The formed h^+ in the valence band by UVA irradiation can also oxidize various materials adsorbed on TiO_2 surface. Hydroxyl radicals and h^+ can oxidize these biomolecules, resulting in the production of oxidized intermediates. The formation of partly oxidized molecules leads to the secondary H_2O_2 production in the presence of metal ions. This H_2O_2 production process may cause a remote H_2O_2 generation in cells.

It has been reported that the photooxidized phenylalanine and tyrosine by TiO_2 produce H_2O_2 in the presence of copper(II) ion [40]. Since TiO_2 photocatalysis induces a hydroxylation of

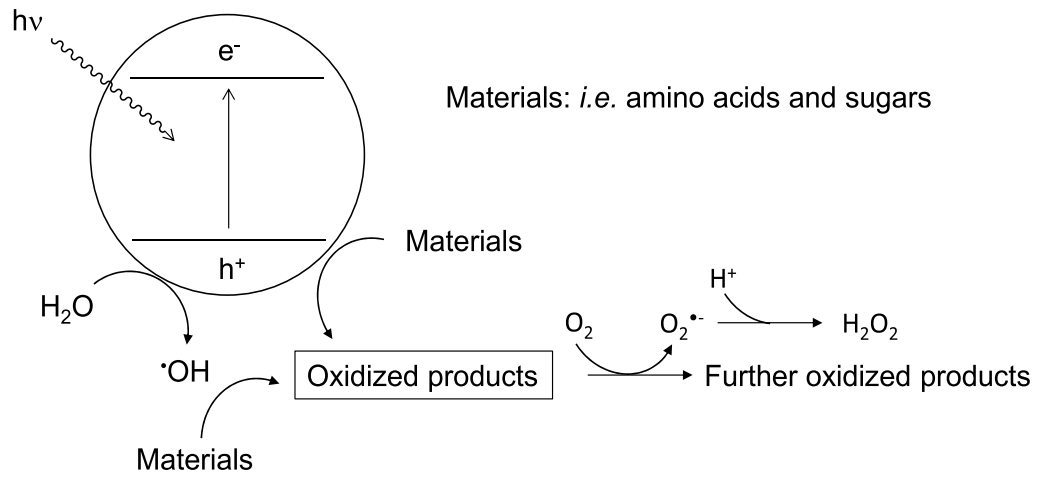

Figure 6. The secondary production of ROS by TiO_2 photocatalysis.

aromatic compounds [42], the formation of benzenediol derivatives from these aromatic amino acids is possible (**Figure 7**). As mentioned above, hydroquinone can produce H_2O_2 through the autoxidation (**Figure 2**) [20, 43]. The amount of H_2O_2 production from the photooxidized phenylalanine is significantly larger than that from tyrosine [40]. The difference of their autoxidation rates should affect the H_2O_2 production. It has been reported that the autoxidation rate of 1,4-form of benzenediol is markedly faster than that of 1,2-form [20]. Phenylalanine can be oxidized into various types of benzenediol, including the 1,4-form; however, tyrosine cannot be converted to the 1,4-form. Consequently, phenylalanine can produce relatively large amount of secondary H_2O_2 through the photocatalysis of TiO_2. Furthermore, other amino acids can be also oxidized by TiO_2 photocatalyst and induce the secondary ROS production. Specifically, photocatalyzed cysteine by anatase form produces significantly large amount of secondary H_2O_2. In the case of sugar oxidation by TiO_2 photocatalyst, the activity of secondary H_2O_2 production by anatase form TiO_2 is larger than that of rutile form [41].

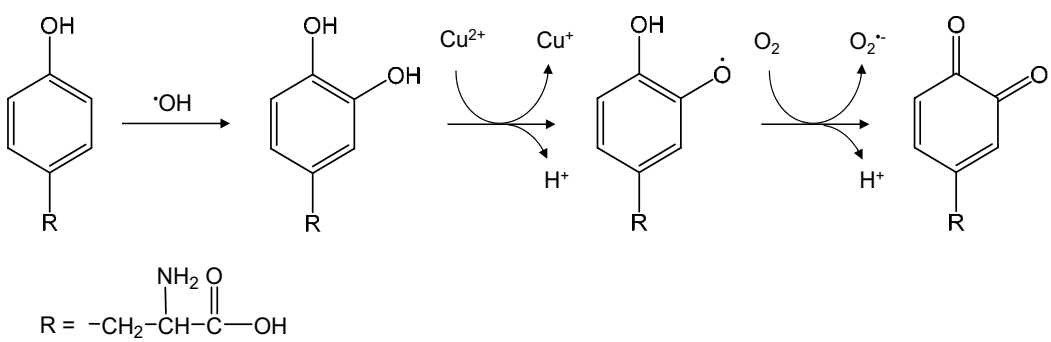

Figure 7. Tyrosine oxidation by TiO_2 photocatalysis and the copper ion mediated ROS production. Phenylalanine is also oxidized by the similar processes, leading to the secondary ROS production.

3. DNA damage by hydrogen peroxide

Hydrogen peroxide itself barely induces DNA damage; however, it can oxidize nucleobases and cleave sugar-phosphate backbone in the presence of metal ions. In this section, the sequence-specific DNA damage by the H_2O_2-derived ROS and its biological effect are briefly introduced.

3.1. Sequence-specific DNA damage by hydrogen peroxide

Hydrogen peroxide causes alkali-labile products at guanine, thymine, and cytosine in the presence of cupper ion (Cu^{2+}) [44]. Since copper ions are associated with chromatin [45] to form stable complexes with DNA [46–49], Cu^{2+} can play an important role in the activation of H_2O_2 in cell nucleus. Polyacrylamide gel electrophoresis studies demonstrated that H_2O_2 itself cannot cleave and oxidize DNA [44]. However, the incubation of DNA with H_2O_2 and Cu^{2+} induce base modifications at guanine, thymine, and cytosine residues. These base modification sites can be cleaved by hot piperidine treatment [20–22, 44]. The derived reactive species from H_2O_2, for example, copper-peroxyl species (Cu(I)-OOH), are responsible for this DNA damage:

$$H_2O_2 + Cu^+ \rightarrow Cu(I) - OOH + H^+ \quad (5)$$

Cu(I)-OOH is not strongly reactive compared with $^{\bullet}OH$; however, its lifetime is relatively long to induce DNA base modification. Single-stranded DNA is easier oxidized by these ROS. Therefore, DNA damage by H_2O_2 is enhanced by denaturation of DNA [44]. Abovementioned chemical compounds, benzenediol [20] and hydrazine [21], induce these base modification in the presence of Cu^{2+}. In the case of relatively low concentration of TiO_2 particles, similar sequence-specific DNA damage was observed after UVA irradiation with Cu^{2+} [32]. DNA damage mediated by H_2O_2 is effectively inhibited by catalase [50], which is an enzyme to decompose H_2O_2 to H_2O and O_2. Chelating molecules for copper ions also effectively suppress this DNA damage. In addition, 3-methylthiopropanal (methional) is an effective inhibitor of Cu(I)-OOH [20, 32, 44]. Cu(I)-OOH cannot be scavenged by free $^{\bullet}OH$ scavengers, such as sugars and alcohols [20, 22, 32, 44]. In the presence of Cu^{2+}, UVA-irradiated NADH also induces DNA damage by the similar process through H_2O_2 production [28]. In general, photosensitized DNA damage could be explained by $^1O_2$ formation mechanism or electron transfer-mediated oxidation [51]. The H_2O_2-mediated DNA is a rare case in the photochemical DNA damage.

Hydrogen peroxide and Cu^{2+} can induce tandem lesion at guanine and thymine residues [32]. Clustered DNA lesions including tandem damage have important mutagenic potential [52–54]. Furthermore, the repair of such DNA damage is more difficult than single-base damage [55–60]. Therefore, oxidative DNA damage through H_2O_2 production may play an important role in carcinogenesis.

In the presence of iron ions (Fe^{2+}), •OH is formed as follows:

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+} \quad (6)$$

Formed •OH induces base oxidation with non-sequence specificity, because •OH can oxidize all nucleobases [44, 61]. In addition, direct cleavage of sugar-phosphate backbone is caused by •OH. Hydroxyl radical-mediated DNA damage was reported by the case of ascorbate with Cu^{2+} [62]. As mentioned above, in the case of TiO_2 photocatalysis, •OH is directly produced from water decomposition [29–32], and DNA damage without sequence specificity can be induced in the absence of metal ions [32]. Relatively high concentration of anatase form of TiO_2 induce non-sequence-specific DNA damage under UVA irradiation without metal ions through •OH production [32]. DNA damage by •OH is effectively inhibited by sugars and alcohols [32, 44]. However, in the presence of metal ions, the addition of •OH scavengers rather enhances DNA damage through the secondary generation of H_2O_2 from the oxidized products of scavengers themselves by •OH [32, 41]. Base modifications can cause carcinogenesis. Because H_2O_2 can penetrate into nuclear membrane, DNA modification can be induced by H_2O_2 originally formed in the sphere of outer cell nucleus through the assistance of metal ions.

3.2. Mutagenicity and cytotoxicity caused by hydrogen peroxide production

As oxidized products of nucleobases by the H_2O_2-mediated mechanism, 8-oxo-7,8-dihydroguanine (8-oxo-G; oxidized guanine, **Figure 8**) [63–65]; 5,6-dihydroxy-5,6-dihydrothymine (OH-thy; oxidized thymine, **Figure 9**) [58, 66, 67]; 5-hydroxyuracil (OH-Ura; oxidized cytosine, **Figure 9**) [67, 68]; 5-hydroxyhydantoin (OH-Hyd; oxidized cytosine, **Figure 9**) [68], and 5-hydroxycytosine (OH-Cys; oxidized cytosine, **Figure 9**) [67] are well-known compounds.

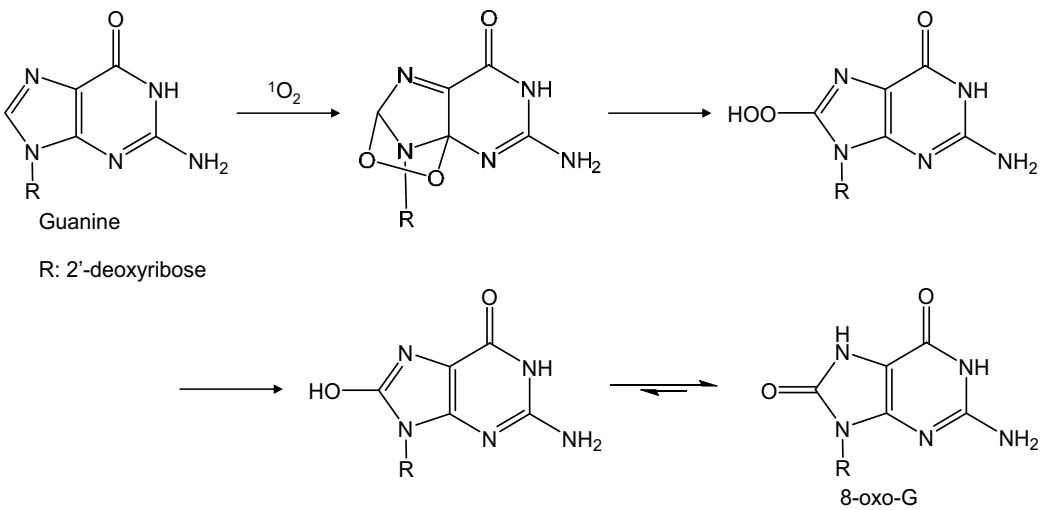

Figure 8. Guanine oxidation by ROS. This scheme is an example of the guanine oxidation by $^1O_2$ to 8-oxo-G. Other H_2O_2-derived ROS, •OH and Cu(I))-OOH, also produce 8-oxo-G through the oxidation of guanine.

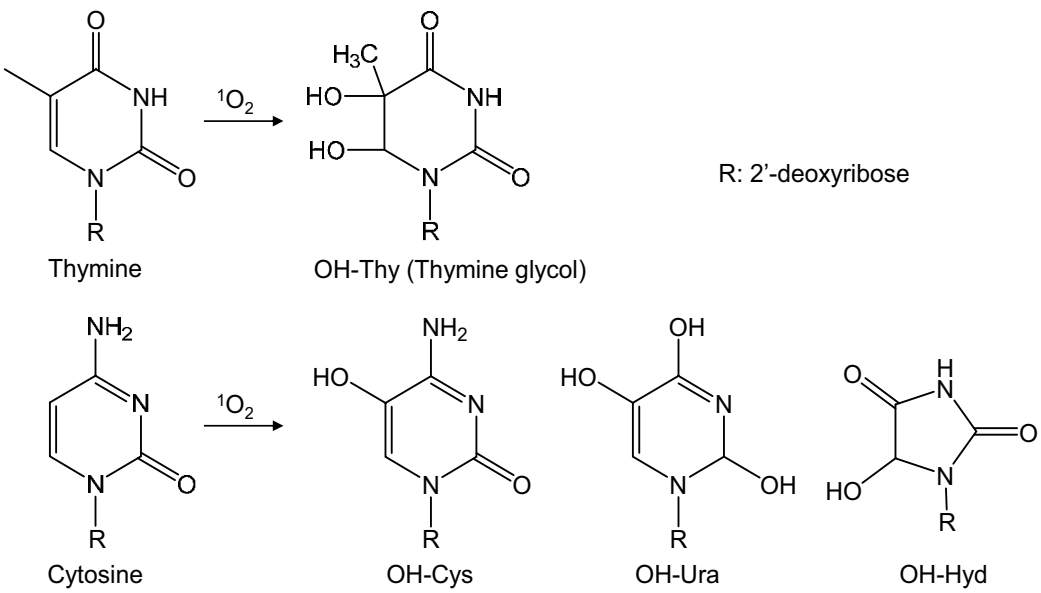

Figure 9. Oxidized products of thymine and cytosine by $^1O_2$.

In a certain case, oxidative DNA damage induces cell death [69, 70]. As a minor oxidized product of guanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G, **Figure 10**) can be formed by H_2O_2 and metal ions [63, 71]. Mutagenicity of Fapy-G is low [72]; however, a related product, methyl-Fapy-G formation, is a lethal lesion [73]. Furthermore, a theoretical study suggested that the formation of Fapy-G contributes to mutation [74]. Cytotoxicity of TiO_2 photocatalyst can be explained by oxidative damage of membrane protein [75–77]. In addition, cellular DNA damage was also reported [78, 79]. Because H_2O_2 has a transparency for nuclear membrane, the cellular DNA damage by TiO_2 photocatalysis can be explained by H_2O_2 production. The formed H_2O_2 through TiO_2 photocatalysis is incorporated into cell nucleus and activated by endogenous metal ions, leading to oxidative DNA damage [32]. Examples of the mutations caused by the oxidized guanines are described in Section 4.

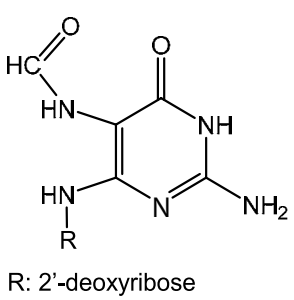

Figure 10. Structure of Fapy-G.

4. Singlet oxygen

In general, the production mechanism of $^1O_2$ involves photochemical processes. Various photooxidation processes can be explained by $^1O_2$ production. In this section, the production mechanism of $^1O_2$, its application, and biomolecule oxidation by $^1O_2$ are briefly introduced.

4.1. General property of singlet oxygen

Singlet oxygen is an excited state of $^3O_2$, ground triplet state of molecular oxygen [16–18]. In general, singlet excited (S_1) states of O_2 are $^1\Delta_g$ and $^1\sum_g^+$; they have excitation energy of 0.98 eV and 1.63 eV above $^3O_2$, respectively [16–18]. Because of the short lifetime of $^1\sum_g^+$ (a few picoseconds), $^1\Delta_g$, the lower S_1 state of O_2, plays an important role in various oxidation reactions. In this chapter, $^1\Delta_g$ is denoted throughout as $^1O_2$. The highest occupied molecular orbital (HOMO) of $^3O_2$ is a semi-occupied molecular orbital (SOMO), whereas this molecular orbital of $^1O_2$ becomes the lowest unoccupied molecular orbital (LUMO) (**Figure 1B**). The oxidative activity of $^1O_2$ is stronger than that of $^3O_2$ due to the vacant molecular orbital. Commonly, $^1O_2$ is produced through photosensitized reaction. Since the excitation energy of $^1O_2$ is relatively small, which corresponds to the energy of photon with the wavelength of 1270 nm (smaller than that of visible light photon), photoexcited states of various dyes can sensitize the generation of $^1O_2$ under visible light or ultraviolet irradiation. Various molecules become photosensitizer (PST) to generate $^1O_2$. In general, the photosensitized reaction of $^1O_2$ generation is an electron exchange energy transfer (the Dexter mechanism) [80]. These processes are presented as follows:

$$PST + h\nu \rightarrow PST^*(S_1) \tag{7}$$

$$PST^*(S_1) \rightarrow PST + \text{fluorescence} \tag{8}$$

$$PST^*(S_1) \rightarrow PST^*(T_1) \tag{9}$$

$$PST^*(T_1) + {}^3O_2 \rightarrow PST + {}^1O_2 \tag{10}$$

where PST*(S_1) and PST*(T_1) are the S_1 and T_1 states of PST, respectively. In general, since the lifetime of PST*(T_1) is markedly longer (several microseconds) than that of PST*(S_1) (several nanoseconds), $^1O_2$ is produced by PST*(T_1). However, the formation of $^1O_2$ by PST*(S_1) is not impossible. The lifetime of $^1O_2$ (τ_Δ) is relatively long (**Table 1**). Generated $^1O_2$ can oxidize various materials, including biomolecules, within its long lifetime. The τ_Δ strongly depends on the surroundings, and a solvent deuterium effect on the reactivity of $^1O_2$ is significant (**Table 1**). For example, the τ_Δ in deuterium oxide (D_2O) is markedly longer than that in H_2O, and the biomolecule oxidation by $^1O_2$ is significantly enhanced in D_2O compared with that in H_2O.

Solvent	Photosensitizer	τ_Δ/μs	Reference
Water (H_2O)	Cationic porphyrin	3.5	[81]
	Rose bengal	3.77	[82]
Phosphate buffer (pH 7.6)	P(V) porphyrin	3.5	[83]
Ethanol (C_2H_5OH)	Rose bengal	15.4	[82]
Ethanol/H_2O (1/1)	Rose bengal	6.37	[82]
Water (D_2O)	Berberine with DNA	72	[84]
	Methylene blue	32	[85]
	Phenalenone	64.4	[86]
	Tris(bipyridine)Ru(II)	59.47	[82]
Chloroform ($CHCl_3$)	Phenalenone	232	[86]
Tetrachloromethane (CCl_4)	Phenalenone	34,000	[86]

Table 1. Solvent dependence of the lifetime of singlet oxygen.

4.2. Photodynamic therapy

One of the most important medicinal applications of $^1O_2$ is photodynamic therapy (PDT) (**Figure 11**) [7–9]. Photodynamic therapy is a promising and less invasive treatment for cancer [7–9] and photosterilization [10–14]. For cancer PDT, in general, porphyrins are used for photosensitizers, for example, porfimer sodium [87] and talaporfin sodium [88]. Photosterilization, antimicrobial PDT, is also carried out using dyes, for example, methylene blue (MB) [11, 14, 89]. The important mechanism of PDT processes including photosterilization is oxidation of biomolecules of cancer cell or bacteria through $^1O_2$ production under visible light irradiation. Visible light, especially longer wavelength visible light (wavelength > 650 nm), is less harmful for the human body and can penetrate into the tissue deeply. As mentioned above, $^1O_2$ can be generated by longer wavelength visible light. Administered photosensitizers, porphyrins, or other dyes produce $^1O_2$ through energy transfer to oxygen molecules with relatively large quantum yield (Φ_Δ).

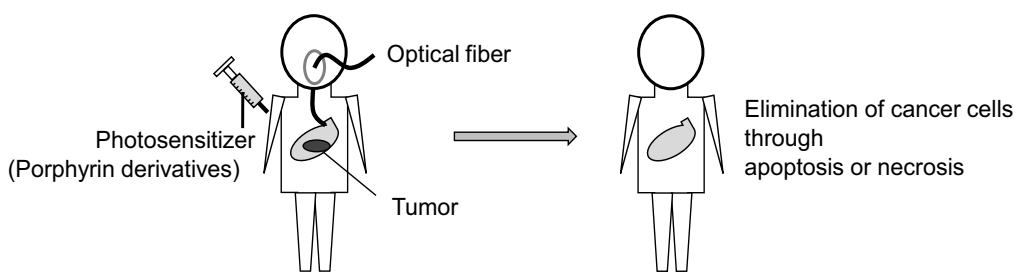

Figure 11. Scheme of the general procedure of PDT.

4.3. Photocatalytic singlet oxygen generation

As mentioned above, TiO_2 photocatalyzes the generation of various ROS. Singlet oxygen can be also produced through the photocatalysis of TiO_2 [31, 90–96]. In general, photogenerated electron in the conduction band reduces the surface-adsorbed oxygen molecules to $O_2^{\bullet-}$. Through the reoxidation of $O_2^{\bullet-}$, $^1O_2$ is formed. The possible reactions of photocatalytic $^1O_2$ productions are as follows:

$$O_2^{\bullet-} + h^+ \rightarrow {}^1O_2 \tag{11}$$

and

$$O_2^{\bullet-} + {}^{\bullet}OH \rightarrow {}^1O_2 + OH^- \tag{12}$$

The photogenerated h^+ in the valence band and $^{\bullet}OH$ can act as the oxidants to produce $^1O_2$. In addition, hydroperoxyl radical ($^{\bullet}OOH$) generated from $O_2^{\bullet-}$ and H^+ also produces $^1O_2$ as follows:

$$O_2^{\bullet-} + {}^{\bullet}OOH + H^+ \rightarrow {}^1O_2 + H_2O_2 \tag{13}$$

The reported values of Φ_Δ are depending on the experimental condition, for example, around 0.2 (0.2, Degussa P25 in water [92], and 0.22, rutile particle in chloroform [95]). Other cases reported relatively small values, for example, 0.003 [96] and 0.02 [94]. In the cases of airborne $^1O_2$, quite small value (10^{-8}–10^{-9}) was reported [93]. It has been reported that the τ_Δ value of $^1O_2$ produced by Degussa P25 aqueous suspension is 5 μs [92]. Other photocatalytic materials, for example, zinc oxide (ZnO) can photocatalyze $^1O_2$ production through the similar reaction of TiO_2 photocatalysis [97]. Recently, carbon quantum dots, which have been paid attention as interesting nano-materials, also photocatalyze $^1O_2$ production [33].

Singlet oxygen is an important ROS for PDT. Other than $^1O_2$, H_2O_2 production can be also applied for PDT mechanism. Photocatalytic materials can produce these ROS under photoirradiation. Therefore, application of photocatalysts, specifically TiO_2 nanoparticles, for PDT has been also studied [29, 98–101]. To realize the TiO_2-utilized PDT, direct administration of small TiO_2 powders into tumor assisted with an optical fiber was proposed [29]. In addition, it was reported that oral-administrated TiO_2 nanoparticles are transported into the tumor of nude mouse skin transplanted from a human prostate cancer cell line [98]. As mentioned above, in general, TiO_2 nanoparticles can be excited by UVA irradiation. To utilize visible light for TiO_2 excitation, upconversion technique was also studied [100].

4.4. DNA oxidation by $^1O_2$ and mutation

Singlet oxygen can oxidize only guanines without sequence specificity; however, it does not have the ability to induce the oxidation of other nucleobases or to cleave the sugar-phosphate backbone [44]. The main oxidized product of guanine by $^1O_2$ is 8-oxo-G (**Figure 8**) [63–65]. Guanines undergo the Diels-Alder reaction by photoproduced $^1O_2$, leading to the formation

of [4 + 2] cycloaddition product with the imidazole ring to produce an endoperoxide. Through the subsequent proton transfer, this peroxide is converted to 8-hydroperoxyguanine [102, 103], which becomes 8-hydroxyguanine [63]. The keto-enol tautomerism produces 8-oxo-G from 8-hydroxyguanine. Because single-stranded DNA is easily oxidized by ROS, 8-oxo-G formation by $^1O_2$ is increased by DNA denaturation [44]. The 8-oxo-G formation causes DNA misreplication (**Figure 12**), which can lead to mutations such as G-C:T-A transversion caused by the stable base-pair formation between 8-oxo-G and adenine [104, 105]. Since 8-oxo-G is more easily oxidized than guanine, 8-oxo-G undergoes further reaction, leading to the formation of imidazolone and oxazolone (**Figure 13**) [63, 106, 107]. Imidazolone forms more stable base pair with guanine than cytosine [106, 107]. Therefore, guanine oxidation by $^1O_2$ may cause G-C:C-G transversion [108, 109] through imidazolone formation, a further oxidized product of 8-oxo-G. Indeed, it has been reported that UVA can induce these mutations [110].

4.5. Protein oxidation by $^1O_2$

Protein oxidation is also induced by $^1O_2$. The following amino acids, tryptophan, tyrosine, cysteine, histidine, and methionine, can be oxidized by $^1O_2$ [111]. In the case of tryptophan oxidation by $^1O_2$, *N*-formylkynurenine (**Figure 14**) is a major oxidized product [112, 113]. The reported reaction rate coefficient between tryptophan and $^1O_2$ is 3.0×10^7 s^{-1} M^{-1} [114]. Oxidation of tryptophan residue in a certain protein can be examined with a fluorometer [115]. For example, human serum albumin (HSA) has one tryptophan residue, and the intrinsic fluorescence of tryptophan at around 350 nm can be diminished by the oxidative damage. Porphyrin phosphorus(V) complexes (**Figure 15**), of which the Φ_Δ is larger than 0.5, can induce oxidative damage to the tryptophan residue of HSA [116]. Photosensitized HSA damage is enhanced in D_2O, in which the lifetime of $^1O_2$ is markedly elongated compared in H_2O (**Table 1**). Furthermore, sodium azide (NaN_3), a strong physical quencher of $^1O_2$ [117], effectively suppresses this HSA damage. From the analysis of the effect of NaN_3 on the HSA damage, the contribution of $^1O_2$-mediated oxidation to the total quantum yield of protein damage

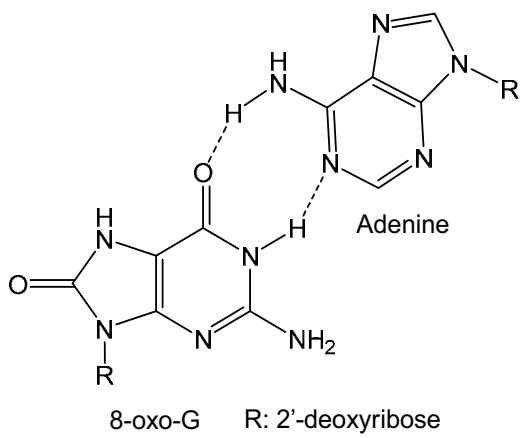

Figure 12. Hydrogen bonding between 8-oxo-G and adenine.

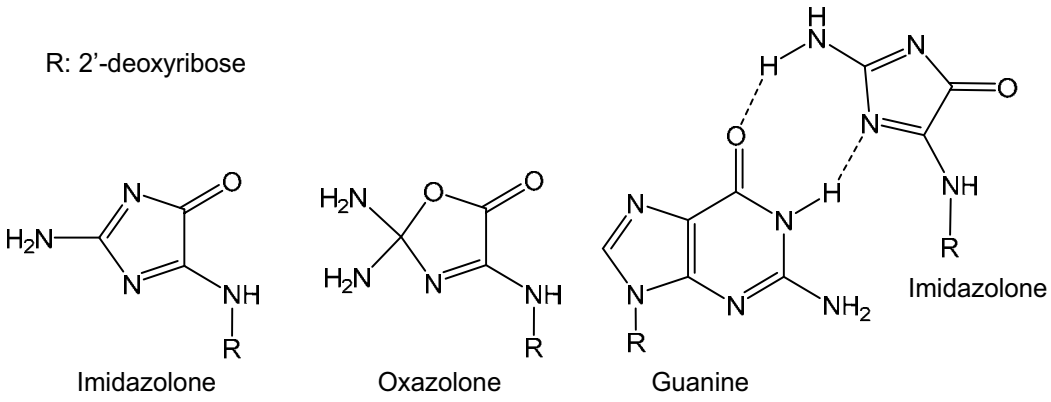

Figure 13. Structures of imidazolone and oxazolone and the hydrogen bonding between guanine and imidazolone.

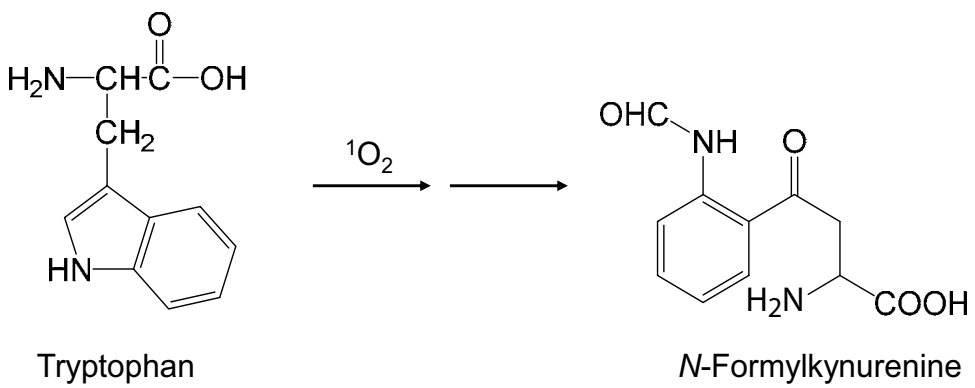

Figure 14. Structures of tryptophan and *N*-formylkynurenine, an oxidized product of tryptophan by $^1O_2$.

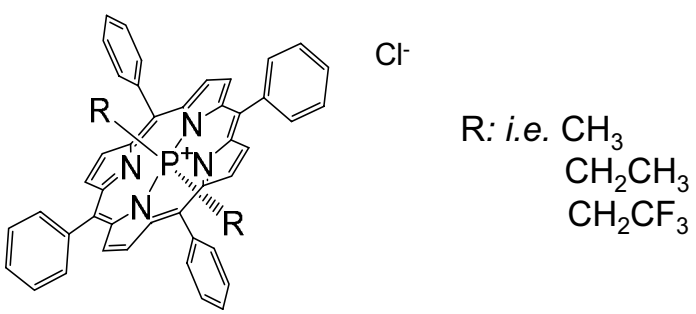

Figure 15. Example of P(V)porphyrin photosensitizer.

can be determined [115]. Photosensitized $^1O_2$ production by porphyrin phosphorus(V) complexes induces the damage of tyrosinase, which is an enzyme to catalyze the hydroxylation of tyrosine, resulting in the deactivation of tyrosinase [118]. Oxidation of the amino acid residue by $^1O_2$ can cause the deactivation of protein function. The protein oxidation photosensitized by porphyrins through ROS production is an important mechanism of PDT.

Photocatalyzed $^1O_2$ production by TiO_2 may not play an important role in the oxidation reaction [31, 94]. Formed $^1O_2$ on the TiO_2 surface is quenched by TiO_2 itself with relatively large quenching rate coefficient (e.g., $2.4 \times 10^9\ M^{-1}\ s^{-1}$ [95]). In the presence of bovine serum albumin, $^1O_2$ produced by TiO_2 photocatalysis is effectively quenched, suggesting the protein oxidation [94]. However, in the case of TiO_2 photocatalyst, other ROS are more important for protein oxidation than $^1O_2$-mediated reaction [29–32].

5. Detection of ROS

ROS detection is an important theme to investigate a biological effect of ROS or evaluation of the activity of PDT photosensitizers [119–122]. Fluorometry is one of the most important and effective methods of ROS detection. For example, 5-carboxyfluorescein-based probe has been developed (**Figure 16**) [123]. This probe can detect H_2O_2 in the living cell. As an inexpensive method, the fluorometry using folic acid (**Figure 17**) was reported [23, 119, 124]. Folic acid can be decomposed by H_2O_2 in the presence of Cu^{2+}, resulting in the fluorescence enhancement. The limit of detection (LOD, at signal/noise = 3) for this method was 0.5 μM H_2O_2. This method is based on the oxidative decomposition of folic acid by Cu(I)-OOH. In the presence of Fe^{2+}, $^{\bullet}OH$ slightly induces the folic acid decomposition; however, the effect of $^{\bullet}OH$ on this folic acid decomposition is negligibly small because of the very short lifetime [125, 126]. In addition, $O_2^{\bullet-}$ does not

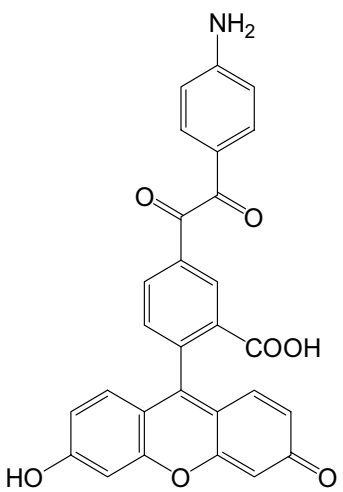

Figure 16. Structure of 5-carboxyfluorescein-based fluorescence probe for H_2O_2 [123].

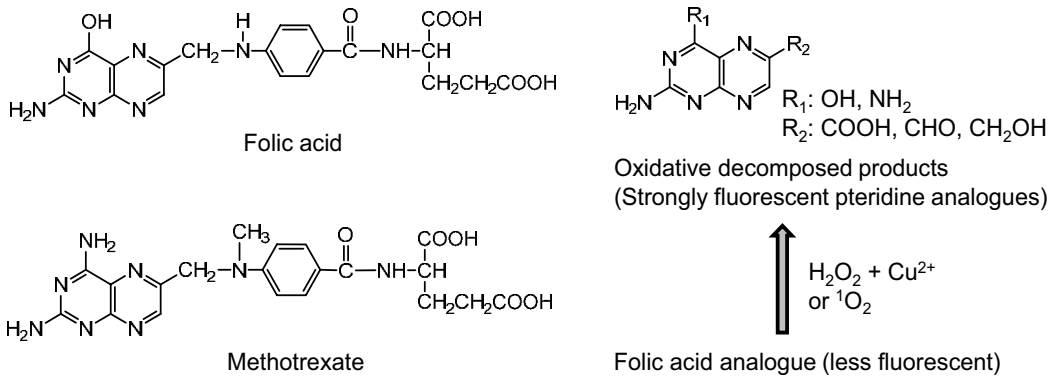

Figure 17. Structures of folic acid and methotrexate and the fluorometry of ROS [119, 124].

have the activity of folic acid decomposition. Using folic acid or its analogue, $^1O_2$ can be also detected [124]. Specifically, in D_2O, folic acid or methotrexate (**Figure 17**), an analogue of folic acid, is effectively decomposed by $^1O_2$, resulting in the fluorescence enhancement [124]. Using this method, the values of Φ_Δ of various water-soluble photosensitizers can be determined.

6. Control of singlet oxygen production

Control of photosensitized $^1O_2$ is an important theme for biology or medicine, for example, to realize target-selective PDT [127] or "theranostics" (therapy and diagnosis) [128]. The pH-dependent control [129] and target-selective control [127, 128, 130–132] methods have been reported. It has been reported that free base porphyrins were synthesized to control their photosensitized $^1O_2$ generating activity by pH (**Figure 18**) [129]. The S_1 state of this porphyrin

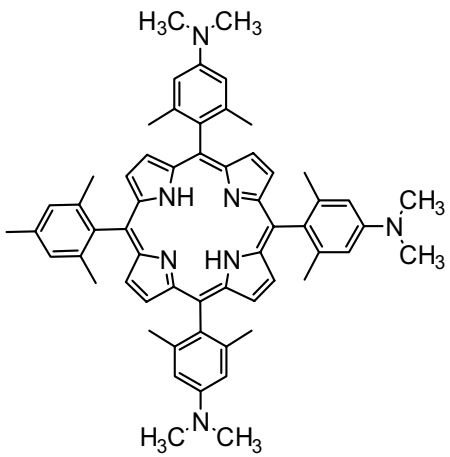

Figure 18. Example of the reported pH-responsive porphyrin [129].

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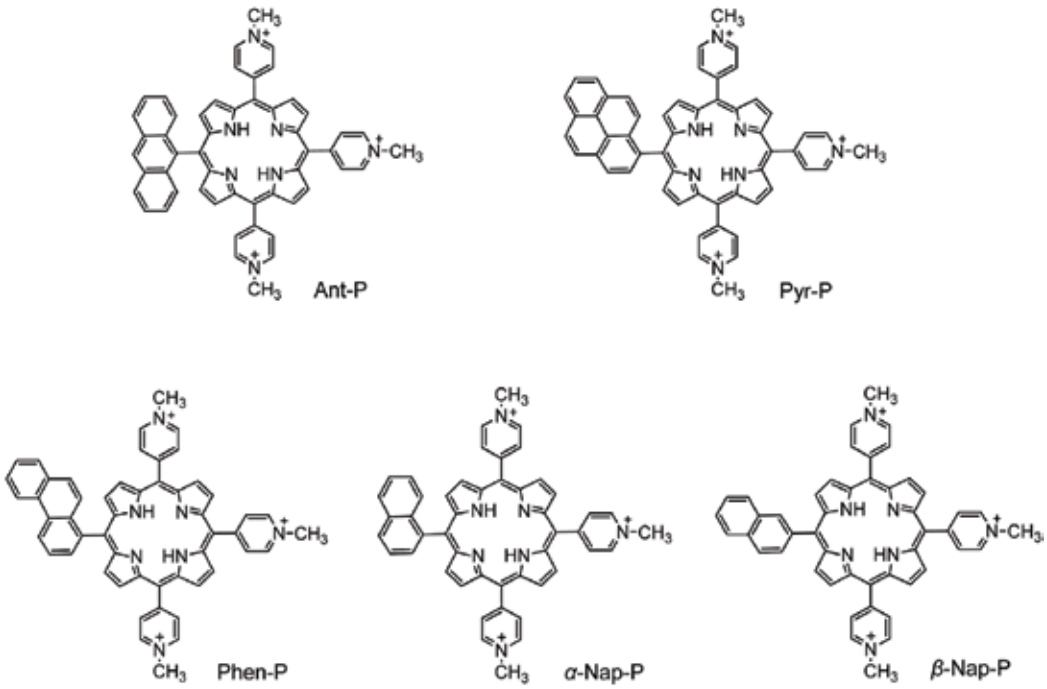

Figure 19. The examples of DNA-targeting porphyrins: Ant-P [81], Pyr-P [130], Phen-P [131], and Nap-Ps [132].

is quenched by the electron-donating moiety in neutral or alkali solution. However, protonation of this electron-donating moiety under acidic condition suppresses the electron transfer, leading to the recovery of the $^1O_2$ production activity of porphyrin ring. Because cancer cell is slightly a more acidic condition compared with normal cells [133–135], this pH-based control of photosensitized $^1O_2$ production can be applied to cancer-selective PDT. DNA-targeting control of photosensitized $^1O_2$ generation has been also reported [127, 128]. For example, electron donor-connecting porphyrins have been studied (**Figure 19**) [81, 130–132]. These compounds can be photoexcited by visible light irradiation, and their S_1 states are effectively quenched through intramolecular electron transfer. The charge-transfer state energy can be raised through the binding interaction with DNA, an anionic polymer, resulting in the inhibition of the intramolecular electron transfer and enhancement of $^1O_2$ generation.

7. Conclusions

Hydrogen peroxide is easily produced from the oxidation processes of chemical compounds by oxygen molecules. In addition, UVA-irradiated NADH and semiconductor photocatalytic materials can also produce H_2O_2. Formed H_2O_2 in cells can be incorporated into cell nucleus and activated by endogenous metal ions. Copper ion induces Cu(I)-OOH formation from H_2O_2,

whereas $^{\bullet}OH$ is produced from H_2O_2 and iron ion. These ROS cause base oxidation, and $^{\bullet}OH$ can induce strand break of DNA. Base modifications lead to carcinogenesis or lethal effect. Photoirradiation to various sensitizing materials induces $^1O_2$ production. Visible light has sufficient energy to produce $^1O_2$. Therefore, $^1O_2$ is easily produced by various dyes under photoirradiation. Photocatalytic $^1O_2$ formation through reoxidation of $O_2^{\bullet-}$ is also possible. Formed $^1O_2$ can oxidize guanine residues of DNA without sequence specificity and several amino acid residues of protein within its lifetime, which depends on the surroundings. Various detection methods of these ROS have been developed. In addition, the target-selective or condition-selective productions of ROS become important strategies for PDT and cancer "theranostics."

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Author details

Kazutaka Hirakawa[1,2]*

*Address all correspondence to: hirakawa.kazutaka@shizuoka.ac.jp

1 Applied Chemistry and Biochemical Engineering Course, Department of Engineering, Graduate School of Integrated Science and Technology, Shizuoka University, Hamamatsu, Shizuoka, Japan

2 Department of Optoelectronics and Nanostructure Science, Graduate School of Science and Technology, Shizuoka University, Hamamatsu, Shizuoka, Japan

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Chapter 10

Soil Remediation Assessment by Detection of Reactive Oxygen Species in Lizard Testis: An Electron Spin Resonance (ESR) Approach

Giulia Guerriero, Gerardino D'Errico, Anna De Maio,
Anna Rita Bianchi,
Oladokun Sulaiman Olanrewaju and
Gaetano Ciarcia

Additional information is available at the end of the chapter

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Abstract

Recent developments in applied research have led to implement novel operative protocols for life-based restoration of contaminated soils, including new monitoring approach. Here, we report the measurements of reactive oxygen species (ROS) content in lizard testis performed in the framework of the project Life Ecoremed. The ROS levels detected by electron spın resonance (ESR) spectroscopy using the spin-trapping technique were analyzed and validated using measurements of total soluble antioxidant capacity and the poly(ADP-ribose) polymerase enzymatic activity, which detect the gonadal antioxidant defense and DNA repair, respectively. The investigations on soil biosentinel *Podarcis sicula* reproductive health gave significant evidence that the ROS level in the testis well correlates with alteration of the antioxidant capacity. In specimens coming from polluted sites, before remediation, a significant increase of ROS content was detected respect to that found in specimens from an unpolluted site. Thereafter, an evident decrease of the ROS levels, corresponding to high levels of total soluble antioxidant capacity and low repair of DNA integrity, has been detected after remediation. Thus, the data relative to all the polluted sites examined support the testis of *Podarcis sicula* as an elective tissue for an innovative and reliable screening method, based on ESR analysis of ROS, in the soil remediation assessment.

Keywords: *Podarcis sicula*, testis, reactive oxygen species, ROS, total soluble antioxidant capacity, poly(ADP-ribosyl)ation, DNA repair, electron spin resonance, ESR, 11 Env/IT/275 Ecoremed, soil remediation assessment

1. Introduction

Soil contamination represents an important source of environmental oxidative stress to terrestrial life and is able to induce the generation of free radicals, including reactive oxygen species, ROS, in living cells (for a review see [1]. Knowledge of the ROS content is therefore vital in order to evaluate environmental risks and, in particular, to study the pollution effects on living vertebrate organisms and their germinal and somatic cells. Some of the works present in the literature in which a quantitative determination of ROS amounts are related to the vertebrate exposure to pollutants are listed in **Table 1**.

ROS inflict, when overexpressed, oxidative damage upon lipids, proteins, DNA and other components of the cell [10]. We began to work on this topic at the laboratory of Comparative Endocrinology lab (EC*lab*) of University Federico II of Naples (Italy) by studying the reproductive health of various animals in presence of metal pollution, in the framework of Life Ecoremed (2011–2017 program). The aim of this research program, which brought together researchers belonging to six faculties (Agriculture, Architecture, Economics, Engineering, Literature and Sciences), was to contribute to the development of a method to implement eco-compatible protocols for agricultural soil remediation in Litorale Domizio Agro Aversano (Campania, Italy); in particular, different biomonitoring systems, including animals, were optimized to evaluate the requalification actions.

Free radicals present in living cells include hydroxyl ($OH^{\bullet}$), superoxide ($O_2^{-\bullet}$), nitric oxide ($NO^{\bullet}$), thyl ($RS^{\bullet}$), and peroxyl ($RO_2^{\bullet}$) species. The term ROS identifies the radicals in which oxygen is the main reactive atom, and is often used also for nonradical species, e.g., hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and ozone (O_3), which can easily lead to free radical production in the organism. Because of the short lifetime and typical low concentration, direct detection of ROS is extremely difficult. As a consequence, indirect methods are usually adopted. They are based on the reaction of ROS with a probe molecule so to obtain a more stable, long-lived chemical specie [11]. Most of the indirect methods for ROS analysis rely on ultraviolet/visible (UV/Vis) or fluorescence spectroscopy. Electron Spin Resonance (ESR) spectroscopy, by using

Vertebrate organism	Tissue ROS effect	Reference
Fish	Damage to cellular constituents	[2]
	Oxidative damage	[3]
Lizard	Oxidative damage	[4]
Frog	Hepatocytes and melanomacrophages alteration	[5]
	Oxidative damage	[6]
Frog tadpole	Alteration of mitochondrial efficiency and reduced growth	[7]
Rat	Reduction of spermatozoa motility	[8]
Boar	Reduction of spermatozoa motility	[9]

Table 1. ROS amounts in vertebrate living cells and their effect on the tissues.

appropriate nitrones as spin traps, is a valid alternative, allowing the quantitative detection of products deriving from ROS oxidative attack to lipids [12–14]. The choice of ROS was motivated since they are of crucial importance for living organisms (see **Table 1**). They are involved in metabolic cell processes but their overexpression might damage seriously cell functionality [1].

ROS effects were evaluated on testis of lizard, an organism phylogenetic close to mammalian. Details on the ROS protocol detection can be found in Ref. [4]. Adult male lizards *Podarcis sicula* were collected, during spermatogonial recrudescence, in different sites of the "Land of Fires", a contaminated area of the Litorale Domizio Agro Aversano (**C**) in accordance with the Italian Legislative Decree No. 152/2006 concerning the Environment's Code [15]. The polluted sites had been a temporary storage of industrial toxic and solid urban waste dumping. Lizards were collected before remediation and after remediation. Chemical analysis of soil samples allowed the baseline pollution levels to be determined prior to the intervention [15, 16]. As a control (**N**), lizards were also collected in a unpolluted site. This study, performed in the framework of subaction C2c "Biomonitoring of oxidative damage and characterization of reproductive health status of selected vertebrate and invertebrate species" has been conducted through 2013–2016. A selective strategy was carried out to identify and follow the biosentinel at risk in contaminated soil. During the first phase, the definition of reliable and reproducible protocols for species identification of different invertebrate and vertebrate biosentinels was reported. During the second phase, the reproductive health of biosentinels before (2013) and after (2015) re-qualification actions was analyzed by morphological, biochemical and molecular approach. As detailed in previous literature work [4] we monitored all metal polluted sites by measuring ROS, total antioxidant capacity and DNA repair in lizard testis before and after remediation.

2. Results and main findings

2.1. Difficulties in ROS quantitative measurements: choice of the optimal technical approach

ROS are difficult to handle due to the short lifetimes and typically low concentrations in terrestrial systems. Their direct observation is only possible on the sub-millisecond timescale, with the relatively stable H_2O_2 being an exception. Indirect methods involve the reaction of ROS with a probe molecule to yield a more stable, long-lived analyte [11]. Such methods typically involve specific chemical derivatization (the spin trapping procedure) or are based on competitive kinetics. Even with these approaches, products of oxidation, which attack biomolecules rather than ROS themselves, are often monitored. Much of the method development for aqueous ROS analysis has focused on ultraviolet/visible (UV/Vis) light spectroscopic techniques and the use of relatively common and hence lower cost probe molecules. Fluorescence and chemiluminescence spectroscopies have also been applied [17]. These strategies are also compatible with methods such as steady-state kinetic analyses, stopped flow methods, time-resolved laser spectroscopy, flash photolysis and pulse radiolysis. Other analytical techniques for ROS detection, such as electron spin resonance (ESR), nuclear magnetic resonance (NMR), derivatization with attendant mass spectrometric (MS) analysis and liquid scintillation counting can also be

quite useful [12]. We used the ESR technique for the assays [18]. This techniques, using appropriate nitrones as spin traps, allows the quantitative detection of products deriving from ROS oxidative attack to lipids [13, 14]. Analysis of the results clarifies regulatory mechanisms that appear as a response to xenobiotic attack, hormone treatment, metals or pesticide exposure, constituting a powerful tool for environmental call [1]. The spin trapping and adduct extraction procedure reported in Ref. [4] are an optimization of a method previously reported in the literature [18]. The analyses were performed on 40 mg of testicular tissue of lizards from the polluted sites (C) pre and post remediation, and of unpolluted site (N). The samples were weighed on an analytical balance and homogenized in physiological saline. The amount of added saline, which was adjusted for each sample, was in all cases lower than 1 mL per gram of tissue. An aqueous solution of the spin-trap N-tert-butyl-α-phenylnitrone, PBN, was prepared (140 mmol/L) and kept in a darkened room. Proper volumes of this solution were added to the homogenized samples at a 1:5 vol/vol ratio. We strongly recommend strict control of the temperature at 4°C and the darkness when working on ROS, in order to limit the risk of side reactions. The samples were allowed to equilibrate for 10 min and then centrifuged for 10 min at 3500 rpm. The supernatant was separated and mixed with HPLC-grade toluene (Sigma) at a 1:1 vol/vol ratio. A small aliquot (10 μL) of the organic phase, which contains the PBN adduct, was transferred in a quartz capillary, which was rapidly vacuum degassed and flame-sealed. The same extraction procedure was used to perform blank experiments. Samples were analyzed by ESR for radical content within 24 h from preparation. Capillaries were inserted in a 3 mm i.d. quartz tube. Measurements were performed at room temperature on a Elexsys E-500 X-band spectrometer (Bruker). Instrumental settings were: microwave frequency, 9.871 GHz; incident microwave power, 6.4 mW; modulation amplitude, 0.1000 mT; modulation frequency, 100 kHz; time constant, 10 ms; scan width, 6.000 mT; magnetic field center, 351.0 mT. The obtained first derivative signals were double integrated to quantitatively determine their intensity [19, 20]. The data were normalized by the weight of the tissue samples subjected to the radical extraction procedure and quantitatively analyzed in terms of relative variations.

2.2. Testis radical content in *Podarcis sicula* before and after remediation

The results of Life Ecoremed research, subaction C2c, show the reliable detection and quantification of radicals deriving from ROS attack to lipids ($LR^{\bullet}$). PBN forms very stable spin adducts with these species, poorly affected by light, heat and oxygen. All the samples analyzed show the typical EPR spectrum of the PBN spin adduct. An example is reported in **Figure 1**.

Two hyperfine coupling constants can be determined from the spectra analysis: a_N (whose mean value was found to be 13.7 ± 0.2 G) and a_H (1.9 ± 0.1 G). The amount of radical species in the samples was estimated by double integration (DI) of the spectra; the obtained values were normalized per weight of the tissue used for the preparation of each sample. The DI values were further normalized by the average value determined for samples coming from the unpolluted site (N), considered as a reference. In this way, it was possible to straight forwardly compare DI values obtained for lizards from different sites. The data indicative of radical content in male gonads of *Podarcis sicula* specimens from unpolluted (N) and polluted sites (C), examined in both pre and post remediation conditions, are shown in **Figure 2**.

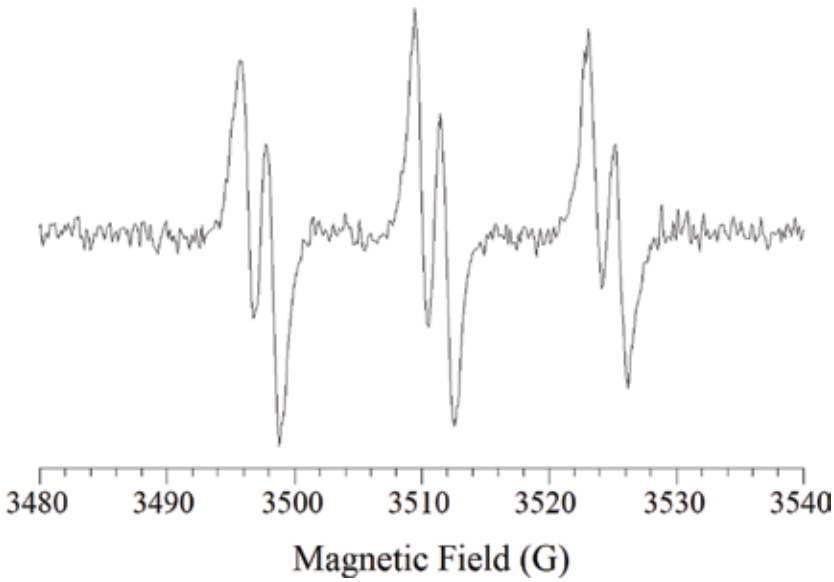

Figure 1. EPR spectrum of PBN adduct of radicals extracted from *Podarcis sicula* testis.

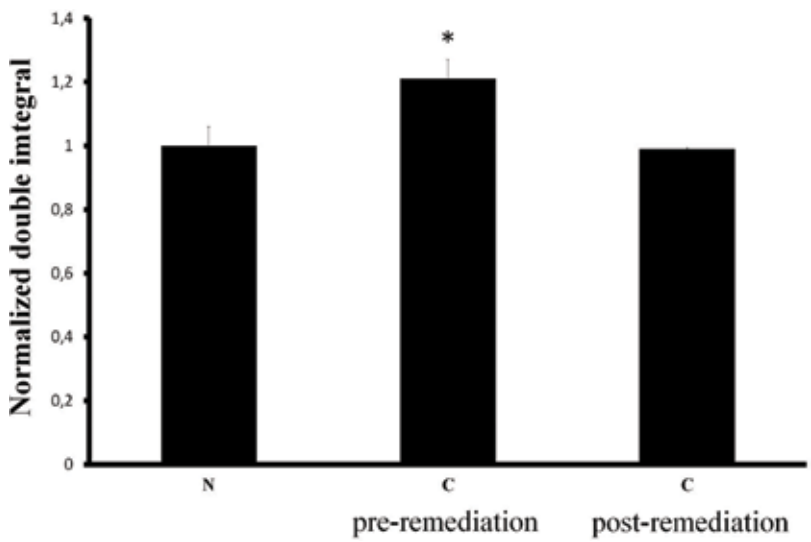

Figure 2. Radical content in *Podarcis sicula* testis collected from C, polluted sites of the Land of Fires, before and after remediation, and N, unpolluted site. The values are expressed as double integral of the ESR spectrum of PBN spin adduct, normalized by the sample weight and expressed relatively to the mean value in the unpolluted site. Bars represent the mean value ± standard deviation. *The significance differences of C pre remediation values compared with the N and C post remediation ($P < 0.001$).

In samples collected before the remediation the EPR measurements detect a concentration significantly higher than that observed after the remediation ($P < 0.001$), which is similar to that observed in the unpolluted site.

The sensitive alteration of ROS was confirmed by the results of total soluble antioxidant capacity and DNA repair [4].

2.3. Total soluble antioxidant capacity in lizard testis

All living organisms and their cells have developed antioxidative defense systems to protect themselves against ROS. At the cellular level, the oxidative stress is strictly associated to the changes in the prooxidant/antioxidant balance due to an overproduction of free radicals and/or to a reduction of the antioxidant defense system. These systems include enzymatic and nonenzymatic antioxidants that are usually effective in blocking the harmful effects of free radical [21]. Nonenzymatic antioxidant compounds [i.e. Vitamin C, Vitamin E, polyphenols], which react directly with oxidizing agents and disarm them, are named "scavengers". For example, vitamin C can directly scavenge $O_2^{-}\cdot$ and ·OH by forming the semidehydroascorbate free radical that is subsequently reduced by GSH, see for review [22]. The accurate assessment of oxidative stress in biological systems is a problem for all investigators working on the role of free radical damage in disease. Numerous biotechnological applications for potential recovery have been described to measure various free radical damage product or antioxidant status in soil and marine polluted areas as genes encoding stress related proteins and/or ultrastructural alterations [1, 23–29].

In the present research we used the method described in [30], and modified in [4], to determine total soluble antioxidant capacity in *Podarcis sicula* testis in recrudescence phase collected from the polluted sites (C) pre and post remediation and from the unpolluted site (N). The assay provides for the formation of phosphate/Mo(V) complex at acidic pH, after reduction of Mo(VI) to Mo(V). All samples (0.5 g), reduced to fine powder, were extracted with water (1 ml/g) for 1 h at room temperature in the dark. The extracts were subsequently centrifuged at 10,000× g for 20 min. This procedure was repeated twice and the two supernatants were combined and kept at 4°C. For the spectrophotometric determinations, aliquots of samples (0.1 ml) were mixed with the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95°C for 90 min, after which the absorbance at 695 nm was measured. A blank solution containing 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample was analyzed under the same conditions of the samples. Total soluble antioxidant capacity was expressed as equivalents (mmoles g-1 fresh weight) of ascorbic acid. For statistical treatment of the data, the Mann-Whitney U test was used.

The results show that, in *Podarcis sicula* testis collected from polluted sites (C) before remediation, total soluble antioxidant capacity is significantly lower than that measured in the sample from unpolluted site (N). Total antioxidant capacity detected in samples collected from polluted site after remediation, instead, is comparable to that of unpolluted site (N) (**Figure 3**).

Thereafter in *Podarcis sicula* testis, collected from polluted sites, the decrease of total soluble antioxidant capacity results correlate to the oxidative stress insurgence, responsible in turn for oxidative DNA damages and PARP activation.

2.4. Repair of DNA damage in lizard testis

The cells are continually exposed to different signals of extrinsic and intrinsic stresses, (i.e. genotoxic, oncogenic, inflammatory, metabolic stresses) [31]; their propagation involves the

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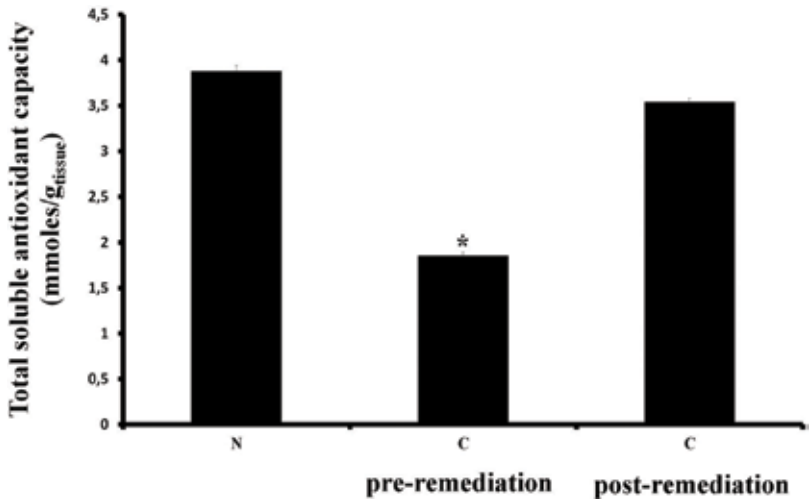

Figure 3. Total soluble antioxidant capacity in the testis of the lizard, *Podarcis sicula*; *N*: unpolluted site, **C**: polluted sites. *The significance differences of C pre remediation values compared with the N and C post remediation ($P < 0.05$).

cross-talk among multiple signaling pathways that lead to defined outcomes. Cell responses to stress determine a series of regulatory processes occurring at genomic, transcriptional, post-transcriptional, translational and post-translational levels. These need a complex network of sensors and effectors from multiple signaling pathways that includes poly(ADP-ribosyl)ation reactions [32]. Poly(ADP-ribosyl)ation is one of the post-translational protein modifications able to influence specific nuclear proteins. Modification of protein with ADP-ribose polymers (pADPr) is a reversible process where pADPr synthesis from NAD+ is catalyzed by pADPr polymerases (PARPs). Following DNA damage, PARPs modify histones, non-histonic and enzymatic proteins by means of long and branched ADP-ribose polymers. These polyanions reversibly alter the functions of accepting proteins, inducing conformational changes, and a net negative charge that, in the case of histones, destabilizes DNA interaction. Poly(ADP-ribosyl)ation is involved in the regulation of numerous cellular functions related to the maintenance of the genomic integrity and the expression and propagation of gene information [33]. It is also implicated in response to abiotic and biotic stresses [34, 35], in stress tolerance [36, 37] and in developmental processes [33, 38]. On the basis of these knowledge, we decided to assay PARP activity in nuclei of *Podarcis sicula* testis in the recrudescence phase collected from polluted sites before remediation, to indirectly verify whether and how the exposure to pollution induce DNA damage and, whether, the remediation was a positive effect on the restore of genomic integrity [4]. The isolation of nuclei was performed at 0–4°C. Briefly, all operations were carried out on ice or at 4°C starting from 500 mg of testicular tissues of lizards from polluted sites (C), before and after remediation, and unpolluted site (N). Tissues were harvested, cut and resuspended in 10 mM TrisHCl (pH 7.0), 1 mM EDTA, 1 mM EGTA, 1 mM $PhMeSO_2F$, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, and 0.5% Triton X-100 (1/4, w/v) (buffer A). The samples were homogenized for 30–40 s at low speed by an Ultra Turrax T8 (IKA-WERKE) and the homogenates filtered through three layers of cheesecloth. The filtrate centrifuged at 1500 × g for 30 min at 4°C and the pellet (containing nuclei) suspended in buffer A was centrifuged as above for three times. The pellets (nuclear fractions) washed with

buffer A without Triton X-100 (buffer B) were suspended in a small volume of buffer B containing 2% glycerol. The enzymatic activity was performed as described essentially in [4, 39] for testis nuclear fractions of lizards from polluted sites (C), before and after remediation, and unpolluted site (N). The reaction mixture (final volume 50 μl) contained 0.5 M Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 10 mM DTT, 0.4 mM [^{32}P]NAD+ (10,000 cpm/nmole) and a defined amount (20 μg proteins) of whole nuclear fraction from examined lizard testis. After incubation for 20 min at 25°C, the transfer onto ice and addition of 20% (w/v) trichloroacetic acid (final concentration) determine the stopping reaction. The mixture filtered through Millipore filters (HAWPP0001, 0.45 μm) was washed with 7% trichloroacetic acid. The activity was detected as acid-insoluble radioactivity by liquid scintillation in a Beckman counter (model LS 1701). One PARP unit is defined as the amount of enzyme required to convert 1 nmol of NAD+/min under standard conditions. The highest PARP activity is measured in nuclei of *Podarcis sicula* testis collected from polluted sites (C) before remediation. In these, indeed, the poly(ADP-ribosyl)ation levels are more than 100 times higher than those measured in nuclei of samples collected from both the polluted sites after remediation and control site, in which a basal PARP activity is detected. The Spearman test confirmed an indirect correlation ($\rho = -1$) between the PARP activity and the antioxidant capacity for all the examined samples. In fact, in samples before remediation, where the antioxidant capacity is lower than control, PARP activity is significantly higher and vice versa in unpolluted site, N, and C post-remediation samples (**Figure 4**).

These interesting evidences suggest that in *Podarcis sicula* testis collected from polluted sites pre-remediation, the exposure to polluted compounds might have caused the significant decrease of total soluble antioxidant capacity caused by ROS increase and consequent insurgence of oxidative stress. In turn, this is responsible for oxidative DNA damage that have induced activation of PARP, biosensor of DNA damage, being involved in DNA repair. In

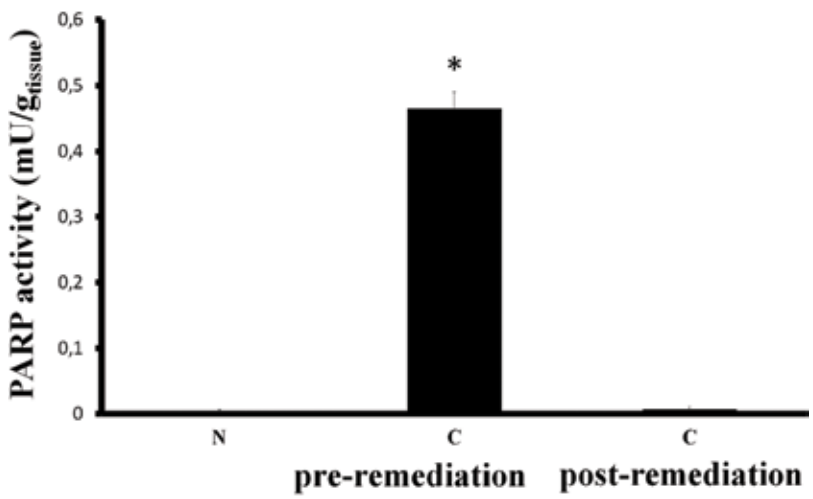

Figure 4. PARP activity in nuclei testis of the lizard, *Podarcis sicula*; **N**: unpolluted site, **C**: polluted sites. *The significance differences of C pre remediation values compared with the N and C post remediation ($P < 0.05$).

testis collected post remediation, DNA repair seems to be occurred, as the total antioxidant capacity and PARP activity levels return to values measured in sample of unpolluted site.

3. General discussion and conclusions

The purpose of the ROS assays performed on animal organism living in metal-contaminated sites is primarily the evaluation of the environmental risk, assessing its variation in case of remediation. The soil, as known, is a key link in the energy flow and nutrient cycling that characterize the ecosystem earth. According to scientific literature, soil biodiversity i.e. can provide useful insights in a number of issues: environmental impact studies and assessments, remediation programs, sustainable land planning and management, food safety and production sustainability, environmental monitoring and assessment programs, desertification or climate change prevention, adaptation and mitigation projects. Numerous studies indicate that soil contamination impacts on the conservation and sustainable exploitation of resources as well as on human reproductive health [40, 41]. The production, in a large scale, of a variety of chemical compounds is causing the deterioration of environmental quality and human health [42]. The accumulation of municipal waste, the use of fertilizers, motor traffic, incinerators, the thermo-electric power plants and many industrial processes have led to a progressive accumulation of xenobiotics [28]. This affects on the regulation of the natural cycle of water, air, organic and mineral substances, finally resulting in a biodiversity impairment. The mechanical and chemico-physical decontamination processes are expensive and tend to remove the biological activity of soil, modifying its intrinsic characteristics. For this reason, researchers have developed many alternative technologies based on biological processes [43].

A reliable monitoring of contaminated soil or marine remediation requires the species identification [39, 44] and their suitable indicators (https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=285170, [22]). Ecoremed, the project of remediation of the Land of Fires, adopted an ecofriendly approach in which many bioindicators with the inclusion of the health evaluation of vertebrate biosentinels have been applied. In our subaction research, we obtained realistic results in monitoring metal-contaminated ecosystems, analyzing the relation between the reproductive health of selected biosentinels and the soil contamination. We first confirmed the possibility to define a protocol to measure the reactive oxygen species (ROS), the total antioxidant capacity and DNA repair in the testis of the *Podarcis sicula*, useful biosentinel [45], collected in contaminated (C) and in non-contaminated soil (N). Once polluted sites had been restored (a process which took 2 years) all parameters were re-evaluated and their variations examined [4]. Interestingly, results converged in demonstrating that lizard testis of the Land of Fires, before remediation, showed free radicals overexpression.

Based on these evidences, in the framework of the Life Ecoremed Project, the present chapter proposes an optimized monitoring strategy for soil decontamination based on ROS detection in vertebrate biosentinels. The presented data are not restricted to specific sites, since we explored various sites localized in the Land of Fires in which the specified biosentinel was found to flourish. We supplemented our review of published studies with a complete data collection about ROS, total soluble antioxidant capacity and poly(ADP-ribosyl)ation on

lizard testis, providing information about protocols used for their determination and brief comments of results obtained. Specific purpose of our studies was to evaluate the risks in recrudescence phase for lizard reproduction health related to the presence of the ubiquitous contaminants in soil compartment. We surveyed each sites for many markers and we realized how important the ROS detection for remediation assessment was and can be.

Other procedures for ROS testing could significantly affect the results, potentially leading to oxidative damage artifacts. As an example, we showed that the lighting conditions maintained during the spin trap experiments favored a realistic quantitative detection. The average hyperfine coupling constants detected in our research, result compatible with trapping of both carbon- and oxygen-centered radicals [46, 47]. It is now well-assessed that spin trapping investigations are not able to discriminate among the different products of lipid oxidation ($ROO^{\bullet}$, $RO^{\bullet}$ and $R^{\bullet}$), since they generate spin adducts with similar coupling constants. Nevertheless, we are interested in their total concentration, whose altered values can be seen as a finger-print of oxidative stress conditions. The comparison between values obtained for lizard testis from different sites and their variations before and after remediation supports the reliability of our ROS content estimates.

The choice of testis was primarily related to the main goal of our study, which was the monitoring of the remediation effects on the reproductive health of biosentinels. The testis is perfectly suited for ESR investigation because it presents abundance of highly unsaturated fatty acids, high rates of cell division and variety of enzymes, thus resulting very vulnerable to ROS overexpression. Lizards from an unpolluted site, collected in the reproductive stage, show the lipid radical amount normally involved in the metabolic cell testis event/reproductive stage through the spermatogonial recrudescence. All metabolic process implies, in normal condition, high rates of mitochondrial oxygen consumption and reactive oxygen species (ROS) generation. As known, ROS overexpression can be harmful, depending on the nature and the concentration as well as the location and length of exposure [1, 10]. The level of lipid radical concentration, in samples from pre remediation soil, is significantly higher with respect to those detected in samples from the unpolluted site, showing a clear correlation with the oxidative stress from the environment. At metabolic concentrations, the ROS are involved in cell physiological processes [2–9] such as control of cell proliferation, playing an important role as messenger in signal transduction pathways; in gonads they may be beneficial or indispensable for gametogenesis processes. The amount of lipid radicals after remediation soil reduces, becoming more similar to that in unpolluted site demonstrating the efficiency of the remediation. High doses and/or inadequate removal of ROS caused by several mechanisms, i.e. bioactivation of xenobiotics, inflammation, increased cellular metabolism, activation of oxidases and oxygenases and loss of antioxidant capacity, cause severe metabolic malfunctions as alterations in gene expression and protein [22, 33, 48, 49]; consequently, we projected to monitor the total antioxidant capacity. As known, to address the risks very common in polluted areas, testis has developed a sophisticated array of antioxidant systems comprising both enzymes and free radical scavengers [23, 50] that can be both diagnostic and prognostic tools [1, 4]. As mentioned earlier, the use of these additional detections was useful to confirm the high levels of ROS detected when the total capacity decreases.

We realized that the increase of ROS and the consequent reduction of antioxidant capacity in *Podarcis sicula* nuclear testis is one of the causes responsible for DNA damage and not all cells in the body have the same degree of genotoxic damage. We also looked into the possibility of extending our research to take into account the DNA repair using PARP activity. Conversely,

the use of the DNA repair by PARP activity contributes to explain why the species where numerous also if their DNA testis were seriously damaged. As known, PARP represents the first molecular response to genomic material damage and is correlated to DNA repair [33, 39]. Regarding this marker, in samples analyzed after remediation, we observe the return of PARP activity and antioxidant capacity values to those measured in the respective controls, suggesting that DNA repair successfully has occurred in all lizard testis examined.

Currently, many bioassay endpoint metrics are species-specific. The approach reported here was applied to other species of lizard of the same genus *Podarcis* as *Podarcis sicula* and *Podarcis muralis* with similar gonadosomatic index (GSI = [testis weight/body weight] × 100), as *Podarcis muralis*, revealing correlation between examined biomarkers (data not shown). Although the hypothesis of effect is generally accepted, we showed these results are in agreement with that found during the investigation of soil remediation of the Life Ecoremed project. In this framework, we think that biomarkers reported here support the value of ROS detection by ESR. Furthermore, the current applied researches demonstrate that antioxidant capacity and DNA repair were sensitive in this species.

Data suggest that, in the testis of *Podarcis sicula*, ROS detection is highly sensitive and involved in the high oxidative insult of each site examined. The analyses done when gonads do not release sperms during spermatogonial recrudescence, let us to eliminate the oxidative stress related to this natural event. Additional studies are ongoing for the evaluation on spermatozoa, mature germinal cell, of lizard separated from testis by centrifugal elutriation. The preliminary investigations related to contaminated soil gave significant evidence of deviations from reactive oxygen species levels in spermatozoa lizard of unpolluted site too.

In conclusion, in the framework of a European remediation project (11 Env/IT/275 Ecoremed) we developed a bioassay to assess the ROS content in a vertebrate biosentinel. ESR measurements of ROS in the biosentinel *Podarcis sicula* testis form the basis of a rapid and reliable assay for decontamination bioassessment and represent a promising tool to be included in the new toxicological screening.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

All authors contributed to conception and design of the experiments. All the authors have given their approval to the final version of the manuscript.

Compliance with ethical standards

The studies were conducted in strict accordance with European (Directive 2010/63) and Italian (Decreto Legislativo n°116/1992) legislation on the care and use of animals for scientific purpose.

Author details

Giulia Guerriero[1,2*†], Gerardino D'Errico[3†], Anna De Maio[1], Anna Rita Bianchi[1], Oladokun Sulaiman Olanrewaju[1,4] and Gaetano Ciarcia[1,2]

*Address all correspondence to: giulia.guerriero@unina.it

1 Department of Biology, Federico II University, Naples, Italy

2 Interdepartmental Research Center for Environment (I.R.C.Env.), Federico II University, Naples, Italy

3 Department of Chemical Sciences, Federico II University, Naples, Italy

4 School of Ocean Engineering, University Malaysia Terengganu, Kuala Terengganu, Malaysia

† These authors contributed equally

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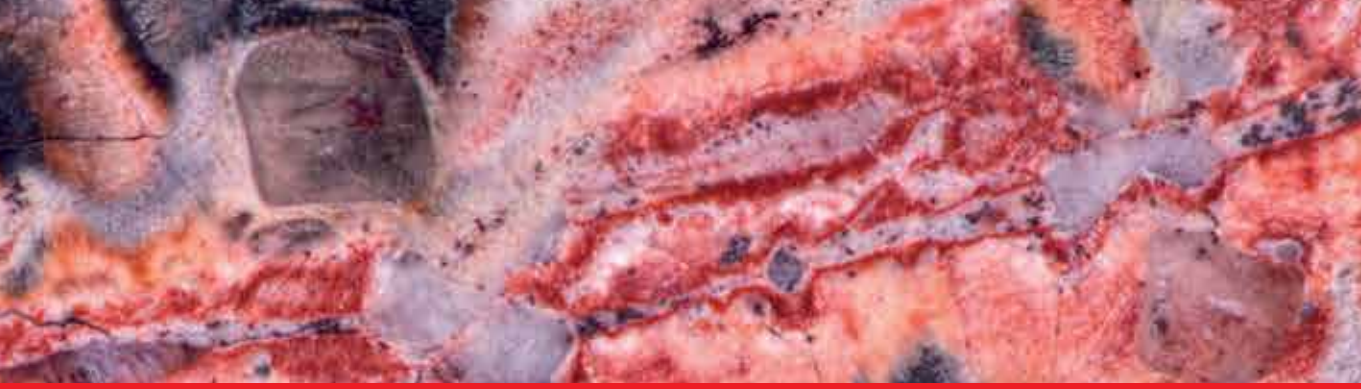

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Oxygen represents only 20% of the Earth's atmosphere, yet it is vital for the survival of aerobic organisms. There is a dark part of the use of oxygen that consists in generating reactive species that are potentially harmful to living organisms. Moreover, reactive oxygen species can combine with nitrogen derivatives and generate many other reactive species. Thus, living organisms are continuously assaulted by reactive species from external or internal sources. However, the real danger comes in the case of high concentrations and prolonged exposure to these species. This book presents an image of the mechanisms of action of reactive species and emphasizes their involvement in diseases. Inflammation and cancer are examined to determine when and how reactive species turn the evolution of a benign process to a malignant one. Some answers may come from recent studies indicating that reactive species are responsible for epigenetic changes.

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