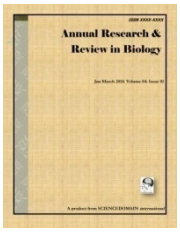

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Role of Antioxidant in Testicular Integrity

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Authors' contributions

This review work was carried out in collaboration between all authors. Author BJD designed the study and wrote the first draft of the manuscript. Authors FO and OTO managed the literature searches. All authors read and approved the final manuscript.

Review Article

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ABSTRACT

Spermatogenesis is an extremely active replicative process capable of generating approximately 1000 sperms a second with correspondingly high rates of mitochondrial oxygen consumption by the germinal epithelium. This process generates reactive oxygen species (ROS) through reactions with drugs and environmental toxins, or when the level of antioxidants is diminished results in oxidative stress. Excessive production of free radicals or ROS can damage sperm, testes and can cause serious chemical damage to biomolecules (DNA, proteins, and unsaturated lipids) which ultimately lead to cell death and cause infertility. The cell has several protective mechanisms that minimize the toxic potential of these reactive oxygen species. These include the low oxygen tension in the tissue and also the elaborate array of antioxidant enzymes and free radical scavengers it contains; to ensure that the spermatogenic and steroidogenic functions of this organ are not impacted by oxidative stress.

Keywords: Testis; oxidative stress; antioxidant; integrity and mechanism.

1. INTRODUCTION

The high rates of cell division inherent in spermatogenesis imply correspondingly high rates of mitochondrial oxygen consumption by the germinal epithelium. Reactive oxygen species (ROS) are formed from the partial reduction of molecular oxygen. These compounds are

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formed continuously as by-products of aerobic metabolism, through reactions with drugs and environmental toxins, or when the level of antioxidants is diminished, all creating the condition of oxidative stress. The highly reactive oxygen intermediates can cause serious chemical damage to DNA, proteins, and unsaturated lipids, and can lead to cell death. These reactive oxygen species have been implicated in a number of pathologic processes, including reperfusion injury, cancer, inflammatory disease, and aging [1].

Excessive production of free radicals or reactive oxygen species (ROS) can damage sperm and testes, and ROS have been extensively studied as one of the mechanisms of infertility. Infertility affects approximately 15% of all couples trying to conceive. Male factor infertility is the sole or contributing factor in roughly half of these cases, and no identifiable cause can be found in over 25% of infertile males [2]. Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Further, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses, such as glutathione [3]. The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis [4] The cell has several protective mechanisms that minimize the toxic potential of these reactive oxygen species [1]. The poor vascularization of the testes means that oxygen tensions are low [5] and that competition for this vital element within the testes is extremely intense. Since both spermatogenesis [6] and Leydig cell steroidogenesis [7; 8] are vulnerable to oxidative stress, the low oxygen tension that characterizes this tissue may be an important component of the mechanisms by which the testes protects itself from free radical-mediated damage. The testes also contain an elaborate array of antioxidant enzymes and free radical scavengers to ensure that the twin spermatogenic and steroidogenic functions of this organ are not impacted by oxidative stress. Antioxidants are classified into two broad divisions, depending on whether they are soluble in water (hydrophilic) or in lipids (hydrophobic). In general, water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid per oxidation. These compounds may be synthesized in the body or obtained from the diet [9]. The different antioxidants are present in a wide range of concentrations in body fluids and tissues, with some such as glutathione or ubiquinone mostly present within the cells. These antioxidant defence systems are of major importance because peroxidative damage is currently regarded as the single most important cause of impaired testicular function underpinning the pathological consequences of a wide range of conditions from testicular torsion to diabetes and xenobiotic exposure [10]. Oxidative stress in the testes is capable of impairing the ability to produce viable spermatozoa capable of initiating and supporting embryonic development [11]. This work presents a review on the role of antioxidants in maintaining testicular integrity and promoting male fertility.

1.1 Oxidant

An oxidant is the substance that is reduced and that, therefore, oxidizes the other component of an oxidation-reduction system [12]. An oxidizing agent (also called an oxidant, oxidizer or oxidiser) can be defined as a substance that removes electrons from another

reactant in a redox chemical reaction. The oxidizing agent is "reduced" by taking electrons onto it and the reactant is "oxidized" by having its electrons taken away. Examples of oxidants include oxygen O_2, ozone O_3 and hydrogen peroxide H_2O_2.

1.2 Pro-Oxidant

Pro-oxidants are compounds or agents capable of generating toxic oxygen *species* [12]. It is a species that causes or promotes oxidation. Pro-oxidants are chemicals that induce oxidative stress, usually through either creating reactive oxygen species or inhibiting antioxidant systems [13].

1.3 Antioxidant

An antioxidant is an agent that inhibits oxidation; any of numerous chemical substances, including certain natural body products and nutrients, that can neutralize the oxidant effect of free radicals and other substances [12]. It is a substance that, when present at a low concentration compared with that of an oxidizable substrate, inhibits oxidation of the substrate [14]. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols [15]. Although oxidation reactions are crucial for life, they can also be damaging; plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. Depending on the circumstances, a compound may exhibit pro- or antioxidant activity. Examples include: polyphenols, thiols, α-tocopherol [15].

1.4 Groups and Sources of Antioxidants

1.4.1 Based on function

- Radical scavenging
  Ascorbic acid
- Scavengers of non-radical oxidants
  Catalase (H_2O_2); thiols (- SH GROUP)
- Compounds that inhibit generation of oxidants
  Metal chelators
- Compounds that induce the production of antioxidants
  Isothiocyanates (sulforaphane

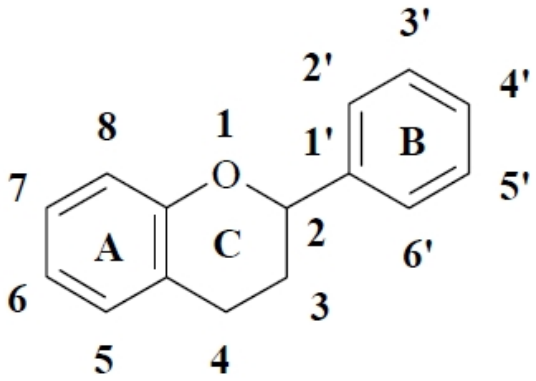
1.4.2 Based on structure

- Enzymatic Antioxidants
  Superoxide Dismutase, Glutathione Peroxidase / Reductase System, Catalase
- Non-enzymatic Antioxidants
  Vitamin E, Vitamin C, Glutathione

- Other Non-enzymatic Antioxidants
  N-acetyl L-cysteine, carotenoids, coenzyme Q10 and carnitines.

1.4.3 Based on locality

- Water soluble
- Lipophilic



General Flavonoid Structure (Orsolya et al., [16])

1.5 Flavonoids

Flavonoids, a group of polyphenolic compounds, can widely be found in fruits and vegetables. They have antioxidant or free radical scavenging properties. The number of flavonoid derivatives is more than 4000 and their antioxidant properties are very different. The antioxidant activity of flavonoids depends strongly on the number and position of hydroxyl groups in the molecule. Dihydroxylated B-ring (catechol structure), presence of unsaturation and of 4-oxo function in the C-ring are also presumed to increase the antioxidant capacity [16].

1.6 Superoxide Dismutase

Superoxide dismutases (SOD) are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen.

The SOD-catalyzed dismutation of superoxide may be written with the following half-reactions:

$$M^{(n+1)+}\text{-SOD} + O_2^- \rightarrow Mn^+\text{-SOD} + O_2$$

$$Mn^+\text{-SOD} + O_2^- + 2H+ \rightarrow M^{(n+1)+}\text{-SOD} + H_2O_2$$

where M = Cu (n=1) ; Mn (n=2) ; Fe (n=2) ; Ni (n=2).

In this reaction the oxidation state of the metal cation oscillates between n and n+1.

Three forms of superoxide dismutase are present in humans, in all other mammals, and most chordates. SOD1 is located in the cytoplasm, SOD2 in the mitochondria, and SOD3 is extracellular. The first is a dimer (consists of two units), whereas the others are tetramers (four subunits). SOD1 and SOD3 contain copper and zinc, whereas SOD2, the mitochondrial enzyme, has manganese in its reactive centre. The genes are located on chromosomes 21, 6, and 4 [17].

1.7 Glutathione Peroxidase

Glutathione peroxidase (GPx) is the general name of an enzyme family with peroxidase activity whose main biological role is to protect tissue from oxidative damage. The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water.

The main reaction that glutathione peroxidase catalyzes is:

$$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$$

Where GSH represents reduced monomeric glutathione and GS–SG represents glutathione disulfide, H_20_2 represent hydrogen peroxide and H_2O represent water. The mechanism involves oxidation of the selenol of a seleno-cysteine residue by hydrogen peroxide. This process gives the derivative with a seleninic acid (RSeOH) group. The selenenic acid is then converted back to the selenol by a two-step process that begins with reaction with GSH to form the GS-SeR and water. A second GSH molecule reduces the GS-SeR intermediate back to the selenol, releasing GS-SG as the by-product. A simplified representation is shown below:

$$RSeH + H_2O_2 \rightarrow RSeOH + H_2O$$

$$RSeOH + GSH \rightarrow GS\text{-}SeR + H_2O$$

$$GS\text{-}SeR + GSH \rightarrow GS\text{-}SG + RSeH$$

Glutathione reductase then reduces the oxidized glutathione to complete the cycle:

$$GS–SG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$$

Mammalian GPx1, GPx2, GPx3, and GPx4 have been shown to be selenium-containing enzymes, whereas GPx6 is a selenoprotein in humans with cysteine-containing homologues in rodents. GPx1, GPx2, and GPx3 are homotetrameric proteins, whereas GPx4 has a monomeric structure. As the integrity of the cellular and subcellular membranes depends heavily on glutathione peroxidase, its antioxidative protective system itself depends heavily on the presence of selenium [18].

1.8 Catalase

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in reproductive reactions. Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrin heme (iron) groups that

allow the enzyme to react with the hydrogen peroxide. The optimum pH for human catalase is approximately 7, and has a fairly broad maximum (the rate of reaction does not change appreciably at pH between 6.8 and 7.5). The pH optimum for other catalases varies between 4 and 11 depending on the species. The optimum temperature also varies by species [19].

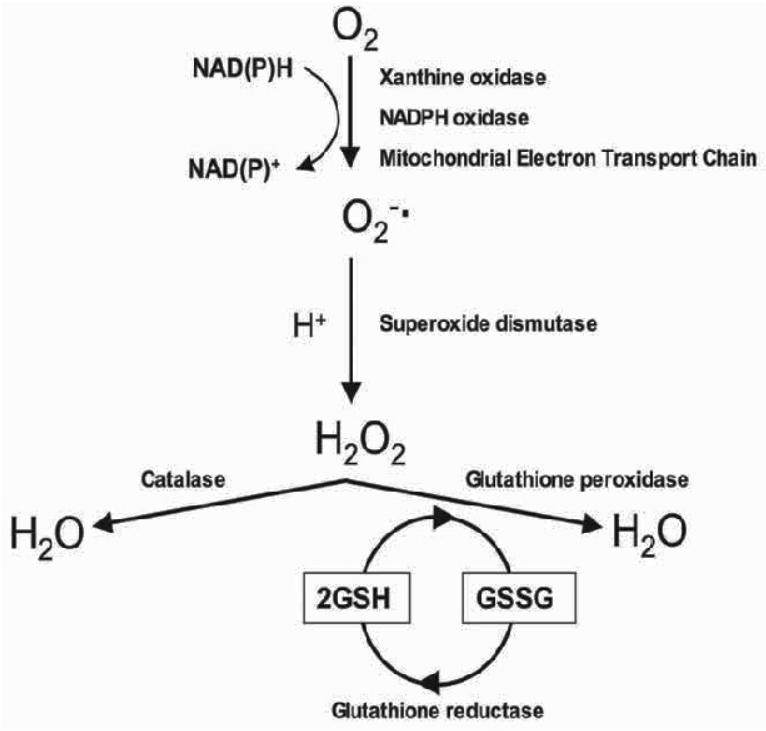


Fig. 1. Reactive oxygen species generation and metabolism *(Aitken, 2007)* [20]

1.9 Mechanism of Action of Antioxidants

The testes have developed a sophisticated array of antioxidant systems comprising both enzymatic and non-enzymatic constituents. Concerning the enzymatic constituents of this defence system, the induction of oxidative stress in the testes precipitates a response characterized by the NF-κB mediated induction of mRNA species for superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities [21]. The fundamental biochemistry of these antioxidant enzymes is summarized in the Fig.1. above and involves the rapid conversion of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2) in the presence of SOD in order to prevent the former from participating in the formation of highly pernicious hydroxyl radicals. The H_2O_2 generated in this manner is a powerful membrane permeant oxidant on its own right that has to be rapidly eliminated from the cell in order to prevent the induction of oxidative damage to lipids, proteins and DNA. The elimination of H_2O_2 is either effected by catalase or glutathione peroxidase, with the latter predominating in the case of the testes [22,23]. GST on the other hand involves a large and complex family of proteins that catalyse the conjugation of reduced glutathione via the sulfhydryl group to electrophilic centres on a wide variety of substrates in preparation for excretion from the cell. This activity is critical in the detoxification of peroxidised lipids as well as the metabolism of xenobiotics.

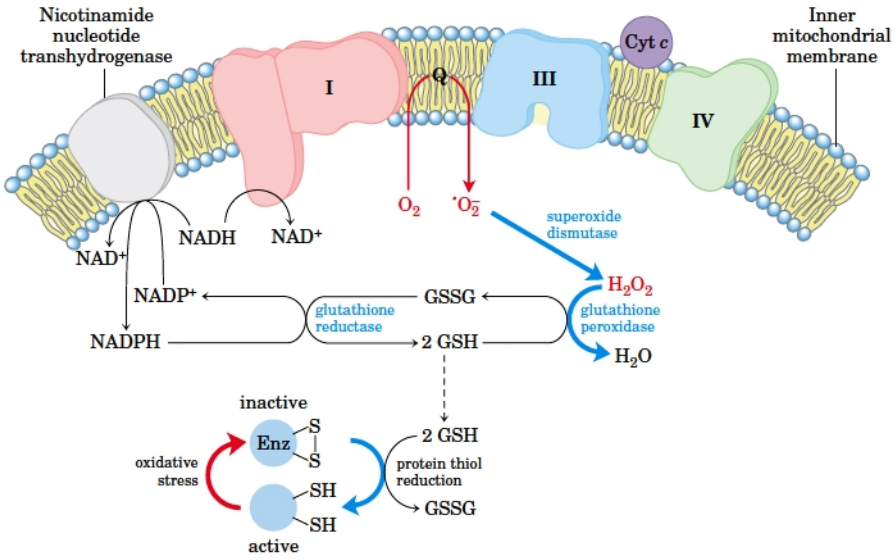


Fig. 2. Mitochondrial Production and Disposal of Superoxide (Lehninger *et al*, 2005)

The Fig.2. above represents the production and disposal of superoxide in the mitochondria. Superoxide radical, $\cdot O_2^-$ is formed in side reactions at Complexes I and III, as the partially reduced ubiquinone radical ($\cdot Q^-$) donates an electron to O_2. The reactions shown in blue defend the cell against the damaging effects of superoxide. Reduced glutathione (GSH) donates electrons for the reduction of hydrogen peroxide (H_2O_2) and of oxidized Cys residues (—S —S—) in proteins, and GSH is regenerated from the oxidized form (GSSG) by reduction with NADPH [24].

2. Antioxidant Enzymes in the Testis

2.1 Enzymatic Antioxidants

2.1.1 Superoxide dismutase

Superoxide dismutase (SOD) scavenges both extracellular and intracellular superoxide anion and prevents lipid peroxidation of the plasma membrane. In order to act against H_2O_2, it must be conjugated with catalase or glutathione peroxidase [25]. SOD also prevents premature hyperactivation and capacitation induced by superoxide radicals before ejaculation [26].

2.1.2 Glutathione peroxidase / reductase system

This system forms an excellent protection against lipid peroxidation of plasma membrane of spermatozoa. It scavenges lipid peroxides thereby arresting the progressive chain reaction of lipid peroxidation. It also scavenges H_2O_2, which is responsible for the initiation of lipid peroxidation. Glutathione reductase (GRD) stimulates the reduction of glutathione disulfide (GSSG) to reduced glutathione (GSH). This ensures a steady supply of the reductive substrate NADPH to GPX. Glucose-6-phosphate dehydrogenase (G6PD) is required for the conversion of $NADP^+$ to its reduced form, NADPH.

2.1.3 Catalase

Catalase detoxifies both intracellular and extracellular H_2O_2 to water and oxygen [12]. In addition, catalase activates nitrous oxide (NO)-induced sperm capacitation, which is a complex mechanism involving H_2O_2 [26].

2.2 Non-Enzymatic Antioxidants

2.2.1 Vitamin E and C

In vitro studies show that vitamin E is a major chain breaking antioxidant in the sperm membrane and it appears to have a dose-dependent protective effect. Vitamin C (ascorbate) is another important chain breaking antioxidant contributing up to 65% of the antioxidant capacity of the seminal plasma [27].

2.2.2 Glutathione

Glutathione is the most abundant non-thiol protein in mammalian cells [28]. A glutathione deficiency can lead to instability of the mid-piece, resulting in defective motility [29,30] It protects plasma membrane from lipid peroxidation, scavenges superoxide, and prevents O_2 formation.

2.2.3 Metallothioneins

Metallothioneins (MTs) belong to the group of intracellular cysteine-rich, metal-binding proteins that have been found in bacteria, plants, invertebrates and vertebrates [31,32, and 33]. These proteins were discovered in 1957 as cadmium-binding proteins isolated from horse kidney [34]. Mammalian MTs may contain 61–68 amino acids, and among them 20 are cysteines [35,36] These unique proteins are involved in diverse intracellular functions [37] but their role in the detoxification of heavy metals and in the maintaining of essential metal ion homeostasis, which is due to their high affinity for these metals, is mostly investigated[38,39] For mammals, MTs bind zinc [40] but with excess copper or cadmium, zinc can be easily replaced by these metals [41]. Cells that contain excessive amounts of MTs are resistant to cadmium toxicity [42], while cell lines that cannot synthesize MTs are sensitive to cadmium [43] Genetic studies using transgenic or knockout mouse models are further evidence of the role of MTs in protection against cadmium toxicity [44, 45] Based on structural models, it can be assumed that the MT molecule is composed of two binding domains, α and β, which are composed of cysteine clusters. Covalent binding of metal atoms involves sulfhydryl cysteine residues. The *N*-terminal part of the peptide is designated as β-domain and has three binding sites for divalent ions, and the C-terminal part (the α-domain) has the ability to bind four divalent metal ions. Four mammalian MT isoforms (MT-1–MT-4) and 13 MT-like human proteins were identified [46]. The differences of constituent forms come mainly from post-translational modifications, small changes in primary structure, type of incorporated metal ion and speed of degradation. Despite the physical-chemical similarity of the forms, their roles and occurrence in tissues vary significantly [47]. MT-1 and MT-2 are present almost in all types of soft tissues [45,48,49] MT-3 is expressed mostly in brain tissue, but also in heart, kidneys and reproductive organs [50, 51] and the MT-4 gene was detected in stratified squamous epithelial cells associated with oral epithelia, esophagus, upper stomach, tail, footpads and neonatal skin [52].

Metallothionein has been shown to scavenge hydroxyl radicals *in vitro*, because of its cysteinyl thiolate groups [53]. Thornalley and Vasak [54] showed that the rabbit liver metallothionein-1, which contains zinc and/or cadmium ions, appeared to scavenge free hydroxyl (•OH) and superoxide (O_2−•) radicals produced by the xanthine/xanthine oxidase reaction. All 20 cysteine sulfur atoms are involved in the radical quenching process, and the rate constant for the reaction of hydroxyl radical with MT is about 340-fold higher than that with GSH [54].

2.2.4 Other non-enzymatic antioxidants

Molecules such as N-acetyl L-cysteine, carotenoids, coenzyme Q10 and carnitines provide excellent antioxidant support. N-acetyl L-cysteine is a precursor of glutathione that improves sperm motility and reduces ROS-induced DNA damage [55,56]. Carotenoids play an important role in protecting the cells and organisms by scavenging the superoxide radicals [57]. Co-enzyme Q10 protects lipids against peroxidative damage [58]. It scavenges superoxide anion as well as peroxides. Carnitine promotes membrane stability and plays an important role in sperm maturation and development [59]

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2.2.5 Low Oxygen tension and oxidative stress in the testes

In the testis, millions of sperm are produced daily. Several observations suggest that this is accomplished in an organ where the safety margins for disturbances in the vasculature could be particularly narrow [60,61]. The testis receives its blood supply by an unusually long artery. The vascular resistance in this vessel is, therefore, high, leaving capillary pressure in the testes lower than that in all other organs and only marginally higher than the venous pressure. The oxygen tension in the testis is low [62,63]. The high metabolic activity in the seminiferous tubules is apparently adapted to this environment of low oxygen and low vascular perfusion pressure, and under normal conditions, the vasculature is able to supply the testis with sufficient amounts of nutrients and oxygen [63,64,65].

Under physiological conditions, the testes are vulnerable to oxidative stress. The testes have a poor vascularization, which means that oxygen tension in this tissue is low *(58)*. Spermatogenesis is an extremely active replicate process. The high rates of cell division inherent in this process imply correspondingly high rates of mitochondrial oxygen consumption by the germinal epithelium. The competition for this element within the testes is extremely intense. Despite the low oxygen tensions that characterize the testicular microenvironment, this tissue remains vulnerable to oxidative stress due to the abundance of highly unsaturated fatty acids and the presence of potential ROS-producing systems. Since both spermatogenesis and Leydig cell steroidogenesis are vulnerable to oxidative stress, the low oxygen tension may be an important component of the mechanisms by which the testes protect themselves from free radical-mediated damage [6,7,8]. Furthermore, the testes have an elaborate array of antioxidant enzymes and free radical scavengers to ensure that the twin spermatogenic and steroidogenic functions of this organ are not impacted by oxidative stress.

Oxidative stress in any tissue results from an imbalance between the production of reactive oxygen species (ROS) and their efficient removal by available antioxidant systems. ROS are small, oxygen-based molecules that are highly reactive because of unpaired electrons *(66)*. The most prominent ROS are the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl ion (OH^-). Oxidative stress triggers a cascade that leads to the production of reactive oxygen species (ROS), accumulation of lipid peroxidation products such as

malondialdehyde (MDA), massive secretion of systemic inflammatory mediators that can result in the development of systemic inflammatory response syndromes, impaired cell function, and multiple organ dysfunctions [67,68,69,70] while the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) play important roles in cell defense against oxidative stress [71]. Antioxidants in cells, such as SOD, catalase (CAT), glutathione (GSH), and GPx protect the organism against the damages of oxidative stress [72].

Excessive amounts of oxygen free radicals cause lipid peroxidation in the cellular and mitochondrial membranes. Peroxidation of the lipids in the membranes changes membrane permeability or disrupts membrane integrity and thus cell integrity [73,74]. Mammalian testes are highly susceptible to oxidative stress. Like all cells living under aerobic conditions, spermatozoa produce ROS, mostly originating from normal metabolic activity. High concentrations of ROS play an important role in the pathophysiology of damage to human spermatozoa [2]. Hence, oxidative stress has been shown to be a major cause of male infertility, and in a large proportion of infertile men, an elevation in the levels of seminal ROS activity was shown. However, spermatozoa and seminal plasma contain a battery of ROS scavengers, including enzymes such as SOD and catalase, and also a variety of substances with antioxidant activities [3,71]

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2.2.6 Antioxidant defence mechanism of the testes

Despite the low oxygen tensions that characterize the testicular micro-environment, this tissue remains vulnerable to oxidative stress due to the abundance of highly unsaturated fatty acids (particularly 20:4 and 22:6) and the presence of potential reactive oxygen species (ROS)-generating systems. ROS generation can be from the mitochondria and a variety of enzymes including the xanthine- and NADPH- oxidases,5,6 and the cytochrome P450s [75]. These enzymes specialize in the professional generation of ROS or produce these toxic metabolites as an inadvertent consequence of their biochemical activity. In order to address this risk, the testes have developed a sophisticated array of antioxidant systems comprising both enzymatic and non-enzymatic constituents [20]

2.2.7 Superoxide dismutase (SOD)

The superoxide anion is produced by a one-electron reduction of an oxygen molecule and initiates a radical chain reaction. Since its discovery, it is believed that SOD, which dismutates superoxide anion to hydrogen peroxide and oxygen molecule, plays a central part in antioxidative reactions. Three isozymes are present in mammals. SOD1 encodes CuZnSOD that contains Cu and Zn as metal cofactors and is mostly cytosolic, while SOD2-encoding MnSOD is a mitochondrial isoform containing Mn. SOD3, encoding the extracellular form, is referred to as ECSOD, is structurally similar to CuZnSOD, and also contains Cu and Zn as metal cofactors [76].

Although many studies have been reported on CuZnSOD since its discovery, there are few papers that suggest its involvement in the male reproductive system. While the SOD1-deficient female is infertile, no phenotype is known in the male reproductive system. A pivotal role of SOD in protection of testicular cells against heat stress-induced apoptosis has been demonstrated *in vivo* and *in vitro* [77,78].

MnSOD is a mitochondrial isoform, but its gene, SOD2, is encoded by nuclear DNA. SOD2 is inducible under various oxidative stress and inflammatory conditions and, hence, the

regulatory mechanism of the gene has been extensively examined. Nuclear factor kappa-B (NF-kB) appears to be the most important transactivating factor responsible for gene induction. Homozygous SOD2-deficient mice suffer severe cardiovascular damage and die soon after birth. No abnormality in the genital tract has been reported for heterozygous mice although some organs of these mice are susceptible to oxidative stress. On the contrary, transgenic male mice that express higher levels of MnSOD are infertile, but the mechanism for this is unknown. Since SOD only dismutates superoxide anion to hydrogen peroxide, the resulting hydrogen peroxide may also cause a toxic effect in testicular cells [76].

ECSOD, which was originally reported as an extracellular isoform in the lung, is present at high levels in the epididymis [79]. ECSOD is also localized in the nuclei in the seminiferous tubules of testis [80]. In this case, the carboxyl terminal stretch of basic amino acids, which bind heparan sulfate to vascular endothelial cells, appears to function as a nuclear import signal. Erectile function is improved by transferring the SOD3 gene to the penis in aged rats [81]. Scavenging superoxide elongates the half-life of nitric oxide (NO), which results in an increase in cGMP levels. It is probable that the elevation of cGMP, which is caused by prolonged NO, relaxes vascular smooth muscle and improves erectile responses. However, no recognizable phenotype in the reproductive system has yet been reported in SOD3 knockout mice [82].

2.2.8 Glutathione peroxidase (GPx)

Glutathione peroxidases detoxify various peroxides using the reduced form of glutathione (GSH) as an electron donor and constitute a large family of groups. These enzymes are classified into two groups in terms of the active site amino acid: one of which contains selenocysteine (Sec) at its active center, while the other does not. Here the former group is described, because the latter group exhibits a lower activity and GPx, in the narrow sense, indicates the former group [76].

Since selenium (Se) deficiency is related to male infertility, the relationship between GPx activity and male fertility has been debated. At least four isozymes belong to selenium-containing GPx in mammals. The cytosolic form, GPx1, is widely distributed in tissues and has been most extensively investigated. GPx1, like other antioxidative enzymes, prevents apoptosis induced by oxidative stress and other stimuli. However, GPx1-knockout mice show no abnormality in phenotype including reproductive capability. GPx2 and GPx3 are gastrointestinal and plasma forms, respectively, and a number of studies on them regarding the reproductive process have appeared [76].

GPx4 encodes an isoform that specifically detoxifies phospholipid hydroperoxide and is expressed at high levels in the testis. Thus, a defect in GPx4 had been suspected as the source of infertility caused by the Se deficiency, although direct evidence for its requirement had been missing for a long period. That GPx4 protein represents about 50 % of the capsule material, which embeds the helix of mitochondria in the midpiece of spermatozoa, has been demonstrated recently [30]. A correlation between male infertility and a GPx4 defect has actually been reported [83]. GPx4-knockout mice show premature embryonal death in the uteri, but the direct cause of the death is not clear [83,84]. A novel member, which has a high sequence identity to GP 4 except for the N-terminal region, is specifically present in sperm nuclei and is considered to act as a protamine thiol peroxidase *(85)*. However, the isoform is actually encoded by the same gene as GPx4 and is produced by an alternate promoter and exon usage [86].

GPx5 is a non-selenium enzyme and, hence, should be classified into the nonselenium-dependent GPx group. However, it is described here because it is named so and is highly associated with the male reproductive system. GPx5 is expressed exclusively in the epididymis and is secreted and present in the caput and cauda epididymides lumens [87]. It constitutes 6 % of the secretory epididymal proteins [88] The binding of GPx5 to sperm membrane has also been reported *(88)*. Thus, the protection of the sperm membrane against per oxidation is a possible function of this epididymis-specific isoform [89]. Since selenium deficiency causes male infertility and Sec-containing GPx is suspected to be a candidate for the defective molecule, non-selenium type GPx5 is speculated to serve as the backup enzyme for the Sec-containing GPx. The activity of nonselenium dependent GPx is low and, hence, its contribution as a GSH-dependent peroxide scavenger is ambiguous.

2.2.9 Catalase (Cat)

Catalase exclusively detoxifies hydrogen peroxide and has no electron donor requirement. Although CAT is a well-known antioxidative enzyme and has been implicated in protection against hydrogen peroxide, its localization is limited to the peroxisome. It plays a role in organs such as liver, but its specific function in male genital tract is unknown [76].

3. Factors that Enhance the Antioxidant Defence Mechanism of the Testes

In addition to the major ROS processing enzymes, the testes rely heavily on small molecular weight antioxidant factors for protection against oxidative damage. These factors include ions and a wide variety of free radical scavengers, the nature of which are reviewed below.

3.1 Zinc

Zinc is an acknowledged antioxidant factor that as well as being a core constituent of free radical scavenging enzymes such as SOD and a recognized protector of sulfhydryl groups is also thought to impair lipid peroxidation by displacing transition metals such as iron and copper from catalytic sites [90]. In keeping with such a central antioxidant role, this element has a profound effect on the level of oxidative stress experienced by the testes. Thus rats fed a zinc deficient diet experience a decrease in testicular antioxidant potential and a concomitant increase of lipid peroxidation in this tissue [91]. Conversely, zinc administration will counteract the oxidative stress created in the testes by exposure to lead as well as the peroxidative damage induced by ischemia-reperfusion as a consequence testicular torsion-detorsion. Zinc administration has also been shown to attenuate the testicular oxidative DNA damage induced by cadmium as well as the decline in sperm production and testosterone secretion induced by this heavy metal [92].

3.2 Vitamins C and E

It has been recognized since the 1940s that vitamin E (α-tocopherol) is a powerful lipophilic antioxidant that is absolutely vital for the maintenance of mammalian spermatogenesis. It is present in particularly high amounts in Sertoli cells and pachytene spermatocytes and to a lesser extent round spermatids [93]. Vitamin C (ascorbic acid) also contributes to the support of spermatogenesis at least in part through its capacity to reduce α-tocopherol and maintain this antioxidant in an active state [94]. Vitamin C is itself maintained in a reduced state by a GSH-dependent dehydroascorbate reductase, which is abundant in the testes [95]. Deficiencies of vitamins C or E leads to a state of oxidative stress in the testes that disrupts

both spermatogenesis and the production of testosterone. Conversely, ascorbate administration to normal animals stimulates both sperm production and testosterone secretion. This vitamin also counteracts the testicular oxidative stress induced by exposure to pro-oxidants such as arsenic, PCBs (Arochlor 1254), cadmium, endosulfan and alcohol. Furthermore, endogenous ascorbate levels decrease dramatically when oxidative stress is induced in the testes by, for example, chronic exposure to lead, chromium, cadmium or aflatoxin. Vitamin E has also been shown to suppress lipid peroxidation in testicular microsomes and mitochondria and to reverse the detrimental effects of oxidative stress on testicular function mediated by exposure to such factors as ozone, iron overload, intensive exercise or exposure to aflatoxin, PCB, cyclophosphamide and formaldehyde. Furthermore, testicular vitamin E levels have also been shown to fall significantly when oxidative stress is induced by exposure to pro-oxidant stimuli such as chromium [96].

3.3 Melatonin and Cytochrome C

The pineal hormone melatonin (N -acetyl, 5-methoxytryptamine) also plays a major role in protecting the testes from oxidative stress; given the significant stimulatory effect of pinealectomy on the oxidative damage recorded in the testes as a consequence of induced hyperthyroidism [97]. Melatonin has two major attributes that set it apart from most other antioxidants. Firstly, it undergoes a two electron oxidation when acting as antioxidant, rather than the one electron oxidation favoured by many free radical scavengers. As a result, this compound cannot redox cycle and inadvertently generate free radicals. Secondly, melatonin is readily soluble in both lipid and aqueous environments and can readily cross the blood-testes barrier to protect the germinal epithelium. Melatonin levels in seminal plasma are depressed in infertile patients exhibiting poor motility, leukocytospermia, varicocele and non-obstructive azoospermia, all of which are conditions associated with oxidative stress in the male tract [98]. Moreover, the intraperitoneal injection of melatonin has been shown to alleviate oxidative stress in the testes following the experimental induction of a left sided varicocele [99].

Another small molecular mass free radical scavenger that has recently been shown to play a major role in reducing H_2O_2 is a testes-specific form of cytochrome C. This cytochrome C isoform is also a powerful activator of apoptosis, providing additional protection to the testes by virtue of its ability to facilitate the depletion of damaged germ cells [4].

4. Role of Antioxidants in Testicular

4.1 Histology Architecture

Oxygen radical scavengers provide significant restoration of testicular function after testicular vascular diseases [100] Both testicular torsion and detorsion result in testicular tissue damage by means of lipid peroxidation, which is evident by an increase in the tissue levels of MDA [9]. Dietary supplementation with garlic extract seems to attenuate the generation of toxic free radicals, as evidenced indirectly by low tissue MDA levels *(9).* In another study by Agarwal *et al.* [101], it was concluded that prevention of testicular damage by free-radical scavengers, can be achieved by oxypurinol or SOD. Melatonin is also a potent antioxidant agent in preventing testicular Ischemia/Reperfusion injury [102].

4.2 Spermatogenesis

Vitamin C (ascorbic acid) also contributes to the support of spermatogenesis at least in part through its capacity to reduce α-tocopherol and maintain this antioxidant in an active state [103]. Vitamin C is itself maintained in a reduced state by a GSH-dependent dehydroascorbate reductase, which is abundant in the testes (95). Deficiencies of vitamins C or E leads to a state of oxidative stress in the testes that disrupts both spermatogenesis and the production of testosterone [104]. Also Selenium, as an antioxidant, is essential for normal testicular function and spermatogenesis. It can reduce free oxidative radicals as a cofactor for antioxidant enzymes [105].

4.3 Leydig Cell/ Steriodogenesis

A study by Nobuo and Nikolas (1999) demonstrated a protective effect of antioxidants on testicular spermatogenic and steroidogenic functions and raised the possibility of administering antioxidants to men with varicoceles who have failed to improve sperm qualitative and quantitative parameters after varicocelectomy [106].

4.4 Sertoli Cells

Melatonin is readily soluble in both lipid and aqueous environments and can readily cross the blood-testes barrier to protect the germinal epithelium. Melatonin levels in seminal plasma are depressed in infertile patients exhibiting poor motility, leukocytospermia, varicocele and non-obstructive azoospermia, all of which are conditions associated with oxidative stress in the male tract [98].

4.5 Sperm Parameters/Quality and Quantity

The binding of GPx5 (Glutathione Peroxidase) to sperm membrane has also been reported *(88).* Thus, the protection of the sperm membrane against peroxidation is a possible function of this epididymis-specific isoform [107]. Also only do antioxidants prevent reduction in sperm motility (mainly vitamin E and C, glutathione, N-acetyl cysteine, SOD, catalase, albumin, taurine, and hypotaurine), these also increase sperm motility (N-acetyl cysteine and coenzyme Q10). A randomized double-blind controlled trial has shown that vitamin E administered orally (300 mg/day) results in a decrease in malondialdehyde (a marker for lipid peroxidation) concentration in spermatozoa and improved sperm motility [108].

4.6 Cell Apoptosis

Apoptosis is basically started with two pathways: "intrinsic" and "extrinsic". Free radicals and radiation, the possible causes of cell apoptosis, are thought to trigger apoptosis via the intrinsic pathway [109]. Apoptosis can be a two-faced companion reinforcing tissue homeostasis and physiological processes as a friend, instigating organ dysfunction and disease as a foe. When apoptosis is improperly activated or regulated in the testis, infertility or even cancer can result. Studies have implicated elevated rates of apoptosis in infertile male patients. Testicular apoptosis serves to deplete excess germ cells and remove abnormal spermatozoa during normal spermatogenesis. Indeed, apoptosis eliminates 75% of germ cells before they become fully mature. In this way, testicular apoptosis monitors germ cell population according to the support capacity of Sertoli cells. While apoptosis is essential for maintaining testicular homeostasis during spermatogenesis, inappropriately

occurring apoptosis has been linked to suboptimal male reproductive function. Excessive apoptosis results from impaired regulation or improper activation, and can affect spermatogenesis and even lead to infertility [110].

Antioxidants are believed to prevent apoptosis that may cause malfunctioning of the testes [111]. Another small molecular mass free radical scavenger that has recently been shown to play a major role in reducing H_2O_2 is a testes-specific form of cytochrome C. This cytochrome C isoform is also a powerful activator of apoptosis, providing additional protection to the testes by virtue of its ability to facilitate the depletion of damaged germ cells *(4)*.

4.7 Erection and Ejaculation

Erectile function is improved by transferring the SOD3 (superoxide dismutase) gene to the penis in aged rats [81]. Scavenging superoxide elongates the half-life of nitric oxide (NO), which results in an increase in cGMP levels. It is probable that the elevation of cGMP, which is caused by prolonged NO, relaxes vascular smooth muscle and improves erectile responses. However, no recognizable phenotype in the reproductive system has yet been reported in SOD3 knockout mice [82].

5. Disease Conditions that Compromise the Antioxidant Mechanism of the Testes

Notwithstanding the antioxidant protection afforded to the testes in order to support its dual functions of steroidogenesis and sperm production, a wide variety of endogenous and exogenous factors are known to perturb these defenses and generate a state of oxidative stress. The following is a review of some of these factors.

5.1 Cryptorchidism

The elevated temperatures associated with experimental cryptorchidism are associated with oxidative stress in the testes and a reduction in SOD and catalase activities [112]. Consistent with these findings, direct exposure of spermatogenic cells to elevated temperatures was found to induce high rates of apoptosis via mechanisms that were associated with elevated levels of H_2O_2 generation and could be ameliorated by the addition of catalase. The clinical significance of this finding can be seen in the high levels of DNA damage and ROS generation seen in the spermatozoa of patients with a history of cryptorchidism [113].

5.2 Testicular Torsion

Testicular torsion is a relatively common, painful condition that must be treated rapidly if the testes are not to suffer permanent damage. Prolonged torsion leads to testicular ischaemia and high levels of oxidative stress in the ipsilateral testes associated with NO and H_2O_2 production, increased lipid peroxide formation, isoprostane accumulation, antioxidant enzyme depletion and increased rates of mitochondria-mediated apoptosis in the germ line [114]. Even short periods of ischaemia, for 3 hours or less, can lead to high levels of oxidative stress in the testes, depletion of testicular glutathione levels and the consequent disruption of spermatogenesis. Significantly, the level of peroxidative damage observed in testicular tissue increases following detorsion, indicating the induction of reperfusion injury

[115]. The biochemical basis for reperfusion injury is thought to involve a key metabolic enzyme, xanthine dehydrogenase, which becomes converted to a xanthine oxidase during ischaemia, due to oxidation of essential -SH groups and/or a limited proteolytic clip. As soon as the tissue is reperfused with blood, the xanthine oxidase is suddenly presented with oxidizable substrate in the form of xanthine/hypoxanthine and starts to generate copious amounts of ROS. The latter then induce high levels of peroxidative damage via mechanisms that are enhanced by the local release of transition metals. Although this scheme of events was developed to explain the tissue injury associated with conditions such as myocardial infarction, it also applies to the testicular injury associated with torsion-detorsion. The general notion that the testicular damage precipitated by temporary ischaemia is associated with oxidative stress is supported by the sudden induction of lipid peroxidation and the concomitant suppression of endogenous antioxidant activities including SOD, catalase and glutathione peroxidase [116]. In addition, the tissue injury induced by testicular torsion/detorsion can be dramatically alleviated by pretreatment with exogenous antioxidants such as selenium, resveratrol, L-carnitine, caffeic acid phenethyl ester and garlic extract [9].

5.3 Varicocele

The impaired venous drainage to the testes seen with varicocele is also associated with the disruption of spermatogenesis via mechanisms involving the induction of oxidative stress. In clinical studies, the presence of a varicocele has been shown to correlate with excess ROS generation by the spermatozoa, high rates of DNA damage in these cells and depleted antioxidant levels in the seminal plasma [117,118,119]. Independent studies have also shown that the testicular expression of 4-hydroxy-2-nonenal modified proteins (another marker of oxidative stress) is significantly higher in patients that responded positively to varicocelectomy, suggesting that surgical treatments are capable of reducing oxidative stress in the testes [120]. Immunocytochemical analyses of 8-hydroxy-2'-deoxyguanosine expression in the testes of varicocele patients also revealed particularly high levels of oxidative DNA damage in the spermatogonia and spermatocytes that correlated well with the severity of the varicosity [121]. The general concept that testicular pathologies associated with varicocele are linked with the induction of oxidative stress has been confirmed in animal models. Thus, creation of experimental bilateral varicocele in rats is associated with increases in lipid peroxidation and NO generation and a corresponding decrease in testicular antioxidant status [10]. Moreover, the pathological consequences of experimental varicocele induction can be significantly reversed by the concomitant administration of an antioxidant, melatonin [99].

5.4 Hyperthyroidism

The induction of hyperthyroidism in rats is associated with oxidative stress in the testes as reflected by increased lipid peroxidation, elevated GSH levels and induction of antioxidant enzymes. The oxidative stress appears to be associated with a thyroxine dependent increase in mitochondrial activity and concomitant leakage of electrons from the mitochondrial electron transport chain [122,123]. The oxidative stress precipitated by hyperthyroidism can be exacerbated by pinealectomy removing melatonin, an important testicular antioxidant, from the redox equation [97] .These data resonate with clinical studies indicating that hyperthyroidism is associated with poor semen quality, particularly impaired motility, that normalize when the patients' thyroid dysfunction is corrected and euthyroidism established [124]. It should also be noted that hypothyroidism can induce oxidative stress in the testes as reflected by enhanced levels of H_2O_2 production and increased carbonyl

generation. Clearly, normal testicular function is highly dependent on a functional thyroid system [125].

5.5 Diabetes

Experimental induction of diabetes in animal models has been shown to impair testicular function and decrease male fertility [97]. Thus, diabetogens such as streptozotocin, enhance ROS generation and induce both lipid peroxidation and protein carbonyl expression in the testes. Moreover the oxidative stress associated with the diabetic condition is associated with DNA damage in the male germ line and high rates of embryonic loss in mated females (dominant lethal effect). These effects could be attenuated by the administration of antioxidants such as ascorbic acid, melatonin, taurine or an herbal mixture containing extracts from *Musa paradisiaca, Tamarindus indica, Eugenia jambolana and Coccinia indica* [126,127,128]. In light of recent data showing an increased level of DNA damage in the spermatozoa of diabetic patients compared with nondiabetic controls, causative links between diabetes, oxidative stress in the male germ line and DNA damage appears both likely and clinically, extremely important [129].

5.6 Infection

Another factor that may cause oxidative stress in the testes is infection. Experimental models of infection, involving the intraperitoneal injection of bacterial lipopolysaccharide (LPS), induced lipid peroxidation in the testes and rapidly depleted this tissue of antioxidant enzyme activity in the form of SOD, catalase and the glutathione peroxidase-reductase couple. This oxidative stress was associated with the transient generation of pro-inflammatory mediators such as interleukin 1β, inducible nitric oxide synthase and cyclo-oxygenase-2 [130]. The same experimental infection model has also been used to demonstrate the particular sensitivity of Leydig cell steroidogenesis to oxidative stress induced by bacterial LPS. In these studies, the oxidative stress induced by LPS stimulated lipid peroxidation in Leydig cell membranes as well as significant reductions in steroidogenic acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase isomerase (3β-HSD) activity. Moreover, these effects were associated with the disruption of Leydig cell mitochondrial function and, specifically, the inhibition of StAR-mediated cholesterol transfer activity [131].

5.7 Physical Exertion

Physical exercise has been shown to up-regulate antioxidant activities in the testes of aging rats and may represent a practical way in which the detrimental effects of age on testicular function can be ameliorated. A similar case could be argued for the ability of moderate exercise to ameliorate the degree of oxidative damage inflicted on the testes by chronic ethanol ingestion. However, excess exercise can have the opposite effect, causing oxidative stress in the testes and generating high levels of lipid peroxidation in association with significant declines in the activities of key antioxidant enzymes including SOD, catalase, GST and GPx. Such stress has a significant inhibitory effect on the both steroidogenesis and germ cell differentiation within the testes. The fact that these effects can be reversed by the administration of an antioxidant, α-tocopherol succinate, confirms the importance of oxidative stress in the aetiology of such exercise-dependent testicular dysfunction [20].

5.8 Reproductive Hormone Imbalance

The immediate endocrine environment of the testes has a major impact on the antioxidant status of this organ. Treatments including exposure to cyclophosphamide or dimethane sulfonate that diminish the intratesticular concentration of testosterone inhibit the testicular expression of antioxidant enzymes such as GPx, SOD and catalase [132]. Furthermore, these suppressive effects on antioxidant expression, as well as the disruption of spermatogenesis, can be reversed by the administration of exogenous gonadotrophin to artificially elevate intratesticular testosterone levels. Suppression of intratesticular testosterone with exogenous steroids, including both androgens and estrogens, similarly resuin the suppression of antioxidant enzyme expression, a concomitant increase in peroxidative damage, the disruption of spermatogenesis and an increase in germ cell apoptosis. Intriguingly, the suppression of antioxidant activity in response to exogenous steroid treatment largely affects the Leydig cells that contain most of the catalase and GPx activities. Testicular SOD activities that are largely confined to the seminiferous tubules did not change dramatically under these circumstances. It is therefore possible that the site of free radical generation in response to gonadotrophin withdrawal involves electron leakage from the inhibited steroidogenic pathway of the Leydig cells. These free radicals then attack the germ cells within the seminiferous tubules leading to extensive apoptosis and the disruption of spermatogenesis [96].

5.9 Retinoids

While fluctuations in Leydig cell steroidogenesis may be one source of free radical generation in the testes, another is the Sertoli cell population. The latter has been shown to generate ROS following stimulation with all trans-retinoic acid (RA), a vital cofactor for spermatogenesis. Exposure of rat Sertoli cells to RA led to activation of ROS generation, lipid peroxidation and, ultimately, a loss of cell viability [133]. There is also some evidence to suggest that retinol might stimulate ROS generation in rat Sertoli cells and that this effect is accompanied by an up-regulation of testicular antioxidant enzymes including SOD, GPx and catalase [134].

5.10 Impact of Xenobiotics

A wide variety of different xenobiotics have also been shown to induce oxidative stress in the testes in concert with the suppression of antioxidant mechanisms. A summary of these testicular toxicants includes Smoking, Nonylphenol, Alcohol, Adriamycin, Chromic acid, Cisplatin, Iron, Cyclophosphamide, Lead, Hexachlorocyclohexane, Cadmium, Trinitrotoluene, Uranium, Aflatoxin, Arsenic, Lindane, Vanadate, Quinalphos, Phthalate esters, Endosulfan, Sulfur dioxide, Diethyl maleate, Sodium fluoride, Monensin, PCB/PCN, Formaldehyde, Methoxychlor, Alloxan, Bisphenol A, Streptozotocin, Acrylamide and Ozone [20]. Heavy paternal smoking, for example, is known to generate oxidative DNA damage in the male germ line in association with a 32% reduction in the α-tocopherol content of the seminal plasma [135]. Experimental exposure of rats to cigarette smoke also induces lipid peroxidation in the testes in association with disturbances in testicular antioxidant enzyme activity. In addition to smoking, excessive alcohol consumption also has a negative effect on testicular function through the induction of oxidative stress and the concomitant disruption of testicular antioxidant status. Given the variety and prevalence of chemical and physical factors that can generate oxidative stress in the male gonad, there is an urgent need to

identify antioxidants that can supplement the tissue's own antioxidant strategies to rescue the testes from the consequences of ROS attack [20].

6. CONCLUSION

Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Further, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. However, the testes contain an elaborate array of antioxidant enzymes and free radical scavengers to ensure that the spermatogenic and steroidogenic functions of this organ are not impacted by oxidative stress. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols, glutathione, vitamin C, vitamin A, and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Therefore maintenance of these antioxidant integrity invariably maintening the integrity of the testicular function and enhances fertility in animals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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