Review Article

Pharmacology of Free Radicals and the Impact of Reactive Oxygen Species on the Testis

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Abstract

The role of free radicals in normal cellular functions and different pathological conditions has been a focus of pharmacological studies in the recent past. Reactive oxygen species (ROS) and free radicals in general are essential for cell signaling and other vital physiological functions; however, excessive amounts can cause alteration in cellular reduction-oxidation (redox) balance, and disrupt normal biological functions. When there is an imbalance between activities of ROS and antioxidant/scavenging defense systems, oxidative stress (OS) occurs. A good number of studies have shown OS is involved in the development of several disease conditions, including male infertility. In the present article, generation of free radicals and their effects, as well as the mechanisms of antioxidant/scavenging defense systems are discussed, with particular focus on the testis. The review also discusses the contribution of OS on testicular dysfunction and briefly focuses on some OS-induced conditions that will alter testicular function.

Keywords: Antioxidant, ROS, Scavenging, Superoxide anion, Testis.
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Introduction

Pharmacological research over the years has made reasonable contributions in virtually every area of medicine including mechanisms of the pathogenesis of various diseases (1-6). In the recent past, on annual basis, many publications on pharmacological research have been focused on the effects of antioxidants in different pathological conditions (2, 4, 9, 11-13). The findings of such studies have revealed the involvement of free radicals (reactive oxygen and nitrogen radicals) in most disease conditions. Increased amounts of reactive oxygen species (oxidative stress) and reactive nitrogen species (nitrosative stress) have now been identified, with definitive evidence, to be prominent features of many acute and chronic diseases, including the normal aging process (1, 2, 7). Oxidative/nitrosative stress is linked to the pathogenesis of: cardiovascular dysfunction, *e.g.*, hypertension, cerebrovascular accidents, and heart failure (4); reproductive dysfunction (6, 8); septic shock (3); cancer (5, 9); aging (10, 14), and many age-related chronic diseases, including atherosclerosis (15); diabetes mellitus (16); rheumathoid arthritis (17), and neurodegenerative diseases (2, 18, 19).

Free radicals and antioxidants

Free radicals: A free radical is defined as any chemical species that contains unpaired electron (s) in its outer orbit (20, 21). Because of these unpaired electrons, free radicals are highly reactive and readily take part in chemical reactions with virtually all cell components (lipids, proteins, complex carbohydrates and nucleic acids) in the body. These reactions occur through a chain of oxidative reactions to cause tissue injury (20). For most biological structures (like lipids, proteins, and nucleic acids), free radical damage is

closely associated with oxidative damage, causing direct cellular injury by inducing lipid and protein peroxidation and damaging nucleic acids (21–23). In most biological systems, the free radicals of interest are often referred to as reactive oxygen species (ROS), as the most biologically significant free radicals are oxygen-centered. ROS produced in cells include superoxide anion (O_2^-), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2), although the latter is not technically considered as a free radical itself because it contains no unpaired electrons (21, 22). However, H_2O_2 is considered a reactive oxygen species because of its ability to generate highly reactive hydroxyl free radicals through interactions with reactive transition metals like iron and copper (22, 24, 25). Reactive nitrogen species (RNS) is another group of free radicals that can alter cellular function (11, 20). As described in the review by Victor et al. (11), RNS refers collectively to nitrogen-centered radicals and includes nitric acid (NO), peroxynitrite ($ONOO^-$), nitrogen dioxide radical ($\cdot NO_2$). Their definition of RNS also includes other oxides of nitrogen and products arising when NO reacts with O_2^-, peroxyl radical ($RO^\cdot$) and alkoxyl radical ($ROO^\cdot$).

ROS play some important roles in a number of physiological processes, including the intracellular killing of bacteria by neutrophil granulocytes (3, 11), detoxification by the liver (26), prostaglandin production and certain cell signaling processes (26–28). In humans, O_2^- is the most commonly produced and well studied ROS, which is generated during the oxidative phosphorylation or one-electron reduction of O_2 in the mitochondria (respiration) as the natural byproduct (29). Molecular oxygen (O_2) is a biradical which contains 2 unpaired electrons with parallel spin configurations. Its complete reduction to H_2O requires sequential transfer of electrons, resulting in the generation of O_2^- and other highly reactive intermediates, including H_2O_2 and ·OH (25, 29). The complete reduction of oxygen is summarized in the following equations:

$O_2 + e^- \longrightarrow O_2^{\cdot-}$ Superoxide radical

$O_2^{\cdot-} + H_2O \longrightarrow HO_2^\cdot + OH^-$ Hydroperoxyl radical

$HO_2^\cdot + e^- + H \longrightarrow H_2O_2$ Hydroperoxide

$H_2O_2 + e^- \longrightarrow OH + OH^-$ Hydroxyl radical

Production of ROS and their mechanisms of biological activities: Phagocytic cells, such as macrophages and neutrophils are also prominent sources of O_2^-. In the presence of invading pathogens like bacteria, phagocytic cells become activated and they generate O_2^- which attacks the invading pathogens as a part of the inflammatory response (11). Superoxide anion is also produced from xanthine by the enzyme xanthine oxidase along with uric acid as waste products of purine metabolism (30). Other intracellular sources of the generation of ROS include reactions involving cytochrome P450 enzymes (31), peroxisomal oxidases (32), and NAD(P)H oxidases (33).

Superoxide anion is important in the body because it generates other free radicals capable of causing cell injury (27, 28). Superoxide anion is impermeable to the cell membrane and mainly affects enzyme function (34). Its mechanism of toxicity involves disassembly of iron-sulphur ([Fe–S]) clusters in proteins through the inactivation of iron regulatory protein-1 (IRP-1), causing release of iron and alterations of -SH residues (32). Superoxide anion reacts with the potent vasodilator and cell-signaling molecule, nitric acid (NO), to form the toxic peroxynitrite ($ONOO^-$) product (28, 34). Superoxide anion can also undergo dismutation to form hydrogen peroxide (H_2O_2), either spontaneously or when catalyzed by the enzyme superoxide dismutase (27). Although, H_2O_2 is not a free radical by definition, it is a biologically important oxidant because it selectively participates in hydroxyl radical generation, an extremely potent radical (21, 24, 34). Hydrogen peroxide undergoes reactions with metal ions like ferrous (Fe^{2+}) or cuprous (Cu^{2+}) to form ferric (Fe^{3+}) or cupric ions (Cu^{3+}) and hydroxyl ions, which is sometimes described as the Fenton reaction (36). Also, because of its nonionized and low charged state, H_2O_2 has a long diffusion distance, since it readily diffuses through hydrophobic membranes as seen with the leakage of H_2O_2 from mitochondria (38). H_2O_2 is broken down to O_2 and water by the antioxidant enzyme catalase (34, 37, 38). In addition to catalase, glutathione peroxidase (GPx) can also break down H_2O_2 and other peroxides that are formed on lipids within the body to yield water and oxidized glutathione (34, 38).

NO is synthesized through the enzymatic conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS), which exists in three known forms ofendothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) which can be up-regulated under certain conditions to induce

oxidetive stress (39). At physiological concentrations, NO controls mitochondrial respiration, causing reversible inhibition of respiration by altering cytochrome C oxidase (Complex IV) activity, which is the terminal enzyme of the mitochondrial respiratory chain (40). NO also binds to soluble guanylate cyclase in the vascular endothelium to control vascular tone (41). Inducible NOS (iNOS) is up-regulated by oxidative stress, producing a burst of NO that far exceeds basal levels which can cause significant cellular injury via different mechanisms:

(1) NO may directly promote excessive peripheral vasodilation, resulting in vascular decomposition (41, 42), and (2) NO may up-regulate the transcription of nuclear factor-κB (NF-κB), thus, initiating an inflammatory signaling pathway that, in turn, triggers numerous inflammatory cytokines (42). Peroxynitrite, obtained from the reaction of O_2^- and NO, has been reported to modify proteins with thiol groups resulting in the generation of nitrosothiols, which can disrupt metal-protein interactions and lead to the generation of other metal-derived free radicals (35). Szabo et al.'s (35) study also showed that peroxynitrite can react with CO_2 to form nitrogen dioxide ($\cdot NO_2$), a radical of less activity than peroxynitrite but of longer diffusion distance.

Formation of superoxide anion

$$O_2 + e^- \xrightarrow{X} \text{Hypoxanthine} \xrightarrow{XO} \text{Uric acid} + O_2^- + H_2O_2$$

X: Xanthine; XO: Xanthine Oxidase

Formation of peroxynitrite and nitrogen dioxide

$$NO + O_2^{\bullet -} \longrightarrow ONOO^-$$

$$ONOO^- + CO_2 \longrightarrow NO_2^{\bullet -} + CO_3^{\bullet -}$$

Formation of hydroxyl ions via Fenton reactions

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet -} + OH^-$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{2+} + OOH^{\bullet} + H^+$$

Hydroxyl radical ($\cdot OH$) is formed not only by the interaction between hydrogen peroxide and the reduced forms of metal ions, *i.e.*, Cu^{2+} and Fe^{2+}, but also by reduction of hydrogen peroxide as well as the interaction of superoxide with hydrogen peroxide (27, 34). The hydroxyl radical is particularly unstable and is the most reactive of the free radical molecules. In addition, it is capable of reacting rapidly and non-specifically with most biological molecules (34, 43). Despite having very short half-lives of nanoseconds, they can cause severe damage to cell and other intracellular structures because they can cause covalent cross-linking of a variety of biological molecules. They cause cell damage by initiating chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins (22, 24, 25, 43). Damage to DNA can cause mutations (23, 25) and possibly lead to cancer (23), if not reversed by DNA repair mechanisms (23). Also, damage to proteins causes enzyme inhibition, denaturation and protein degradation (43).

Chemical chain reaction: The process of chemical chain reaction has been described by Catala (44) to involve 3 stages: initiation, propagation, and termination. During initiation, a sufficiently reactive ROS attacks on a peroxide-free lipid system to abstract a hydrogen (H) atom from its methylene group ($-CH_2-$), as these hydrogens have very high mobility. This attack easily generates free radicals (conjugated dienes, peroxyl radical). Hydroxyl radical is the most efficient ROS for this attack, whereas H_2O_2 and O_2^- are insufficiently reactive. A peroxyl radical is also able to abstract H from another lipid molecule (adjacent fatty acid), especially in the presence of metals such as copper or iron, thus causing an autocatalytic chain reaction. The peroxyl radical combines with H to give a lipid hydroperoxide (or peroxide). This reaction characterizes the propagation stage. Polyunsaturated fatty acids (such as those found in cell membranes) are particularly vulnerable to this process of initiation and propagation because of the multiple unsaturation points found in their structure. Oxidative stress-induced peroxidation of membrane lipids eventually generates a variety of mainly α, β unsaturated reactive aldehydes, such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 2 propenal (acrolein) and isoprostanes as terminal products, which also cause tissue damage (45). Oxidative damage of membranes results in alterations in the degree of membrane fluidity which can lead to compromised membrane integrity, inactivation of membrane-bound receptors and enzymes. This may in turn impair normal cellular function and increase tissue permeability (1). Furthermore, the terminal products of lipid peroxidation may act as "second cytotoxic messengers" to induce cellular damage. Some of these products exhibit facile reactivity with various biomolecules, including proteins, DNA and phospholipids, thus generating stable

products at the end of a series of reactions that are thought to contribute to the pathogenesis of many diseases (46).

Scavenging/antioxidant systems: Antioxidants can be described as substances capable of counteracting the damaging effects of oxidation in body tissues. Antioxidants terminate oxidative chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by donating electrons to the free radicals and become oxidized themselves; hence antioxidants are often reducing agents and include enzymatic and non enzymatic compounds (47). The body has a number of interrelated antioxidant mechanisms (enzymatic and non enzymatic) developed to protect the body against oxidative damage and to maintain redox homeostasis and repair damage which occurs (34, 47).

Enzymatic systems: In mammalian cells, the enzymatic defense system consists mainly of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (9, 20, 47, 48). SOD enzymes are a family of metalloenzymes, found in virtually every oxygen-based organism, and their major function is to catalyze the dismutation of O_2^- to H_2O_2 (48), as described earlier. This reaction is generally considered to be the body's primary antioxidant defense because it prevents further generation of free radicals. Depending on the metal ion cofactor (copper, zinc or manganese), three isoforms of SOD are recognized to be important, which are also distributed differently in the body. In humans, manganese (Mn) SOD is present in the mitochondrion, copper-zinc (Cu/Zn) SOD is present in the cytosol, while a third form of SOD in extracellular fluids also contains copper-zinc (Cu/Zn) in its active sites (48, 49). Extracellular SOD is found in the interstitial spaces of tissues and also in extracellular fluids, accounting for the majority of the SOD activity in plasma, lymph and synovial fluid. In humans, SOD occurs in high concentrations in brain, liver, heart, erythrocytes, kidney and spleen (48).

Catalase and glutathione peroxidase (a selenium-containing enzyme), both work to detoxify O_2^- reactive radicals by catalyzing the formation of water from H_2O_2, derived from O_2^- as shown in the reactions below. Glutathione peroxidase requires the presence of reduced glutathione for its action. Glutathione reductase (GSR) reduces glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant (50). For every mole of oxidized glutathione (GSSG), one mole of NADPH is required to reduce GSSG to GSH.

Common oxygen radical scavengers in mammalian systems

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O$$

$$H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

Non enzyme molecules: In addition to the above enzyme systems, there are a variety of other non enzyme antioxidant molecules which play key roles in the body defense mechanisms. The molecules include gluthathione, α-tocopherol (vitamin E), ascorbic acid (vitamin C), uric acid, bilirubin, melatonin and plasma proteins. In general, water-soluble antioxidants (*e.g.*, ascorbic acid) react with oxidants in the cell cytoplasm and the blood plasma, while lipid-soluble antioxidants (*e.g.*, α-tocopherol) protect cell membranes from lipid peroxidation (51).

Glutathione is a water-soluble tripeptidethiol found in millimolar concentrations in the cell cytosol and other aqueous phases and exists in reduced (GSH) and oxidized (GSSG) states (50). The reduced state (GSH) contains thiol group which readily interacts with free radicals, especially the $^{\cdot}OH$, by donating a hydrogen atom. This reaction provides protection by neutralizing reactive $^{\cdot}OH$ radicals that are thought to be a major source of free radical pathology, including cancer. So, GSH is considered to be one of the most important cellular antioxidants to maintain cellular redox state (50, 52). In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase, which in turn reduces other metabolites and enzyme systems, such as ascorbate (in the glutathione-ascorbate cycle), glutathione peroxidases and glutaredoxins and reacts directly with oxidants (50, 53).

Alpha-tocopherol (vitamin E) is the most important lipid-soluble antioxidant and stabilizes cell membranes by preventing lipid peroxidation. Vitamin E protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction, thus removing free radical intermediates and terminating the propagation reaction (54, 55). Vitamin E also acts by scavenging reactive oxygen species before they

can damage cells (54). Oxidised α-tocopheroxyl radicals produced in this process may be recycled back to the active reduced form through reduction by ascorbate, retinol or ubiquinol (54, 56). Another important antioxidant vitamin is vitamin C (ascorbic acid), which has been shown by Niki et al. (56) and Padayatty et al. (57) as a potent and effective hydrophilic antioxidant. Vitamin C is an electron donor (*i.e.*, reducing agent), and by donating its electrons, it prevents other compounds from being oxidized, while it is oxidized in the process. Thus, one of the mechanisms of antioxidant action of vitamin C is through the scavenging or quenching of free radicals. Vitamin C is maintained in its reduced form by reaction with glutathione, which can be catalyzed by protein disulfide isomerase and glutaredoxins (58). In addition, the antioxidative effects of vitamins C and E have been shown to be complimentary or synergistic (56, 59).

Uric acid and bilirubin are both metabolites with high antioxidant properties. Uric acid is present in high concentration in human blood and provides about half of the total antioxidant capacity of human serum (60). Uric acid is a metabolite of purine, produced from xanthine by the enzyme xanthine oxidase. High levels of uric acid, seen in some conditions such as stroke and heart attacks, is considered to be due to uric acid activation as a defense mechanism against oxidative stress, or uric acid acting as a pro-oxidant and contributing to the damage caused in these diseases (61, 62). Bilirubin interacts with and neutralizes ROS, and becomes oxidized to biliverdin. In a previous study, it has been shown that bilirubin undergoes redox cycling with biliverdin, which is subsequently reduced by biliverdin reductase to regenerate bilirubin (63). Melatonin is another powerful free radical scavenger which protects nucleus DNA, membrane lipids and cytosolic proteins against oxidative damage (64). Melatonin has also been shown to activate antioxidant enzyme systems including GPx, catalase and SOD (65). Unlike other antioxidants, melatonin does not undergo redox cycling and is therefore referred to as a terminal (or suicidal) antioxidant (66). Some plasma proteins such as transferrin, ferritin and albumin also play significant roles in protecting the body against oxidative stress. Transferrin and ferritin contribute to antioxidant defense by chelating transition metals (*e.g.*, Fe and Cu) and preventing them from catalyzing the production of free radicals in the cell (36), while serum albumin readily donates electrons to free radicals via its thiol group and becomes oxidized (67). Other antioxidant compounds include retinol, taurine (68), ubiquinone (69), polyphenols such as, carotenoids and flavonoids (70), and thioredoxin and metallothionein, which can be up-regulated during oxidative stress (71).

Oxidative stress: When the production of damaging free radicals exceeds the capacity of the body's antioxidant defenses to detoxify them, oxidative stress (OS) occurs. Thus, oxidative/nitrosative stress is a disturbance in the balance between the production of reactive oxygen species/reactive nitrogen species (ROS/RNS) and antioxidant defenses, which may lead to tissue injury (1, 20, 22). The resultant cellular injury caused by oxidative stress has been linked to several clinical disorders already discussed earlier.

Mechanisms of free radicals mediated testicular dysfunction

The male reproductive system: The male reproductive system consists of the testes and accessory organs. The testis, which is the major male reproductive organ, is primarily responsible for spermatogenesis and androgen production. Spermatogenesis is the process of transformation of male germ cells into spermatozoa, which occurs mainly in the avascular seminiferous tubules. Spermatogenesis is a continual process involving mitosis of the male germ cells, which results in extensive morphological changes in cell shape and ultimately undergoes meiosis to produce the haploid spermatozoa (72, 73). The process requires high intra-testicular levels of testosterone hormone (74), produced by the Leydig cells of the testis (75). It has been shown that testosterone secretion is directly dependent on circulating levels of leutinizing hormone (LH), which is secreted by the anterior pituitary gland (75–77). The anterior pituitary gland also secretes follicle stimulating hormone, FSH (76, 77), which binds with specific FSH receptors attached to the Sertoli cells in the seminiferous tubules, causing these cells to grow and secrete various spermatogenic substances like nutrients, minerals and growth factors required for the normal development of germ cells. In addition, prolactin, also secreted by the anterior pituitary, and inhibin, secreted by the Sertoli cells have been shown to play regulatory roles necessary for the normal development of sperm cells (76–78). Furthermore, the release of these hormones is regulated through a feedback mechanism involving

the hypothalamus. It is well recognized that initiation of testicular function is dependent on the hypothalamic secretion of gonadotropin releasing hormone (GnRH) which in turn stimulates FSH and LH to act on the testis (76, 77). The testis in turn, through the secretion of hormones produced in the Sertoli (*e.g.*, inhibin) and Leydig cells, exerts a negative feedback control on the production of gonadotropic hormones (FSH and LH). Thus, testicular function is tightly controlled by both local and endocrine cells via interrelationships between the hypothalamus, pituitary and testis (the hypothalamic-pituitary-testicular axis).

Normal reproductive function is vital for procreation and sexual satisfaction in humans. Thus, reproductive dysfunction (infertility) is a major health challenge and proper understanding of the pathophysiology is vital for effective treatment of this pathological condition. Although, fertility may decrease with increase in age, infertility often occurs as a result of reproductive dysfunction and this is more common in males than females (79, 80), with at least 20 percent having an underlying endocrine disorder, involving testosterone and FSH (80). Several reports from current research have implicated oxidative/nitrosative stress in male reproductive dysfunction (81–84). Impairment of normal spermatogenesis and sperm function are the most common causes of male factor infertility (80, 81). Normal sperm production and sperm quality, which include motility, capacitation, acrosome reaction, egg penetration and decondensation of sperm head, are essential processes necessary to achieve fertilization. However, a number of studies have shown oxidative stress-related mechanisms for the impairment of these processes (83–86).

ROS generation and antioxidant systems in the testis: Sufficient evidence suggests that low levels of endogenous ROS is required for the regulation of vital sperm functions, such as capacitation, acrosome reaction, and sperm-oocyte fusion (87, 88). However, germinal sperm cells are highly sensitive and easily compromised by high levels of ROS (81). Thus, maintenance of redox balance in the testicular environment by the involvement of free radical-scavenging/antioxidant system is critical for normal testicular function.

ROS may be generated by human spermatozoa either via reactions involving the NADPH oxidase system at the level of the sperm plasma membrane (89), or the NADH-dependent oxido-reductase (diphorase) in the mitochondria (90), as was observed in the spermatozoa of infertile men (91). Sources of high ROS generation have been identified by different researchers to include immature and abnormal spermatozoa (88, 92), contaminating leukocytes (93, 94), and low scavenging/antioxidant activities in serum/seminal plasma (95, 96). Activated leukocytes in semen, in response to a variety of stimuli including inflammation and infection, can produce relatively high (up to 100-fold higher) amounts of ROS than normal semen via several mechanisms (97). Activated leukocytes increase NADPH production via the hexose monophosphate shunt, while metabolic processes of both activated polymorphonuclear (PMN) leukocytes and macrophages lead to respiratory burst and production of high levels of ROS (98). ROS can also be generated in semen *in vitro* during sperm analysis, via sperm processing methods, such as excessive centrifugation, cryopreservation/thawing, and when there is low scavenging/antioxidant activities in sperm-processing media (83, 95).

In order to maintain redox homeostasis in the testis, and to protect sperm from oxidative damage, seminal plasma contains a number of effective enzymatic and non enzymatic antioxidant systems. Enzymatic antioxidants that have been identified in seminal plasma include SOD (99, 100), the glutathione peroxidase/glutathione reductase (GPx/GSR) system (101), and catalase (101). In similar studies, non enzymatic antioxidants that have been identified in seminal plasma include ascorbate (102, 103), α-tocopherol (104), urate (105, 106), pyruvate (107), glutathione (108, 109), taurine and hypotaurine (110). It has been reported that some of these antioxidants also enhance sperm viability/motility (107) as well as normal sperm morphology (106). Furthermore, seminal plasma antioxidants concentrations have been shown to be significantly higher in fertile men than those in infertile men (103, 105, 111).

Pathogenesis (mechanisms) of testicular dysfunction: High levels of ROS (superoxide, hydroxyl, hydrogen peroxide, nitric oxide, and peroxynitrite) adversely affect normal sperm production and quality (motility, viability, and function) by interacting with membrane lipids, proteins, nuclear and mitochondrial DNA (112, 113).

Peroxidation of sperm membrane lipids: Mammalian spermatozoa membranes are rich in polyunsaturated fatty acids (PUFA), which is responsible for sperm fluidity, but at the same time make sperm very susceptible to free radical-induced

peroxidative damage (99). Orientation of unsaturated fatty acids in the plasma membrane creates the fluidity necessary for spermatozoa to perform its normal physiological functions (*e.g.*, acrosome reaction and sperm-egg fusion during fertilization), which involve variety of secretory events (114). Similar studies also reveal that the functioning of membrane-bound ATPases (ion pumps) to maintain normal intracellular concentrations of nutrients and ions (such as sodium or calcium) is critically dependent on membrane fluidity (114, 115). It therefore implies that the loss of membrane fluidity via ROS-induced perturbation of membrane structure/function impairs membrane pump functions, resulting in the disturbance of the cellular ionic homeostasis and cellular calcium utilization. Impairment of cellular Ca^{2+} homeostasis has been reported to affect sperm motility (116), and may lead to cell death. Cellular Ca^{2+} homeostasis can further be affected by low levels of NADH and glutathione due to increased glutathione peroxidase activity during lipid peroxidation to remove lipid peroxidation metabolites, as reported by Alvarez and Storey (117).

Peroxidation of sperm proteins: Besides affecting membrane components and fluidity, ROS-induced peroxidation of critical thiol groups in proteins will alter structure and function of spermatozoa with an increased susceptibility to attack by macrophages (118). Turner and Lysiak (83) in their review on oxidative stress-induced testicular dysfunction reported that H_2O_2 can diffuse across the membranes of sperm into the cells and inhibit enzyme activities, such as glucose-6-phosphate-dehydrogenase (G6PD). G6PD controls the rate of glucose flux through the hexose monophosphate shunt, which in turn, controls the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase (119). NADPH is also required for the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH) by glutathione reductase (34, 53). Inhibition of G6PD leads to a decrease in the availability of NADPH and a concomitant accumulation of GSSG and reduced level of GSH. This can reduce the antioxidant defenses of the spermatozoa and increase peroxidation of membrane phospholipids (120).

Peroxidation of sperm DNA: Oxidative stress in the testis has also been shown to cause peroxidative damage to integrity of sperm DNA (121, 122), which has become an area of focus in male infertility studies. Similar studies have identified damage to DNA, characterized by a high incidence of base modification, DNA fragmentation, chromatin cross-linking and DNA strand breaks in spermatozoa of infertile men (121, 123, 124). The DNA damage that was observed in those studies was attributed to high levels of ROS in spermatozoa. Duran et al. (125) and Meseguer et al. (126) have shown that DNA damage of sperm cell can cause low quality of sperm and fertilization problems since spermatozoa requires intact DNA during fertilization process. Oxidative damage to mitochondrial DNA (mtDNA) is also known to occur in spermatozoa and other aerobic cells that are rich in mitochondria. Multiple mtDNA deletions in spermatozoa could arise through a free radical-driven event occurring at the spermatogonial cell stage and has been linked to reproductive failure in some men (127).

ROS-induced DNA damage increases the rate of germ cell apoptosis (123), described as programmed cell death. Apoptosis results from the activation of an intracellular program that leads to cell death without the induction of an inflammatory response, and its induction has been linked to cytokine-induced stress-kinase stimulation of E-selectin expression in the testicular vascular endothelium. This leads to testicular neutrophil recruitment and an increase in intratesticular ROS, which in turn causes peroxidative damage to cell membranes and the initiation of germ cell apoptosis (128). Although, germ cell apoptosis is important during normal spermatogenesis to eliminate excess immature sperm cells (129), when the process is up-regulated, as seen under conditions of oxidative stress (*e.g.*, toxin exposure, cryptorchidism, testicular torsion etc), testicular function becomes highly compromised (130, 131). As reported by Cudicini et al. (132) and Lysiak et al. (133), germ cell apoptosis particularly affects the seminiferous epithelium and during severe induction, Sertoli cells engulf large numbers of dying germ cells, which may overwhelm normal Sertoli cell processes. This initiates a switch-on of pro-inflammatory cytokines expression involving NF-κB, such as IL-1 and IL-6.

Testicular vascular function: Normal microvascular blood flow in the testis is very vital for testicular function as the lack of adequate blood flow is reported to result in ischemia and cell necrosis (134). In most experimental animals, variation in microvascular blood flow is caused by vasomo-

tion (134). Defined as rhythmic oscillations in vascular tone, vasomotion is caused by local changes in vessels and many tissues. Although, the physiological importance of vasomotion remains uncertain, there is some evidence indicating that it might serve as a protective mechanism when perfusion is compromised, *i.e.*, under conditions of ischemia (135). Lysiak et al. (136) have reported in their study that testicular injury occurring in ROS-mediated ischemia-reperfusion (IR) causes alterations in testicular vasomotion in rats. Also, Collin et al. (134) have demonstrated that testicular vasomotor can be inhibited by ROS-induced decline in testosterone in the rat. Furthermore, Lysiak et al. (136) also revealed that IR events will increase the expression of the vascular relaxing compound (NO), via the up-regulation of iNOS and eNOS in the testis. When produced, NO has been shown to influence vasomotion as well (137). NO is also thought to participate in other events that promote testicular injury, such as expression of cell adhesion molecules (CAMs) on the luminal surface of the vascular endothelium of rats (138), and formation of peroxynitrites (35). CAMs play a key role in IR-induced injury in the testis and other tissues because they are key modulators of leukocyte recruitment. It has been generally observed that the recruitment of leukocytes is the forerunner of much of the subsequent IR pathology in organs (139) and in the testis, specifically (140, 141).

***Testicular endocrine function*:** Testicular oxidative stress causes reduction in testicular testosterone production, either as a result of injury on the Leydig cells or on endocrine structures, such as the anterior pituitary (142, 143). Normal process of steroidogenesis generates ROS, which occurs largely from mitochondrial respiration and the catalytic reactions of the steroidogenic cytochrome P450 enzymes (144). ROS generated in this way has been identified to cause oxidative damage to mitochondrial membranes of sperm cells and contribute to the inhibition of subsequent steroid production if unchecked by intracellular antioxidants (145). In another study, it has been shown that increased amounts of NO in the testis (with the consequent formation of peroxynitrites) decreases testosterone secretion (146).

Conditions that induce testicular oxidative stress: Several conditions have been identified to promote oxidative stress in the testis which lead to infertility including aging, pathological states and exposures to some toxicants.

Aging: Oxidative stress is thought to be involved in the aging process in aerobic organisms (10). Age-related oxidative stress has been established to cause up-regulation of proinflammatory gene expression via the activation of redox-sensitive transcriptional factors and linked with male infertility and age-related pathology (2, 10, 14, 146). Luo et al. (145) and Cao et al. (147) have shown in their studies that aging is associated with reduction in testicular antioxidant capacity, while Zirkin and Chen (142) have reported that aging is associated with decrease in testosterone production or steroidogenesis.

Pathological states: Various studies have shown that the pathophysiology of certain pathological conditions of the testis is linked with increase in oxidative stress in the testis. Orchitis, testicular torsion, varicocele, and cryptorchidism are among disease conditions that have been identified to cause increase in ROS levels in the testis (148–152). Orchitis is characterized by localized testicular infections or systemic inflammations, while testicular torsion results from twisting of the spermatic cord, with resultant ischemia. Varicocele occurs due to dilation of the pampiniform venous plexus above and around the testicle (particularly the left testicle), and is characterized by elevated scrotal temperature due to impaired circulation. Cryptorchidism is described as undescended testes, and occurs with implicit increase in temperature. Genomic studies of the testis have revealed that localized testicular infections or inflammatory processes cause spermatogenic failure in humans (148). In other studies, testicular oxidative stress and damage have been observed in contralateral testis of rats undergoing experimental torsion (149), and in rats with varicocele (150). Ishikawa et al. (151) and Misro et al. (152) have also shown over expression of free radicals and germ cell damage in experimental cryptorchidism.

Exposure to xenobiotics: It has been documented that testicular oxidative stress occurs during exposures to pesticides (153), industrial chemicals (154, 155) and metals, such as high doses of iron (156), cadmium (6, 157) and lead (158). In addition, several therapeutic medications, such as antineoplastic drugs (159, 160), antibacterials (161), antimalarials (162–165), calcium channel blockers (166), and antidepressants (167) have been reported to impair testicular function via increase in OS in the testes. Disturbances in redox homeostasis have also been observed with irradiation

(168), excessive alcohol consumption (169), and tobacco smoke (170, 171).

Conclusion

Excessive amounts of ROS affect redox balance and results in oxidative stress. Oxidative stress adversely affects cellular functions in various ways and has been evidently linked to the development of testicular dysfunction and a number of other diseases. Antioxidants preserve adequate function of cells against disturbances of homeostasis, including processes involving oxidative stress. Furthermore, antioxidant supplementation would theoretically protect or prevent peroxidative damage to testicular structures and may be helpful in male infertility. This concept of improving fertility potential of infertile patients under high oxidative stress by use of certain antioxidants has been debated in the past but has now gained considerable attention in cases of testicular dysfunction and artificial reproductive therapy. Thus, the development and patenting of potent antioxidants and inhibitors of important proinflammatory mediators, as well as developing suitable delivery systems for them may be of profound benefit to medicine generally.

Conflict of Interest

The author declares no conflict of interest.

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