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Edited by Pinar Atukeren

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Meet the editor

Dr. Pinar Atukeren was born in Istanbul on May 1975. She is a professor in Biochemistry at the Department of Medical Biochemistry, Cerrahpasa Medical Faculty, Istanbul University. She received her master's degree in 1999 and PhD degree in 2005 at the same department. She received the National Research Grant Award for successful students from the Scientific and Technical Research Council of Turkey. Her research interests are oxidative and nitrosative stress, free radicals and antioxidants, neurodegenerative diseases, brain tumors, and aging. She has published many papers in SCI and SCIE journals and both international and national books and book chapters.

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Preface

Oxidative stress plays a crucial role in the pathophysiology of various diseases when there is a disruption of the intracellular redox balance and the homeostatic balance between cellular oxidants and antioxidants. Reactive oxygen species (ROS), although essential for normal physiologic processes, are deleterious when produced in excess. Free radicals include not only ROS but also reactive nitrogen species (RNS) such as nitric oxide and peroxynitrite similarly leading to nitrosative stress. These molecules react with molecular targets including proteins, lipids, and nucleic acids contributing to mitochondrial injury and cellular dysfunction.

This book intends to provide the readers with an extensive overview of the novel approaches and prospects based on oxidative and nitrosative stress in the pathophysiology of various diseases and in the current treatment strategies with antioxidants, and it is the one to which the authors have made significant contributions in all chapters.

I believe that this book will provide a good grounding in pointing the way to new disciplines that will contribute to the evolution of strategies for creating, analyzing, and presenting the medical information in the future stimulating our colleagues at all multidisciplinary levels.

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Chapter 1

Oxidative Stress: Noxious but Also Vital

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Abstract

The imbalance between reactive oxygen species (ROS) production and antioxidant defenses determines the condition called oxidative stress. When there is an increase in ROS production or a decrease in the antioxidant defenses, this systemic antioxidant/pro-oxidant imbalance may lead to the accumulation of oxidative damage, which, in turn, may lead to a modification of biomolecules. These consist of reactions resulting in protein adducts, DNA oxidation, and formation of lipid peroxides, which, in turn, reduce the cellular functional capacity and increase the risk of disease development. The body has natural scavenging systems against free radicals and other reactive species. However, sometimes the endogenous antioxidant capacity is exceeded by the production of ROS. When this occurs, exogenous antioxidants exert important function for the human health. These bioactive compounds act preventing and neutralizing the formation of new reactive species and free radicals. In some cases, an increase of ROS can help the host to resolve an infection or even to control the tumor growth. Finally, the levels of ROS can be perceived by signal transduction pathways involving known targets (i.e., p53, Ras, and NF-κB) and regulate physiopathological events such as the cellular cycle, apoptosis, and inflammation.

Keywords: reactive species, cellular oxidation, antioxidant system, health, disease

1. Cellular respiration and generation of reactive species in the mitochondria: implications in cell viability and aging

Oxidative phosphorylation is the center of energy metabolism in plants, animals and several microbial life forms [1]. In eukaryotes, this process occurs in mitochondria. The mitochondria is a cytoplasmic organelle surrounded by two membranes, outer and inner membrane, which main function is the production of most of the phosphate compounds necessary for the energetic balance of the cell. In addition, other functions such as the regulation of the body's heat generation [2–4] programmed cell death [5–7], reactive oxygen species (ROS) generation and cell signaling [8] is also associated with mitochondria. Cellular vitality is directly related to mitochondria, and mitochondrial dysfunctions are frequent causes of accidental cell death [5, 9–11], cancer [12, 13], diabetes [14–16] and neurodegenerative diseases [17–19], among others.

The characterization of the respiratory electron chain could be performed in studies using the fractionation of its components by certain detergents that at low concentrations break the interactions between proteins and lipids in the membranes, leaving associations between proteins intact [20]. In electron transport chain, through this process, four protein complexes were found. They were named complex I (or NADH-Ubiquinone oxidoreductase), complex II (succinate dehydrogenase), complex III (Ubiquinol -cytochrome c oxidoreductase, or complex bc_1) e complex IV (cytochrome c oxidase). The complex V is also known as ATP synthase. Despite glycerophosphate dehydrogenase (glycerol-3-phosphate dehydrogenase) and ETF–ubiquinone oxidoreductase have not complex nomenclature, they are connect to the electron transport chain, as complex I and complex II, i.e., delivering electron to ubiquinone [21].

The redox carriers within the respiratory chain consists of flavoprotein containing tightly bound FAD or FMN as prosthetic groups, protein-bound couper, ironsulphur (nonhaem iron) proteins and cytochromes, with haem prosthetic groups. The ubiquinone also participated in electron transport chain as a free and diffusible cofactor [20]. While electron transport occurs through the mitochondrial complexes, complexes I, II, and III pump protons from mitochondrial matrix to the intermembrane space. The energy associated to this process is used to the production of ATP by ATP synthase (**Figure 1**) [22].

1.1. Reactive species in mitochondria

The ROS comprise a variety of molecules derived from molecular oxygen, including oxygen radicals and non-radical oxygen derivate. The major intracellular site of ROS formation in most tissues is mitochondria [23, 24]. Within mitochondria, the electron transport chain continuously generates water from O_2 through the electronic reduction at the cytochrome c oxidase level (**Figure 1**). These electrons reach cytochrome c oxidase by sequential transfer from the reduction of other components, and are initially removed from NADH and $FADH_2$. During this transfer, a small amount of electrons are lost at intermediate stages in the electron transport chain, mainly in the complex I and complex III [25–27] in mammals, leading to a monoelectronic reduction of O_2 [28].

This monoelectronic reduction of O_2 results in the formation of anion superoxide radical. While complex I releases superoxide only in the mitochondrial matrix, complex III releases

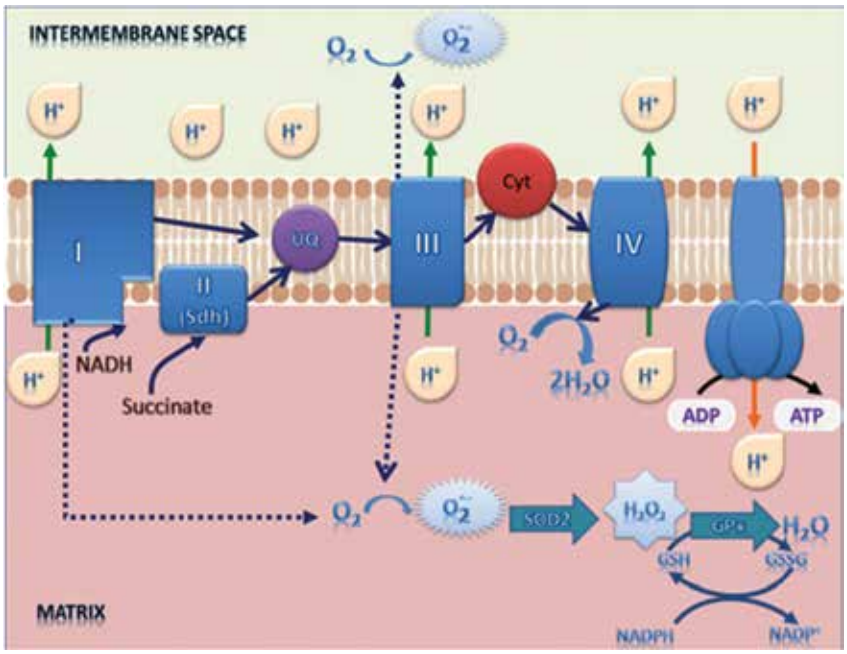

Figure 1. Electron transport chain, ROS, and antioxidant defense. The electron transport chain receives electrons from reduced compounds, as NADH in complex I (also called NADH coenzyme Q reductase) and succinate or $FADH_2$ in complex II (succinate dehydrogenase) and transfers them successively to coenzyme Q or ubiquinone, complex III, complex IV and finally to molecular oxygen with the formation of water. Concomitant with electron transport, protons are transferred from the mitochondrial matrix to the intermembrane space by complexes I (in mammals, but not in yeasts), complex III and IV. The difference in electrochemical potential between intermembrane space and matrix is used by ATP synthase to produce ATP from ADP and inorganic phosphate. During the passage of electrons through the complexes, a small fraction is leaked to oxygen at intermediate points, producing the superoxide radical anion, which in the mitochondrial matrix can be removed by SOD2 forming H_2O_2. Hydrogen peroxide can be converted to water in the mitochondrial matrix by glutathione peroxidase (mammals). Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; UQ, ubiquinone; Cyt, cytochrome c; SDH, succinate dehydrogenase; I, complex I or NADH coenzyme Q reductase; II, complex II or succinate dehydrogenase; III, complex III; IV, complex IV or cytochrome c oxidase; $O_2^{\bullet-}$, superoxide radical anion; SOD2, superoxide dismutase 2 or mitochondrial MnSOD; GPx, glutathione peroxidase. Modified from Ref. [22].

superoxide in both sides of inner mitochondrial membrane [29]. Complex II could theoretically generate superoxide, due presence of flavoprotein in its structure. However, the redox centers are arranged in a manner that aids the prevention of ROS by avoiding the access of O_2 to the flavoprotein. This may explain the reason why this complex does not show a ROS formation by itself [30], but only due reverse electron transfer, i.e., when electrons flow from succinate to ubiquinone and back to complex I [31].

In addition to the electron transport chain, recent studies in mammalian tissues have shown that proteins belonging to the α-ketoglutarate dehydrogenase complex located in the mitochondrial matrix are also a source of ROS in a mechanism stimulated by the low concentration of NAD^+ [32, 33]. In *Saccharomyces cerevisiae*, the deletion of the LPD1 gene, which leads to the inactivation of the enzyme dihydrolipoyl dehydrogenase, E3 subunit of the pyruvate dehydrogenase complex, also leads to a decrease in ROS production. This finding shows the importance of other mitochondrial proteins, other than those associated with the electron transport chain, in the regulation of redox balance [34].

The term reactive specie is not restricted to oxygen, but is also include others, as reactive nitrogen (RNS). Nitric oxide is a membrane permeable free radical that participates in a multiple process in the cells as signaling molecule, but also can contribute in cell oxidative damage. Its effect depends on NO levels and localization in the cell microenvironment [35, 36]. When nitric oxide is present in environment, as in mitochondrial matrix, the reaction of this free radical with superoxide can form others RNS, as peroxynitrite.

Besides mitochondria electron chain and enzyme linked to mitochondrial dehydrogenase complexes, other sources of ROS in cells include enzymes, as NADPH oxidases, cytochrome P450, cyclooxygenases, and the system xanthine/xanthine oxidase. Autoxidation is another example of source of ROS that in cells occurs when a biochemical compound is exposure to O_2, as it occurs in $FADH_2$, L-DOPA and in nitric oxide synthase with generation of superoxide. The auto oxidation can be catalyzed by metallic ions, finally, harm proteins, in which O_2 bind Fe^{2+} could lead to superoxide, as in hemoglobin [37].

1.2. Mitochondria and reactive species: physiological level, oxidative stress, and its implications

ROS and RNS are normally produced in metabolism and have an important role as signaling molecules regulating diverse physiological cell events, as cell signaling, metabolism and regulation of transcription factors [35, 38–42].

The steady state of reactive species will depend on their generation, reactivity and removal by antioxidant defenses. When the level of reactive species generation is much larger than their removal it is said that there is a condition called oxidative stress, i.e., an imbalance between reactive species and antioxidants in favor of reactive species. The maintenance of cell redox state is important to cell viability [43]. The increased level of reactive species can lead to oxidative damage to a vast number of biological molecules, as DNA [44–46], proteins [47], lipids [48], including membranes [3] leading to a range of pathologies, as cancer [36], neurological disease [49], cardiac disease [50, 51], inflammation process [52] and aging.

There is a grand amount of theories about aging process, at least 300 theories according Medvedev [53]. In 1956, Harman proposed in his "free radical theory of aging" that the damage of biomolecules that occurs during aging is due oxidative stress, ROS increments [54]. Mitochondria, as the major site of ROS production, have been associated with aging process [55, 56]. Moreover, studies with caloric restriction in yeast and mammals have shown that the mitochondria, ROS, and RNS have an important role in the aging process [34, 56–61].

2. Protein adducts, DNA oxidation and epigenetic regulation, and effects on biological membranes

During oxidative stress, ROS can attack molecules at electron-dense sites or abstract protons, producing secondary radical species, which undergo conformational change generating more stable products. The molecules that are vulnerable to these deleterious modifications include

the lipids, proteins and nucleic acids. In other words, when the generation of reactive species exceeds antioxidant capacity, the cellular macromolecules also become targets of oxidation by these species. The possible consequences originated from this extensive oxidation, including an increased risk for cardiovascular disease, cancer and neurodegenerative disease (as detailed in Section 4).

2.1. Protein adducts

Under oxidative stress conditions, proteins suffer extensive modification [62–65]. Basically, ROS can oxidize amino acids cysteine and methionine, resulting in the production of dithiol and methionine sulfoxide crosslinks, respectively [66]. Moreover, reactive species also can cause protein modification by nitration of tyrosine and by nitrosation of amino acids with thiol group. These changes often result in the alteration of function or inhibition of enzyme activities. The protein adducts have been observed in several pathologic conditions [67, 68], suggesting their deleterious effects. However, whether these endogenous modifications are produced in a controlled manner, they may also control physiological responses [69, 70].

It is important to stress that the presence of proteins containing nitrotyrosine residues, for example, has been a biomarker of damage by reactive species [67, 68]. The tyrosine nitration occurs by addition of NO_2 to the ortho position of the phenolic ring of this amino acid. In fact, this NO_2 group is obtained from peroxynitrite ($ONOO^-$), a very strong oxidant [71]. During oxidative stress conditions, especially in inflammatory processes, a proportion of $O_2^{\bullet -}$ reacts with NO to form $ONOO^-$. This last is a much more powerful oxidant than $O_2^{\bullet -}$ and, beyond the tyrosine residues, can damage several classes of molecules. $ONOO^-$, its protonated form peroxynitrous acid (ONOOH), and its secondary radical product, react with electron-rich groups, such as sulfhydryls, ironsulphur centers, zinc-thiolates and active site sulfhydryl in tyrosine phosphatases [67, 68, 72, 73].

The thiol group (-SH) of cysteine, for example, it is another relevant protein targets of ROS. Disulfide bond is important in protein structure and function [74], and recently its role in redox signaling has also been evidenced [75]. The reaction of H_2O_2 with the deprotonated thiol group of cysteine produces a sulfenic acid (R-SOH). This last may be oxidized again producing a sulfinic acid ($R\text{-}SO_2H$). With high levels of stress oxidative, cysteines can further be oxidized to a sulfonic acid ($R\text{-}SO_3H$) [70, 76]. While sulfenic and sulfinic acids can be enzymatically reversible by the glutathione and thioredoxin enzyme systems [77] (Details about antioxidant mechanisms in next section), the sulfonic acid in cysteine residues seems to represent an irreversible protein damage.

2.2. DNA oxidation

The reactive species react directly with nucleic acids producing oxidative damage. Since oxidative DNA damage is a major threat to genetic integrity, causing mutations and modifications in gene expression pattern, it has been implicated in a wide variety of diseases, including cancer, cardiovascular and neurodegeneration disease, as well as aging process [46, 73].

The nitrogenous bases as well as the sugar suffer radical attacks, causing several base alterations and strand breaks [78]. In fact, around 80 different bases have been observed in DNA exposed to oxidants [79]. In this context, •OH is the most important reactive species that attacks DNA, since it reacts with the four bases and sugar moiety of the DNA backbone [78, 80] with a reaction rate limited by diffusion (4.5×10^9 to 9×10^9 $M^{-1}s^{-1}$) [79]. •OH attacks carbo-carbon double bonds of bases due to the high electron density. These attacks produce the hydroxylation at C5 and C6 of pyrimidines and C4, C5 and C8 of purines [78, 80]. These secondary radicals are subjected to other oxidation and reduction reactions, producing a wide DNA lesions, including the well characterized derivatives, 7,8-dihydro-8-oxodeoxyguanine (8-oxoG) and 2,6-diamino-4-hydroxy-5-formamido-pyrimidine (FapyGua) [71]. 8-oxo-G is the most stable of these altered bases and can give rise to mutations due to insert Adenine (A) opposite 8-oxo-G during DNA replication, instead of the Cytosine (C) [46, 71].

Another mutation produced by oxidative damage is C to thymine (T) transition, mainly due to the cytosine-derived products uracil glycol and 5-hydroxyuracil mispairing with A, instead of the G [71]. Although other pathways also induce this mutation, it is important to stress that C to T transition is the most frequent mutations found in cancers and in the tumor suppressor gene p53 [81, 82].

2.3. Effects on biological membranes

Under conditions of oxidative stress occur an oxidative process termed lipid peroxidation that affects lipids containing multiple double bonds, such as fatty acids, phospholipids, glycolipids and cholesterol, modifying properties of cellular membranes [73, 83]. This degenerative process is believed to contribute to aging and several diseases, such as atherosclerosis, Alzheimer's disease, peptic ulcer disease, and cancer [84, 85].

Cellular membranes are especially vulnerable to lipid peroxidation not only because of their high levels of unsaturated fatty acids, but also because of their connection with molecules capable of producing reactive species. They attack mainly the unsaturated fatty acids which contain carbon-carbon double bonds and CH_2 groups with particularly reactive hydrogen, and start radical peroxidation chain reactions [86]. These chain reactions are going to terminate when primary or secondary radicals directly react. Lipid peroxidation is accelerated by the presence of Fe^{2+} and Cu^{2+} ions [87, 88]. It is important to stress that lipid peroxides are unstable derivatives from the oxidation of unsaturated fatty acids and decompose to form reactive carbonyl molecules, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) [85, 89]. These two products are abundant biomarkers of lipid peroxidation [85, 90].

Membrane-bound proteins are also involved in the process of lipid peroxidation. Aldehyde products, such as MDA and 4-HNE, react with amine and thiol groups of membrane protein, causing several damages, including inactivation of enzymes. Conformational changes of membrane molecules also include lipid–lipid cross-links and lipid–protein cross-links [91, 92].

Moreover, lipid peroxidation modifies the global biophysical properties of the membranes. This process affects the packing of lipids and the permeability to solutes, which in turn, changes its function, including the membrane potential. Furthermore, the process

of peroxidation can inhibit the activity of protein transporters and ion channels [89, 91]. The increase of the permeability also seems to occur in internal mitochondrial membrane, uncoupling respiratory-chain phosphorylation [93]. Finally, the lipid peroxidation leads the severe damages: modification of membrane permeability, enzymatic inhibitions, inactivation of transporters [37, 92].

3. Endogenous/exogenous defense mechanisms

The exposure cells and tissues to the harmful effects of free radicals cause a cascade of reactions and induces activation of some strategies to damage prevent, repair mechanism to alleviate the oxidative damages, physical protection mechanism against damage, and the final most important is the antioxidant defense mechanisms [94, 95].

The antioxidant defenses are the first line of choice to take care of the stress. Endogenous antioxidant defenses include antioxidant enzymes and non-enzymatic molecules that are usually distributed within the cytoplasm and various cell organelles [94]. The exogenous antioxidants are present in consumed fruits, vegetables, juice, tea, coffee, nuts and cereal products [95].

The concept of biological antioxidant refers to any compound present at a lower concentration which is able to either delay or prevent the oxidation of the substrate. Antioxidant functions imply lowering oxidative stress, DNA mutations, malignant transformations, as well as other parameters of cell damage [96]. Antioxidants reactions can deplete molecular oxygen or decreasing its local concentration, removing pro oxidative metal ions, trapping aggressive ROS such as superoxide anion radical or hydrogen peroxide, scavenging chain initiating radicals like hydroxyl OH·, alkoxyl RO· or peroxyl ROO·, breaking the chain of a radical sequence or quenching singlet oxygen ($^1O_2$) [97].

The antioxidants include some high molecular weight (SOD, GPx, catalase, albumin, transferrin, and metallothionein) and some low molecular weight substances (uric acid, ascorbic acid, lipoic acid, glutathione, ubiquinol, tocopherol/vitamin E, flavonoids). Natural food-derived components have received great attention in the last 2 decades, and several biological activities showing promising anti-inflammatory, antioxidant, and anti-apoptotic-modulatory potential have been identified. These enzymatic and nonenzymatic antioxidant systems are necessary for sustaining life by maintaining a delicate intracellular redox balance and minimizing undesirable cellular damage caused by ROS [94, 97, 98].

3.1. Enzymatic antioxidant system

Antioxidant enzymes catalyze ROS conversion directly via an active-site metal ion or through pathways involving the donation of an electron from the moiety-conserved redox couples thioredoxin and glutathione, which require continuous regeneration of the reduced species [99]. Superoxide and H_2O_2 metabolizing enzymes are generally considered to be the primary antioxidant enzyme defense system in the body [98].

The SOD is a family of enzymes catalyzing dismutation of superoxide into oxygen and H_2O_2. Three types of superoxide dismutases can be encountered in mammalian tissues: copper-zinc containing superoxide dismutase (SOD1) present in the cytosol, manganese containing superoxide dismutase (SOD2) found in the mitochondrial matrix and extracellular superoxide dismutase (SOD3). All three are highly expressed, mainly in the renal tubules of healthy kidneys [15, 98, 100]. The final product of the SOD activity - H_2O_2, is then converted into water and oxygen by the catalase (CAT). This enzyme is a homotetrameric protein containing four iron heme and largely located in the peroxisomes [15, 100].

Other important enzymatic antioxidants in the first line of defense include glutathione peroxidase (GPX) and myeloperoxidase (MPO) enzymes. The GPX is a selenium-containing enzyme, catalyzes both the reduction of H_2O_2, and organic hydroperoxides to water or corresponding alcohols. Reduced glutathione functions as effective electron donor in the process, as free thiol groups are oxidized to disulfide bonds: $H_2O_2 + 2GSH \rightarrow GS\text{-}SG + 2H_2O$ [97]. The MPO, a heme peroxidase, abundant in granules of human inflammatory cells, catalyzes the conversion of H_2O_2 to HClO with the production of ROS. The ROS production is associated with cardiovascular disease, chronic obstructive pulmonary disease, and Alzheimer's disease. Oxidant species derived from MPO lead to the production of specific oxidation products, such as 3-Cl-Tyr. This can be used as biomarker in several diseases, as above described, and its levels correlate with MPO [100].

Other enzymes could be cited by our antioxidant activity, such as Peroxiredoxin Family (PRX). These enzymes are a family of abundantly present 20–30 kDa peroxidases that are excessively reactive with H_2O_2. So, they are likely to be critical for both oxidative stress protection as well as redox signaling [98]. The antioxidant enzymes may possibly offer novel treatment options for redox-related diseases, provided that the molecular mechanisms are known and can be specifically targeted. Besides that, inhibiting a given antioxidant enzyme or specifically silencing its gene expression may help treat disorders related to a gain of enzymatic function [98] and this fact can will help the researchers to explore future options in enzymatic antioxidant system and diseases.

3.2. Nonenzymatic antioxidant systems

Among the nonenzymatic antioxidant compounds, the principals are obtained from dietary as the class of phenolic compounds, vitamins C and E, and carotenoids [101]. Phenolic compounds represent a large group of secondary metabolites [102], among them flavonoids, phenolic acids, tannins and tocopherols as the most common natural source phenolic antioxidants [103].

The phenolic compounds are composed of one or more aromatic rings with varying degrees of hydroxylation, methoxylation and glycosylation, and various studies have associated the structure of phenolic compounds with their antioxidant properties [102, 104]. The antioxidant activity generally increases with the degree of hydroxylation in aromatic rings and decreases with C-3 methoxylation [105, 106]. The antioxidant activity is based on the availability of electrons to neutralize the free radicals; in addition, it is related to the number and nature of the hydroxylation pattern in the aromatic ring and the ability to act as a hydrogen donor [106].

The flavonoid group is the most diverse within phenolic compounds, with two aromatic rings associated via C-C bonds by a 3C oxygenated heterocycle. Flavonoids have antioxidant and chelating properties, inactivate ROS, acting against the oxidation of low density lipoproteins (LDL) and improving inflammation of the blood vessels. They also reduce the activity of the xanthine oxidase enzymes and the nicotinamide adenine dinucleotide phosphate oxidase, enzymes that stimulate the production of ROS [107].

In cellular compartments, flavonoids function as antioxidants inactivating free radicals both in hydrophilic and lipophilic compartments. For example, the antioxidant activity of phenolic compounds present in spices (cinnamon, sweet weed and mustard) differs between aqueous and lipid systems [108].

Vitamins C and E act together to inhibit lipid peroxidation and protect the cell against oxidative damage, as DNA damage. The antioxidant activity of vitamin C involves the transfer of an electron to the free radical and the consequent formation of the radical ascorbate [109]. In addition, vitamin C acts synergistically with vitamin E, which regenerate the vitamin C has better antioxidant activity in hydrophilic media, and in aqueous phase of extracellular fluids, it is able to neutralize ROS in the aqueous phase before they can attack lipids. Vitamin E is an important fat soluble antioxidant, acting as the chain breaking antioxidant within the cell membrane and playing an important role in the protection of membrane fatty acids against lipid peroxidation [110].

Vitamins C and E inhibit lipid peroxidation and protect against oxidative damage by their scavenging actions of ROS, as well as by modulateing numerous enzymatic complexes involved in the production of ROS, endothelial function and aggregation of platelets. These vitamins can also regulate NADPH oxidase, the most important source of $O_2^{\bullet -}$ in the cardiovascular system. It has been reported that ascorbic acid and α-tocopherol, derivated from vitamin C and E respectively, may involved in the transcriptional modulation of NADPH oxidase [111].

The most common carotenoids are xanthophylls and carotenes. Carotenoids can neutralize singlet oxygen by quenching it or can break the chain reaction of free radicals, or scavenging it, not so effective action (scavenging). The structure of the free radical is the main factor that determines if the carotenoid will have quenching or scavenging action. It also depends on the region where the radical is in heterogeneous biological tissue, aqueous or lipid region (plasma, blood, heart, liver, brain etc.), and the structure of the carotenoids (number of conjugated, cyclic or acyclic double bonds), polar or nonpolar groups, redox properties [112–114].

The physical quenching is the transfer of excitation energy from the singlet oxygen to the carotenoid. The oxygen returns to ground state and the carotenoid is in the excited triplet state, the energy is dissipated producing stable carotenoid and thermal energy and the carotenoid can undergo other cycles of singlet oxygen quenching [112, 115].

The chemical quenching the carotenoid combines with oxygen or is oxidized, leading to its destruction and producing a variety of oxidized products. Carotenoids can also extinguish the triplet-excited state of chlorophyll or other excited sensitizers, thus preventing the formation of singlet oxygen [112]. The free radical scavenging can occur in three ways, by electron transfer, by hydrogen abstraction, and by addition [112, 116].

4. Interaction between reactive species, enzymes, and antioxidant molecules in health and disease

All living cells have molecular tools to perceive and respond properly to environmental cues. All the cascades of intracellular reactions involved in promoting a biochemical response are denoted as signal transduction. There are well known receptor types or systems of signal transduction such as the G protein-coupled receptors (GPCR), tyrosine kinase receptors (TKR), ion channels, cell adhesion receptors, nuclear receptors and guanylyl-cyclases. Since cells often need to deal with many signals at the same time, the final biochemical response is a result of the integrations of many simultaneous cascades produced by one or more systems.

Before we move on exploring the targets of ROS in health and disease, an important question is raised: "Which are the main sources of cellular ROS?" Enzymes such as NADPH oxidases (Nox), xanthine oxidase (XO), lipoxygenase, MPO and uncoupled nitric oxide synthase are involved in the production of the anion radical superoxide ($O_2^{\cdot -}$). Furthermore, the mitochondrial aerobic respiration contributes with a huge amount of $O_2^{\cdot -}$. Peroxynitrite ($ONOO^-$) is formed by the reaction of nitric oxide and superoxide and is thought to contribute to eNOS uncoupling [69]. The majority of $O_2^{\cdot -}$ generated within the mitochondrial matrix or the cytosol is dismutated to H_2O_2 by the SOD antioxidant enzyme. Moreover, metal exposure can mediate the generation of H_2O_2, $O_2^{\cdot -}$, and even the hydroxyl radical ($OH^{\cdot}$), mainly via the Fenton or the Haber-Weiss reactions [117].

Some ROS such as $O_2^{\cdot -}$ and $HO^{\cdot}$ are highly reactive and have a brief half life. For this reason they are not considered signaling molecules, but intermediates of nonselective nature. On the other hand, H_2O_2 is relatively stable and can both mediate intracellular signaling and also serve to paracrine signaling (i.e., cell-to-cell communication involving nearby cells), since it can cross biological membranes [118].

Up to date, several proteins have been recognized as downstream targets of ROS, such as kinases, phosphatases, mitogen-activated protein kinases (MAPK), small G proteins, transcription factors, microRNAs, and phospholipases. In this section, we do not intend to deeply review the literature, but to show an overview of important targets and exemplify their involvement in the signal transduction by ROS in health and disease.

ROS can induce alterations in the intracellular and extracellular processes, for example, in the PI3K/AKT signaling. The lipid phosphatidylinositol 3,4,5-triphosphate (PIP3) has a function as a second messenger and is not present in the quiescent cells, but it rises within seconds to minutes when there is a stimuli. PIP3 is produced by the phosphorylation of the phosphatidylinositol 4,5-bisphosphate (PIP2) catalyzed by the phosphatidylinositol 3-kinase (PI3K). This enzyme is activated by ROS through two different pathways, or directly, throught amplications of downstream PI3K pathway, or indirectly by inhibition of the phosphatase and tensing homolog deleted on chromosome 10 (PTEN). PTEN is responsible for the degradation of PIP3 signaling, since it catalyzes the hydrolysis of phosphate in the 3′ position on PIP3 to produce PIP2 [119]. ROS, mainly, H_2O_2, can oxidize and inhibit PTEN, which culminates in an increase in the PIP3 production, that acts in cell signaling, through activation of proteins, as serine/threonine protein kinase, AKT/PKB, among others [120, 121]. The AKT activation provides the transcription of

several targets, such as GSK3, BAD, FOXO, p53, NF-kB, mTOR/p70S6K1 and HIF-1 [122, 123]. In this way, ROS increase the final cascade response in cell, i.e., cell cycle progression, proliferation, anti-apoptosis, invasion, autophagy and angiogenesis [124]. The PI3K/AKT pathway hyper activated by ROS might favor carcinogenesis in the end of the process.

An important class of redox regulated proteins is the Src family of nonreceptor tyrosine kinases (SFKs), a group of structurally related kinases that catalyze the phosphorylation of tyrosine in downstream targets to regulate cellular functions coupling receptors such as the TKR, the cell adhesion molecules (CAMs), and the GPCR to the cellular signaling machinery [125]. For example, during focal adhesion while the extracellular matrix (ECM) contact triggers a slight or partial activation of SFKs, the ROS production is associated with a strong oxidative-dependent activation and recruitment of Src kinases to cell membranes. The redox-activation of SFKs can induce sustained PI3K, protein kinase C (PKC), and extracellular regulated kinase (ERK) activation and, thereby, create conditions for tumor cell growth, invasion, angiogenesis, and resistance to apoptosis [126]. In a variety of human cancers an increased activity of Src kinases have been described, as well as activation of important Src downstream targets such as PI3K/Akt, focal adhesion kinase (FAK), paxillin, p130Cas, signal transducer and activator of transcription 3 (STAT3) and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) [127–130].

Carcinogenesis is also related with activator protein-1 (AP-1) transcription factor activation. Among other systems, ROS are recognized as activators of AP-1; however, the signaling transduction events involved are not totally understood. Chromium, cobalt, cadmium and vanadium are metals involved in the activation of AP-1 through signaling cascades involving the production of ROS and comprised of proteins and enzymes such as thioredoxin (Trx), redox factor-1 (Ref-1), ERK/MAPK, NADPH oxidase, I kappa B kinase (IKK), p38, JNK/c-jun [117, 131, 132].

ROS are important for the regulation of vascular tone, however an excess of reactive species might be associated with pathological dysfunction. Endothelial nitric oxide synthase (eNOS) regulates smooth muscle cells (SMC) relaxation through the production of the second messenger nitric oxide (NO) from L-arginine, which activates guanylyl cyclases to initiate the conversion of GTP to cyclic guanosine monophosphate (cGMP), which allosterically activates the cGMP-dependent protein kinases (PKGs). The enzyme eNOS can be uncoupled and, consequently, change its profile from NO synthesis to $O_2^{\cdot -}$ production instead. Two major events are involved in eNOS uncoupling. First, an increase of ROS might generate the peroxynitrite ($ONOO^-$) through the reaction of NO and $O_2^{\cdot -}$. The anion $ONOO^-$ reacts with and oxidizes tetrahydrobiopterin (THB/BH_4), a cofactor of eNOS [133]. Second, an increased ratio of oxidized glutathione (GSSG)/reduced glutathione (GSH) cause reversible S-glutathionylation and uncoupling of eNOS [134]. Paradoxically, H_2O_2 produced by NADPH oxidase increases eNOS expression and NO production, but this effect is not believed to counteract the effects of oxidative stress [135].

Interestingly, in a scenario of reduced NO levels, in which it would be expected a lack of input signals to PKG activation (e.g., cGMP), the H_2O_2 can cause vasodilation through PKG oxidation [136]. Another target of ROS is the small GTPase RhoA, which when oxidized activates its downstream partner Rho kinase (ROCK), leading to inhibitory phosphorylation of myosin light chain (MLC) phosphatase and, ultimately, to SMC contraction [137, 138]. For a more

explored involvement of ROS in the regulation of signal transduction in the cardiovascular system, check the review of Brown and Griendling [118].

The activating or deactivating switch, in which a group of kinases is active or a group of phosphatases is active, provokes different downstream cascades with consequences in the cellular response. As we described above, several kinases are susceptible to ROS reactions, but also phosphatases are vulnerable to ROS, since they react with a group of amino acids presents in different enzymes. The reaction between ROS and phosphatases causes the oxidation and inhibition of those enzymes, increasing the kinases signaling [139]. Another phosphatase inhibited by ROS is PTEN, which increases the PIP3 signaling, as described above.

A vascular injury promotes an increase in the expression of platelet derived growth factor (PDGF) and PDGF receptor, which in turn cause stimulation for the vascular smooth muscle cells to migrate [140]. The activation of the PDGF receptor is controlled by the action of low molecular weight protein tyrosine phosphatase (LMW-PTP). The Cys12 and Cys17 in LMW-PTP is susceptible to a reaction with ROS resulting in a disulfide bond, and so its inactivation [141]. Therefore, without the LMW-PTP deactivation upon PDGF receptor, its signal is amplified, which generates migration. Oxidized LMW-PTP also increases the Rho family signal, since PDGF receptor is stimulated, and it binds to phospholipase C, Src, and PI3K. As described before, PI3K catalyzes the reaction and formation of PIP3. The Rho-guanine nucleotide exchange factors are activated by PIP3, which triggers Rho-GTPase family members' activation (Rho, Rac, and cdc42). As Nox family is activated by Rac, it produces ROS. Therefore, this process is kept by a positive feedback: generated ROS oxide Rho in a redox sensitive motif and restrain the LMW-PTP action [118, 138].

Phospholipases are enzymes that hydrolyze phospholipids and generate second messengers involved in the regulation of many physiological functions. Phospholipase A2 (PLA2) cleaves the fatty acyl group at the sn-2 position of the glycerol backbone, releasing arachidonic acid (AA) and lysophospholipid. It was attributed a role for the Ca^{2+}-independent PLA2 (iPLA2) isoform in the excessive production of $O_2^{\cdot -}$ by primed neutrophils of patients with poorly controlled diabetes. This study suggested that hyperglycemia is related to the activation of iPLA2 and AA formation which, in part, regulate NADPH oxidase activity (i.e., generation of $O_2^{\cdot -}$) [142].

PLA2 activation has also been related to alterations implicated in the pathogenesis of neurodegenerative diseases, such as neuronal excitation, cognitive and behavioral function, oxidative and nitrosative stress [143]. Phospholipase C (PLC) is a well-known enzyme especially involved in the signaling transduction of GPCR coupled to $G_{q/11}$ protein and some G protein $\beta\gamma$ subunits (PLC-β), but also in RTK (PLC-γ and PLC-ε), Ras and Rho small GTPases (PLC-ε) and Ca^{2+} (PLC-δ) signaling pathways, which involves the generation of the phosphate-containing head group inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) through the hydrolysis of the membrane phospholipid PIP2 [144]. The activation of PLC-γ1 was shown to have an important protective function during mouse embryonic fibroblasts (MEF) response to oxidative stress (H_2O_2) treatment [145]. A further study suggested that this function of PLC-γ1 involved the PKC-dependent phosphorylation of Bcl-2 and inhibition of caspase-3 [146]. Phospholipase D (PLD) cleaves a phosphodiester bold in membrane-bound lipids, similarly to PLC. However, its activity generates phosphatidic acid (PA) and an alcohol, usually choline or

ethanolamine [147]. A link between oxidative stress and PLD has been proposed by Kim et al. [148], in a study that suggests that H_2O_2 induces rat vascular smooth muscle cells tyrosine kinase activity, and PLD1-dependent PKC-α activation.

In the innate immune system, mononuclear monocytes/macrophages eliminate pathogen, antigen and cellular components through generation of ROS/RNS [149]. When there is an imbalance in the equilibrium between oxidative/nitrosative stress and cellular requirements, the stress can generates pathological complications. Among others, rheumatoid arthritis is an autoimmune disease that has oxidative/nitrosative stress as one of the causes. The cellular immune system is vulnerable to reactions caused by ROS, which in turn can affect the regular physiological process and activates inflammatory signaling pathways that produce pro-inflammatory cytokines, chemokines and prostaglandins. The inflammatory mechanism involves synovial cellular infiltrate and peripheral blood inflammatory cells following by polymorphonuclear neutrophils and lymphocytes culminating in the joint damage [150, 151]. The signaling cascade occurs via activation of NFkB for synthesizing pro-inflammatory cytokines and chemokines [149]. The Th1 cytokines are one of the most important because can provide the development of autoimmune disorders. These cytokines can directly or indirectly promote oxidative stress in the cells, intensifying the rheumatoid arthritis.

Prostaglandins have a pivotal role in the formation of the inflammatory response, since they mediate pathogenic mechanisms and provide the development of the cardinal signs of acute inflammation. Their biosynthesis involves the initial enzyme, phospholipase A2 (PLA2). PLA2 catalyzes the conversion of membrane phospholipids in AA. Then, cyclooxygenases convert AA into prostaglandins. Prostaglandin E2, in particular, rises vasoactive components (histamine, bradykinin, and nitric oxide), hence generating edema, pain and hyperalgesia at the local inflammatory sites, and so the inflammation [152]. ROS stimulate this process through the activation of cyclooxygenases. Prostaglandins, also, activate NADPH oxidase, which produces superoxide anion radical [153]. Therefore, this system becomes cyclic, ROS activate cyclooxygenases and so the prostaglandins biosynthesis, further prostaglandins trigger NAPH oxidases, increasing ROS.

The microRNA (miRNA) is a small noncoding endogenous RNA, that has an important role, since it regulates gene expression. Its function can be modified depending on epigenetic changes, chromosomal abnormalities and oxidative stress. It has been found that miRNA can respond to ROS, implying in its ability to activate certain genes transcription during stress, and this is prominent in cancer cells, which was correlated to the adaptation of these cells to unfavorable and/or hypoxic environment [130, 154, 155]. However, studies showed that some types of miRNAs can regulate gene expression of protective proteins and antioxidant enzymes [156, 157]. Some ROS dependent miRNAs play a role as oncogenic (miR21 and miR155), but interesting miR21 also targets SOD, which can be interpreted that this miRNA regulate the ROS levels in the cell. When miR21 is stimulated, it also affects the immune system through the chemokine CXCL10. CXCL10 adjusts innate and adaptive immune response by activating T lymphocytes, macrophages and inflammatory dendritic cells. The miR155 also has opposite actions, it can be oncogenic (the targets are BCL2, FOXO3a, RhoA) or tumor suppressor (the targets are TGF-beta/SMAD) [158]. The literature about miR155 is vast, and we suggest the articles by Higgs and Slack [158] and Mattiske et al. [159] for a deep reading. Besides these two

miRNAs cited above, others miRNAs are upregulated by ROS, such as miR23, miR200, miR210, etc., affecting migration, invasion; tumor growth, angiogenesis; cell cycle, DNA damage (among others), respectively [126].

In addition to the miRNAs that are ROS upregulated as cited above, there are ROS downregulated miRNAs important in the carcinogenic process, such as miR34 family. Some miR34 members regulate p53 causing a cell cycle arrest in G1 and apoptosis when DNA is impaired. The miR34a, for example, induce tumor suppression and metastasis inhibition. Another miRNA, miR124, has been shown to be affected by H_2O_2 [160]. This miRNA is correlated to the regulation of tumor cell proliferation, migration and drug resistance through its action upon R-Ras, PI3-KCA, AKT2, ROCK1, Src, DNA methyltransferases and others. The miR199a is also downregulated by ROS, some of its targets are ERBB2, ERBB3, IKKB, HIF-1alfa, ApoE, CCR7, having an effect upon cell proliferation, invasion, metabolism and metastasis [126, 161]. This is just a

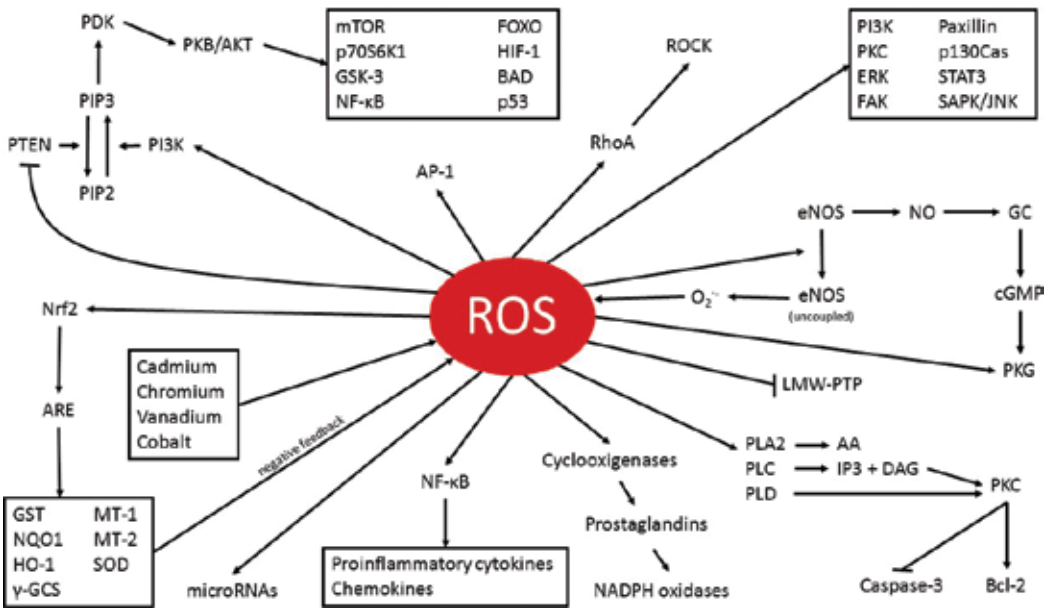

Figure 2. Examples of molecular targets involved in the signal transduction mediated by reactive oxygen species. Abbreviations: AA, arachidonic acid; AP1, activator protein 1; ARE, antioxidant-responsive element; BAD, Bcl-2-associated death promoter; Bcl-2, B-cell lymphoma 2 protein; cGMP, cyclic guanosine monophosphate; DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; ERK, extracellular-signal regulated kinase; FAK, focal adhesion kinase; FOXO, Forkhead box protein O; GC, guanylyl cyclase; GSK-3, glycogen synthase kinase 3; GST, glutathione S-transferases; HIF-1, hypoxia-inducible factor 1; HO-1, heme oxygenase 1; IP3, inositol 1,4,5-triphosphate; LMW-PTP, low molecular weight phosphotyrosine protein phosphatase; MT-1, metallothionein-1; MT-2, metallothionein-2; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-kappa B; NO, nitric oxide; NQO1, NAD(P)H:quinone oxidoreductase; Nrf2, nuclear-factor erythroid-2 related factor; $O_2^{\cdot-}$, superoxide anion radical; p130Cas, p130 Crk-associated substrate; p53, p53 tumor suppressor protein; p70S6K1, p70S6 kinase 1; PDK, phosphoinositide-dependent kinase; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-triphosphate; PKB/AKT, protein kinase B; PKC, protein kinase C; PKC, protein kinase C; PKG, cGMP-dependent protein kinases; PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RhoA, Ras homolog family member A; ROCK, Rho-associated protein kinase; SAPK/JNK, stress-activated protein kinase or c-Jun N-terminal kinase; SOD, superoxide dismutase; STAT3, signal transducer and activator of transcription 3; γ-GCS, gamma-glutamylcysteine synthetase.

summary of some important miRNAs and their responses in carcinogenesis, for more information check the review Mu and Liu [126].

As previously discussed in this chapter, cells have a repertoire of antioxidant molecules and enzymes as a defense mechanism to an increase in ROS production. However, oxidative stress takes place when the antioxidant capacity is overwhelmed by reactive species production. In this scenario, to maintain cell homeostasis and/or terminate the ROS signal transduction there are some stress sensors that regulate the translation of antioxidant proteins. The antioxidant responsive element (ARE) is a region of non-coding DNA (short consensus sequence) which is localized upstream and regulates the transcription of many antioxidant neighboring genes such as glutathione S-transferases (GST), NAD(P)H:quinone oxidoreductase (NQO1) [162], heme oxygenase 1 (HO-1), γ-glutamylcysteine synthetase (γ-GCS) [163], metallothionein-1 and -2 (MT-1 and MT-2) [164], and SOD [165].

It was shown that ARE induction protected against oxidative stress mediated by 6- hydroxydopamine in vitro, a mitochondrial inhibitor used to model Parkinson's disease [166]. The nuclear-factor erythroid-2 related factor (Nrf2) is a central transcription factor involved in the upregulation of ARE-containing genes and, consequently, synthesis of proteins with antioxidant function. However, there are also nuclear factors that negatively regulate ARE-mediated gene expression, such as Mafs (MafG and MAfK), large Maf (c-Maf), c-Fos, and Fra1 [163].

Finally, in this section, we showed an overview of processes regulated by fluctuating levels of ROS and their molecular sensors. Furthermore, we showed that in response to oxidative stress and to maintain homeostasis, cells can upregulate the synthesis of antioxidant defenses (**Figure 2**).

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Chapter 2

The Role of Oxidative Stress and Systemic Inflammation in Kidney Disease and Its Associated Cardiovascular Risk

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Additional information is available at the end of the chapter

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Abstract

Chronic kidney disease (CKD) is a major global health burden, with a prevalence of 10–15% and high mortality rates. In particular, CKD portends a disproportionately high risk of cardiovascular disease beyond the traditional cardiovascular risk factors, with pathophysiological factors such as oxidative stress, inflammation and hyperuricaemia considered to exert an additional role in accelerated atherosclerosis. The presence of heightened oxidative stress and systemic inflammation in CKD is associated with increased mortality. The possible underlying mechanisms include gut dysbiosis, dialysis factors, infections, metabolic acidosis and hyperuricaemia. The state of oxidative stress and systemic inflammation are closely linked and perpetuate each other resulting in progression of CKD and cardiovascular disease. Potential interventions to alleviate the oxidative stress and inflammation in CKD include lifestyle modifications including dietary changes and exercise, optimization of dialysis procedure and pharmacotherapeutic agents including antioxidants. They present a potentially highly effective approach to add to the currently available traditional risk-modification strategies. To date, the majority of the published trials have had a small number of participants with a short duration of follow up. Therefore, no robust evidence has been established. Larger trials with meaningful clinical outcomes and longer follow up are required to evaluate such potential therapies.

Keywords: cardiovascular disease, chronic kidney disease, endotoxin, nitric oxide, oxidative stress, reactive oxygen species, systemic inflammation

1. Introduction

Chronic kidney disease (CKD), defined as an estimated or measured glomerular filtration rate (GFR) <60 mL/min/1.73 m^2 and/or evidence of kidney damage (usually manifested as proteinuria/albuminuria) for at least 3 months [1], is a major and growing public health burden [2]. It is currently estimated that approximately 10–15% of the world's population suffer from CKD and accounts for 4% of the deaths worldwide [3] and has progressively risen from the 21st to the 17th commonest cause of global years of life lost between 2005 and 2015 [4].

The presence of CKD carries a disproportionately increased risk of cardiovascular disease, which increases with progressive declines in GFR and/or increases in albuminuria/proteinuria [1]. Indeed, CKD is considered the most potent known risk factor for cardiovascular disease [5]. Part of this risk is explained by the frequent clustering of traditional cardiovascular risk factors, such as diabetes mellitus, hypertension, obesity, smoking, dyslipidaemia and depression in patients with CKD. However, traditional cardiovascular risk factors account for less than half of the observed excess cardiovascular risk [6]. Consequently, investigators have evaluated the roles of a range of non-traditional cardiovascular risk factors in patients with CKD, including oxidative stress, inflammation and hyperuricaemia (**Table 1**).

Oxidative stress is a particularly promising avenue of investigation. It is becoming well established that CKD is a state of elevated oxidative stress. This is evidenced by the presence of elevated reactive oxygen and nitrogen species, oxidized end products and reduced levels of antioxidants in patients with CKD [7–10]. Systemic inflammation is also up-regulated in CKD evidenced by higher concentrations of inflammatory markers including C-reactive protein (CRP) and interleukin-6 (IL-6) which have been associated with higher mortality in CKD patients and patients with end stage kidney disease (ESKD) [11, 12]. Oxidative stress and chronically elevated inflammatory state may contribute to accelerated atherosclerosis via direct endothelial injury and alteration in nitrogen handling. Reactive oxygen species (ROS) can also cause direct glomerular and tubulointerstitial injury in the kidneys, resulting in further progression of CKD.

The therapeutic options currently available and shown to be beneficial for CKD are limited to anti-hypertensive agents, particularly renin-angiotensin-aldosterone system (RAAS) blockers. However, these agents are only partially effective, typically lowering the risk of renal and

Traditional risk factors	Non-traditional risk factors
Age	Oxidative stress
Hypertension	Mitochondrial dysfunction
Hyperlipidaemia	Systemic inflammation
Tobacco use	Hyperhomocysteinaemia
Diabetes	Hyperuricaemia
Obesity	Thrombosis
	Adipocyte dysfunction

Table 1. The traditional and non-traditional risk factors for development of cardiovascular disease.

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cardiovascular end-points by approximately 20%. There is therefore a pressing need to develop novel, and more effective therapeutic strategies. Therapies targeting oxidative stress and inflammation are promising and may be adjuncts to current therapies targeting traditional risk factors.

This chapter reviews the pathophysiologic mechanisms underlying the heightened oxidative stress and systemic inflammation in CKD, and their association with mortality. Current evidence on various antioxidant and anti-inflammatory therapies are also reviewed.

2. Oxidative stress in patients with CKD

Oxidative stress is a state of excessive pathological pro-oxidant activities relative to antioxidant defense mechanisms. The majority of oxidizing agents are reactive oxygen species (ROS), but others include reactive nitrogen species (RNS), chlorine and carbonyl species.

In CKD, there is accumulating evidence that excess oxidative activity exists with deficient antioxidant protection. Studies in dialysis populations show higher levels of end products of oxidation, such as protein carbonyl, oxidized lipoproteins, F2-isoproteins, advanced oxidation protein products (AOPPs), thiobarbituric acid reactive substances (TBARS), 8-hydroxyl-2′-deoxyguanosine (8OHdG). The free radical superoxide anion production by the pro-oxidant enzyme NADPH oxidase (NOX) is found to be elevated in haemodialysis patients [7]. At the same time, it has been shown that dialysis patients have reduced antioxidant activities of superoxide dismutase (SOD), glutathione, and reduced levels of antioxidants such as vitamin A, C, E, zinc (Zn) and selenium (Se) [7, 8]. In a cross-sectional study of 159 patients (28 to 36 patients in each of the CKD stages 1 to 5) compared with 30 healthy controls, Yilmaz et al. [9] also found that a progressive increase in the levels of oxidative stress marker, malondialdehyde (MDA), while concentrations of antioxidant elements, including SOD, glutathione peroxidase (GSH-Px), Zn, copper (Cu) and Se, fell with increasing levels of kidney dysfunction. The most profound redox imbalances were seen in haemodialysis patients. Therefore, the level of oxidative stress in CKD patients appears to escalate with declining renal function.

These perturbations in oxidant-antioxidant balance begin early in the course of CKD. Fortuño et al. [13] showed increased levels of NADPH-generated ROS in patients with early stage (stages 1 and 2) CKD. Similarly, Yilmaz et al. [9] reported lower levels of activity of the antioxidant enzymes, SOD and GSH-Px, in patients with CKD, including those in stages 1–2.

In kidney transplant recipients, there was an increase in oxidative stress, as evidenced by a rise in MDA immediately after allograft reperfusion [14]. Over the following 2 weeks after transplant, there was a continued rise in MDA, although this was somewhat counterbalanced by a concomitant rise in antioxidant levels, such as GSH-Px. Other oxidative and inflammatory markers, such as IL-6, CRP, tumour necrosis factor-α (TNF-α) and protein carbonyls, significantly declined by 2 months after transplantation [15]. The level of improvement in oxidative stress depended on the level of graft function, such that complete resolution was only possible if renal function returned to normal. At 1 year after transplant, the patients with higher serum creatinine concentrations also displayed higher levels of oxidative stress and

inflammation, such as IL-6, MDA, and transforming growth factor beta (TGF-β), compared to those with normal serum creatinine levels [15].

2.1. Mechanisms of increased oxidative stress

A number of factors have been identified which may contribute to the state of heightened oxidative stress in people with CKD.

2.1.1. Gut dysbiosis

A symbiotic relationship exists between the host and its bacterial flora (also known as microbiota), which are predominantly found in the gastrointestinal tract. In addition to regulating the nutrient absorption and protecting the host from pathological bacteria, the gut microbiota has been increasingly linked to progression of CKD through several mechanisms, including generation of uraemic toxins, enhanced intestinal permeability to endotoxins and alteration of nitrogen handling, all of which contribute to elevated oxidative state.

The breakdown of tyrosine and phenylalanine by the intestinal bacteria produces p-cresyl sulphate (PCS), and the breakdown of tryptophan produces the end product of indoxyl sulphate (IS) in the liver. These two nitrogenous end products have been extensively studied for their role in CKD. They are highly protein-bound in plasma, but as their levels become elevated in patients with CKD, the toxic free fraction level also rises [16]. The serum levels of these toxins have been found to correlate with the extent of damage observed in renal glomerular and tubular cells, tubulointerstitial damage and increased production of reactive oxygen species [16, 17].

Factors influencing the amount of toxins produced include the balance between carbohydrate (in the form of dietary fibre) and amino acids in the large intestine, the intestinal transit time, and the permeability of the gastrointestinal tract. Production of uremic toxins is influenced by substrate availability for fermentation in the colon, notably the balance between carbohydrate (fermentable fibre), and amino acids. A low concentration of fermentable fibre alters the composition of intestinal bacteria to favour more proteolytic bacterial species resulting in the higher production of nitrogenous waste and uraemic toxins. The slower intestinal transit time predisposes to bacteria overgrowth with subsequent production of pro-inflammatory toxins [18].

In addition to the increased production of uraemic toxins in CKD patients, there is evidence that changes in the composition of intestinal bacteria compromise the integrity of intestinal barrier resulting in increased intestinal permeability. This is supported by findings of depletion of the tight junction proteins in the gastrointestinal tract of uraemic patients and drops in transepithelial electrical resistance in colonocytes exposed to the plasma of uraemic patients [19]. Suppression of anti-inflammatory nuclear factor erythroid 2-related factor 2 (NRF2) has also been noted. This may contribute to gut inflammation and reduced expression of epithelial tight junctions, resulting in increased gut permeability [20]. Translocation of bacterial fragments and endotoxins due to increased intestinal permeability may activate the pro-inflammatory pathways in the systemic circulation. For example, increased plasma concentration of lipopolysaccharides due to increased intestinal permeability activates the toll-like receptors (TLR) 2 and TLR4, which in turn activates the nuclear transcription factor kB

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(NF-kB). NF-kB is the master initiator of proinflammatory processes and induces the production of cytokines IL-1, IL-6 and TNF-α [21]. The influx of proinflammatory cytokines causes generation of reactive species (**Figure 1**).

2.1.2. Hyperglycaemia

Hyperglycaemia in diabetic kidney disease has direct and indirect roles in increasing oxidative stress. Advanced glycation end products (AGEs) are formed when the carbonyl component is added non-enzymatically to the free amino acid group of proteins or lipids in the presence of chronic hyperglycaemia. The effects of AGEs are executed via various receptors for AGE (RAGE), which in turn activate transcription factors, such as NF-KB, AP1 and SP1, to activate various oxidative pathways. The pathways activated by AGEs cause production of reactive oxygen species with the end results of mesangial expansion, glomerular basement membrane (GBM) thickening and endothelial cell dysfunction. First, AGEs activate the protein kinase C (PKC) pathway in the glomeruli producing ROS and downstream pathologic changes. The second enzymatic pathway is the activation of NOX which directly generates free radicals. In addition to the enzymatic pathways, hyperglycaemia can directly stimulate the formation of TGF-β, again resulting in mesangial expansion, renal hypertrophy and glomerulosclerosis. The presence of increased renin-angiotensin-aldosterone system (RAAS) activity increases the level of angiotensin II which is a potent initiator of inflammatory processes and subsequent oxidative stress [10, 22].

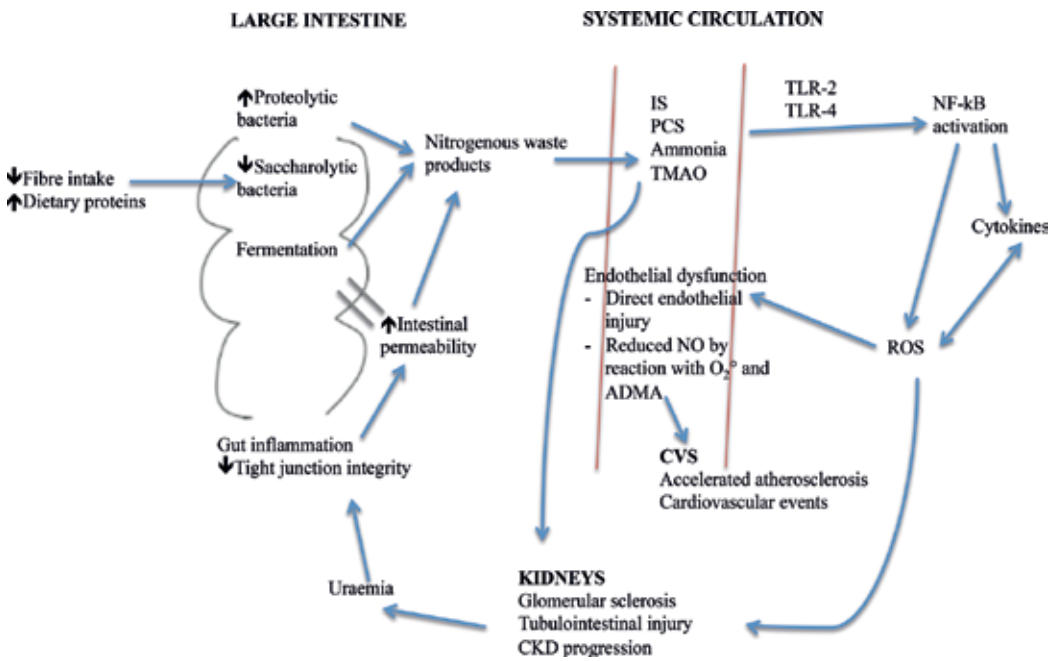

Figure 1. The role of microbiota in the progression of chronic kidney disease and accelerated atherosclerosis. IS, Indoxyl sulphate; PCS, p-cresyl sulphate; TMAO, trimethylamine N-oxide; TLR, toll-like receptor, NF-kB, nuclear factor kB; ROS, reactive oxygen species; NO, nitric oxide; ADMA, asymmetric dimethylarginine; CVS, cardiovascular system.

2.1.3. Dialysis

The dialysis procedure itself accentuates the heightened state of oxidative stress observed in CKD patients. In haemodialysis patients, the mechanisms of oxidative stress include the use of bioincompatible membranes, contamination of dialysate with bacterial endotoxins, occult infection of clotted vascular access and potential loss of antioxidants during the dialysis procedure. Wu et al. [23] demonstrated that levels of myeloperoxidase (MPO), AOPP and 8-OHdG were significantly higher in patients dialysed with regenerated cellulose membranes compared to those dialysed with synthetic polysulphone membranes. However, even with the use of biocompatible membranes, the haemodialysis procedure can still increase systemic levels of reactive oxygen species by 14-fold during one session [24].

It is known that the endotoxins in the dialysate affect the level of cytokines produced by the peripheral leucocytes. Studies have shown that lower levels of endotoxin contamination correlate with lower levels of inflammatory cytokines and oxidative stress markers. In a meta-analysis of 31 studies involving 1580 dialysis patients, the use of ultrapure dialysate has been found to reduce the oxidative stress markers, pentosidine, MPO and oxidized LDL cholesterol [25].

The loss of antioxidants, especially water-soluble vitamins such as vitamin C, has been demonstrated during the dialysis procedure [24, 26].

2.1.4. Inflammation

CKD has also been noted to be a systemic inflammatory state, which is intertwined with oxidative stress. Inflammatory cells stimulate the release of reactive species at the site of inflammation. Conversely, oxidized end products and ROS stimulate phagocytic cells, such as macrophages and neutrophils, to release inflammatory cytokines as well as more ROS, thereby creating a positive feedback loop of inflammation and oxidative stress state. When the phagocytic cells release ROS, they also induce nearby non-phagocytic cells to release inflammatory cytokines. Studies of oxidative states in people with CKD commonly include the investigation of inflammatory cytokines as the two pathways are intimately inter-related.

Multiple pathways have been identified that highlight the mechanisms of interplay between inflammation and oxidative stress (**Figure 2**). The master regulator of the inflammatory process is NF-kB transcription factor. Reactive oxygen species, such as H_2O_2, activate NF-kB which induces production of an array of inflammatory cytokines as well as activates NOX. These in turn stimulate further release of reactive species. Free radical-induced DNA base modifications can also act via NF-kB to activate the inflammatory processes.

NOX are responsible for free radical production by cells. They can be activated by inflammatory mediators, such as TGF, angiotensin II and TNF-α via other redox sensitive signal transduction pathways, such as c-Jun N-terminal kinase (JNK).

Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain 3 (NLRP3) is an inflammasome complex involved in the production of cytokines, such as IL-1β and IL-18. Oxidative stress can activate NLRP3 via oxidized mitochondrial DNA and thioredoxin-interacting proteins. Damaged mitochondria can directly release ROS, which perpetuate oxidative stress and inflammation.

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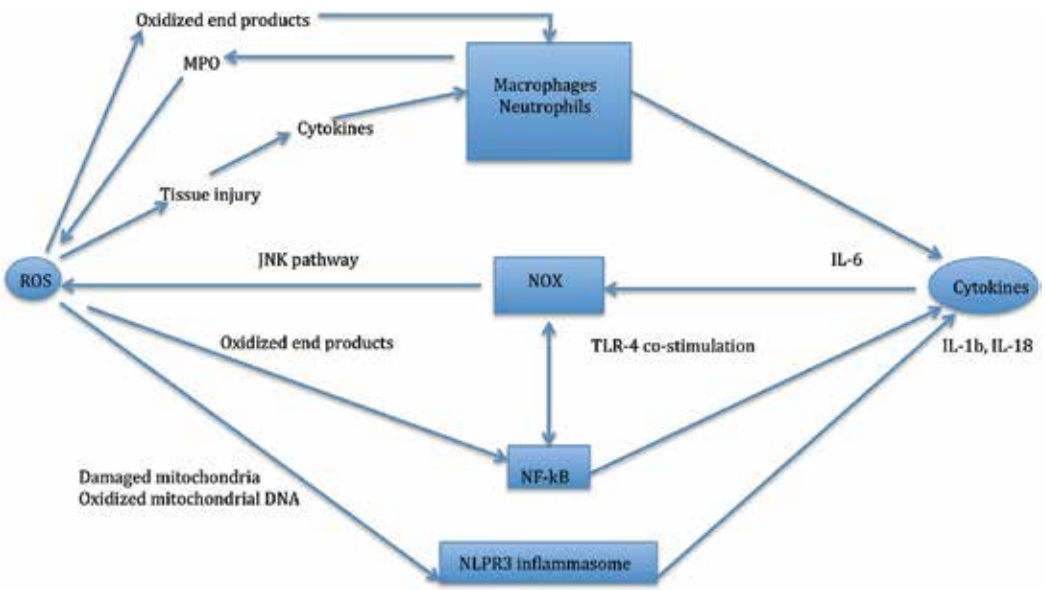

Figure 2. Interrelation between ROS (reactive oxygen species) and oxidized end products signifying the oxidative stress, and cytokines as part of the inflammatory process. ROS, reactive oxygen species; MPO, myeloperoxidase; NOX, NADPH-oxidases; NF-kB, nuclear factor kB; NLRP3, nod-like receptor protein 3; TLR4, toll-like receptor 4; JNK, c-Jun N-terminal; IL, interleukins.

Therefore an event inciting increased oxidative stress can result in an inflammatory response which further propagates the oxidative state, and vice versa.

2.2. Association of oxidative stress with CKD progression and cardiovascular disease (CVD)

A number of mechanisms have been reported to underpin the association between increased oxidative stress and progression of CKD and CVD in patients with CKD.

2.2.1. Oxidative stress markers and vascular disease

Increased oxidized low density lipoprotein (LDL) levels have been recognized as a risk factor for cardiovascular disease in uraemic patients [27, 28]. Oxidized LDLs stimulate the release of inflammatory cytokines from macrophages with subsequent vascular phenotypic changes and platelet aggregation [29], which result in endothelial dysfunction. Oxidized LDL also stimulates further oxidative activity by enhancing the activity of NOX in endothelial cells, juxtaglomerular cells and mesangial cells. Increased levels of ox-LDL in CKD patients correlate positively with carotid atherosclerosis and mortality [30].

Similarly, in patients with CKD, increased carotid intima-media thickness, a marker of atherosclerosis, has been strongly positively correlated with increased levels and activities of NOX, MDA, AOPP, TBARS, 8-OHdG and MPO [24, 31–34] and negatively correlated with SOD and GSH-Px levels [34].

Reactive oxygen species may also promote direct injury of vascular endothelial cells by inducing significant increases in nucleic acid damage in CKD patients [35].

2.2.2. Direct toxicity of oxidized end products

Oxidative stress (induced by H_2O_2) in experimental models of human proximal tubular epithelial cells is demonstrated to cause apoptosis and reduce mitosis of the renal cells. The results are highest when combined with induction of cell senescence, which itself can be induced by oxidative stress [36].

Gut-derived uraemic toxins, in addition to promoting oxidative stress, may exert direct toxicity to renal and cardiovascular tissues. In animal models, IS is implicated in the causation of vascular smooth muscle proliferation, aortic calcification, and vascular wall thickening thereby contributing to increased cardiovascular risk [16]. At the same time, it is also associated with reduced renal function, increased glomerular hypertrophy and glomerular sclerosis, signifying nephrotoxicity [16]. Similar findings were noted in human observational studies, where strong associations were observed between IS, free IS, PCS with cardiovascular disease [16, 37]. In a cross-sectional study involving 149 patients with CKD stages 3 and 4, Rossi et al. [38] investigated the potential mechanisms of toxicity of these uraemic toxins. It was found that the free forms of IS and PCS correlated positively with the level of inflammatory markers (IFN-γ, IL-6 and TNF-α) and negatively with the antioxidant GSH-Px. The total PCS concentration correlated positively with carotid-femoral pulse wave velocity as a measure of arterial stiffness. These findings suggest roles for elevated inflammatory and oxidative stress states as the causes of vascular and renal dysfunction.

Trimethylamine-N-oxide (TMAO), a metabolic end product of choline that is metabolized by the gut microbiota, has also been found to be correlated with an increased risk of cardiovascular disease [39].

2.2.3. Alterations in nitrogen handling

Nitric oxide is a free radical that plays an important role in regulation of endothelial function and regional blood flow by acting as a smooth muscle relaxant. The major pathway for nitric oxide production is by conversion of L-arginine by endothelial nitric oxide synthase (NOS) enzyme. Asymmetric dimethyl arginine (ADMA) is an inhibitor of NOS by competing with L-arginine and therefore reducing the availability of nitric oxide. It plays a significant contributory role in the development of endothelial dysfunction, and also correlates with proteinuria and progression of renal disease [40].

Yilmaz et al. [9] studied 159 patients with CKD stages 1–5 compared to 30 healthy controls. In addition to findings of reduced antioxidant activity and increased oxidative end products, it was also found that the levels of ADMA were higher in CKD patients compared to controls and were negatively correlated with renal function. The levels of oxidized LDL and ADMA were also found to be inversely related to brachial artery endothelium vasodilation. These findings raise the possibility that the elevated oxidative stress present in patients with CKD results from impaired endothelial function due to the inhibition of NOS by ADMA.

Additionally, reactive oxygen species degrade the critical co-factor of NOS causing functional impairment. Super-oxide anion also reacts with nitric oxide forming peroxynitrite thereby

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reducing availability of nitric oxide to the vascular smooth muscle. Peroxynitrite, due to its oxidative and nitrosative properties, perpetuates the vascular injury [40].

3. Systemic inflammation in patients with CKD

Systemic inflammation, most commonly evidenced by an elevation of CRP, is frequently observed in people with CKD, even early stage disease. In CKD, this systemic inflammation has been ascribed to a complex biological reaction in response to exposure to inciting exogenous stimuli such as bacterial pathogens or endogenous stimuli such as injured cells. It is mainly characterized by involvement of the cells of innate immune system and release of acute phase proteins and cytokines.

The most studied inflammatory marker in CKD patients is CRP. Elevated levels of CRP and the presence of a chronic inflammatory process in haemodialysis patients was first observed in 1980s with evidence accumulating over time of the heightened inflammatory state in CKD. CRP is produced by the liver as part of anti-inflammatory response and the rate of metabolism is nearly constant. Earlier studies involved mainly in haemodialysis patients where the elevation was noted consistently. The interest then expanded to non-dialysis ESKD patients and peritoneal dialysis (PD) patients. It is found that CRP is elevated even in ESRD patients receiving conservative care and also in PD patients [41]. This inflammatory state has in turn been linked with progression of CKD and CVD.

More recently, other biomarkers of inflammation have been studied. In the large Chronic Renal Insufficiency Cohort (CRIC) study [42], inflammatory markers (IL-1β, IL-1receptor antagonist, TNF-α, and fibrinogen and in addition to CRP) were found to be negatively correlated with kidney function and positively correlated with albuminuria (a marker of kidney damage). Another study showed an inverse correlation between IL-6 and kidney function [12]. The pattern of elevated pro-inflammatory cytokines (IL-1, IL-6, TNF-α) with low anti-inflammatory cytokines (IL2, IL4, IL5, IL12, CH50 and T cell number) has also been described in haemodialysis patients [43]. CRP, IL-6 and IL-10 levels are significantly higher in ESKD patients compared to controls and the levels did not change significantly after initiation of maintenance haemodialysis [44]. In another study, it was noted that CRP levels actually increased after initiation of haemodialysis [41].

Other markers of inflammatory status involve adipokines. There are proinflammatory adipokines and anti-inflammatory adipokines secreted by adipose tissue. Finally, the National Health and Nutrition Examination Survey (NHANES) demonstrated that levels of pro-inflammatory adipokines were higher in CKD patients than in the general population and that and the ratio of pro- to anti- inflammatory adipokines predicted mortality in PD patients [45].

3.1. Mechanisms of increased systemic inflammation

3.1.1. Gut dysbiosis

In addition to promoting oxidative stress, gut dysbiosis may also promote systemic inflammation in patients with CKD. Endotoxemia activates TLR4 on endothelial cells and macrophages

thereby leading to activation of NF-kB pathway and ultimately resulting in the production of inflammatory cytokines, chemokines, adhesion molecules, reactive oxygen species and systemic inflammation [39]. Gut-derived uraemic toxins, IS and PCS, are also associated with elevated levels of inflammatory markers such as IL-6, TNF-α, and interferon-γ (IFN- γ) [38]. Higher levels of IS and PCS have been documented in patients with CKD compared to the healthy population [46] and positively correlated with endothelial dysfunction [37].

3.1.2. Infection

Localized or systemic infections are more common in patients with CKD and in turn activate inflammatory and oxidative stress pathways. Dialysis patients are also at higher risk of infections due to presence of foreign bodies such as venous catheters, PD catheters or arteriovenous grafts, predisposing to blood stream infections. Periodontitis is also much more common in patients with CKD compared to normal population and has been linked with heightened cardiovascular risk. In an analysis of 861 CKD patients from NHANES data with a median follow up of 14.3 years, periodontitis was found to be associated with higher mortality. The presence of periodontitis increased the 10-year all-cause mortality from 32% (95% CI 29–35%) to 41% (36–47%), comparable to addition of diabetes to CKD at 43% (95% CI 38–49%) [47].

3.1.3. Dialysis

CKD patients display increased levels of inflammatory markers even prior to initiation of dialysis. Following commencement of dialysis, studies have variably shown either no change or a worsening of inflammatory and oxidative state [41, 44]. In haemodialysis patients, traces of lipopolysaccharides from dialysate contamination may stimulate the inflammatory process, which may be ameliorated by the use of ultrapure dialysate [48]. The presence of a foreign body, such as an arteriovenous graft, has also been shown to be associated with higher CRP levels and lower albumin levels, indicating a chronic inflammatory process [49]. Dialysis membranes may also have an impact, with cuprophane membranes eliciting higher levels of inflammatory biomarkers than other biocompatible membranes (polyamide or polycarbonate) [50]. Similarly, in PD patients, inflammation may be triggered by PD catheters and dialysis fluids [51].

3.1.4. Metabolic acidosis

Metabolic acidosis is increasingly more common with more advanced stages of CKD due to impaired renal excretion of acid generated by the body's metabolic processes. In patients with stage 2–4 CKD in the CRIC study, each mmol/L reduction in plasma bicarbonate concentration was associated with a 3% increased risk of progression to ESKD, although there was no association with mortality [52]. In haemodialysis patients, metabolic acidosis has been associated with increased circulating levels of the pro-inflammatory cytokine, IL-6, which was counterbalanced to some extent by increased levels of the anti-inflammatory cytokine, IL-10, and likely reflected a counter-regulatory mechanism [53]. On the other hand, another study by Ori [54] reported increased levels of IL-6 and reduced levels of IL10 indicating greatly augmented inflammatory status.

3.1.5. Vitamin D deficiency

In CKD, vitamin D levels are invariably reduced to various extents. In addition to its role in bone mineral metabolism, vitamin D has been associated with immunologic regulatory and antioxidant functions. For example, vitamin D has been found to potentiate the antioxidant effect of alpha-Klotho protein by increasing its gene expression [55]. Consequently, vitamin D receptor knockout mice have been reported to have augmented DNA damage and increased production of NADPH-dependent superoxide anion production [55]. In mouse models of HIV nephropathy, the downregulation of vitamin D receptor expression was observed together with increased reactive oxygen species generation and DNA damage, which was improved by vitamin D agonist supplementation [55].

3.1.6. Oxidative stress

A pathologic condition that increases oxidative stress, e.g. ischemia reperfusion injury, simultaneously and subsequently activates inflammatory pathways resulting in a state of both elevated inflammation and oxidative stress. The prominent feature is the two way cross-talk between NOX, NF-kB, inflammasomes and phagocytic cells such as macrophages, resulting in production of both ROS and inflammatory cytokines. The knowledge of underlying pathways and mechanisms are evolving due to ongoing research in this area.

3.2. Association of inflammation with CKD progression and CVD

In parallel with oxidative stress markers, increases in inflammatory markers have been associated with adverse cardiovascular and mortality outcomes. For example, plasma IL-6 levels have been recognized as an independent predictor of cardiovascular events, atherosclerosis progression and all-cause mortality [44]. Similarly, higher CRP levels correlate with increased carotid artery intima-media thickness in predialysis patients [56] and with increased mortality in both haemodialysis patients [57] and peritoneal patients [58]. Furthermore, Wanner et al. [11] found that a single CRP measurement can predict overall and cardiovascular mortality in the following 4 years. Haemodialysis patients with CRP levels in the highest quartile were associated with a 2.4- to 4.6-fold higher risk of all-cause mortality and a 1.7- to 5.5-fold higher cardiovascular mortality compared to those with CRP levels in the lowest quartile [11, 59]. Similarly, in a prospective observational study involving 62 haemodialysis patients, total mortality was 37.1% and cardiovascular mortality 16.1% at the 2 year follow up. All-cause and cardiovascular mortality were significantly increased at CRP levels above 5 mg/L [60].

4. Potential therapeutic strategies targeting oxidative stress and inflammation

With the increasing understanding of the mechanisms underpinning oxidative stress and systemic inflammation in CKD, various interventions have been explored to address these issues (**Table 2**). The challenging aspect of this complex pathological state is that inflammation and

oxidative stress are two very intertwined processes. A primary disorder with elevated oxidative stress would inevitably end up with increased inflammatory state and vice versa. Since multiple cellular components, pathways and end products are involved in the pathogenesis and perpetuation of oxidative stress and inflammation, it has been difficult to attain a clinically meaningful impact by therapy targeted at just one aspect. With the exception of RAAS inhibition that has been well established to improve renal and cardiovascular outcomes in patients with CKD, the evidence for the majority of other interventions has been limited by small studies with short follow up duration, high degrees of heterogeneity and limited data available on patient-level outcomes.

4.1. Lifestyle modifications

4.1.1. Dietary interventions

Dietary management is an integral part of the treatment of CKD, especially in its advanced stages. The current scope of dietary modification includes fluid restriction, sodium, potassium and phosphate restriction, and achievement and maintenance of a healthy body weight.

It has been shown that the uraemic toxins, IS and PCS, are produced by the breakdown of nitrogenous waste products by gut bacteria and are associated with negative renal and cardiovascular outcomes due to systemic inflammation and oxidative stress. Dietary modifications have been explored in order to reduce the production of uraemic toxins by the gut bacteria. It is found that the balance between protein and dietary fibre intake appears to have a more significant impact on the production of uraemic toxins than the absolute intake of either protein or fibre alone. Rossi et al. [61] performed a cross-sectional study of 40 CKD stage 4 and non-dialysis CKD 5 patients and correlated the protein-fibre index with serum IS and PCS levels. It was found that the ratio of the protein to fibre intake had significant associations with the serum IS and PCS levels. The total dietary fibre intake had significant associations with PCS but not with IS and the total protein intake had no association with either toxin.

The benefit of dietary fibre supplementation has also been explored in order to increase the carbohydrate content in the colon and to reduce protein fermentation. In a randomized controlled study involving 56 haemodialysis patients, fibre supplementation was found to significantly reduce the level of IS, and to a lesser extent the level of PCS at 6-week follow up [62]. In another non-randomized prospective study, significant reductions in PCS but not IS were found in haemodialysis patients when given dietary fibre supplements [63]. In a large population based cohort study involving 1110 community-dwelling elderly men (around 45% of participants with GFR < 60 ml/min/1.73 m^2), dietary fibre intake was found to be associated with higher GFR, lower CRP and lower mortality at 10 year follow-up [64]. In a meta-analysis of 14 studies on dietary fibre supplementation in CKD patients, reduction in serum creatinine and urea was noted [65]. However, the trials are very small (n = 3–22) and the dose of fibre supplementation was also highly variable. While the available data suggest dietary fibre supplementation may slow the progression of CKD, robust studies with meaningful clinical end points are lacking.

Nitrogenous waste products can be reduced by dietary protein restriction, which in turn would favour the growth of saccharolytic bacteria over proteolytic bacteria in the colon.

Lifestyle modifications

1. Dietary interventions

- Increased dietary fibre intake
- Protein restriction
- Increased fibre-to-protein ratio
- Sodium restriction
- Fluid restriction
- Pomegranates
- Soy milk

2. Prebiotics and probiotics

3. Exercise

Optimization of dialysis procedure

1. Use of ultrapure dialysate

2. Modification of dialysis membranes

- Biocompatible membranes
- High-flux membranes
- Vitamin E-coated membranes

3. Modification in dialysis technique and frequency

- Online haemodiafiltration
- Short daily, extended, frequent dialysis sessions

Pharmacologic therapies

1. Oral absorbents

2. Allopurinol

3. N-acetyl cysteine

4. Omega-3-polyunsaturated fatty acids

5. Bardoxolone

6. Statins

7. Cytokine therapies

Antioxidants

1. Vitamin E

2. Vitamin C

3. L-carnitine

4. Coenzyme Q-10

5. Miscellaneous antioxidants

- Vitamin A, selenium, zinc, methionine, alpha-lipoic acid, curcumin

Table 2. Interventions in studies to improve oxidative stress and systemic inflammation in chronic kidney disease.

However, the benefit of protein restriction per se in CKD patients is overshadowed by the risk of malnutrition and its complications.

Dietary sodium restriction has been explored as a potential strategy for reducing systemic inflammation either directly or indirectly via reducing fluid overload and extracellular volume expansion [66–68]. In a randomized controlled study involving 53 haemodialysis patients, significant reductions in CRP, IL-6 and TNF-α were found in the sodium-restricted group at 8 weeks, which persisted at 16 weeks [69]. However, in a randomized trial by Campbell et al. [70] in patients with stage 3 or 4 CKD, no differences in inflammatory markers were observed after 2 weeks. The apparent disparity in the results of these two studies may be explained by the different stages of renal failure and the duration of intervention.

Pomegranates contain polyphenols, which are known to have antioxidant and anti-inflammatory properties. The number of trials studying on the effect of pomegranate extract or juice on inflammatory markers has been increasing over the last 15 years [71]. There have been a few human trials with varying durations of follow up between 1 day and 12 months. Observed changes in inflammatory markers were variable but the randomized controlled study with longest duration of follow-up of 12 months demonstrated reductions in all inflammatory markers in haemodialysis patients [72].

Soy milk contains isoflavones that are a subgroup of polyphenols with antioxidant properties. Although there have only been small studies with short follow up durations, soy milk has been reported to reduce inflammatory markers and improve renal function deterioration, proteinuria and lipid profile [73].

4.1.2. Prebiotics and probiotics

A number of studies have been conducted to explore the effect of supplementation of synbiotics (a combination of prebiotics and probiotics) on clinical outcomes in both dialysis and non-dialysis CKD patients. These studies have generally noted reductions in uraemic toxins (dimethylamine, nitrosodimethylamine, blood urea nitrogen, plasma p-cresol) and improvement in gastrointestinal scores and quality of life, although reductions in uric acid and alterations in microbiota have not been consistently identified [16, 39]. In a recent randomized, placebo-controlled cross over trial (SYNERGY) [74], 37 patients with stage 4 or 5 CKD (non-dialysis) received synbiotic treatment or placebo for 6 weeks, followed by a washout period of 4 weeks and a cross over to the alternative treatment arm for a further 6 weeks. The prebiotic component consisted of high-molecular weight inulin, fructo-oligosaccharides and galacto-oligosaccharides. The probiotic component consisted of nine different bacterial strains including *lactobacillus*, *bifidobacteria* and *streptococcus* genera. The administration of synbiotics significantly reduced serum PCS levels and favourably modified the gut bacteria. In a pre-specified analysis in which patients who received antibiotics during the study were excluded, serum IS levels were also significantly reduced by the symbiotic intervention. No significant changes in anti-inflammatory markers were noted. Thus, the study provided proof of concept that synbiotics can modify the gut microbiota and serum uraemic toxin levels in people with CKD. A larger randomized, placebo-controlled trial with 12-month follow up is currently underway which may yield more definite answers regarding the clinical utility of the synbiotic therapy.

4.1.3. Exercise

Physical exercise has been reported to improve proteinuria and progression of renal function in non-dialysis CKD patients [75] and improve cardiac function and other cardiovascular risk factors in haemodialysis patients. Although multiple pathways can be involved, reduction in the systemic inflammatory state and modulation of immune function may be a major contributing factor for these benefits.

An improvement in inflammatory markers has been noted after bouts of acute exercise or regular long-term exercise with both resistance training or aerobic exercise programs. Viana et al. [76] studied 15 predialysis CKD patients and found that 30 min of walking promoted an anti-inflammatory milieu by increasing the anti-inflammatory cytokine IL-10. An increase in serum IL-6 concentration was also noted, although the authors concluded that the muscle-derived IL-6 in the study exerted anti-inflammatory effects rather than the pro-inflammatory IL-6β. The group also followed up the patients after 6 months of a regular walking exercise program (30 min per day for 5 days a week) and found that the increased IL-10 levels were sustained with a reduction in the ratio of IL-6 to IL-10. T cell and monocyte activation were down-regulated without an effect on the numbers likely representing the modulation of a chronically elevated inflammatory state. In a randomized controlled trial involving 26 non-dialysis CKD patients, resistance exercises for 45 min 3 times a week were also found to reduce CRP and IL-6 after 12 weeks, together with improvements in muscle mass and endurance [77].

4.2. Optimization of dialysis

Given the significantly elevated oxidative stress and systemic inflammatory levels observed in dialysis patients, different modifications to conventional dialytic therapy have been investigated. These interventions include the use of ultrapure dialysate to reduce endotoxin load, changes in membrane types (such as biocompatible membranes, high-flux membranes, and vitamin E coated membranes), modifications in the dialytic techniques (such as on-line haemodiafiltration) and alteration of dialysis frequency (such as short daily dialysis or nocturnal dialysis).

To reduce the inflammation induced by bacterial endotoxins in the dialysate, the use of ultrapure dialysate was investigated. In the meta-analysis by Susantitaphong et al. [25], 31 studies were analysed which included 16 single-arm studies, 5 non-randomized studies and 10 randomized controlled trials. It was found that the use of ultrapure dialysate compared to conventional dialysate significantly reduced CRP and IL-6 levels in all studies. Single arm studies showed a decrease in TNF-α and IL-1 although the controlled trials failed to show a significant decrease in these markers. In terms of oxidative stress state, all studies showed significant decreases in pentosidine, MPO and ox-LDL levels. These findings support the use of ultrapure dialysate as a standard of care in dialysis therapy.

Sequelae of bioincompatible membranes in activation of complement and inflammatory pathways have been long recognized [78]. The historic use of bio-incompatible cellulose-based membranes has now been replaced by use of biocompatible synthetic membranes such as polysulphone and polypropylene.

Due to its antioxidant properties, vitamin E has been used as a membrane surface modifier to improve biocompatibility and confer additional benefit with respect to oxidative stress.

A recent meta-analysis conducted by D'Arrigo et al. [79] included 60 studies, 23 of which were randomized controlled studies and 37 were non-randomized studies. Improvement in oxidative stress was evidenced by a decrease in MDA and TBARS, without any change in other parameters, such as SOD or NOX. Reduction in ox-LDL levels became significant after improving heterogeneity by excluding three parallel studies. The use of vitamin E-coated membranes reduced the IL-6 levels without any change in CRP.

The clearance of pro-oxidant middle molecules, such as uraemic toxins, in haemodialysis may be enhanced by online haemodiafiltration. Using data from a large randomized controlled trial CONvective TRAnsport Study (CONTRAST) investigating the mortality and cardiovascular outcomes of online haemodiafiltration versus conventional low flux haemodialysis, Den Hoedt et al. [80] analysed a sub-group of 405 patients for changes in inflammatory markers after 3 years. Significant differences in the CRP and IL-6 levels became apparent after 6 months of the study. However, mortality and cardiovascular benefits were not noted in the main study.

Increasing dialysate flow rate, utilizing super-flux membranes or adding a sorbent to dialysate could also potentially improve the clearance of gut-derived, protein-bound uraemic toxins. A small study found that increasing the dialyzer mass transfer area coefficient by using two dialyzers in series improved the clearance of protein-bound molecules compared to the conventional use of a single dialyzer. Another small study also showed that by increasing the dialyzer size and dialysate flow in nocturnal haemodialysis patients, the clearance of IS and PCS were enhanced [16].

In terms of dialysis duration and frequency, short daily dialysis (3 h sessions for 6 days a week) was associated with significantly reduced levels of CRP compared with conventional dialysis (4 h sessions for 3 days a week) with corresponding improvement in erythropoietin stimulating agent sensitivity [81]. In a meta-analysis by Susantitaphong [82], there was improvement in cardiac parameters, such as left ventricular mass index (LVMI), left ventricular ejection fraction (LVEF) and blood pressure, in patients using frequent (2–8 h, > thrice weekly) or extended (>4 h, thrice weekly) haemodialysis, compared to a conventional (≤4 h, thrice weekly) haemodialysis schedule. Beyond utilization of ultrapure dialysate and high-flux online haemodiafiltration, the addition of further convective permeability by utilizing hyper high flux membranes did not confer any extra benefits in oxidative stress markers [83].

4.3. Pharmacologic therapies

4.3.1. Oral absorbents

AST-120 is the only oral charcoal absorbent available to reduce the absorption of indole in the gastrointestinal tract, thereby reducing the systemic concentrations of IS. It is possible that other uraemic toxins are also absorbed. It is widely used in CKD patients in a number of Asian countries to prolong the time to initiation of dialysis. Initial retrospective and prospective studies showed an enhanced clearance of the toxins as well as improved clinical outcomes with AST-120 [16]. However, in two randomized controlled trials (EPPIC-1 and EPPIC-2) [84] involving 2035 patients with moderate to severe CKD (sCr 2–5 mg/dL for men and 1.5–5 mg/dL for women at screening) in 13 countries, AST-120 did not significantly alter the primary composite end point of dialysis initiation, transplantation or doubling of serum creatinine compared

with placebo (HR 1.03, 95% CI 0.84–1.27, p = 0.78 in EPPIC-1 and HR-0.91, 95% CI 0.74–1.12, p = 0.37). Notwithstanding their apparent lack of efficacy in these large trials, the main limitations of orally administered absorbents is poor compliance due to pill burden with the need to take 30 pills a day, and gastrointestinal side effects, such as constipation, diarrhoea, nausea, abdominal distension and flatulence.

4.3.2. Allopurinol

Uric acid is the final product of purine metabolism. Production of uric acid is catalysed by the enzyme xanthine oxidase, which also generates reactive oxygen species in the process. Hyperuricaemia has been found to be associated with increased RAAS activity [85], hypertension [86], endothelial dysfunction [87] and cardiovascular disease [88]. It is also associated with higher mortality in patients with non-dialysis CKD patients [89] as well as haemodialysis patients [90]. A number of single centre studies have shown that inhibition of xanthine oxidase by allopurinol or febuxostat may slow the progression of renal disease and improve cardiovascular outcomes [91, 92]. In the meta-analysis by Bose et al. [93] involving eight randomized controlled studies of allopurinol treatment found that no significant changes in glomerular filtration rate (GFR) in five studies, but improvement in serum creatinine concentrations in three studies. There was appreciable heterogeneity in the studies in terms of baseline GFR (three studies with baseline GFR > 67 mL/min/1.73 m^2), follow up duration (4–24 months) and etiology of CKD. They were all single centre studies with small sample sizes (n = 36–113). Since the publication of the meta-analysis, a long-term follow up outcome of a previous trial by Goicoechea et al. [94], and four additional randomized trials have been published. Goicoechea et al. [94] found that there was reduction in the number of renal and cardiovascular events in the allopurinol arm compared to the control group (HR 0.32 and HR 0.43 respectively) at 5 years of follow up. However, definitive conclusions regarding the safety and efficacy of urate lowering therapies in CKD cannot be drawn at this stage.

Currently there are three multi-centre, randomized, double-blinded prospective trials being conducted. The first trial, *the CKD-FIX Trial: Controlled trial of slowing of Kidney Disease progression From the Inhibition of Xanthine oxidase*, will involve 620 patients with CKD stage 3 or 4 and albuminuria and decline in GFR of at least 3 ml/min/1.73 m^2 in the preceding 12 months. Hyperuricaemia is not mandatory for inclusion. The intervention will be allopurinol dose escalated from 100 mg daily to 300 mg daily in a stepwise manner according to patient tolerance. The follow up period will be 104 weeks and the primary outcome measure of changes in GFR will be assessed. The second trial, *FEATHER Trial: FEbuxostat versus placebo rAndomized controlled Trial regarding reduced renal function in patients with Hyperuricemia complicated by chRonic kidney disease stage 3*, will titrate febuxostat dose from 10 to 40 mg in the first 9 weeks in 400 participants with CKD stage 3. Hyperuricaemia with serum uric acid 7.1–10 mg/dL is required for inclusion in the study. The primary outcome measure of GFR slope will be assessed. The third trial, *The Preventing Early Renal Function Loss (PERL) Allopurinol Study*, will include subjects with type1 diabetes with albuminuria, GFR 45–100 ml/min/1.73 m^2 and serum uric acid ≥4.5 mg/dL. The primary outcome measure of GFR will be measured at the end of the three-year study period. At the conclusion of the current trials, it is hoped that more robust evidence will be obtained regarding the effect of uric acid lowering therapy on progression of renal disease in patients with pre-existing CKD.

4.3.3. N-acetyl cysteine

N-acetyl cysteine provides L-cysteine, which is the rate-limiting precursor to glutathione synthesis, thereby enhancing antioxidant defenses. It also acts as a scavenger of free radicals. Even though there is evidence of reduction in oxidative activity in animal models and dialysis patients [95–97], a small randomized controlled trial in patients with proteinuria and early CKD showed no difference in proteinuria between patients treated with N-acetyl cysteine and placebo [98].

4.3.4. Omega-3-polyunsaturated fatty acids

Eicosapentaenoic and docosahexaenoic acids are the two major bioactive omega-3 fatty acids mainly derived from dietary sources. In animal models, they have been shown to improve the anti-oxidant systems and reduce inflammation and tubulointerstitial fibrosis [99]. Numerous studies have been conducted in dialysis patients investigating their effects on inflammatory markers, nutritional status and lipid profile. In haemodialysis patients, there is evidence of inhibition of up-regulation of endothelial chemokines [100] and reduction of all-cause mortality [101]. However, a study conducted in continuous ambulatory peritoneal dialysis (CAPD) patients did not show any significant changes in SOD and reduced glutathione (GH) levels [102]. In non-dialysis CKD patients, IL-1β and TBARS were reduced and SOD and GH levels were improved, but no effect on IL-6 and TNF-α was noted [103, 104].

4.3.5. Bardoxolone

Nuclear factor erythroid 2-related factor (Nrf-2) is a nuclear transcription factor which generates the production of antioxidant enzymes via induction of antioxidant response element (ARE) genes. It is activated by increased oxidative stress, such as reactive oxygen and nitrogen species, and induces the ARE gene. This in turn results in production of reducing factors such as NADPH and the elimination of reactive oxygen species by antioxidant enzymes such as SOD and GSH-Px [105]. In animal models, reduced activity of Nrf-2 produced tubular injury and progressive fibrosis which can be ameliorated by administration of Nrf-2 activators [106]. In a randomized controlled trial involving 227 diabetic CKD patients, improvement in the renal function was noted at 24 weeks which persisted at 52 weeks in the intervention group with Nrf-2 activator, bardoxolone methyl [107]. Based on these initial findings, a prospective, randomized controlled trial was conducted in 2185 patients with type 2 diabetes mellitus and stage 4 CKD with the intervention of bardoxolone methyl (20 mg daily per os) versus placebo [108]. At 9 months, the patients receiving bardoxolone methyl experienced a significantly higher rate of heart failure-related hospitalizations or deaths (hazard ratio 1.83, 95% CI 1.32–2.55, $p < 0.001$) prompting premature termination of the trial. Bardoxolone methyl did not reduce the risk of the primary composite end-point of ESKD or death from cardiovascular causes (HR 0.98, 95% CI 0.70—1.37, $p = 0.92$). Although further trials are underway addressing this aspect, the potential therapeutic role of bardoxolone methyl in patients with CKD appears limited.

4.3.6. Statins

Statins, 3-hydroxyl-3-methylglutaryl coenzyme A reductase inhibitors, are well-established treatment for hypercholesterolemia. New evidence has recently emerged that statins may have

an additional role in improving systemic inflammation. In a meta-analysis by Deng et al. [109], nine randomized controlled trials involving 3098 dialysis patients were analysed. Three studies assessed CRP and six studies utilized hs-CRP. One study also included IL-6 and TNF-α in the assessment. All the studies found significant reductions in CRP and hsCRP in the patients treated with statins, while the control group experienced an increase or no change in inflammatory markers. IL-6 levels did not change but there was a significant decrease in TNF-α in one study. Overall, there is evidence that improvement in systematic inflammatory status achieved by statins may play a contributory role in primary and secondary prevention of atherosclerosis.

4.3.7. Cytokine therapies

Attempts have been made to directly target pro-inflammatory cytokines by investigating the role of anti-IL-1, anti-IL-6 and TNF-α. In a small, randomized controlled trial with 22 haemodialysis patients, administration of an IL-1β antagonist improved the levels of hs-CRP and IL-6 and anti-inflammatory adiponectin after 4 weeks [110]. Currently, the available evidence of the direct anticytokine therapy is limited.

4.4. Antioxidants

A number of small trials have been conducted using antioxidants of various types, such as vitamins, naturally occurring dietary extracts, and trace elements. Of the current available information, the Cochrane systematic review in 2012, prior to the BEACON study, concluded that antioxidants confer a significant reduction in serum creatinine, changes in GFR and risk of ESKD but no difference in cardiovascular outcomes [111]. However, there was a high degree of heterogeneity in the meta-analysis. In another meta-analysis on diabetic kidney disease, it was found that there was a significant reduction in albuminuria, but no evidence in other renal outcomes [112].

4.4.1. Vitamin E

The vitamin E family consists of tocophenols in saturated form, and tocotrienols in unsaturated form with a side chain of an isoprenoid. They both play a role in reducing oxidative stress by scavenging free radicals, inhibiting pro-inflammatory pathways and increasing levels of other antioxidants. Meta-analyses of studies in haemodialysis patients using vitamin-E coated dialysis membranes, and oral supplements in CKD patients failed to improve any clinical outcomes although some studies did show improvements in oxidative markers [79, 112, 113]. In a randomized controlled study, *Secondary prevention with antioxidants of cardiovascular disease in end stage renal disease* [114], 196 haemodialysis patients were randomized to vitamin E group receiving 800 IU/day or placebo and followed up for 519 days. At the end of the study period, there was a 40% reduction in cardiovascular end points in the group receiving vitamin E, mainly driven by a reduction in the incidence of myocardial infarction. In contrast, a post-hoc analysis of another randomized controlled study, *Heart Outcomes Prevention Evaluation* [115], involving 993 patients with mild to moderate renal insufficiency, found no benefit from administration of vitamin E 400 IU/day (RR 1.03, CI 0.79–1.34, p- = 0.82). The apparent disparity in findings between the two studies may be due to the differences in the degree of renal impairment and dose of vitamin E. Nevertheless, the role of vitamin E in mitigating cardiovascular risk in CKD patients remains uncertain at this point in time.

4.4.2. Vitamin C

Vitamin C exerts its antioxidant properties by acting as an electron donor to free radicals. A number of small studies have found improvements in inflammatory markers such as CRP [116], hsCRP [117], and 8-OHdG [118] in haemodialysis patients. In a meta-analysis of randomized controlled trials examining use of antioxidants in diabetic kidney disease, vitamin C reduced albuminuria in some studies but had no effect on GFR [111]. All the studies were small (n = 14–29) with short durations of follow up (4 weeks to 12 months) and generally were of suboptimal methodologic quality. Consequently, no conclusions can currently be drawn regarding the safety and efficacy of vitamin C therapy in patients with CKD.

4.4.3. L-carnitine

Carnitine is an endogenous product of amino acid metabolism produced in the liver. It acts as a transporter of long chain fatty acids across the mitochondrial membrane by reversibly substituting the acyl group of Coenzyme A, forming acyl-carnitine. Once the fatty acids have been transported into the mitochondrial matrix, acyl-carnitine dissociates to form L-carnitine again and coenzyme A is regenerated. L-carnitine has been noted to reduce the oxidative stress through increased glutathione levels, increased GSH-Px activity, and a decreased MDA levels. In a meta-analysis by Chen et al. [119] of 49 RCTs involving 1734 haemodialysis patients, L-carnitine was found to improve CRP and LDL levels. However, in another meta-analysis by Yang et al. [120] involving 25 RCTs, contradictory evidence was found in that L-carnitine did not appreciably alter inflammation, oxidative stress, hyperlipidaemia or quality of life. Currently, there is insufficient evidence to support L-carnitine administration to CKD patients.

4.4.4. Coenzyme Q10

Coenzyme Q10 is a ubiquinone which contains one quinine group and 10 isoprenyl units. It acts as an enzyme co-factor in inner mitochondrial cell membranes protecting against damage from free radicals produced by oxidative phosphorylation. It also stabilizes the cell membranes as an electron and proton carrier and restores vitamin E in its antioxidant form. Early studies in rat models of diabetic nephropathy showed reduced mesangial expansion and tubulointerstitial fibrosis after administration of mitochondrial-targeted coenzyme Q10 [121]. Two recent studies in dialysis patients showed significant reductions in F2-isoprostanes and isofurans at high doses of 1200 g and 1800 g respectively [122, 123]. Further studies exploring the effects of Coenzyme Q10 on disease progression in CKD patients and cardiovascular complications in dialysis patients will be valuable.

4.4.5. Miscellaneous antioxidants

Studies have been conducted to explore the role of other antioxidants, such as vitamin A, selenium, zinc, methionine, alpha-lipoic acid, and curcumin. However, thus far, there is no strong evidence to support their routine use in clinical practice.

5. Future directions

CKD constitutes a state of increased oxidative stress and systemic inflammation. These processes are pathogenetically interrelated and there is increasing evidence that they may contribute to CKD progression and a disproportionately increased cardiovascular risk through the promotion of endothelial dysfunction, atherosclerosis and vascular calcification. The various mechanisms of action of this increased oxidative stress are increasingly being elucidated. Interventions to reduce oxidative stress and inflammation in these patients present novel, and potentially effective approaches to add to the currently available traditional risk-modification strategies. Due to the complex interrelation between reactive oxygen species and inflammatory markers, it is possible that simultaneous, multiple targeted approaches may be required to effectively address the pathological changes in CKD and its associated cardiovascular risk. Larger trials with meaningful clinical outcomes and longer follow up are required to further evaluate such potential therapies.

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Chapter 3

Role of Oxidative/Nitrosative Stress in Diarrhea and Constipation

Kaïs Rtibi, Hichem Sebai and Lamjed Marzouki

Additional information is available at the end of the chapter

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Abstract

Oxidative/nitrosative stress, a pervasive condition of increased amounts of reactive and nitrogen species, is responsible for a variety of degenerative processes in some human diseases such as gastrointestinal affections. Diarrhea is one such infection that has long been recognized as one of the most important health problems in developing countries. Constipation is a delay or difficulty in evacuating the stool. In this respect, several studies were performed and have shown that the diarrhea pathophysiology and constipation were accompanied by accumulation of biomarkers of oxidative/nitrosative stress as well as the depletion of antioxidant system. In this chapter, we discuss about the recent advances that propose a major role of oxidative/nitrosative stress on diarrhea pathogenesis and constipation.

Keywords: oxidative/nitrosative stress, reactive and nitrogen species, diarrhea pathogenesis

1. Introduction

Reactive oxygen species (ROS)/reactive nitrogen species (RNS) are produced as the by-products of the normal metabolic mechanism in all aerobic organisms [1]. The augmentation of oxidative/nitrosative stress normally describes a situation in which cellular antioxidant capacities are incapable to scavenge the ROS and RNS engendered as a result of massive generation of ROS/RNS, loss of antioxidant defenses, or both. The ROS/RNS cause disruptions in the cellular macromolecules such as the oxidative degradation of lipids, DNA lesion and proteins alteration [2].

Constipation is defined as infrequent or difficult evacuation of feces leading to water absorption, hardening of stool in colon, and excessive straining. This gastrointestinal disorder is a risk factor of colorectal cancer [3].

Diarrhea is usually a result of gastrointestinal infection, which can be induced by various microorganisms such as viruses, bacteria, and parasites. Despite different pathophysiological changes in different types of diarrheas, there are four major mechanisms responsible for this gastrointestinal disruption in electrolyte and water exchange, that is, elevated luminal osmolarity, increased electrolyte secretion, decreased electrolyte absorption, and accelerated intestinal motility [4].

Therefore, the objective of this chapter is to discuss, based on the literature, the contribution of oxidative/nitrosative stress in gastrointestinal disorders such as constipation and diarrhea.

2. Oxidative/nitrosative stress and gastrointestinal disorders

Alterations in the digestive tract such as constipation and diarrhea are caused by many external agents and factors. These disturbances are accompanied by the installation of oxidative/nitrosative stress, which can cause various disruptions in gastrointestinal intestinal function (**Figure 1**).

2.1. Oxidative stress and diarrhea

Many literature studies suggest the involvement of oxidative stress in the aggravation of diverse perturbations, including gastrointestinal infectious diseases produced by pathogens. These results indicate that Rotavirus induces a generation of ROS and deficiency in the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio [5]. Added to that, it has been shown also that diarrhea induced by bacterial infections was combined with an oxidative injury. Indeed, during the steps of salmonellosis, ROS are also generated which provokes a depletion of glutathione in intestinal epithelial cells [6].

Other researches have shown the implication of oxidative stress in castor oil-induced diarrhea. Therefore, recent studies have shown that acute administration of castor increased the formation of malondialdehyde (MDA) in the gastrointestinal tract mucosa indicating an increase in lipid peroxidation. This process presents a possible mechanism of tissue alteration by oxygen reactive derivatives [7, 8]. Furthermore, current findings showed that intestinal hypersecretion was also accompanied by H_2O_2 generation in mucosal intestine. H_2O_2 can lead to the formation of toxic ($^{\bullet}OH$) which oxidizes important cellular components and induces the depletion of glutathione. Oxidative damage of lipids provokes a membrane fluidity alteration, disruption in ion transport, loss of membrane integrity, and finally, cellular function disturbance [9].

Other studies reported that diarrhea was able to induce deleterious effects on the sulfhydryl (—SH) group and generation of protein carbonyls. These effects can be explained by the proteins oxidation process, which leads to the dysfunction of many enzymes [10].

Enzymatic antioxidants including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) have an important role in the prevention of oxidative damage by reactive oxygen species. SOD plays a crucial action in dismutation of superoxide radicals to H_2O and oxygen. On the other hand, catalase protects the cells from toxic effects of ROS by transforming

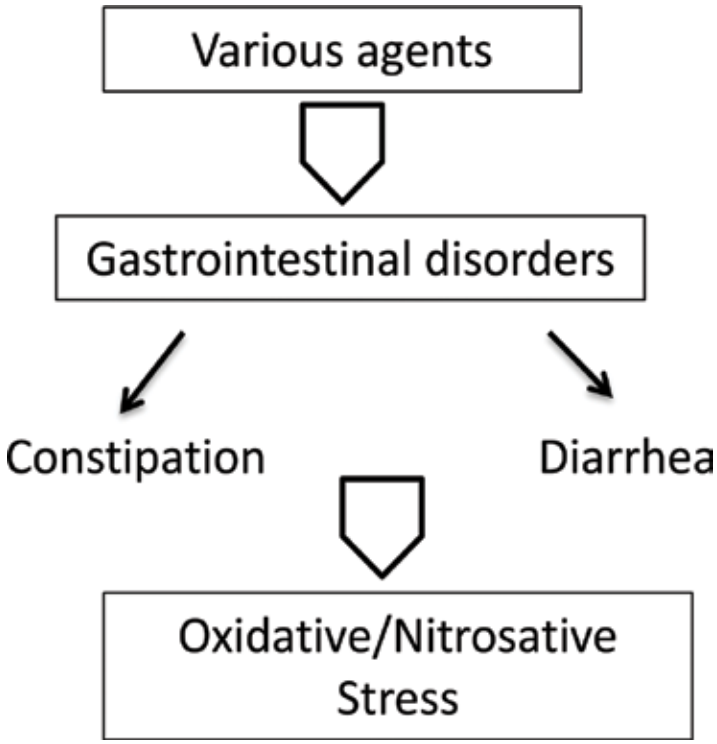

Figure 1. Contribution of oxidative/nitrosative stress in gastrointestinal disorders including diarrhea pathophysiology and constipation pathogenesis.

H_2O_2 to H_2O and O_2 [11]. In addition, glutathione peroxidase has a high affinity for hydrogen peroxide; it therefore allows for the removal of hydrogen peroxide, even when present at a low concentration. In this respect, numerous studies have reported that castor oil-induced diarrhea causes a depletion of antioxidant activities of SOD, CAT, and GPx, which explains the overproduction of ROS [12, 13].

2.2. Oxidative stress and constipation

On the other hand, several studies have reported an increased oxidative stress and imbalance in antioxidant enzymes following the administration of antineoplastic agents that induced the constipation. In this respect, the use of vinblastine was provoked by the installation of constipation which is associated with a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses in intestinal mucosal barrier. This mechanism was evaluated by lipid peroxidation, protein oxidation, and damaging actions on sulfhydryl groups. Disorders in the normal redox state of cells can induce toxic activities through the generation of free radical reactive oxygen species that induce cell injury and alter these cellular macromolecules [14]. These obtained results are in agreement with those found by Li et al. [15] who revealed that the level of MDA augmented in constipated rats. In addition, other previous reports indicate that chronic constipation can cause potential oxidative stress in children and depletion of antioxidant enzyme activities [16].

2.3. Nitrosative stress and diarrhea

The castor oil-induced diarrhea model and intestinal mucosal injury responses may involve the nitric oxide that caused an enhancement of epithelial layer permeability to calcium ions, leading to an accumulation of intracellular Ca^{2+} and improvement of calmudin activation of NO synthetase action. At this level, the NO could cause the hypersecretion process in the small bowel. It was later proved in many research studies that NO and prostaglandins are strongly involved in the inflammatory pathway produced by castor oil [17].

3. Conclusion

These data clearly demonstrate the implication of oxidative/nitrosative stress in gastrointestinal disorders such as diarrhea and constipation.

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Chapter 4

Role of Reactive Oxygen Species in Male Reproduction

Sabiha Fatima

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Abstract

The production of reactive oxygen species (ROS) is a normal physiological event in the male germ line. ROS are a double-edged sword, despite its role as key signaling molecules in physiological processes such as capacitation and hyperactivation, its overproduction which overwhelms the body's antioxidant defenses is thought to affect male fertility and normal embryonic development. The excess generation of ROS in semen by exogenous and endogenous factors has been recognized as detrimental etiologies for male infertilities. Spermatozoa are vulnerable to ROS attack because they are rich in mitochondria, have abundance of substrates for free radical attack and their capacity to protect themselves from oxidative stress is limited. The cytotoxic aldehydes generated as a result of lipid peroxidation are known to form adduct with the mitochondrial protein involved in electron transport chain and stimulate generation of ROS in mitochondria. ROS and their metabolites can lead to oxidative DNA damage in mitochondria and nucleus that eventually culminates in DNA fragmentation. The presence for large amount of damaged DNA is a major characteristic of defective human spermatozoa, which affect the fertility and pregnancy outcome. Thus, as a comprehensive approach, treatment of oxidative stress should involve strategies to reduce stress-provoking conditions to help reverse sperm dysfunction.

Keywords: ROS, oxidative stress, male infertility, DNA damage

1. Introduction

Infertility is a disorder affecting 10–15% couples of reproductive age worldwide [1, 2]. It is defined as the inability of a couple to achieve spontaneous pregnancy after 1 year of regular, unprotected sexual intercourse [3]. The inability to have children affects the infertile couples psychologically and it may lead to depression, suicidal tendencies and other pathological and psychological conditions [4, 5]. Although, fertility may decrease with increase in age, but often occurs as a result of anatomic defects, endocrinopathies, immunologic problems, gene

mutation, ejaculatory failures or radiation, chemotherapy and environmental exposures [6–9]. In approximately half of all the cases of infertility, male factor is the sole or major contributing factor with no identifiable cause found in over 25% of infertile males [10, 11]. In approximately 40–50% of the male infertility cases, oxidative stress-related mechanisms are found to be responsible for the impairment of the sperm function and fertilization [12]. Oxidative stress is a disturbance in the balance between the systemic manifestation of reactive oxygen species (ROS) and the ability of the body to counteract their harmful effects through neutralization by antioxidant defense mechanism [13]. ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$) are highly reactive oxidizing agents produced continuously during metabolic processes [14]. Oxidative processes related to spermatozoa are particularly of interest as they exhibit a double-edged sword role in these cells (**Figure 1**). The physiological level of ROS is necessary to regulate a critical redox-sensitive processes such as capacitation and hyperactivation without which fertilization is impossible [15]. While its supraphysiological level affects normal spermatogenesis and sperm functions such as motility, capacitation, acrosome reaction, egg penetration and decondensation of sperm head, which is essential to achieve fertilization. Spermatogenesis is a metabolically active biological process during which haploid spermatozoa are produced in the seminiferous tubules. During this process O_2^- are generated as a natural by-product of cellular respiration. The germ cells undergoing differentiation to spermatids in testes are protected from oxidative stress by its nurse cells called sertoli cells which possess high level of antioxidant enzymes such as superoxide dismutase (SOD) as well as the reductase, transferase, and peroxidase activities of the glutathione cycle [16]. Once the spermatozoa are released from the germinal epithelium, they become vulnerable to oxidative attack as they are no longer protected by defense mechanism of sertoli cells [13, 17]. Excess ROS can lead to cellular injury by damaging DNA, lipids, and

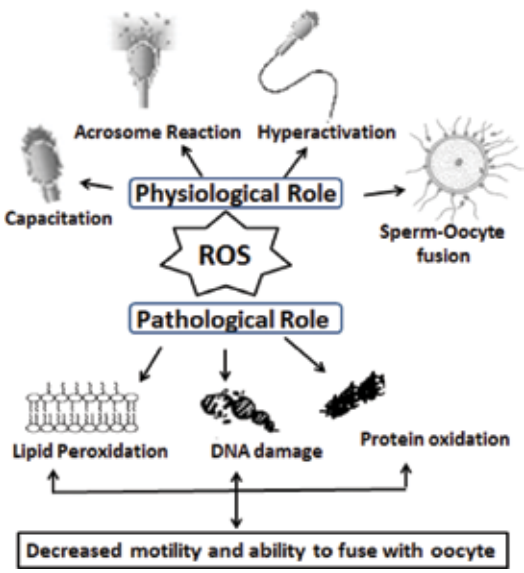

Figure 1. Physiological and pathological role of ROS in male reproduction.

proteins in the cells [18]. Thus, the ROS must be maintained at physiological levels for optimal sperm function, the maintenance of cellular homeostasis, and redox-sensitive signal transduction mechanisms affecting fertility.

2. ROS and sperm physiology

During their transit through the epididymis, spermatozoa progressively acquire the ability to move but lack fertilizing capacity [19]. They acquire the ability to fertilize in the female tract through a series of physiological changes called 'capacitation' which involves hyperactivation, acrosomal reaction, and sperm-oocyte fusion. Mammalian sperm capacitation is a redox regulated process which requires the production of different types of ROS to promote the fertilization of spermatozoa to the mature oocytes [20, 21]. The primary ROS generated in human spermatozoa is the O_2^- which appears to play a role in this process [22]. This one-electron reduction product of oxygen generated reacts with itself via dismutation reaction, which is greatly accelerated by SOD, to generate H_2O_2. It has been reported that the capacitating populations of mammalian spermatozoa generate ROS mainly by two mechanisms: the membrane nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme complex that is contained in the plasma membrane; and the mitochondrial nicotinamide adenine dinucleotide (NADH)-dependent oxido-reductase [23, 24]. The NADPH required by NADPH oxidase can be supplied by dehydrogenases located both in the plasma membrane and the cytosol. Studies have suggested the activation of sperm plasma membrane oxidase during capacitation and acrosome reaction [25, 26]. In mammalian spermatozoa, NADPH oxidase 5 (NOX5) are actively involved in generating O_2^- [27]. The mitochondria located in the mid-piece region of the sperm generates a low level of ROS during steady-state respiration but have the potential to accelerate this activity when these gametes enter the intrinsic apoptotic pathway [28, 29]. In addition to H_2O_2 and O_2^-, a variety of secondary cytotoxic radicals which are reported to stimulate sperm capitation includes nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$) [30, 31]. The O_2^- generated from these two sources is thought to combine with $^{\bullet}NO$ produced by nitric oxide synthase (NOS) and result in the formation of powerful oxidant ONOO–, which mediates the oxidation of cholesterol to oxysterols. The oxysterols then exit the plasma membrane dramatically to enhance membrane fluidity [31, 32]. Further, the combined action of ONOO– and H_2O_2 concomitantly lead to the inhibition of tyrosine phosphatase activity while the combination of O_2^-, bicarbonate (HCO_3^-), and calcium ions (Ca^{2+}) activates soluble adenylyl cyclase, thereby stimulating cAMP production and the activation of protein kinase A (PKA) [33–35]. Activated PKA phosphorylates and inhibits protein phosphatase and activates tyrosine kinase that leads to an increase in actin polymerization, an essential process required for the development of hyperactivated motility [36, 37]. Only hyperactivated spermatozoa have increased motility to undergo acrosome reaction and acquire the characteristics required for successful fertilization. The role of low concentrations of OH^- in the initiation of hyperactivation in vitro has been well documented [38]. The hyperactivated spermatozoon traverse the cumulus oophorus surrounding ovulated eggs, it then binds and penetrate to the zonapellucida (ZP) of the oocyte and initiates an exocytotic release of proteolytic enzymes, creating a

pore in ZP's extracellular matrix. For successful fertilization, the spermatozoa then penetrate this physical zona barrier and fuse with the oocyte [39, 40]. Thus, ROS during the capacitation and acrosome reaction has been shown to increase the membrane fluidity and rates of sperm-oocyte fusion (**Figure 1**).

3. Human spermatozoa are vulnerable to oxidative stress

During spermatogenesis, germ cells produce high levels of reactive oxygen species, but fortunately a complex of antioxidant defense system and DNA repair system exists in the testis that protects genome integrity in differentiating sperm [16]. In normal spermatogenesis, the developing spermatozoa extrude most of the cytoplasm by the action of sertoli cells to change to a condensed, elongated form [41]. The lack of cytoplasm results in decreased intrinsic antioxidant defense due to the loss of most of antioxidant enzymes, rendering the cells less protected against ROS by the time they are discharged into the epididymis [42, 43]. Further, they also lack the necessary cytoplasmic-enzyme repair systems, thus they have very limited capacity for detection and repair of DNA damage [44]. Therefore, during their transit and storage into the epididymis or post-ejaculation they have no DNA repair mechanism, and thus cannot synthesize DNA, RNA, or translate proteins (such as repair enzymes) [45, 46]. The mammalian spermatozoa are vulnerable to oxidative stress not only because of their inherent free radical generating activity and lack of endogenous antioxidant protection, but also due to the abundant substrates that these cells possess for free radical attack. In mature spermatozoa, the small cytoplasm with limited defense remains confined to the mid-piece region in the vicinity of the mitochondria. As a result, the plasma membrane richly endowed with high concentrations of polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA) (22:6) and arachidonic acids (20:4) containing six and four carbon-carbon double bonds per molecule, surrounding the acrosome and the tail are not protected by the intracellular antioxidants [47–49]. In human spermatozoa, approximately 50% of the fatty acids are composed of DHA which is thought to play a major role in regulating spermatogenesis and membrane fluidity [18]. The presence of double bond in PUFAs adjacent to a methylene group weakens the methyl carbon-hydrogen bonds and makes hydrogen more susceptible to abstraction and thus vulnerable to oxidation [44, 50].

Sperm mitochondrial DNA has long been postulated as a sources and often likely target of ROS oxidation as they are not protected by histones and has a very limited capacity for DNA repair with complete lack of nucleotide-excision repair pathways [51]. It is estimated that the mitochondrial DNA exhibits the mutation rate two orders of magnitude higher than that of nuclear DNA. Thus any quantitative or qualitative aberrations in mitochondrial DNA will result in the increased ROS generation which will affect the cellular functioning of the cell [52].

Despite its vulnerability to oxidative stress, maturing sperms spontaneously generate ROS during their progress through the epididymis, as its normal metabolite that aids it to acquire full fertility competence [53]. The lack of intrinsic antioxidant protection forces these cells to dependent on defense provided by seminal and epididymal enzymatic and non-enzymatic

antioxidant mechanisms. These mechanisms compensate for the deficiency in cytoplasmic enzymes in sperm [54, 55]. Thus the sperms which spend long period as an isolated cells both in male and female genital tracts (approximately 3 weeks), these limited defenses can be easily overwhelmed with an increased generation of ROS [56].

4. ROS scavenging capacity of semen

Spermatozoa like other aerobic cells are dependent on cellular respiration process which supports its life. But excessive generation of its metabolites, such as ROS, can modify cell functions. Hence, under normal condition male reproductive system must continuously inactivate ROS to maintain a balance between ROS production and its scavenging mechanism in order to keep only the small amount necessary to maintain normal cell function. Thus, in order to maintain the redox homeostasis, the mature spermatozoa with limited antioxidant defense capacity are mainly dependent on seminal plasma which is well endowed with an array of effective enzymatic and non-enzymatic antioxidant defense mechanisms [57, 58].

The main enzymatic antioxidants in the semen include superoxide dismutase (SOD), catalase, and glutathione peroxidase/glutathione reductase (GPX/GRD) system [59]. SOD is metalloenzymes which is present in both intracellular and extracellular forms [60]. SOD spontaneously dismutase O_2^- to form H_2O_2 and catalase catalyzes the decomposition of H_2O_2 to O_2 and water (H_2O) thus preventing the lipid peroxidation of the sperm plasma membrane. Another enzyme of the antioxidant system in the semen is glutathione peroxidase (GPX), which catalyzes the reduction of hydrogen peroxide and organic peroxides, including the peroxides of phospholipids [61]. Spermatozoa have limited supply of catalase and GPX, while SOD is the main enzymatic antioxidant which protects it from oxidative stress [62]. Beside the enzymes antioxidant protective mechanism, seminal plasma is also employed by the low molecular weight, non-enzymatic antioxidants that assist enzyme activity. These include ascorbic acid (vitamin C), tocopherol (vitamin E), vitamin A, pantothenic acid, coenzyme Q10, carnitine, amino acids (taurine, hypotaurine) zinc, selenium albumin, and urate. These agents principally act by directly neutralizing free radical activity chemically and some of these antioxidants are reported to enhance sperm viability/motility as well as normal sperm morphology and required for spermatogenesis, development of spermatozoa [63, 64]. The seminal plasma antioxidants concentrations have been shown to be significantly higher in fertile men than those in infertile men [65, 66].

5. Sources of ROS in seminal plasma

Oxidants in seminal plasma originate from numerous extrinsic and intrinsic sources. The human ejaculate is composed of various types of cells, which include mature and immature cells, round cells from extraordinary degrees of spermatogenesis, leukocytes, and epithelial cells [67, 68]. Of those, leukocytes, specially neutrophils and macrophages and immature

spermatozoa are taken into consideration as the primary endogenous assets of ROS [69], while numerous life style elements including immoderate smoking and alcohol intake, and environmental elements inclusive of radiation and pollution can contribute as exogenous sources of ROS (**Figure 2**) [70, 71]. Exposure to radiation and toxins induces ROS production which impairs spermatogenesis and leads to DNA damage in human spermatozoa, which further decreases the motility and vitality of sperm cells as well as their concentration depending on the duration of exposure [72]. Cigarette smoking is found to be correlated with leukocytospermia. It has been reported that smoking can elevate the leukocyte concentration by 48% and ROS by 107% in seminal plasma [73].

5.1. Immature/abnormal spermatozoa

One of the major cellular sources of ROS in the semen is sperm cells [74]. When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective, and spermatozoa are released from the germinal epithelium carrying surplus cytoplasmic residues in the midpiece [75]. These residues are rich in the cytoplasmic enzymes such as superoxide dismutase, lactic acid dehydrogenase, glucose-6-phosphate dehydrogenase (G6PDH), and creatine kinase [69, 76]. However among these enzymes, the key enzyme was thought to be G6PDH, which would be expected to enhance the intracellular availability of NADPH via the hexose monophosphate shunt. NADPH is used to fuel the generation of ROS via NADPH oxidase activity [27, 77]

5.2. Leukocytes

The main source of ROS inside semen is leukocytes. Infection or chronic inflammation may activate the leukocytes to release 1000-times more ROS than spermatozoa [78]. This high production of ROS by leukocytes plays an important role in the cellular defense system

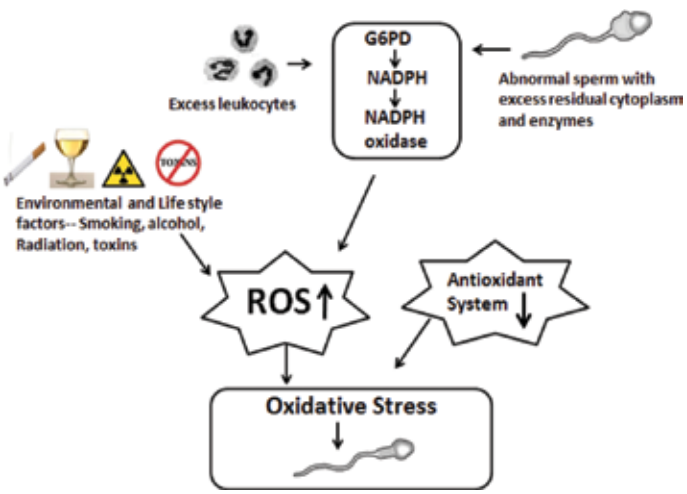

Figure 2. Extrinsic and intrinsic factors of ROS generation in seminal plasma.

against infections as well as inflammation [78]. However, the high concentrations of ROS may overwhelm seminal antioxidant defenses and damage the sperm cell [79]. Essentially, the cellular mechanisms for the generation of ROS within leukocytes and spermatozoa are same, in leukocytes, the release of the large amounts of superoxide into phagocytic vesicles for killing the pathogens [80, 81].

6. Impact of oxidative stress on spermatozoa

The exact mechanism of oxidative stress-induced decline in sperm function remains unknown but is mainly attributed to peroxidative damage to axoneme and depletion of intracellular ATP levels, followed by generation of 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) owing to the oxidation of lipid membrane components and oxidation of DNA followed by fragmentation of both nuclear and mitochondrial DNA [82].

6.1. Lipid peroxidation

In PUFAs, the hydroxyl radicals attack lipids containing carbon-carbon double bond and promote the hydrogen abstraction from carbon to generate a carbon-centered lipid radical ($2CH^{-\bullet}$) that then combines with oxygen to generate lipid peroxyl radicals (ROO•) [83]. The ROO• radicals subsequently attacks another lipid molecule, abstract a hydrogen atom in order to stabilize itself as the lipid hydroperoxide but in the process generates another carbon-centered lipid radical that perpetuates the cascade of chemical reactions called lipid peroxidation. The process results in the generation of small molecular mass electrophilic lipid aldehydes such as 4-hydroxynonenal (4HNE), acrolein, and malondialdehyde [84]. Lipid peroxidation (LPO) is extremely harmful to spermatozoa, having a dramatic effect on both sperm movement and the competence of these cells for fertilization (**Figure 3**). Immature human sperm cells contain high levels of DHA in the cytoplasmatic droplet and showed more susceptibility to LPO than normal matured sperm with lower DHA levels [85].

Added to this vulnerability, it has been shown that cytotoxic aldehydes generated as the result of oxidative stress has the ability to of triggering ROS generation by the sperm mitochondria in a self-perpetuating cycle; the greater the level of unsaturation, the greater the level of the stimulatory effect. The defective human spermatozoa contain abnormally high cellular contents of free polyunsaturated fatty acids, the levels of which are positively correlated with mitochondrial superoxide generation. The lipid aldehydes, 4HNE or acrolein bind covalently to the nucleophilic centers of vulnerable proteins, such as succinic acid dehydrogenase and form a protein adducts in the mitochondrial electron transport chain (ETC) that results in the leakage of electrons which disturbs the normal flow of electrons and reduction of oxygen to water [86, 87]. The leakage of electrons from the ETC results in the reduction of oxygen to generate O_2^-, which then by mitochondrial superoxide dismutase rapidly dismutates to H_2O_2 [88]. The excess of cytoplasm in the immature or defective spermatozoa contain superabundance of cytoplasmic enzymes. The retention of excess of SOD can only be an asset for any cell seeking to protect itself from oxidative stress if it is accompanied by a corresponding increase in the

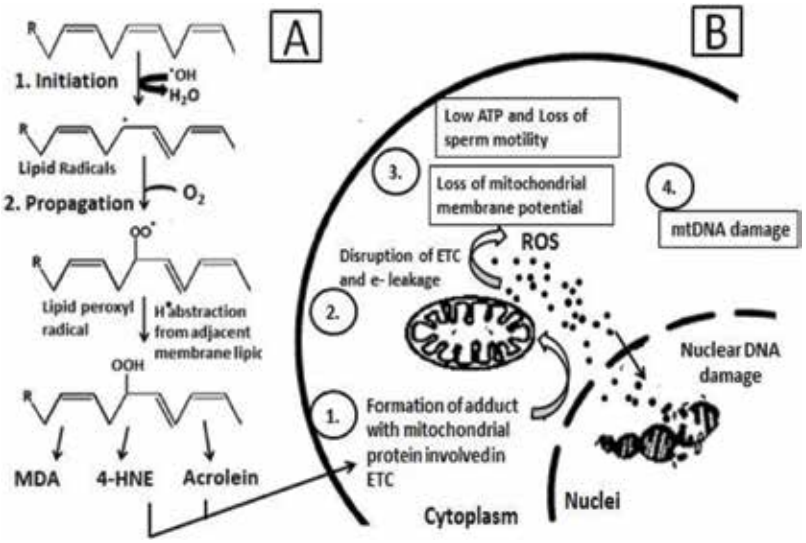

Figure 3. (A) ROS-induced initiation and propagation of lipid peroxidation (LPO) generates lipid hydroperoxides plus a new carbon-centered radical that continues the chain reaction. (B) Mitochondrial ROS generation. The electrophilic aldehydes generated as a by-product of LPO process bind to protein of electron transport chain and further promote mitochondrial ROS generation. This process results in loss of mitochondrial membrane potential, low ATP generation, loss of sperm motility, oxidative DNA damage followed by DNA fragmentation.

presence of enzymes such as glutathione peroxidase or catalase that can scavenge H_2O_2. But excess of SOD and limited supply of glutathione peroxidase or catalase in human spermatozoa simply turns a short-lived, membrane-impermeant, relatively inert free radical O_2^- into a long-lived, membrane-permeate reactive oxidant, H_2O_2 [89, 90]. The damage of protein and membrane lipids due to elevated levels of ROS in mitochondria might affect the process of oxidative phosphorylation causing depletion of intracellular ATP levels leading to axonemal damage, decreased sperm viability, and increased mid-piece sperm morphological defects with deleterious effects on sperm capacitation and acrosome reaction and decline of motility and fertility [91]. The mitochondrial function as a measure of inner mitochondrial membrane potential is found to be decreased in the spermatozoa of infertile men with elevated levels of ROS production and is positively correlated with the sperm concentration [92].

6.2. DNA damage in spermatozoa

Mitochondrial DNA is particularly vulnerable to free radical attack because it is essentially unprotected and has a very limited capacity for DNA repair [93]. Sperm nuclear DNA, on the other hand, is much resistant to damage because it is tightly compacted by replacing histones with small, positively charged molecules known as protamine [94, 95]. Sperm DNA maturation and appropriate packaging are vital steps in the proper development of spermatozoa.

During the late spermatogenesis in the mammalian germinal epithelium, the differentiating spertamids are highly susceptible to DNA damage due to important changes in the cytoarchitecture

and dramatic remodeling of the chromatin during which most of the histones are removed from the DNA and are first replaced by transition proteins TP1 and TP2, and then by protamines P1 and P2 which are approximately half the size of histones. P1 and P2 are normally expressed in a 1:1 ratio in human sperm, and provide a tight packaging of the sperm DNA. The chromatin remodeling is facilitated by the coordinated loosening of the chromatin by histone hyperacetylation and by the DNA topoisomerase II (topo II), which produce temporary stand breaks in the sperm DNA to relieve torsional stress that results from supercoiling [96, 97]. This forms the basic packaging unit of sperm chromatin, a toroid, which is further compacted by the intramolecular and intermolecular disulfide cross-links between cysteine residues present in protamines. The tight packaging of the sperm DNA enables the entire haploid genome to be condensed and packed in a sperm head measuring 5 × 2.5 μm. This level of protect and ensures that the paternal genome is delivered in a form that allows developing embryo to accurately express genetic information Normally, these temporary strand breaks are repaired by nuclear poly (ADP-ribose) polymersases (PARP) and topoisomerase II prior to completion of spermiogenesis and ejaculation [98]. However in pathological cases, the error in chromatin remodeling and repair mechanism leads to the generation of high level of nicked and poorly protaminated nuclear DNA with relatively high nucleohistone content or abnormally high and low P1/P2 ratios [99–101]. Thus, defect in the chromatin remodeling process causes DNA damage in spermatids during spermiogenesis, this creates a state of vulnerability whereby spermatozoa become increasingly susceptible to oxidative damage.

7. Causes of DNA damage in spermatozoa

When the protection of DNA in spermatozoa, which is dependent on its close association with cysteine rich protaminesis is lost, the cells become very susceptible to oxidative DNA damage induced by several extrinsic and intrinsic factors. Deoxygenated guanine (dG) is more susceptible to oxidation than other nucleosides in DNA due to its low oxidation potential [102]. The enzyme 8-oxoguanine glycosylase 1 (OGG1) immediately clips the 8OHdG residues out of the DNA generating an abasic site, But due to the absence of base excision repair enzyme, the spermatozoa are ejaculated carrying a abasic sites in their DNA [103]. Studies have reported that the spermatozoa of subfertile patients contain particularly high levels of 8-hydroxy-2′-deoxyguanosine (8OHdG), the major oxidized base adduct formed when DNA is subjected to attack by ROS [104].

DNA repair does occur during spermiogenesis but stops post-spermiogenesis because spermatozoa are transcriptionally and translationally silent. They cannot undergo programmed cell death called apoptosis, due to their inherent physical architecture, the endonucleases released from the mitochondria have no access to the DNA. Thus, abortive apoptosis initiated post-meiotically, when the ability to drive the spermiogenesis process to completion is declined and the stand breaks are not repaired due to impairment in the repair process results in high levels of DNA fragmented sperm in the ejaculate [105]. Sperm with DNA fragmentation still has thepotential to fertilize and some types of stand DNA breaks in sperm can be repaired by oocytes, before the initiation of the first cleavage division, and generate normal offspring, but that

depends on the type and level of chromatin damage and the capacity of the oocyte to repair it [106]. DNA-strand breaks are extremely harmful lesions if not repaired and can lead to genomic instability and cell death. In natural conception, percentage of DNA damage has been negatively correlated to the rate of fertilization. If post fertilization oocyte make mistake in the repair process, deletions or sequence errors may be introduced, then it fabricates the possibility for *de novo* mutations, which could have a profound impact on the health and well-being of the offspring [107]. Sperm DNA damage in context to assisted reproductive technique (ART) has important clinical implications. Sperm selected for ART mostly originates from environment experiencing oxidative stress and high percentage of these sperms may have damaged DNA. If such sperms are used clinically in the form of therapy then can lead to substantial risk in pregnancy outcome. In case of intrauterine insemination (IUI) and in vitro fertilization (IVF), the use of these spermatozoa may not be cause of concern. But in case of intracellular sperm injection (ICSI), this natural selection barrier is bypassed and the spermatozoa with damaged DNA are directly injected into oocytes. Studies have reported that DNA damaged spermatozoa used in ICSI have some capacity for fertilization, but percentage of DNA damage has been negatively correlated to the rate of fertilization [108]. ROS-mediated DNA damage may be linked to an increase in early embryo death, infertility in the offspring, and high incidence of childhood cancer [109, 110]. We propose that extrinsic and intrinsic sources of ROS could make a significant contribution to the induction of OS and DNA damage in spermatozoa which can decrease pregnancy rate and affect the fertility outcome, further additional studies are clearly needed to validate this concept.

8. Management of infertility caused by oxidative stress

Oxidative stress plays an important role in the pathophysiology of male infertility, which is caused due to pathological level of ROS and the loss of antioxidant protection for the spermatozoa. There are many factors which can induce oxidative stress and can alter seminal parameters and rate of fertilization. Thorough examination and management of some of these factors may protect the ROS-induced DNA damage and improve a couple's chances of conception either naturally or via assisted reproduction.

8.1. Behavior and life style modification

Various behaviors and lifestyles factors such as alcohol consumption, cigarette smoking, obesity, excess exposure to environmental toxicants, and psychological stress are negatively correlated with spermatogenesis and may cause oxidative stress and reduction in sperm quality [111]. The increased consumption of simple sugars and high-fat food and physical inactivity are leading causes of the growing obesity. It is suggested that abnormal hormonal regulation, dysregulation of adipocytokine, and ROS generation lead to suboptimal semen quality these patients [112]. Several systemic diseases, such as diabetes mellitus, infection, and cancer are known to cause oxidative stress-induced male infertility [113, 114]. There are studies which have shown positive correlation of exercise with improvements in semen parameters, sperm DNA integrity, and pregnancy rate [115]. Nevertheless, modification in behavior and unhealthy living, regular exercise, stress free jobs, and treatment of a patient's underlying pathology should be the first steps to reduce or eliminate stress-provoking conditions to reverse sperm dysfunction.

8.2. Dietary antioxidants

As many studies suggested that oxidative stress is a major cause of unexplained male infertility, antioxidant therapy would be expected to have a therapeutic effect in such cases. There are evidences which have suggested that oral antioxidants and herbal products can also boost male reproductive functions [116, 117]. But, despite of known effect of antioxidant on oxidative stress, very few studies conducted have any validity due to small sample size, difference in dosage and duration of therapy, and lack controls [118]. In order to make the study valid, patient's selection criteria for the trial should be based on the evidence indication oxidative stress as a key element in their pathology, a thorough diagnosis is required to determine patients that need to be supplemented. However, if this strategy is pursued, great care must be taken in selecting the most appropriate antioxidants for clinical use. Since ROS plays an important role in regulating the signal transduction cascades that drive sperm capacitation, we should ensure that any antioxidants employed in vitro do not compromise the fertilizing potential of these cells [119].

The study of *in vitro* antioxidants is highly relevant in the era of assisted reproduction because sperm preparation techniques in ART are potential generators of exogenous stresses that make human spermatozoa vulnerable to oxidative stress and DNA damage.

9. Conclusion

Oxidative stress has been recognized as a major contributory factor to male infertility. Spermatozoa are professional generator of ROS as physiological level of ROS is necessary to regulate critical redox-sensitive processes such as capacitation, hyperactivation, acrosome reactions, and signaling processes to ensure appropriate fertilization. On the other hand, many endogenous and exogenous factors can elevate ROS production which can overwhelm their antioxidant mechanism. This results in male infertility via mechanisms involving the induction of peroxidative damage to the sperm plasma membrane, DNA damage, which significantly impairs sperm function. Lack of repair mechanism and abortive apoptosis in mature spermatozoa results in high levels of DNA fragmented sperm in the ejaculate. In natural conception, oocytes can repair some of stand DNA breaks, but that depends on the type and level of chromatin damage and the capacity of the oocyte to repair it. If post fertilization oocyte make mistake in the repair process it may lead to failure in fertilization. But if fertilization occurs, then it creates the possibility for *de novo* mutations, which could have a profound impact on the health and well-being of the offspring. When the natural balance between ROS and antioxidants is disturbed, the first restorative measure to be taken should be changes in lifestyle, maintaining a healthy and balanced diet, and antioxidant supplementation may then be taken together to improve the patient's health outcomes.

9.1. Suggestion

The conventional seminological parameter in infertile cases reflects the functional competence of the spermatozoa and the fertilizing potential of the ejaculate, but the underlying mechanisms of male fertility is not known. Thus in order to enrich the diagnostic value of this

fundamental form of investigation, the detailed examination of sperm DNA damage may be incorporated as a potentially valuable tool to investigate the functional integrity of the spermatozoa at the molecular level.

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Chapter 5

Particularities of Oxidative Stress in Newborns

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Additional information is available at the end of the chapter

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Abstract

The oxidative stress at newborns is augmented by different conditions like preterm birth, asphyxia, respiratory distress, and intraventricular hemorrhage. Preterm neonates associate a more pronounced oxidative stress than healthy term newborns. Several neonatal conditions like respiratory distress (RDS), asphyxia, intraventricular hemorrhage, bronchopulmonary dysplasia, retinopathy, and necrotizing enterocolitis will increase the oxidative stress. The harmful effects of free radicals are linked to their capacity to react with polyunsaturated fatty acids of cell membranes, proteins, and nucleic acids. Free radicals will produce protein alteration with function loss and lipid peroxidation.

Keywords: oxidative stress, new born, prematurity

1. Introduction

Oxidative stress represents all injuries caused by reactive oxygen species (ROS) on biomolecules, inducing the destruction of membranes, enzymes, receptors, as well as alteration of cell function. The consequence of oxidative stress is a disruption of the physiological balance between pro-oxidants and antioxidants [1, 2].

Newborn possesses defense mechanisms such us molecules protection, limitation of ROS production, and mechanisms for repair and adaptation to endogenous and exogenous ROS overproduction.

Reactive oxygen species are involved in physiological processes such as physical exercise, hyperbarism, regulation of vascular tone, stimulation of cell growth and proliferation, stimulation of erythropoietin secretion, the learning and memory process, as well as in pathological processes: inflammation, aging, carcinogenesis [1–3].

In neonatal pathology, there are multiple circumstances that are associated with oxidative stress. An excess of reactive oxygen species in the context of immature, deficient antioxidant defense mechanisms may cause multisystemic diseases [2, 3].

1.1. Effects of reactive oxygen species on biomolecules

Reactive oxygen species will act on molecules, inducing their deterioration as follows:

- DNA lesions—action on the bases in the DNA structure—thymine, cytosine, adenine, guanine, deoxyribose, followed by cell damage and mutations
- Alteration of NADPH—inhibition of the nucleotide coenzyme activity
- Lipids and proteins—action on the covalent bond in their structure
- Glycoproteins—action on hyaluronic acid in their structure
- Lipid peroxidation—structural and functional changes in cell membranes

1.1.1. Effects of oxidative stress on proteins and amino acids

Reactive oxygen species (ROS) act on the side chain of amino acids. Through oxidation of amino acids in the structure of proteins, the following changes are induced: protein fragmentation, aggregation, and proteolytic degradation. Aldehydes resulting from lipid peroxidation and glycosylation will also act on proteins. The consequence will be the functional alteration of proteins, with the loss of their contractile, enzymatic, and transport function.

1.1.2. Effects of oxidative stress on lipids

The most extensively studied action on lipids is their cellular and extracellular peroxidation. Lipids and lipoproteins are involved, particularly those mainly composed of polyunsaturated fatty acids (PUFA) such as linoleic and arachidonic acids, abundantly in cholesterol esters, lecithin, and erythrocytic phospholipids. Lipoproteins can be oxidized by two pathways:

- Specific enzymatic oxidation—with the formation of prostaglandins, thromboxane, prostacyclin, leukotrienes, and isoprostanes
- Nonspecific enzymatic oxidation—a peroxidation process with the formation of products with a damaging effect.

As a result of lipid peroxidation, a disorganization of the cell structure occurs, lipids being part of the membrane structure. A more rigid membrane will develop, with implications on essential membrane proteins, such as Na^+-K^+-dependent ATPase. Thus, a change in the ion pump rate will occur.

Lipid peroxides alter the properties of cellular, mitochondrial, and lysosomal membranes, with the disappearance of osmotic, chemical, and electrical gradients. Thus, cell excitability

and metabolic processes are disturbed, and morphological lesions occur. In the nerve fiber, the myelin sheath is attacked. At pulmonary level, there is an alteration of endothelial membrane permeability, with the movement of lipids from the vascular space to the extravascular (interstitial) and intracellular space, and the development of pulmonary edema.

1.1.3. Effects of oxidative stress on carbohydrates

Glucose and monosaccharide oxidation leads to the formation of activated molecules that may interact with other molecules, generating new compounds. By polysaccharide oxidation, structural changes in deoxyribonucleic acid (DNA) may occur.

1.1.4. Effects of oxidative stress on nucleic acids

The number of oxidative attacks on DNA in humans is 10,000/cell. Thus, chromosome fragmentation occurs. Double-chain DNA is much more vulnerable than single-chain DNA. Altered fragments are eliminated as purine and pyrimidine bases by urinary excretion and they can be dosed. Oxidative DNA lesions accumulate with age. Aging and carcinogenesis are explained by incomplete DNA repair after oxidative attacks.

The superoxide anion resulting from the xanthine/xanthine oxidase system induces DNA breaks. The hydroxyl radical through its instantaneous reaction with nucleic acids also causes DNA breaks. These breaks should be normally repaired by cell enzymes, but due to their low fidelity, the repair process is inadequate through the inclusion of inappropriate bases in the DNA structure.

Oxygenated water also has effects on DNA. Through its reaction with DNA-bound metal ions, oxygenated water will induce the formation of hydroxyl radical, which will immediately fragment the DNA molecule.

2. Oxidative stress in newborns

The literature currently speaks about free radical disease in newborns, a term introduced by Sullivan [2], which includes the following pathogenic conditions: bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), intra-periventricular hemorrhage (IPVH), necrotizing enterocolitis (NEC), and renal failure [1]. Premature newborns with a gestational age of less than 30 weeks and a weight of less than 1500 g, respectively, have a major risk to develop these disorders. At cerebral level, there is a predisposition to oxidative stress, due to the high amount of polyunsaturated fatty acids in the immature brain, particularly in neuronal membranes, but also due to the relatively high amount of protein-unbound iron [41]. Immaturity associated with premature birth and also oxygen therapy used for the treatment of respiratory distress significantly increase oxidative stress in premature newborns. The knowledge of oxygen toxicity mechanisms is important both for their prevention and to ensure a harmonious development of newborns in general and premature newborns in particular, given their low antioxidant defense capacity [1, 3].

2.1. Implication of oxidative stress in pulmonary disorders

Respiratory distress due to surfactant deficiency is one of the most frequent disorders in premature newborns. The higher the degree of prematurity, the higher is the incidence and severity of this disease. Due to the immature lung structure, surfactant deficiency, pulmonary fluid retention, and poorly developed bone and muscle structures, there is an increased susceptibility of the premature to pulmonary lesions. The mechanism of these pulmonary lesions is based on alveolar instability and pulmonary atelectasis.

Mechanical ventilation, in addition to its role of ensuring an adequate oxygen supply to the body, also has undesired effects. Thus, it interferes with inflammatory cell metabolism and pulmonary mediators. Pulmonary vascularization is supplied from the heart but is also a reservoir of neutrophils—1/3 of the total number of neutrophils outside the bone marrow is found in the lungs [38].

Animal experiments have evidenced that in the context of mechanical ventilation, the number of neutrophils and the level of mediators, platelet activating factors, thromboxane B2 in pulmonary lavage fluid, and TNF-alpha in alveolar macrophages increase [11]. In premature animals with RDS, mechanical ventilation will cause an increase in pulmonary inflammation mediators, an increase in granulocytes, and cytokine activation [8]. Ventilatory support may affect the alveolar-capillary barrier and induces a release of inflammation mediators from the alveolar space into the circulation. The translocation of endotoxins from the aerial space to the circulatory space will generate a systemic inflammatory response [8, 9].

Mechanical ventilation along with oxygen supplementation will generate oxidative stress, with protein oxidation under the action of ROS. The carbonyl group of proteins will react with 2,4-dinitrophenylhydrazine, and 2,4-dinitrophenyl-hydrazone will form, which is measured by spectrophotometry [16]. Prematures who develop bronchopulmonary dysplasia (BPD) have significantly higher levels of protein carbonyls during the first week of life [1, 16].

Newborns requiring mechanical ventilation are more exposed to free radical production as a result of oxygen therapy exposure, inflammatory response, and ischemia reperfusion [10, 49].

Experimentally, it has been demonstrated on animals that the synthesis of enzymatic and consequently, antioxidant systems occurs at the end of gestation. Hemolysis after birth associated with a low level of iron-binding antioxidants can favor protein damage [5].

Protein carbonylation in the tracheobronchial fluid is a marker of oxidative stress at pulmonary level, while the exhalation of lipid peroxidation products (ethane, pentane) is a marker of lipid peroxidation in the entire body. As a result, a high level of exhaled peroxidation products is associated with extrapulmonary morbidity [9, 10].

Enzymes protecting against oxidant agents in the lungs, such as catalase, can be the target of oxidative attack inducing their inactivation. Liposoluble free radical scavengers such as tocopherol or beta-carotene have a protective effect against oxidative attack on lipids, but not on proteins. At the end of the first week of life, a depletion of these scavengers occurs as a result of the rapid increase in ROS production under the action of pulmonary inflammatory response. Thus, lipid peroxidation products are released through oxidation of polyunsaturated

fatty acids. During the first 3–4 days of life, pulmonary proteins are the first target of free radicals. In the context of RDS, edema develops as a result of increased membrane permeability. Due to its high protein content, its presence in the lung will make it the ideal target for the initiation of oxidative attack by ROS [30, 31]. The inactivation of α_1-proteases under the action of oxidative attack will disrupt the balance of the pulmonary protease-antiprotease system. In infants with RDS, ROS will interact with the surfactant as well as with other proteins and lipid structures, delaying the normalization of pulmonary function [2, 5, 38].

Patients with respiratory distress have high levels of hydrogen peroxide in the pulmonary condensate. Oxidized glutathione and the altered alpha-1 protein also show high levels in the pulmonary fluid. In addition, the antioxidant proteins catalase and ferritin are elevated, which could represent a compensatory response. Oxidative stress markers are also increased in patients with sepsis, in those infected with HIV [40].

With the increase in the survival rate of newborns with extreme prematurity, the incidence of chronic pulmonary disorders has also increased, not only as an undesired consequence of RDS, but also of mechanical ventilation used for its treatment. The most frequent chronic pulmonary disease of premature infants with RDS and a history of mechanical ventilation is bronchopulmonary dysplasia. This is a chronic lung disease developing in newborns treated with oxygen and mechanical ventilation for a primary pulmonary disease. It affects between 20 and 60% of prematures, but it may also occur in term infants with severe respiratory distress [8].

Its etiopathogeny is complex. There is the hypothesis that bronchopulmonary dysplasia might start as an acute inflammation, which subsequently turns into a chronic lung disease under the action of free radicals [2, 5].

Preliminary studies have shown that pulmonary lesions can be improved by administration of antioxidants such as superoxide dismutase (SOD). The function of SOD is to convert the extremely toxic superoxide radical to less toxic hydrogen peroxide and water. Superoxide dismutase is also present naturally, but not as synthetic surfactant. Genetic engineering has demonstrated that in alveolar cells, SOD survives for a longer time period. An experimental animal with an SOD gene disrupted by genetic engineering, exposed to hyperoxia, will survive for a shorter time and will have more pulmonary lesions than an animal with an intact gene. Superoxide dismutase plays a major role in the prevention of pulmonary lesions in the context of hyperoxia. If SOD activity is increased in the newborn lung, inflammatory changes and pulmonary lesions can be prevented [9, 20, 21].

The incidence of bronchopulmonary dysplasia has increased not only with the extensive use of positive pressure ventilation for the treatment of neonatal respiratory distress, but also due to the increase of the survival rate by modern intensive care techniques in newborns with extreme prematurity. The Neonatal Research Network reported a 68% incidence of BPD in prematures with a gestational age of 22–28 WA.

Today, it is known that barotrauma, particularly at high inspiratory pressures, is a key factor in the development of pulmonary lesions independently of any other lesions generated by oxygen therapy. Epithelial alterations in the airways occur, as well as with an increase in capillary permeability with extravasation of protein substances. In addition to the implication

of high inspiratory pressure in the genesis of pulmonary lesions, increased tidal volume also plays an important role. High but also significantly decreased tidal volume may generate an accumulation of neutrophils and a release of toxic agents such as proteases and free radicals, as well as proinflammatory cytokines. An analysis of pathological anatomy data has allowed to evidence a correlation between the incidence of interstitial emphysema during the first week of life and the incidence of interstitial fibrosis or proliferation in newborns with bronchopulmonary dysplasia surviving for more than 28 days [22, 24].

Another factor that plays a role in the development of BPD is inflammation. Proinflammatory cytokines were detected in the tracheal aspiration fluid during the first 1–2 weeks of life in newborns who subsequently developed BPD. Recently, it has been demonstrated that amnionitis and fetal infection are risk factors for BPD; consequently, inflammation, even prenatal, plays a role in its genesis [3].

Premature infants, due to their pulmonary immaturity, have a high risk for BPD, because they require additional oxygen supply for a longer time period in the treatment of the lung disease, their intracellular antioxidant defense capacity is affected, and they have an increased susceptibility to infections.

Studies have demonstrated the presence of a high level of lipid peroxidation products on days 1–2 of life in newborns who develop BPD. The presence of a high level of protein peroxidation products in the tracheal aspirate of infants weighing less than 1500 g compared to those with a higher weight has also been demonstrated. This confirms the fact that antioxidant defense decreases with the increase of immaturity [9, 15, 16].

There is also a close correlation between the protein oxidation level and activated neutrophils. This supports the presence of a relationship among neutrophil accumulation, oxidative stress, and the development of BPD [1].

In randomized trials for the study of STOP-ROP, some authors monitored oxygen exposure and the evolution of retrolental fibroplasia and BPD [36]. Thus, in groups with 89–94% O_2 Sat, compared to those with 96–99% O_2 Sat, the influence of saturation on the progression of retrolental fibroplasia was not significant. In contrast, in the group with higher saturation, the BPD progression rate was higher (13.2%) compared to subjects exposed to lower saturation levels (8.5%) [23, 39].

Exposure to a FiO_2 of 100% on the first day practically doubles the risk of BPD. Excessive oxygen administration and/or barotrauma may increase the risk of BPD. However, a high PaO_2 is a cofactor, not a causal agent in the development of BPD [36].

Preterm birth associates vitamin A deficiency, which is important in pulmonary epithelial lesion repair. A number of studies have evidenced a significant reduction in the incidence of bronchopulmonary dysplasia under conditions of vitamin A administration (Shenai et al.) [34, 37], but there are also studies that could not demonstrate this fact (Perason et al.) [32, 33].

Selenium deficiency can also be considered in association with low glutathione levels. Darlow et al. found a significant relationship between plasma selenium levels and the incidence of bronchopulmonary dysplasia at the age of 28 days, but could not clarify whether these were a cause or an effect of BPD.

A study conducted in our department, which assessed lipid peroxidation by measuring malondialdehyde (MDA) levels on the first and third days of life found that MDA values in newborns with respiratory distress increased with the increase in the severity of respiratory distress (**Table 1**).

MDA values in the mentioned study tended to decrease on day 3 compared to day 1, but without a statistically significant difference.

Also, the lipid peroxidation process was more intense in the study group-newborns with different pathologies generating oxidative stress compared with the control group of healthy term newborns, without any oxidative-stress inducing disorders (**Figures 1** and **2**).

In the same study, we also monitored protein peroxidation in newborns with respiratory distress, and we found the presence of a significant correlation between protein carbonyl values on the third day of life and respiratory distress ($r = 0.56$; $p < 0.05$). For the evaluation of protein peroxidation in the mentioned study, we measured protein carbonyls on the first and third days of life using the Reznick spectrophotometric method with dinitrophenylhydrazine. The protein substrate in the lung is an optimal target for the action of ROS and the triggering of oxidation reactions of these proteins. In fact, it was demonstrated that in newborns with RDS, this protein oxidation process under the action of ROS also contributes to the pathogenesis of BPD. Under the action of reactive oxygen species, peroxidation of proteins and other lipid and protein structures occurs. Thus, the normalization of pulmonary function is delayed. Surfactant administration before the initiation of mechanical ventilation limits oxidative injury induced by mechanical ventilation.

2.2. Effects of oxidative stress at endothelial level

The effects of ROS at endothelial level during the neonatal period are found in the following morbid conditions: septic shock, systemic inflammation, acute ischemia, inducing considerable oxidative stress [5]. Reactive oxygen species contribute to the development of ischemic and inflammatory vascular lesions by an important efflux of oxidants to the ischemic tissue and the induction of new lesions in tissues and organs in the next reperfusion stage [29].

		RankSum1	RankSum2	U	p	n1	n2
RDS mild vs. severe	MDA DOL 1	255.0	273.0	84.0	0.1159	18	14
	MDA DOL 3	281.0	247.0	110.0	0.5611	18	14
RDS mild vs. moderate	MDA DOL 1	307.5	638.5	136.5	0.0283	18	25
	MDA DOL 3	379.0	567.0	208.0	0.6877	18	25
RDS severe vs. moderate	MDA DOL 1	75.5	114.5	23.5	0.1740	6	13
	MDA DOL 3	79.0	111.0	20.0	0.0956	6	13
MV yes vs. no	MDA DOL 1	376.5	1453.5	285.5	0.7229	13	47
	MDA DOL 3	407.5	1422.5	294.5	0.8454	13	47

RDS = respiratory distress syndrome, MV = mechanical ventilation, RankSum = rank sum, p = test sign, and n = group size.

Table 1. MDA values according to RDS severity.

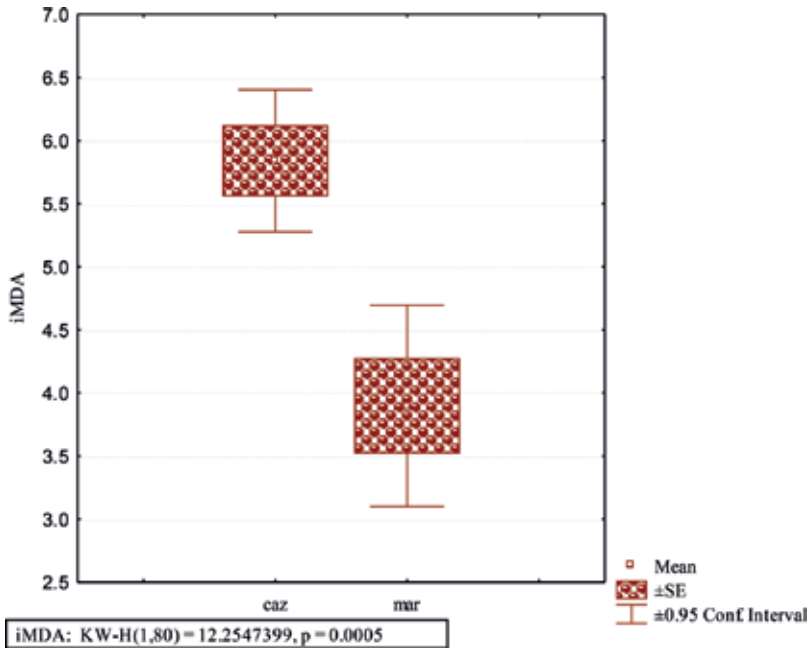

Figure 1. MDA values of case group DOL 1 vs. control group (caz = case, mar = control).

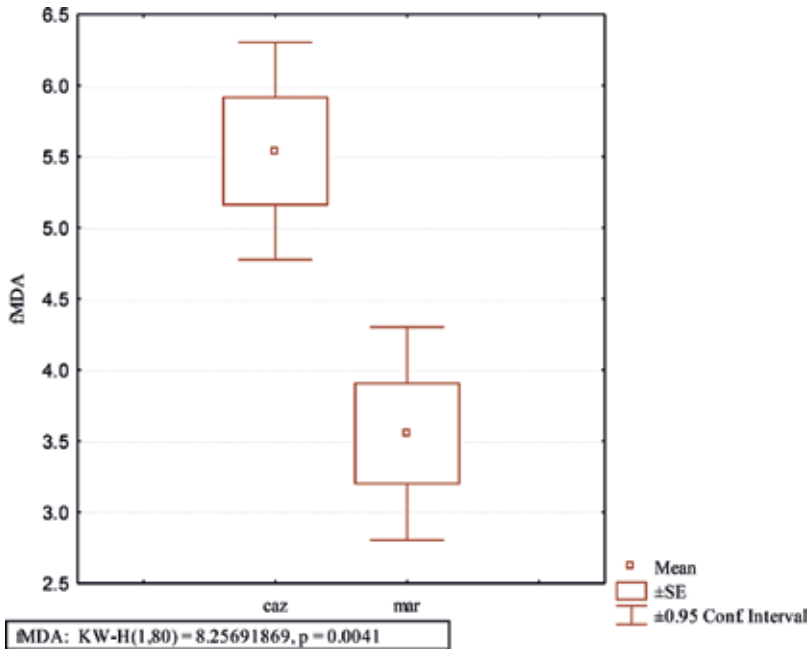

Figure 2. MDA values of case group DOL 3 vs. control group (caz = case, mar = control).

The vascular surface has the role to control critical processes for the functioning of organs. During inflammatory processes, the endothelium is exposed to oxidation [25]. It has been demonstrated that TNF-alpha stimulates the release of oxygenated water at the contact area between neutrophils and the endothelial cell, inducing endothelial cell retraction.

The exposure of endothelial cells to significant concentrations of exogenous oxidants will cause specific physiological effects. Endogenous oxidants have been recognized as a physiological signal component, probably triggered by lung injury, with the release of TNF-alpha, interleukin, growth factor β, and platelet growth factors. These will stimulate tyrosine phosphorylation, with the activation of ERK, extracellular signal-regulated kinase. DNA synthesis will occur accompanied by an increase in hydrogen peroxide levels. Oxidants mediate the effects of growth factors β and insulin, which will stimulate mitogen-activated proteins [27–28].

2.2.1. Oxidative stress in retinopathy of prematurity

The harmful effects of ROS at endothelial level also manifest in ROP [10, 23]. In the pathogenesis of ROP, the following factors are involved: self-regulation, which is absent in newborns, hyperoxygenation of the retina, which is frequent because antioxidant defense is reduced, particularly in the premature. Hyperoxygenation induces peroxidation of vasoactive isoprostanes. Vasoconstriction and vascular cell toxicity with ischemia and vascular proliferation occur [4, 7].

Glutathione is the most important intracellular antioxidant; however, its synthesis is reduced during intrauterine life and in prematures. In the vitreous fluid of the premature at risk for retinopathy, there is a high level of hypoxanthine with a role in the formation of free radicals [1, 12]. Papp et al. [6] found that the oxidized glutathione/reduced glutathione ratio is more than double in prematures with retinopathy compared to those without this disease. This is why the problem of using this ratio as a screening method for the detection of ocular involvement in premature infants was posed. The same authors found that in infants with active disease aged less than 3 months, reduced glutathione values were lower, and those of oxidized glutathione were higher, the greatest decrease in reduced glutathione occurring after the in vitro alteration of oxidative status.

2.2.2. Effects of oxidative stress in ulceronecrotic enterocolitis

In ulceronecrotic enterocolitis, there are multiple pathogenic mechanisms. One of the pathogenic links is hypoxic ischemic injury. Hypoxic ischemic injury in the mesentery is followed by a cascade of events, with intestinal mucosal reperfusion injury. Cytotoxic damage of vascular endothelial cells occurs, which in turn will cause ischemia and new cytotoxic effects through the formation of free radicals [41].

The regulation of mesenteric blood flow includes a reflex self-regulation mechanism. The peripheral autonomic nervous system plays a role in the regulation of mesenteric blood flow.

The initiated ischemia will induce transcapillary fluid passage and local edema. Reperfusion will exacerbate transcapillary fluid production and induce tissue destruction. This event is

mediated by factors released from the ischemic intestine, including endotoxins, histamine, prostaglandins, and superoxide anion, which result from oxygen metabolization. Superoxide will induce lipid peroxidation with the disruption of the integrity of capillaries and epithelial cells. Mucosal lesions characterized by edema, hemorrhage, ulceration, and necrosis may be induced experimentally by a combination of oxidants (hypoxanthine and xanthine oxidase) [14]. Superoxide dismutase can experimentally prevent or attenuate the described lesions. The protective effect of SOD was experimentally observed in animals with superior mesenteric artery occlusion. The sequential development of intestinal enzymes: SOD and xanthine oxidase can be a determining factor of neonatal intestinal susceptibility to ischemic mucosal injury [13, 45]. Regarding NEC prevention, many studies performed on animals show the beneficial effect of melatonin in preventing oxidative stress involved in the development of NEC lesions. Melatonin acts by reducing the level of inflammatory cytokines and by stimulating the activity of antioxidant enzymes. A decrease in TNF-α and IL-1β levels was found in animals treated with melatonin. Melatonin also counteracts the reduction of intestinal motility generated by lipopolysaccharides. Melatonin is an indolamine produced by the pineal gland, having serotonin as a precursor. It is synthesized by serotonin-rich enterochromaffin cells in the digestive tract. This synthesis takes place postprandially in the digestive tube and its level is 100 times higher than blood levels [45, 46].

The pathophysiological mechanism of NEC is based on a hypoxic-ischemic process similar to that found in postasphyxia brain injury. A number of studies have demonstrated that the association of melatonin with misoprostol, a gastric mucosal protective agent, is more beneficial than melatonin therapy alone. These studies on animals found that the administration of 10 mg/kg body weight melatonin for 3 days to animals with induced NEC-like lesions significantly reduced the severity of the disease by decreasing cytokine levels and stimulating antioxidant enzyme activity [45, 48].

2.2.3. Effects of oxidative stress in neonatal asphyxia and hypoxic ischemic encephalopathy

In the brain, there are some particularities that increase the vulnerability of this tissue to oxidative stress: cell membranes are rich in polyunsaturated fatty acids, the brain is poor in catalase and SOD, and there are some brain areas rich in iron. Nerve cell injury in the context of asphyxia will induce iron release [13]. Given the low antioxidant defense, the release of low molecular mass iron will allow the formation of hydroxyl radical and lipid peroxidation. By lipid peroxidation, free radicals induce molecular damage, including endothelial factor destruction. The low level of antioxidants in the serum seems to be directly involved in the genesis of cerebral hemorrhage [17]. Transferrin and ceruloplasmin levels can be indicators of the risk of cerebral hemorrhage in newborns with asphyxia at birth [17, 18]. In prematures with asphyxia, a decrease in these enzymes will precede cerebral hemorrhage. Ceruloplasmin acts as a strong ferroxidase, catalyzing iron oxidation to less reactive ions. The antioxidant defense capacity can be exceeded, making transferrin inadequate for binding, with the release of low molecular mass iron, which will subsequently induce lipid peroxidation. In case of iron overloading or severe oxidative stress, the antioxidant defense capacity is exceeded, making nontransferrin bonds available, with the release of low molecular mass iron, followed by lipid peroxidation. This may occur even if transferrin is not completely iron saturated [42].

Hypoxic ischemic brain injury is a long process that starts with the occurrence of the insult and continues during the recovery period, after reperfusion. This reperfusion process represents a paradoxical tissue response: the appearance of oxidative lesions in a hypoxic tissue, poorly perfused after circulatory stabilization, at the time of its perfusion with oxygenated blood. This stage of reperfusion will cause an excessive production of free radicals generating new lesions after the initial oxidative attack. In the premature, an increased risk of cerebral ischemia persists postnatally, during the first week of life [19, 26, 27].

In the reperfusion stage, ROS with a cell damaging effect are produced, in the first place by the conversion of xanthine dehydrogenase to xanthine oxidase. This process will result in the formation of thiol groups or proteolysis by activation of proteases in energetically depressed cells. In the reoxygenation stage, hypoxanthine is converted to uric acid under the action of xanthine oxidase, and superoxide and peroxide are released through the mediation of xanthine oxidase by molecular oxygen binding. Hydroxyl radical generated through the Fenton reaction will also be released, due to the catalytic effect of metals such as iron [42]. Perinatal hypoxic stress is a frequent cause of morbidity, mortality, and neurological damage in survivors. In the perinatal hypoxic context, several factors play a role in the pathogenesis of lesions: hypoxia with initial ROS formation, followed by ischemia-reperfusion, a stage at which arachidonic acid and phagocytes will be activated under the action of inflammation mediators. Thus, a vicious circle is created. ROS will be formed, followed by tissue lesions and the genesis of new free radicals [42, 43].

At CNS level, under asphyxia conditions, encephalopathy lesions are induced by activation of leukocytes or glial cells and release of new free radicals. Hypoxic lesions will be perpetuated by release of protein-unbound iron. Endothelial lesions, hemostasis abnormalities, and inflammatory lesions occur, as well as with an increase in anaerobic metabolism, lactic acid levels, brain damage as a result of oligodendroglial vulnerability, astrocyte dysfunction, N-methyl-D-aspartate receptor abnormalities, and synaptic damage [28, 29].

Mitochondrial DNA damage induces changes in respiratory chain proteins, with the formation of new free radicals and subsequent cell lesions. Neonatal cerebral hypoxia stimulates activin secretion, which in turn stimulates erythropoietin, resulting in the production of nucleated red blood cells [35, 43].

The types of lesions that occur in the brain as a result of hypoxia are variable. In the extremely immature brain, preoligodendrocytes and cell precursors of oligodendrocytes are affected. As the brain matures, the resistance of oligodendrocytes to oxidative stress increases due to an increase in antioxidant defense, as well as to the protein structure involved in programmed cell death.

After the hypoxic episode, there is an increase in the density of glutamate receptors, and mitochondrial calcium accumulation occurs, which will trigger apoptosis. Caspase 6 and, subsequently, caspase 3 are activated. NMDA receptor activation depresses the mitochondrial respiratory process and induces apoptosis—a process which is not found in adults, known as the NMDA paradox [41, 48].

Studies conducted by Peeters and Schulte regarding the activity of glutathione peroxidase in the cerebrospinal fluid of newborns with asphyxia and the neuron-specific enolase value evidenced a correlation between the glutathione peroxidase value and gestational age, as well

as between the neuronal enolase value and the neurological evolution of perinatal asphyxia cases. The influence of the genetic factor on postischemic evolution was also demonstrated, as well as on the presence of a correlation with patient's sex, males being more predisposed to develop lesions compared to females.

With the stabilization of newborns with asphyxia at birth, in the reoxygenation stage, the exposure of the diseased cell to a new oxidative attack follows. The use of 100% oxygen in resuscitation is an important source for the formation of ROS. Hyperoxia causes an increase in the activity of antioxidant enzymes such as catalase or superoxide dismutase, as well as an activation of enzymes in the glutathione reductase cycle: GSH-reductase and GSH-S-transferase. Some studies demonstrated that the urinary level of N-acetyl-glucosaminidase is correlated with the value of oxidized glutathione and is higher in newborns exposed to a FiO_2 of 100%. Based on these findings, resuscitation guidelines were changed in 2010, indicating to start neonatal resuscitation with atmospheric air, followed by an increase by titration in the FiO_2 value depending on the improvement of blood oxygen saturation [1, 3, 43].

In the study conducted in our unit, we found that lipid peroxidation in newborns with asphyxia was maintained at a high level on the third day compared to the first day of life, without a statistically significant difference. This high plateau value on day 3 was attributed to perinatal hypoxic stress and to the reoxygenation-reperfusion process. None of the newborns with asphyxia received hypothermia treatment, because at the time of the study, this therapeutic method was not available in our unit. The analysis of lipid peroxidation after neonatal asphyxia by gestational age groups evidenced more intense peroxidation in premature infants compared to term infants with asphyxia. We explained this process by the association of neonatal asphyxia with respiratory distress in the case of prematures, which involved oxygen therapy and respiratory support for its treatment, factors that increased oxidative stress and implicitly, malondialdehyde values. Regarding protein peroxidation, its markers are found in high amounts in the plasma of prematures with asphyxia. Their high values are maintained until the seventh day of life [43]. In newborns with hypoxic ischemic encephalopathy in our study, protein peroxidation was more intense compared to the other newborns. Hypoxic injury is aggravated by protein-unbound iron release. In its presence, as part of the Fenton reaction, hydroxyl radicals are released, which will exert a strong toxic effect on the brain. ROS toxicity in newborns is enhanced by the increased production of ROS, their rapid tissue growth, and impaired antioxidant defense. In the developing brain, endothelial lesions, hemostasis abnormalities, inflammatory reaction, an increase of anaerobic metabolism occur, which are followed by lactic acid accumulation. Oligodendroglial injury, astrocyte dysfunction, and synaptic abnormalities will result [43, 44].

Oxidative stress markers have an important predictive value for neuronal injury in newborns at risk. The correlation between the values of oxidative stress markers and imaging electrophysiological brain changes, near-infrared spectroscopy (NIRS) aspects, and the degree of neurological impairment is not currently described in the literature. This is why further studies are required in this respect. Also, research on therapies protecting against cerebral oxidative stress after perinatal asphyxia has not reported a clearly beneficial medication at this stage of knowledge. The only therapy currently applied with positive, beneficial results in perinatal asphyxia is controlled hypothermia. In the near future, studies are needed which

should attempt to identify free radical scavengers that can be administered to newborns and could be useful in limiting oxidative stress, particularly in prematures, in whom antioxidant defense is impaired due to the end of pregnancy before term [44, 47].

3. Conclusion

Oxidative stress at newborns has role in pathogenesis of different neonatal diseases. The oxidative stress is more severe in preterm than in term neonates. The antioxidant defense of preterm less developed than in term neonates, mainly the enzymatic antioxidant defense.

In the near future, studies are needed which should attempt to identify free radical scavengers that can be administered to newborns and could be useful in limiting oxidative stress, particularly in prematures, in whom antioxidant defense is impaired due to the end of pregnancy before term.

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Chapter 6

Oxidative Stress in Hadrontherapy

Carine Laurent

Additional information is available at the end of the chapter

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Abstract

Conventional radiotherapy has shown its efficiency since decades with large progresses during the 1990s. However, for 15–20% of treated patients, there is no prognosis improvement either due to tumor radiation resistance and/or to side effects on normal tissues representing the limiting dose given during a radiotherapy protocol. A new modality of radiation therapy has emerged representing a technological breakthrough: hadrontherapy. This regroups mainly proton and carbon ion therapy. Dose deposit is in favor of hadrons compared to photons as it occurs at a precise depth in human body sparing upstream and downstream normal tissues. Mechanisms of action of photons and hadrons are different. When photons mainly act by water radiolysis—producing e^-_{aq}, $H^\bullet$, $^\bullet OH$, H_2O_2, $O_2^{\bullet-}$…, carbon ions and protons mainly act by direct effects, i.e. by direct transfer of ion energy to biological macromolecules. Moreover, efficiency of carbon ions is considered threefold higher (1.1 for protons) than X-rays in killing tumor cells, whereas it is considered lower for normal cells. These findings suggest strong advantages of hadrontherapy compared to conventional radiotherapy. However, some recent studies tend to show a stronger increase in oxidative stress in normal cells after protons or carbon ions than X-rays.

Keywords: hadrontherapy, oxidative stress, carbon ions, protons, DNA damage, tumor killing efficiency, normal tissue toxicity, senescence, inflammation

1. Introduction

Oxidative stress is of major interest in killing tumor cells. In this way, radiation therapy is one of the most used modality for cancer treatment (**Figure 1**). Ionizing radiations lead to the production of deleterious reactive oxygen species that overcome antioxidant systems resulting in tumor cell death. On the 14 million of new cancer cases each year in the world, about half of them will benefit from this treatment [1].

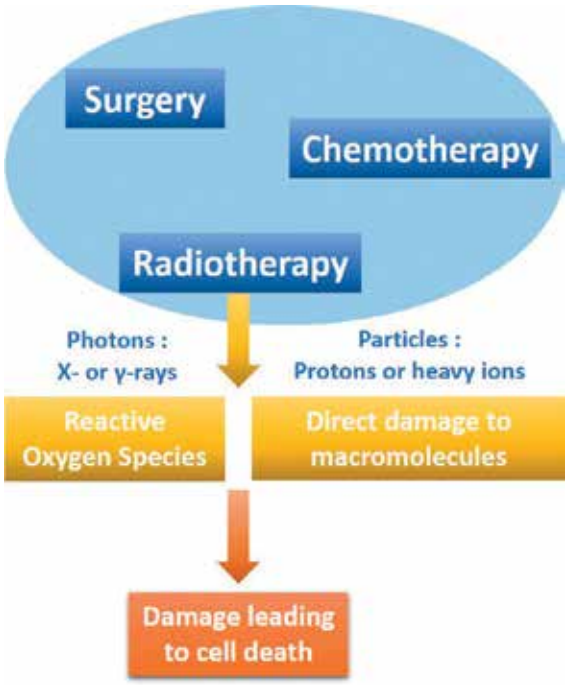

Figure 1. Cancer treatment: focus on radiotherapy.

Conventional radiotherapy—by photons (γ- or X-rays)—has known a revolution since the 1990s, mainly thanks to progresses in imagery, computer sciences and robotics. In this way, new modalities of radiation therapy occurred: intensity-modulated radiotherapy (IMXRT), image-guided radiation therapy (IGRT) and respiratory-gated radiotherapy: the 4D radiotherapy. These new kinds of treatments allowed to overcome the main difficulties encountered in conventional radiotherapy: the exponential dose deposit which leads to an overdose in normal tissues upstream and downstream from the tumor (**Figure 2**, left panel) [2].

In parallel, therapy by accelerated hadrons was developed since the 1950s (Berkeley, United States). Hadronic particles regroup neutrons, protons, pions, antiprotons, helium, lithium, boron, carbon and oxygen ions. The major interest of protons and heavy ions (mass greater than helium) lies in the profile of dose deposit: the Bragg peak (**Figure 2**, right panel). Contrary to conventional radiations, dose distribution is in favor of normal surrounding tissues as the maximum of dose is deposited at a precise depth in the matter with a larger peak for protons than for carbon ions. However, a plateau phase does exist upstream from the peak, resulting in a small proportion of dose deposition in normal tissues preceding the tumor, as well as a fragmentation tail downstream from the peak (except for protons which cannot fragment in smaller particles). Moreover, in the case of heavy ions, their fragmentation when encountering matter lead to secondary particles, which properties are different in terms of LET (linear transfer energy) and biological effects. In addition, to treat the whole tumor volume, hadron beam energy and direction are modified to spread the peak: SOBP, Spread Out Bragg Peak (**Figure 2**, right panel). This leads to an addition of plateau phases as well as fragmentation

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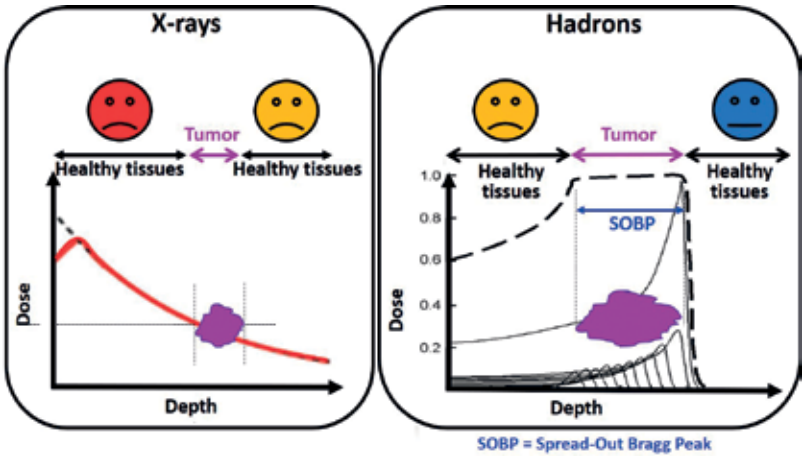

Figure 2. Dose deposit after X-rays in comparison to hadrons. Left panel: Dose deposit of X-rays according to depth in human body. Right panel: Dose deposit of hadrons according to depth in human body. Bragg peak: Continuous black line. SOBP: Dotted line.

tails. In this way, normal surrounding tissues received a percentage of dose that could be non-negligible according to the tumor size and localization.

Radiations lead to a wide range of oxidative damage to DNA, lipids and proteins. Effects of photons were widely studied *in vitro* and *in vivo* since decades. When photons mainly act by indirect effects, i.e. water radiolysis—producing e^-_{aq}, $H^\bullet$, $^\bullet OH$, H_2, H_2O_2, H^+, OH^-, $O_2^{\bullet-}$…, carbon ions and protons mainly act by direct effects, i.e. by direct transfer of ion energy to biological macromolecules.

We propose to develop involvements of oxidative stress in: (i) tumor cell killing efficiency of hadrontherapy and (ii) side effects of hadrontherapy—secondary tumors and normal tissue injury.

2. Oxidative stress and tumor cell killing efficiency of hadrontherapy

The main advantages of the use of hadrons in comparison with photons are their superior dose localization, their efficiency against radioresistant and hypoxic tumors and the ability to shorten treatment planning.

2.1. Interest of protons and carbon ions in clinics

Due to their high charge, heavy ions lead to concentrate ionizations when they cross matter. These concentrate ionizations result in concentrate oxidative damage. On the contrary, when photons (low LET) encounter matter, they produce low ionization densities. Tumor cell killing is more efficient with hadrons as, for example, clusters of DNA damage are produced leading to difficult DNA repair in comparison with photons producing more easily repaired SSB (single-strand breaks). Efficiency—RBE for Relative Biological Efficiency—of carbon ions

is considered twofold to threefold higher (1.1 for protons) than X-rays in killing tumor cells. These RBE are calculated for a percentage of clonogenic survival of 10%. However, experiments leading to these values were performed under a broad range of conditions, among other things: LET or cell cycle phase—cell irradiation at confluence stage or during exponential phase. In this way, higher RBE than 3 were found—up to 5, for example, 3.3 for normal human skin fibroblasts exposed at confluence stage to mimic skin physiology to carbon ions in the plateau phase before Bragg peak as it would be the case during radiotherapy [3]. Biological interactions of protons and carbon ions being a lot more complex than photons, and to improve hadrontherapy, there is a need of a better knowledge of biological effects, at early and late times, of hadrons according to LET, fractionation, cell type, oxygenation, cell cycle phase, etc. (for review [4]). Due to the favorable dose deposit profile, this kind of therapy is recommended for unresectable and radioresistant tumors. Until now, more than 110,000 patients have been treated by proton therapy and 15,000 patients by carbon ion therapy.

Concerning protons, numbers of pediatric tumors were treated by protons as the dose deposit should be favorable for normal surrounding tissues: medulloblastoma [5, 6], rhabdomyosarcoma [7, 8], craniopharyngioma [9], etc. There is a trend to extend the indications for proton therapy from already treated skull base [10, 11] and brain [12–14] tumors to prostate [15–17], lung [18–20], head and neck (for review, [21]), gastrointestinal (for review, [22]) and breast [23] cancers. Compared to conventional radiotherapy, proton therapy obtained the same results in terms of tumor local control (for review, [24]). The superiority of protons is still discussed, except in large ocular melanomas, chordomas and chondrosarcomas [25].

The main experienced facilities providing carbon ion beams and treating a big number of patients are: NIRS (Japan) and GSI and then HIT (Germany). The main indications were, as for protons, not only pediatric cancers but also bone and soft tissue sarcomas; head and neck cancers; pancreas, prostate and cervix cancers; hepatocellular carcinomas and glioblastoma (for review, [26–28]). Carbon ion therapy present significant advantages, but, due to a lack of available data in the literature, clinical evidences are still lacking.

2.2. DNA damage and repair, mitotic catastrophe

Hadrons are considered as acting mainly by direct effects. Carbon ions are particularly deleterious in terms of cell survival, viability and apoptosis, even on very radioresistant tumor cell lines [29, 30]. This efficiency to kill tumor cells could come from the type of damage produced by carbon ions: DSB (double-strand breaks) and clustered DNA damage considered as difficult to repair. Clusters of damage could be a criterion explaining ion irradiation efficiency as it was shown that cluster number increases with LET. However, Hada et al. [31] have shown that DNA damage (DSB, abasic sites, oxidized bases) number decreased in genomic DNA irradiated at high LET and DSB was more frequent than other damage after charged particles, even low-LET protons, than after X-rays. In the same manner, Heilmann et al. [32] demonstrated that carbon ion irradiation (LET from 14 to 400 keV/μm) did not generate more DSB than X-rays (kV) with a maximum of about 38 DSB/Gy/cell. A possible explanation for the strong RBE of carbon ions could be related to DNA damage repair. Moertel et al. [29] showed that residual DSB were more numerous in ion-irradiated human glioblastoma cells than in

X-ray-irradiated cells. Moreover, Weyrather et al. [33] highlighted that carbon ion RBE was related to cell repair capacity. The role of HR (homologous recombination) was highlighted after proton irradiation as deficiency in this pathway leads to a sensitization of cells to protons [34]. DNA repair by the Ku-dependent NHEJ (non-homologous end joining) pathway was shown as inhibited by high-LET irradiation [35]: the yield of DSB should be the same after low- or high-LET irradiation but high-LET induced smaller fragments inhibiting the efficient binding of Ku to DSB fragment. However, there are reports of a primordial role of NHEJ after carbon ions as inhibition of DNA-PKcs led to a sensitization of cancer cells to carbon ions [36]. Recent report pointed out another response to DNA damage after carbon ion irradiation: mitotic catastrophe. Kobayashi et al. [37] demonstrated that mitotic catastrophe phenomenon was induced in a larger manner after carbon ions than after X-rays in 20 human cancer cell lines, whereas apoptosis and senescence were unchanged between both radiation types.

2.3. Oxygen effect and carbon ions

Hypoxic tumor cells are reoxygenated during radiotherapy treatment, and this reoxygenation plays an important role on treatment efficacy [38]. Hypoxic tumors resist to X-rays, whereas carbon ion exposure remains efficient [39]. The same study showed that this could come from a faster reoxygenation of tumors after carbon ion irradiation compared to X-rays. This was confirmed by Oya et al. [40] and also by Fukawa et al. [41] by pO2 measurements in mouse fibrosarcomas. Recently, Wozny et al. [42] have shown that hypoxia-induced factor HIF-1α, whose role was demonstrated in radioresistance to conventional radiotherapy, is expressed earlier in carbon irradiated cancer stem cells—subpopulation of head and neck squamous cell carcinoma—localized in tumor hypoxic areas. In the presence of oxygen, ROS quantity increases leading possibly to a major oxidative stress and so to a stronger attack of biological macromolecules. Oxygen effect could play a major role in the difference observed between carbon ion and photon responses as hypoxia leads to a decrease in DSB repair capacity [43]. Hirayama et al. [44] observed a decrease in DNA damage after hypoxia but this was much less significant after carbon ion than after X-ray irradiation. Moreover, the same study demonstrated that repaired DSB percentage was unchanged after carbon ion irradiation in hypoxic conditions, which is not the case for X-rays. The authors concluded that DSB repair plays an important role in oxygen effect as this effect was decreased after carbon ion irradiation compared to X-rays. This could be related to a stable effect of oxygen on DSB during the time after carbon ion irradiation, whereas it decreases after X-ray irradiation.

2.4. Role of oxidative stress in hadrontherapy efficiency

Studies are controversial concerning protons. The use of edaravone, a radical scavenger, did not decrease DNA DSB formation in MOLT-4 tumor cells after protons as it was the case for X-rays leading to conclude that radical-induced indirect DNA damage was lower with protons than with X-rays [45]. However, Baran et al. [46] showed that proton irradiation led to a disruption of the electron flow in the complex I of the mitochondrial respiratory chain in human leukemia Jurkat T cell, and the use of antioxidants in HeLa cancer cell line allowed an attenuation of the enhancement of radiation-activated gene expression [47].

Concerning carbon ions, studies performed at high radiation doses (30 Gy) on murine squamous cell carcinoma and fibrosarcoma transplanted in mouse allowed to provide evidence of a strong upregulation of stress-responsive and cell communication genes after carbon ion irradiation compared to γ-rays [48]. Moreover, glutathione depletion in human squamous cell carcinoma cell lines potentiates the effects of carbon ion irradiation [49]. In this way, heavy ions do not act only by direct interaction with biological macromolecules but also by an induction of oxidative phenomena.

3. Oxidative stress and side effects of hadrontherapy

Radiotherapy aims to destroy cancer cells by the use of photons, protons or heavy ions. But this is a double-edged sword as it can also kill normal cells. Two types of side effects can appear: deterministic (pneumonitis, gastrointestinal or cutaneous syndrome, etc.) or stochastic (carcinogenesis and genetic effects). Indeed, dose deposit is exponential for photons so that the maximum of the dose is given at the entrance in the body before reaching tumor. In this way, normal tissues—present upstream and downstream from the tumor—receive ionizing radiations leading to ROS (reactive oxygen species) production. When normal cells are unable to detoxify these ROS, there is an imbalance leading to oxidative stress. Signaling pathways leading to inflammation maintain this process, therefore participating to side effects on normal tissues. It is considered that 5–10% of the general population exhibit acute or late adverse effects after radiotherapy. For example, pneumonitis is observed in 5–15% of patients irradiated for breast, lung and mediastinal tumors [50]. By the use of hadrons, organs at risk present around the tumor could be spared, and biological efficiency is considered higher in tumors than in normal tissues. In this way, treatment time could be shortened by hypofractionation of the total radiotherapy dose: 3 weeks compared to 6–7 weeks.

3.1. Toxicity encountered in patients after proton or carbon ion therapy

Toxicities of radiation therapy can not only occur at skin level (dermatitis, telangiectasia, etc.), cardiovascular and pulmonary level (pneumonitis, cardiovascular disease, etc.), gastrointestinal level (xerostomia, mucositis, esophagitis, enteritis, proctitis, emesis) and genitourinary level (cystitis, erectile dysfunction, vaginal dryness and stenosis, infertility and teratogenicity), but also at psychological level with fatigue and depression (for review [51]).

Proton therapy studies reported approximately the same proportion of early toxicities than photon therapy. However, comparative studies to photons are still necessary when possible. Recent reports tend to show a decrease in early and late toxicity: Romessser et al. [52] reported that proton therapy for head and neck cancers had significantly lower rates of early grade 2 (grade represents the degree of gravity of toxicity) or greater acute dysgeusia (5.6 vs. 65.2%), mucositis (16.7 vs. 52.2%) and nausea (11.1 vs. 56.5%). Yock et al. [53] reported ototoxicity and neuroendocrine deficit, but no cardiac, pulmonary or gastrointestinal late effects after treatment of medulloblastomas by protons, with a median follow-up of 7 years.

First studies of patients undergoing carbon ion therapy and presenting side effects were reported during the end of the 2000s (for review, [26]). Comparative studies on toxicities

of carbon ion therapy versus conventional radiotherapy are still missing. Concerning bone and soft tissue sarcomas, toxicities were mostly decreased compared to conventional radiotherapy. A report showed that, on 78 patients treated by carbon ion therapy for unresectable osteosarcomas, grade 3 acute and late skin reactions were seen in 3 and 4 patients, respectively, and grade 4 skin and soft tissue reaction occurred in 3 patients [54]. However, for an escalation dose protocol, toxicities were considerably increased: 34 on 35 patients present acute skin reactions and 26 on 27 patients late skin reactions up to grade 4 [55]. For unresectable sarcomas: on 47 patients treated for non-sacral spinal sarcomas, 1 patient presented grades 3 and 4 late skin reaction and 1 patient grade 3 spinal cord reaction [56]; 6 patients and 2 patients on 188 patients with sacral chordomas presented grade 3 peripheral nerve and grade 4 skin toxicity, respectively [57]; and 4 patients on 75 patients treated for non-skull base chondrosarcomas report grade 3 or 4 late skin and soft tissue reactions [58]. Except bone and soft tissue sarcomas, most of toxicity was encountered for cervical cancers: a dose escalation protocol led to 18% of major gastrointestinal toxicity [59], and in another study, 8 patients on 29 developed bladder complications and 4 patients presented grade 4 rectal toxicities [60]. Clinical trials are in progress to register toxicities in the different facilities providing carbon ion therapy [26].

Induction of secondary tumors was also reported. Concerning protons, Chung et al. [61] studied 558 patients treated by protons and 558 treated by photons: second malignancies occurred in 5.2% of proton patients compared to 7.5% of photons. They concluded that proton therapy was not associated with a significantly increased risk of secondary malignancies compared with photon therapy, but the follow-up of these patients was only around 6 years after radiation therapy. This reduced risk of secondary malignancies due to proton therapy was confirmed by Sethi et al. [62], whereas there are no enough long-term reports after carbon therapy. Indeed, concerning carbon ions, literature on secondary tumors is still poor but a study pointed out that 30% of patients treated for cervical cancers developed distant metastases [63]; a case was reported of a brain tumor induced by heavy particle radiotherapy [64]. Preclinical studies, recently performed on mice exposed to carbon ions in comparison to photons, revealed that interstitial chromosome deletions were more increased in secondary cancers induced by carbon exposure [65]. They contradict previous results of Ando et al. [66] showing the same induction in carbon locally irradiated mice of secondary tumors after γ-rays.

3.2. DNA damage and repair, mitotic catastrophe

Production of clusters of DNA damage can lead to mitotic catastrophe in fast or slow renewal normal tissues then leading to early or late toxicities. A lower immediate increase in DNA damage measured by alkaline comet assay was observed in confluent primary cultures of skin fibroblasts after carbon ion versus X-ray irradiation but a late increase in DNA damage was observed only after carbon ions whereas it was not the case after X-rays [3]. The lower immediate increase could be explained by the production of smaller fragments after carbon ions compared to X-rays whereas the late production of DNA damage after carbon ions could come from DNA repair. Indeed, micronucleus frequency—described as a result of impaired repair of DNA double-strand breaks—was 1.7-fold increased 24 hours after carbon irradiation compared to X-rays (unpublished results) and this increase persisted 2 weeks after irradiation (unpublished results) where a late wave of oxidative damage was observed [3].

Results obtained by Antonelli et al. [67] on quantification of γ-H2AX foci after carbon ion vs. X-ray irradiation in lung fibroblasts showed a longer persistence of γ-H2AX foci after carbon ion irradiation which is in agreement with a more difficult repair of DNA complex damage. Moreover, Gustafsson et al. [68] studied, in normal human skin fibroblasts, clustered DSB and non-DSB lesions which convert into DSB during preparation for pulsed-field gel electrophoresis and their results showed a similar increase after carbon ion or low-LET irradiation. It was recently shown that clustered DSB perturb normal human fibroblast DNA repair after high LET irradiation [69]. In confluent normal fibroblasts, accumulations of p53 at early times and p21 at late times were 2–3 times higher after carbon ions than after X-rays [70]. DNA repair proteins (hMRE11, p21, PCNA) were accumulated along ion trajectory in normal human fibroblasts and this was dependent of chromatin compaction [71].

3.3. Role of oxidative stress in hadrontherapy toxicity

Highest toxicity of carbon ions, and in a lower extent of protons, could come from indirect effects of irradiation, i.e. due to a stronger concentration of reactive oxygen and nitrogen species that cells would not be able to detoxify. However, only few studies were interested in oxidative phenomena occurring after carbon ion or proton irradiation.

Wan et al. [72] showed that ROS production in human epithelial cells occurred in the same proportion after proton or X-ray irradiation. Whole body proton irradiation of mice also led to an early differential modulation of oxidative stress gene expression in liver: only proton irradiation led to an increase in Prdx6 and Sod3, mainly, whereas other genes were common to photon irradiation [73]. Chang et al. [74] demonstrated that whole body proton irradiation of C57BL/6 J mice leads to a late increase in ROS production, NOX4 transcription and DNA damage in hematopoietic stem cells from irradiated mice. Proton irradiation of rat eye led to an upregulation of oxidative stress and apoptosis gene expression [75]. Baluchamy et al. [76] concluded that, after proton irradiation, mouse brain presented modifications in expression of genes related to oxidative stress which could lead to programmed cell death. Moreover, the use of antioxidants allowed to protect against biological effects of protons not only *in vitro* [77] but also *in vivo* [78], which tends to demonstrate the importance of oxidative stress. Transgenic mice overexpressing human mitochondrial catalase presented protective effects on low-dose proton-induced brain injury [79]. In the same manner, neuroprotective effects of reducing mitochondrial ROS were also shown by Liao et al. [80] in proton irradiated mice not only at low dose but also at a higher dose of 2 Gy. SOD mimetic was also shown efficient in reducing oxidative damage in retinal cells from proton eye-irradiated rats [81] and in ameliorating acute and chronic proctitis in focal proton irradiated rat rectum [82].

After carbon ion irradiation, an increase in oxidative stress was observed in confluent irradiated primary cultures of normal human skin fibroblasts with an increase in biological macromolecule damage and a decrease in antioxidant enzyme activities in comparison with X-rays [3, 83]. This trend was confirmed by Dettmering et al. [84]: an increase in superoxide anion production was measured in normal human fibroblasts and the maximum level was obtained at a lower dose after carbon irradiation than after X-rays. In human hematopoietic stem/progenitor cells (HSPCs), carbon irradiation led to a strong increase in heme oxygenase-1

and NAD(P)H dehydrogenase-quinone 1 expression [85]. *In vivo*, mouse whole-body carbon irradiation was shown to decrease glutathione level and to increase MDA content in testis one week after irradiation [86]. At longer term - 2 months after exposure - and in comparison to gamma-rays, mouse whole body irradiation led, in intestine and colon, to: (i) a persistent increase in ROS, mitochondrial cardiolipin oxidation and lipid damage; (ii) a late decrease in antioxidant enzyme activities [87]. The use of other antioxidants indirectly pointed out an important role of oxidative phenomena. Indeed, some antioxidants allowed to decrease effects of carbon ions in normal cells or tissues: curcumin ameliorates cognitive deficits in carbon-irradiated mice via SOD increase, MDA decrease and upregulation of important genes in oxidative stress pathways like heme oxygenase-1 and NAD(P)H quinine oxidoreductase 1 [88]; melatonin reduced carbon-induced apoptosis in mouse carbon-irradiated testes [89] and brain [90] via a decrease in carbonyl and MDA content and an increase in SOD and catalase activities; Dragon's blood decreased hydrogen peroxide and MDA levels and increased SOD activity and glutathione content in carbon-irradiated rat brain [91]. These last experiments provide indirect proofs of the major role of oxidative stress in hadrontherapy toxicity.

3.4. Stress-induced premature senescence

In normal human fibroblasts, radiation exposure lead to a G1 cell cycle arrest evolving in quiescence or senescence [92]. Premature senescence, or SIPS (stress-induced premature senescence), differs from replicative senescence. SIPS phenomenon was generally observed in fibroblasts exposed to prolonged or repeated stresses [93, 94] and was also shown after X-ray exposure [95, 96]. Naka et al. [96] showed, by the use of ATM mutated fibroblasts, that pathway leading to premature senescence in fibroblasts after oxidative stress or X-ray exposure could also be ATM-dependent and could act via p38MAPK and p16INK4A. After carbon ion exposure, a higher accumulation of p21 in carbon-irradiated confluent normal fibroblasts was observed at late times compared to X-rays [70]. In normal human lung fibroblasts, carbon ion irradiation led to a faster senescence than γ-rays [97] However, this phenomenon of premature senescence was observed in the same proportion as for X-rays in the progeny of human fibroblasts after an immediate cell cycle arrest and senescence reappeared and persisted after 5 months after exposure [98]. Our experiments on confluent primary cultures of normal human skin fibroblasts showed a lower proportion of senescence-associated β-galactosidase cells 3 weeks after carbon ion exposure compared to X-rays (unpublished results) (**Figure 3**).

3.5. Inflammation and late toxicity

Schematically, acute side effects in normal tissues would be generally related to a loss of fast renewal cells, whereas late effects would appear due to several more complex phenomena as the loss of low renewal cells, progressive ischemia due to the loss of microvascularization endothelial cells and the development of late fibrosis, mainly due to inflammatory processes [99, 100]. After irradiation, it is known that cytokines, which are important mediators of late radiation-induced effects, are not only secreted at early times after irradiation but also at later times-months or years after exposure. Normal tissues monocytes and macrophages produce proinflammatory cytokines like IL-1, IL-6 and TNF-α, which attract macrophages and

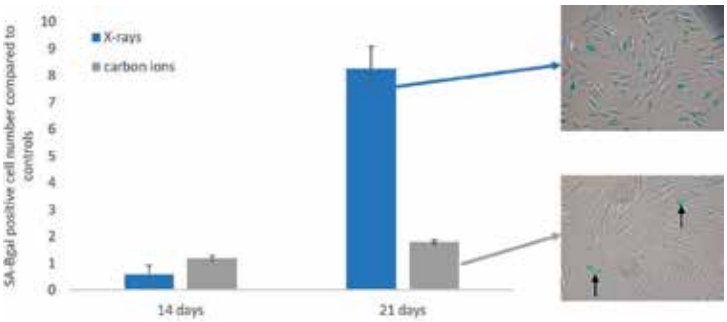

Figure 3. Premature senescence in normal human skin fibroblasts exposed to carbon ions or X-rays at an isosurvival dose (unpublished results). Cells were irradiated at confluence and kept until 14 or 21 days post irradiation. After fixation, SA-β-galactosidase staining was performed (citric acid/sodium phosphate 40 mM, NaCl 150 mM, $MgCl_2$ 2 mM, potassium ferrocyanide 5 mM, potassium ferricyanide 5 mM and X-gal 1 mg/mL). SA-β-galactosidase activity was determined by counting blue cells using a microscope. Data represent mean percentage of β-gal positive cells ± SEM.

lymphocytes. Activated macrophages and stimulated stromal cells synthetize fibrogenic cytokines such as TGF-β and PDGF modulating fibroblast proliferation-differentiation balance and protein synthesis and degradation via metalloproteinases (MMP) and their inhibitors (TIMP) (for review, [99, 101]). In this way, specificity of proton or carbon ion irradiation concerning these pathways is of main interest to modulate late effects of hadrontherapy. Fournier et al. [102] showed an accumulation of fibrocytes and extracellular matrix proteins in normal human foreskin fibroblasts exposed to carbon ions. However, a lowered increase in IL-6 was observed in normal human skin fibroblasts exposed to carbon ion compared to X-ray irradiation [3]. The use of Dragon's blood, which presents antioxidant and anti-inflammatory properties, did not allow to reduce TNF-α, IFN-γ and IL-6 levels in carbon-irradiated rat brain as it was the case for γ-rays [91]. A recent report on carbon-irradiated normal human skin models showed similar inflammatory processes than after the same dose of X-rays [103].

3.6. Bystander effects

Bystander effect, i.e. biological effects to cells which were not irradiated via signals coming from irradiated cells, could be at the origin of normal surrounding tissue injury and to, for example, abscopal effects. Oxidative stress signal pathways could play an important role in these effects.

Indeed, confluent human skin fibroblasts were shown to present a persistent oxidative stress after exposure of 0.036–0.4% of them to proton or X-ray microbeam but this was not the case for carbon ions [104]. However, when normal cell cultures exposed to low-LET protons were co-cultured with unirradiated cells and after 20 population doublings, no changes in survival, chromosomal damage, protein oxidation and lipid peroxidation were observed [105]. This was not the case for higher LET (iron and silicon ions) for which a higher level of oxidative damage, a decrease in antioxidant enzyme activities and an alteration of mitochondiral proteins - encoded by mitochondiral DNA - were observed [105].

Recently, Autsavapromporn et al. [106] have shown that glioblastoma cell carbon irradiation led to damage in unirradiated normal fibroblasts. Moreover, a single dose of carbon irradiation

	8-oxodG concentration (nM)	
Time after irradiation	**24 hours**	**14 days**
X-ray control	0.775 +/- 0.140	0.817 +/- 0.036
X-ray irradiated	1.980 +/- 0.064	2.175 +/- 0.073
C-ion control	0.822 +/- 0.103	0.830 +/- 0.084
C-ion irradiated	3.103 +/- 0.296	3.153 +/- 0.262

Table 1. 8-oxodG concentration in normal human skin fibroblast culture supernatants exposed to carbon ions or X-rays at an isosurvival dose (unpublished results).

led to less damage than a fractionated dose. However, after 20 population doublings, there were more damage on cells irradiated in one time than in several fractions. Dose hypofractionation, which is presented as a major advantage of carbon therapy, could therefore engender more late effects to bystander normal tissues. Inflammatory pathways playing an important role in oxidative stress persistence in normal tissues after irradiation thus leading to normal tissue injury, the study of bystander effects on the secretion of inflammatory cytokines is of major interest. Carbon microbeam irradiation of a low proportion (0.45%) of immune cells led to decreased cytokine levels [107]. Oxidized extracellular DNA could also be a signaling factor in bystander effects. 8-oxodG is the main oxidatively generated DNA lesion and is formed either by direct oxidation or can be incorporated in DNA from oxidized nucleotide pool by DNA polymerase. Its extracellular presence can be due to DNA repair, cell death, mitochondrial turnover, cellular uptake or salvage of DNA damage products. Carbon-irradiated confluent skin fibroblasts exhibited a 1.5-fold increase in extracellular 8-oxodG 24 hours and 2 weeks after C-ion beam exposure compared to X-rays (see **Table 1**, personal unpublished data). In this way, the role of bystander effects in carbon or proton therapy remains unclear and needs further investigations.

Cell culture supernatants were purified by solid phase extraction, and samples were adjusted for the standard addition method in order to correct for the matrix effects contributed by the culture medium constituents as reported previously [108]. An optimized method for the quantification of 8-oxodG has been applied. HPLC-ECD signals were recorded in the culture supernatants spiked with the external standard. Data represent mean 8-oxodG concentration ± SEM.

4. Conclusion

Mainly due to the cost of hadrontherapy facilities, there are too few studies dealing with biological effects of protons, carbon ions or other particles on tumors and normal tissues. In addition, a large proportion of these works did not compare carbon ion effects to X-ray effects. Advantages of hadrons, mostly on tumors, are often highlighted but particular attention should be paid on side effects of hadrons, especially hypofractionation which could lead to major injuries in normal tissues. Killing efficiency of carbon ions is often considered lower for normal cells than for tumor cells. However, some recent studies tend to show a strong increase in oxidative stress in normal cells after protons [74, 79] or carbon ions [3, 84].

Figure 4. Proposed schema of biological effects of hadron irradiation leading to tumor killing and normal tissue toxicity.

According to the literature, **Figure 4** proposed a schema of biological effects leading to tumor cell death or to normal cell toxicity.

Further investigations are needed to better understand toxicity of protons and carbon ions. Prediction of side effects for each patient should be of major interest in order to adapt radiotherapy protocol and/or to prevent deleterious effects due to normal tissue irradiation or to bystander phenomena. The use of antioxidants, which were demonstrated as efficient in reducing late effects of protons and carbon ions, could be of major interest in preserving normal tissue during proton or carbon ion therapy. Another guideline for reflection is related to the drawbacks of protons and carbon ions: they could lead to an interest of other ions as helium ions which should lead to less toxicity in normal tissues but are also less efficient on cancer cells and which do not present the same interest as carbon ions in killing tumor cells in hypoxic conditions. In conclusion, due to complex effects of hadrons when encountering normal tissues and tumors, there is a strong need in preclinical studies—at early and late times post-irradiation and in comparison to photons—to determine biological effects of SOBP, ion fragmentation, LET distribution in depth, hypofractionation, beam scanning, etc.

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Chapter 7

Erythrocyte Nitric Oxide

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Additional information is available at the end of the chapter

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Abstract

Nitric oxide (NO) is a vasoactive molecule that, by stimulated and functional vascular endothelial cells, is released to the lumen of the vessel and into the surrounding smooth muscle cells. Once in the lumen, NO is captured by red blood cells and scavenged inside through hemoglobin and derived as NO metabolites. The delivery ability of erythrocytes allowing the NO efflux also occurs. Manipulation of NO levels inside the erythrocyte through different external (acetylcholine, acetylcholinesterase inhibitors, fibrinogen and CD47 4N1K peptide) and internal (redox and protein phosphorylation levels) stimuli will be described. The values of NO efflux from the erythrocytes and its association with the data quantified in the hemorheology properties and in clinical parameters obtained from patients with vascular diseases will also be present. The in vivo animal experimental studies highlighting the ability of NO efflux (delivered) from the erythrocytes where is scavenged and its influence in inflammatory and hemorheological responses will be addressed. So, the aim of this chapter is to present the knowledge obtained about the NO signal transduction mechanism in erythrocytes and the association between erythrocyte availability in NO with clinical biomarkers obtained in inflammatory vascular diseases. A final question is raised—namely, could NO be considered a hemorheological parameter?

Keywords: erythrocyte, nitric oxide, deformability, intravital microscopy, pathophysiology

1. Introduction

Vascular endothelium cells behave like "meeting points" between white blood cells and mediator factor participants in the steps of inflammatory response allowing "cross-talk" with blood, red blood cells (RBCs), platelets, fibrinogen, lipoproteins and other blood biomolecule components [1]. Endothelial cells (ECs) under influence of mechanical, physical and chemical stimuli are prone to secrete vasoactive molecules into the lumen vessels and smooth muscle cells [2].

The endothelium-derived factor secreted by vascular endothelial cells, known by its vasodilator property, was further identified as nitric oxide (NO) by Robert F. Furchgott, Louis J. Ignarro and Ferid Murad who received the Nobel Prize in 1998 [3, 4].

Ignarro's spectral analysis of hemoglobin (Hb) evidenced that when this biomolecule was exposed to endothelial cells' stimulate by acetylcholine (ACh), NO is liberated and a shift of the Hb absorption curve occurs, establishing for the first time the NO binding to Hb and an indirect link between red blood cells (RBCs) and NO [4].

Other authors evidenced the ability of RBCs to rescue NO liberated from endothelium cells and the need to liberate it according the tissues oxygen partial pressure [5, 6]. Experiments conducted in vitro using RBCs under normoxia conditions submitting to low oxygen tension (hypoxia) showed liberation of oxygen and NO binding to deoxygenated hemoglobin demonstrating consequently allosteric structure transitions of the Hb molecule [5]. The role of erythrocyte membrane band 3 protein into NO through erythrocyte was studied [5]. The bioavailability of RBCs in NO may be the trigger or the consequence of the involvement of the RBC's hemoglobin as the oxygen sensor [5, 6]. NO binds to oxygenated Hb in its thiol group of cysteine β93 at high-tissue oxygen pressure (PaO_2) originating as S-nitrosohemoglobin (SNO-HbO_2), while at low PaO_2, NO binds to the iron ion of the hemoglobin; heme group generates nitrosylhemoglobin molecules [7, 8]. Regarding the efflux of NO from erythrocytes, the transfer of NO between SNO-HbO_2 and the thiol group of band 3 protein was verified [9–11]. The transnitrosation reaction could occur with the thiol group of other biomolecules [9–11]. Using inhibitors of protein tyrosine kinase (PTK) p72 syk, Src Lyn and of SHP-2 protein tyrosine phosphatase (PTP), in vitro studies have evidenced, respectively, band 3 protein phosphorylation and dephosphorylation at tyrosine residues [12]. The dephosphorylation of band 3 is associated with oxyhemoglobin and glycolytic enzymes binding which, upon band 3 phosphorylation, delivers oxygen and glyceraldehyde dehydrogenase, aldolase and phosphofructokinase closer to the cytosol [13]. Glutathione (GSH) is a redox biomolecule resulting from the reaction between the three peptides glycine, glutamic and cysteine showing its thiol group that can bind directly NO transferred from SNO-HbO_2, originating from nitrosothiol such as S-nitrosoglutathione (GSNO) [14]. The GSNO is a transient reservoir of NO because it is essential to be in its reduced state for the regeneration of NADPH to NADP levels of the erythrocyte. However, the inactivation of glutathione reductase induced by oxidative stress influences the concentration of GSH which is needed for regeneration of oxidized proteins [15]. For instance, dithiothreitol (DTT) is a thiol-reducing agent capable of regenerating disulfide-containing proteins and establishing an interchangeable thiol-disulfide reaction with glutathione [16]. Beyond that, DTT's presence induces changes on enzyme activity states, for example, of the PTP and PTK [17].

If auto-oxidation of hemoglobin occurs the peroxide anion will be produced, which generates peroxynitrite after reaction with NO [18]. The decomposition of peroxynitrite molecules leads to nitrite (NO^{2-}) and nitrate (NO^{3-}) which are designated NO derivative molecules (NO^x) whose concentrations are changed by external RBC-binding biomolecules as shown [19, 20]. It was evidenced that NO release from SNOHb could bind to thiol groups and be exported from RBCs as nitrosothiol or may be so as oxidation generates nitrate [19]. The NO in the presence of oxyhemoglobin molecules induces methemoglobin and nitrate formation [20]. The hemoglobin reductase with the NADH produced in the glycolytic pathway maintains the methemoglobin concentration [21].

Endothelial cells and lymphocytes are able, by the participation of choline acetyltransferase, to synthesize ACh which is released into the plasma through vesicular acetylcholine transporter [22–24].

Depending on the degree of endothelium integrity the circulating ACh induced vasodilation or vasoconstriction according to the amount of nitric oxide synthesized and released [3]. The NO released from endothelial cells and platelets is scavenged by erythrocyte and blood cell-free hemoglobin [25]. In order to identify the signal transduction mechanism of the erythrocyte ability to scavenge or deliver NO, in vitro studies mimicking normal and inflammatory conditions were performed and are presented in the next section. This section also includes ex vivo studies showing the quantification of the NO efflux values, performed with RBCs obtained from blood samples of healthy donors and patients with vascular inflammatory diseases and their association with clinical biomarkers. Also, *in vivo* studies' conduct in animal models of hypertension and inflammation are included in the next section to show erythrocyte NO availability, contribution and association with inflammatory vascular diseases. From all data obtained and herein described we are able to conclude the erythrocyte NO translocation across the erythrocyte membrane as a hemorheological parameter.

2. Erythrocyte nitric oxide studies

2.1. In vitro

Erythrocyte membrane acetylcholinesterase (AChE) is a hydrolytic enzyme with a rare kinetic profile with an optimum substrate concentration (So) from on AChE activity decrease with the augment of its substrate ACh [26, 27]. At lower or higher So values, the AChE-ACh enzyme complex forms are active or the less active ones, respectively [26, 27].

Based on the fact that SNO-Hb and GSNO have been considered reservoirs of NO and ACh is an endogenous compound with vasoactive properties, present in blood circulation, we raised three questions, whether ACh induces changes on erythrocyte deformability, if there is NO inside erythrocyte and whether it could be mobilized to the outside. In order to answer these, human erythrocyte suspensions, in the presence of ACh, were loaded with the permeable non-fluorescent probe diamine fluorescein-2 diacetate (DAF-2 Da). Intra-erythrocyte fluorescence intensity of triazolofluorescein (DAF-2 T) was visualized, by fluorescence microscopy, as a result of the reaction between NO and the 4, 5- diamine fluorescein [28]. So, inside the erythrocyte, there is NO when stimulated with ACh and also there is an increase in the levels of NO^{2-} and NO^{3-} [28]. When erythrocytes are in the presence of ACh, erythrocyte deformability, during the impairment of oxygen hemoglobin affinity and of erythrocyte aggregation (EA), has been verified [29]. The presence of an active complex (AChE-ACh) in red blood cells is able to trigger band 3 protein phosphorylation when PTP is inhibited, with a higher mobilization of NO-derived metabolites [30]. This complex is unable to induce band 3 phosphorylation upon p53/56lyn and p72syk inhibition, providing a lower degree of NO efflux and NOx mobilization [30]. This mobilization is enhanced with phosphorylated but not a dephosphorylated band 3 protein. The maximum translocation of NO efflux from RBC achieved upon acetylcholine stimulation and band 3 phosphorylation was related to the higher levels of the methemoglobin,

[L-lactate], concentration ratio between cyclic guanylyl cyclase (cGMP) and cyclic adenosine monophosphate (cAMP) and lower oxygen affinity to hemoglobin value and of oxyhemoglobin concentration [30]. At variance, the effect of the AChE inhibitor velnacrine maleate (VM) induced a higher degree of [NO] efflux/[NOx] mobilization through the AChE-VM inhibitor complex in the presence of p53/56lyn and p72syk inhibitors [30]. When in the case of erythrocyte membrane band 3 protein dephosphorylated state, the inactive complex form of the AChE promotes higher NO efflux than the AChE active complex form [30]. But the opposite was observed with erythrocyte membrane band 3 protein phosphorylation [30]. When experiments were done with the AChE strong inhibitor, VM, an almost inactive complex, results and induces lower NO efflux from erythrocytes and higher GSNO and peroxynitrite concentration values than those obtained with the active complex form AChE-ACh [30].

Additional studies were performed taking into account the identification of the type of a G-protein involved in the erythrocyte ACh/NO signaling pathway. It was evidenced that at the N-terminal band 3 protein domain only $G_{\alpha i1/2}$ binds. $G_{\alpha i1/2}$ and the G_{β} are associated with band 3 protein at the C-terminal site domain independently of the band 3 phosphorylation degree [31]. This chapter confirmed our previous hypothesis of the potential involvement of a heterotrimeric G protein in signal events mediated by the erythrocyte membrane AChE-ACh complex or AChE-inhibitor complex band 3 protein interactions with the participation of adenylyl cyclase inhibition [20, 31].

The quantification of NO efflux from the erythrocyte was assessed, by the first time for us, using the amino-IV sensor by the amperometric method which is described [32, 33]. The nitric oxide release from RBC in presence of ACh is sense by an electrode which oxidize NO at the working platinum electrode, resulting on electric current. The redox current is proportional to the NO concentration outside the membrane and it was continuously monitored with a computer. The AChE-ACh active complex activates PKC which phosphorylates PTP and PTK switching them to inactive and activate enzymes states, resulting in band-3 protein phosphorylation by PTK active form without with consequently NO release [32–34].

Beyond the AChE's strong inhibitor velnacrine, the moderate AChE inhibitor timolol was used, forming a less active AChE-timolol complex, and a lower erythrocyte efflux from NO was quantified in relation to those values obtained with AChE-ACh [35–37].

For the first time we evidenced that when erythrocytes were in the presence of ACh or timolol, the efflux of GSNO was lower with AChE-timolol than with AChE-ACh, both values being higher than in their absence [38].

It was evidenced by those in vitro studies that AChE's active and less active molecular conformations induce increased or decreased NO efflux from erythrocytes, respectively [38].

In the presence of SpermineNONOate, one among other NO donors, there is an increase in erythrocyte deformability and oxygen hemoglobin affinity (29).

The plasma levels of ACh increase in inflammatory pathologies like fibrinogen (Fib), a plasma molecule predominantly produced by the liver [39, 40]. From many years, it was recognized that Fib behavior in vascular domains, where blood circulation is under low shear stress, acts as the most influent molecule in erythrocyte aggregation (EA) [41]. The association between Fib and EA has been verified in several pathological conditions [42]. Only in this twenty-first

century was CD47 established as a binding target in the erythrocyte membrane for the soluble form of fibrinogen [43].

It was shown that for soluble Fib, in physiological concentrations, the NO efflux from erythrocytes decreased with increased GSNO, nitrite and nitrate levels [44]. The scavenging NO RBC ability to reduce efflux was surpassed showing normal values when both 4N1K (the CD47 peptide analog of thrombospondin binding site) and high fibrinogen levels are present or when 4N1K is absent [45]. These data show the dependence of lower cyclic adenosine monophosphate (cAMP) associated with adenylate cyclase (AC) inhibition by $CD47G_{\alpha i}$ [45]. When phosphorylation of the erythrocyte membrane protein band 3 is induced in the presence of high fibrinogen concentration and in the absence or presence of 4N1K, the NO efflux increases [46, 47]. The NO efflux from erythrocytes at high fibrinogen concentration is dependent on band 3 protein phosphorylation which was confirmed in the experiments where the erythrocyte casein kinase 2 (a cytosol protein that phosphorylates the band 3 protein) inhibitor was used, showing unchanged levels of NO efflux in relation to its absence [27, 48].

During inflammation high levels of both acetylcholine and fibrinogen are presented and normal values of NO efflux from erythrocytes have been observed in vitro [39, 49]. Besides, a higher NO efflux from RBC will be expected resulting of the presence of ACh and high fibrinogen concentration, normal values were obtained; the AChE-ACh molecular conformational state activates PKC which inhibits PDE 3 with increase of cAMP concentration that normalize the lower levels of cAMP derived from the inhibition of AC [35, 38, 45, 49]. Interestingly, the presence of forskolin (activator of the enzyme AC) in an in vitro model of hyperfibrinogenemia did not change the levels of NO efflux from erythrocytes, because the PDE3 is functional to hydrolase cAMP [27, 48].

When stimulating the erythrocyte redox thiol status by loading dithiothreitol (DTT), there is a decreased NO efflux concomitant with increased levels of nitrite, nitrate and GSNO [50]. It is well known that dithiothreitol induces band 3 dephosphorylation and a dephosphorylated state accounts for the AChE inhibitors and fibrinogen effects on red cells [17, 30, 49].

High concentrations of oxidized LDL when in the presence of blood samples of healthy human erythrocytes increase its ability to scavenge NO [51]. The same behavior was obtained in another study conducted with blood samples taken from healthy humans and exposed or not (control aliquot) to two different concentrations of LDL/HDL; no changes in NO efflux values from the erythrocyte, no alterations on intra-erythrocyte peroxynitrite concentrations and an unaltered deformability profile, at all shear stresses, were observed. At variance the levels of intra-erythrocyte NO derivative molecules nitrite, nitrate and GSNO showed significantly increased values when compared with control aliquots. The unchanged deformability values obtained at lower and high shear stresses for all treated blood sample aliquots with LDL/HDL are indicative of membrane stability, internal viscosity maintenance and normal interactions of membrane peripheral and cytoskeleton [52]. The absence of erythrocyte membrane instability obtained in blood sample aliquots under LDL/HDL addition is confirmed by the unchanged nitrogen reactive species concentration of peroxynitrite, as evidence by the normal levels obtained for peroxynitrite is an index of auto-oxidation of oxyhemoglobin [52, 53]. The addition of different concentrations of the lipoprotein sub-fractions' LDL/HDL seems not to favor hemoglobin auto-oxidation. Superoxide anion will be formed from the

auto-oxidation of hemoglobin, but without its generation plus unchanged values of peroxynitrite concentrations it was evidenced that when the thiol status of erythrocyte was maintained in normal range, no alterations were verified in erythrocyte deformability [50].

The NO efflux from erythrocytes is gender independent [54] at variance with higher women's RBC AChE enzyme activity than men as previously evidenced [54]. Timolol maleate is a topically therapeutic drug used in glaucoma patients that, when incubated with blood samples of patients with this nerve optical disease, they did not induce significant differences in NO efflux from erythrocytes and nor in GSNO concentration values inside RBCs when compared to the absence of timolol in the blood aliquots of those erythrocytes [55]. Both NO efflux and GSNO values obtained were significantly higher than those quantified in blood samples of healthy persons [55]. Erythrocytes' NO metabolism in glaucoma patients are not affected by timolol treatment [55].

The same amperometric NO sensor, mentioned above, was used in confluent human umbilical endothelial cells (HUVECs) in which we have demonstrated the existence of membrane-bound acetylcholinesterase and higher NO production in the presence of ACh in relation to velnacrine [56–58]. The activation of the signal transduction mechanism induced by the AChE-ACh active complex revealed high values of [cAMP] and [cGMP] which are lowered by the AChE-VM inactive complex [58].

2.2. Ex vivo

Hemorheology is the science which studies blood deformation and its components' interaction with vessel walls, occurring inside blood vessels of macro and microcirculation. In the past, the longitudinal and follow-up clinical studies done, ex vivo, have as an objective the characterization of the intravascular profile of different diseases according to hemorheological parameters and inflammatory factors [41, 48, 59]. To accomplish this aim, blood samples were taken from patients with acute myocardial infarction, glaucoma, Bechet, renal diseases whether submitted or not to chronic hemodialysis or kidney transplant and diabetic retinopathy degree, and an association between the laboratorial data and the clinical parameter values was observed [41, 48, 59].

Erythrocytes scavenge and liberate oxygen and NO at high and low local tissue oxygen partial pressure, respectively [60, 61]. Erythrocyte deformability is a biorheological influent factor on blood viscosity, cellular oxygenation and a biomarker of acute and chronic inflammation [62].

Patients with hypercholesterolemia, hypertension and renal transplant present lower ability to reversible change its shape (decrease erythrocyte deformability values) and higher values of NO efflux from erythrocytes when stimulated with ACh [63]. In the same study, an inverse relationship between erythrocyte deformability values and NO efflux concentrations from erythrocytes obtained from blood samples of those patients, was evidenced [63]. In all samples lower values of NO efflux were verified in relation to those of healthy persons—what could be considered as a compensatory mechanism to avoid more wall vessel damage [63]. We will present in vivo studies later in this section.

Disturbed blood rheology in patients with systemic lupus erythematosus (SLE) and patients with rheumatoid arthritis (RA) that could contribute to atherosclerosis is described [64–66].

So, a study was performed to evaluate the associations between hemorheology parameters including the erythrocyte NO rescue ability of RBCs and the cardiovascular risk factors, inflammatory parameters and subclinical atherosclerosis. Erythrocyte NO efflux was significantly associated with both carotid intima-media thickness (cIMT) and the presence of plaques (negative association) and was an independent predictor of cIMT [67]. Erythrocyte NO production can be looked at as a compensatory mechanism [67]. As mentioned above, under low tissue oxygen partial tension, erythrocytes release NO bound to hemoglobin, promoting vasodilation [61, 62]. Besides, NO could represent a protective factor against atherosclerosis; it could be produced in large amounts by the inducible nitric oxide synthase (iNOS) which is characteristic of the dysfunctional endothelium which combining with superoxide anion generates peroxynitrite molecules that have oxidant properties; this NO derivative worse the dysfunctional endothelial wall hindering it to return to be functional [68].

The data of the hemorheological and inflammatory evaluations performed ex vivo in blood samples of women with SLE suggested greater risk of arterial thrombosis and prediction of higher mortality than humans with normal blood viscosity and fibrinogen values [67, 69]. Both SLE and RA patients showed high erythrocyte aggregation independent of the medication undertaken by SLE patients. This ex vivo study shows that hemorheological parameters are independently associated with the early stages of atherosclerosis in SLE and RA patients. Additionally, it documents disturbed and unfavorable hemorheological features in association with disease activity and with traditional CV risk factors contributing to atherogenesis in inflammatory rheumatic diseases. So, the evaluation of NO and also of hemorheological parameters must be done in order to predict the development course of the autoimmune disease in RA and SLA patients [67].

In patients with amyotrophic lateral sclerosis (ALS) that is a neurodegenerative disease of the motor system, our aim was to assess RBCs' biochemical and hemorheological parameters and identify novel biomarkers of one of the most painful and fast mortal disease after diagnosis [70]. The erythrocyte deformability and AChE activity of blood samples were increased in patients with ALS in comparison to healthy donors [70]. This ex vivo study conducted with blood samples of ALS patients showed lower values of NO efflux from RBCs and nitrites than those obtained in healthy humans [70]. Due to variability between the duration of this disease until death, the higher NO quartile values are associated with the worse respiratory function [70]. A positive relation of quartiles values were obtained between AChE enzyme activity and nitrites levels [70]. Both erythrocyte NO and AChE were suggested as biomarkers of ALS and further potential therapeutic targets [70].

Another very sad complex situation with high mortality covering healthy humans from all ages and under a variety of situations from a simple infection or travel accident to a post-surgery complication is sepsis [71]. Several pathophysiological mechanisms from unbalanced pro−/anti -inflammatory, hypo and hyper-insulinemia, to hypo- and hyper-glycemia are simultaneously deregulated in intensive care units (ICUs). Follow-up studies carried out in ICU are urgently needed [71]. We have verified that in septic shock patients before dead, at 24 in IUC showed higher efflux of NO from erythrocytes and worse blood circulation observed by hemodynamic parameters namely high unequal blood flow and high microvascular flow index quantified in sub lingual microcirculation [72].

2.3. In vivo studies

The ACh molecule has a ubiquity property with an anti-inflammatory effect, decreasing leukocytes' adherence and plasma TNF-α concentration evidenced in an in vivo animal model of lipopolysaccharide-induced inflammation [73, 74].

The influence of NO on the hemoglobin affinity to oxygen and on biorheology properties of the erythrocytes was shown in healthy and in ill humans as described earlier. NO influx into erythrocytes induced by spermineNONOate or the efflux stimulates by ACh increased the reversible discocyte shape, or erythrocyte deformability, under shear stress values characteristic of the microcirculation network as shown [29].

The angiotensin II AT1-receptor antagonist, valsartan, is able to restore, in hypertensive Sprague–Dawley rats which are LNAME dependent, their systolic blood pressure (SBP) to the physiological values, as well as normalize the whole blood viscosity (WBV) values increased during the hypertensive time [75]. The NO efflux from erythrocytes decreases in parallel to WBV and SBP returning to normal values after valsartan application [75]. Regarding erythrocyte deformability values that decreased during the hypertensive state of the animals, lower or normal values at lower and higher shear stress, respectively, were maintained after systolic blood pressure recovery by valsartan [75]. This in vivo animal experimental model of hypertension demonstrated the relation between the endothelial cells and the erythrocytes' availability of NO, beavering as a compensatory mechanism vascular disturbance [75]. This could be considered as an antagonist effect to the occurrence of reactive nitrogen species (RNS) and to the amplification of oxidative reactive species (ORS).

The in vivo mice model of acute inflammation induced by intra-scrotal injection of the platelet-activator factor (PAF, a phospholipid mediator of inflammation) showed that the NO efflux from erythrocytes decreases with acute-phase response development [76] The end of the acute inflammatory response visualized by intravital microscopy showed normalization of the number of labeled neutrophils rolling and adherent, rolling velocity and vessel diameter values [76]. NO normal values rewound with inflammation recover, besides the maintenance of decreased RBCs' deformability [76]. There are PA receptors (PAF-R) constitutively present on platelets, leukocytes and endothelial cells but are absent in red blood cells; besides, PAF stimulates the breakdown of sphingomyelin on RBCs in isotonic conditions [77]. Therefore, PAF may cause changes in the physicochemical structure of the erythrocyte membrane, which in turn may cause changes in RBC deformability maintained after the recovery of the initial phase of inflammatory response [77]. The NO efflux from erythrocyte behavior in this model of acute inflammatory response during 6 h in mice reinforces the idea of the NO rescue inside erythrocyte as a compensatory mechanism in low-grade or chronic inflammation of those diseases reported earlier [63, 77].

3. Conclusions

The nitric oxide mobilization inside or outside the erythrocyte is possible under either the action of the non-neuronal cholinergic components, acetylcholinesterase and acetylcholine, or the manipulation of band 3 protein phosphorylation degree. Higher NO efflux occurs under the influence of the AChE-ACh complex as well as, simultaneously, with the band 3 protein

phosphorylation. At variance, higher NO mobilization inside the erythrocytes happens under simultaneous influence of AChE-velnacrine and band 3 protein dephosphorylation.

At normal acetylcholine plasma levels, the erythrocyte NO efflux increases by a signal pathway dependent of membrane band 3 protein phosphorylation, $G_{i\alpha\beta}$ protein, AC, acetylcholinesterase enzyme activity and its molecular conformations, PKC and PD3.

The erythrocyte aggregation tendency is impaired by the presence of the AChE-ACh complex and is reinforced by higher thiol redox status inside the erythrocyte. The erythrocyte aggregation is impaired by changes on band 3 phosphorylation/dephosphorylation equilibrium; besides, higher values are associated with a higher phosphorylation degree. On the contrary, ED increases in the presence of the AChE-ACh complex.

The ability of RBC to scavenge NO may be considered as a compensatory mechanism acting against the overproduced NO by the endothelial-inducible NO synthase when the vascular endothelium is dysfunctional [50].

Under unstimulated erythrocytes, GSNO efflux did not occur, and it is regarded as a potentially therapeutical agent, acting as a store or donor of NO.

It is possible to modulate erythrocyte availability in NO by plasma fibrinogen in a nonlinear, dose-dependent manner in human erythrocytes. Lower intra-erythrocyte cAMP is an influent condition to the NO efflux in an in vitro model of hyperfibrinogenemia. These results may be considered a useful therapeutic approach for the storage of blood that is used in transfusions. Fibrinogen-C47-triggered erythrocyte GSNO and decreased NO efflux may, if verified in vivo, be associated with coagulopathy and hypotension under acute phase states. These effects show on NO-derived molecules, allowing intra-erythrocyte NO scavenging as a protector under inflammatory conditions as we evidenced in an animal model of acute inflammation and verified ex vivo in a vascular inflammatory disease with low-grade or chronic inflammation. An anti-reactive nitrogen role can be attributed to ox-LDL for its contribution in the erythrocyte-scavenged ability for nitric oxide. From all studies reviewed here, we can suggest NO efflux or influx from or into RBCs and the internal mobilization between the NOx as a hemorheological parameter participating in the erythrocyte deformability in dependence of the structural protein conformations or phosphorylation degree components of the membrane or the internal RBC compartment.

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Conflict of interest

The authors declare no conflict of interest.

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Oxidative stress plays a crucial role in the pathophysiology of various diseases when there is a disruption of the intracellular redox balance and the homeostatic balance between cellular oxidants and antioxidants. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) react with molecular targets including proteins, lipids, and nucleic acids contributing to mitochondrial injury and cellular dysfunction. This book intends to provide the readers with an extensive overview of the novel approaches and prospects based on oxidative and nitrosative stress in the pathophysiology of various diseases and in the current treatment strategies with antioxidants.

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