Chapter 2

Reactive Oxygen Species (ROS) and Male Fertility

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Additional information is available at the end of the chapter

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Abstract

Oxidative energy production is inevitably associated with the generation of reactive oxygen species (ROS), excessive concentrations of which can lead to cellular pathology. A free radical may be defined as any molecule that has one or more unpaired electrons. The superoxide anion, the hydroxyl radical, and the hypochlorite radical are some of the highest reactive radicals of oxygen. Owing to their high reactivity and to their capability of initiating an uncontrolled cascade of chain reactions, ROS produce extensive protein damage and cytoskeletal modifications and inhibit cellular mechanisms. Aerobic organisms are equipped with a powerful battery of mechanisms that protect them from the adverse effects of lipid peroxidation (LPO) and other manifestations of oxygen toxicity. Defective sperm function frequently causes male infertility, due to abnormal flagella movement, failure to recognize the zona, and inhibition of sperm-oocyte fusion. ROS are fundamental mediators of physiological sperm function, such as signal transduction mechanisms that have an effect on fertility. ROS can have positive effects on sperm and the concentration functions depending on the nature and the concentration of the ROS involved. They are necessary in regulating the hyperactivation and the ability of the spermatozoa to undergo acrosome reaction. An increased amount of superoxide anion (O_2^-) is one of the first steps required by the spermatozoa for induction and development of hyperactivation and capacitation. Numerous studies have shown that oxidative stress plays an important role in the pathophysiology of infertility and assisted fertility. The paternal genome is of primary importance in the normal embryo and fetal development. ROS-induced sperm damage during sperm translation, such as signal transduction through the seminiferous tubules and epididymis, is one of the most important mechanisms leading to sperm DNA damage. Male germ cells are extremely

vulnerable to oxidative stress as the sperm membrane is rich in unsaturated fatty acids and lacks the capacity for DNA repair. Spermatozoa are particularly susceptible to ROS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (PUFA) and their cytoplasm contains low concentrations of the scavenging enzymes. Many clinical and research institutes are investigating the usefulness of antioxidant supplementation and their role in prevention of the infertility problems. Incubation under oxygen in vitro was detrimental to human spermatozoa, decreasing motility and viability. Since then, many reports have associated ROS with impaired sperm function, including decreased motility, abnormal morphology, and decreased sperm-egg penetration. Increasing knowledge of the mechanisms whereby ROS and endogenous antioxidant systems influence reproductive processes can assist to optimize the application of exogenous antioxidants to fertility treatment.

Keywords: ROS, Fertility, Oxidative stress

1. Introduction

1.1. Mammalian testis and reproduction

The primary sex organs of the male reproductive system are the two testes in which sperm is produced [1, 2]; the testis contains seminiferous tubules that consist of germinal epithelium and peritubular tissue [2, 3]. The epithelium contains two basic cell types, the somatic and germinal cells [4]. At different developmental stages, germ cells, including spermatogonial stem cells and differentiated cells formed during and following meiosis, are primary and secondary spermatocytes and spermatids, respectively.

These cells are located within invaginations of somatic Sertoli cells, with which maintain an intimate and cooperative relationship [3, 4]. Sertoli cells form the blood-testis barrier and are implicated in phagocytosis, secretion of testicular fluid for sperm transport, production of endocrine and paracrine substances that regulate spermatogenesis, and secretion of androgen-binding protein [5].

The development of the testis is a paradigm for the development of other organs, incorporating mechanisms for determining organ shape, size, internal architecture, vascularization, and interaction with other tissues physically, hormonally, and neurally. In the testis's development, several cells are bipotential, since the genital ridges must be able to differentiate into testes or ovaries depending on signals received; the differentiation of these cell lineages does not proceed independently, but it follows from differentiation of Sertoli cells, which then orchestrate the behavior of all other cell types [6]. Finally, the testis is built from a combination of innate precursors and immigrant cells such as germ cells.

Testosterone-secreting Leydig cells are found in the intertubular tissue surrounding the capillaries and have an important role in the spermatogenesis and the differentiation of sexual

organs and secondary male sex characteristics. The Leydig cell is a polyhedral epithelioid cell with a single ovoid nucleus that contains one to three nucleoli and abundant dark-staining peripheral heterochromatin. The acidophilic cytoplasm contains many membrane-bound lipid droplets and a large amount of smooth endoplasmic reticulum. Testicular Leydig cells are the principal source of androgens in the male.

Spermatogenesis occurs in the seminiferous tubules, and it is a dynamic and metabolically active biological process during which haploid spermatozoa are produced through a gradual transformation of germ cells. These cells migrate from the basal compartment toward the luminal regions of the tubules, passing the blood-testis barrier.

The secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary, under the influence of gonadotropin-releasing hormone (GnRH) released by the hypothalamus, affects the male reproductive function. LH stimulates Leydig cells to produce testosterone, which exerts a negative feedback on GnRH and gonadotropin secretion. FSH stimulates Sertoli cell proliferation, a necessary step for the maturation of germ cells, given that the number of Sertoli cells largely determines the number of germ cells that can be correctly nurtured in the testis. During spermatogenesis, FSH and testosterone act in synergy [7, 8].

The early development of gonads has a higher energy requirement than ovaries [9]. The presence of many mitochondria in male germ cells highlights their importance in testicular metabolism [10, 11]. The germ cells's survival in the adult testis is dependent from carbohydrate metabolism, including glycolysis and mitochondrial oxidative phosphorylation. During spermatogenesis, many changes in the energy metabolism of germ cells are involved, mainly due to the blood-testis barrier and changes to the surrounding medium.

The spermatogonia, mature sperm, and the somatic Sertoli cells show high glycolytic activity, whereas spermatocytes and spermatids produce adenosine triphosphate (ATP) by mitochondrial oxidative phosphorylation [12, 13]. During spermatogenesis, three types of mitochondria are identified: the mitochondria in Sertoli cells, spermatogonia, and preleptotene and leptotene spermatocytes; the intermediate form in zygotene spermatocytes; and the condensed form in pachytene spermatocytes, secondary spermatocytes, and early spermatids [7]. The physiological death of germ cells via apoptosis occurs in the spermatogenic process and can be increased by hormone deprivation, heat, and toxin exposure [3]. Therefore, mitochondria play a central and important role in Leydig cell steroidogenesis.

1.2. ROS and male fertility

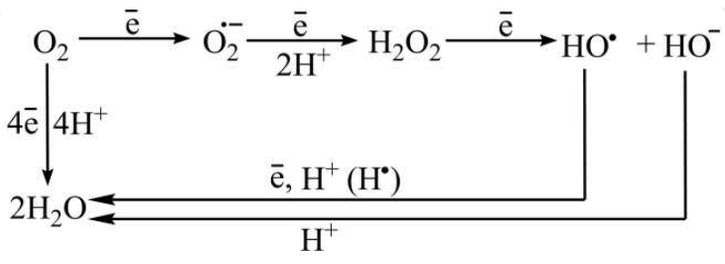
Oxygen is essential for animal life. Most of the body's energy is produced by the enzymatically controlled reaction of oxygen with hydrogen in oxidative phosphorylation occurring in the mitochondria during oxidative respiration. In controlled reaction steps, hydrogen is provided in the form of reducing equivalent and the energy produced is conserved in the form of high-energy phosphates. A four-electron reduction of molecular oxygen to water involving cytochrome oxidase occurs in the mitochondria. During this stepwise, enzymatic reduction of oxygen, free radicals are formed [14].

Free radicals were first described more than a century ago [15]; more than 30 years later, it was showed that all oxidation reactions involving organic molecules would be mediated by free radicals [16]. Then, free radicals were found in biological systems and were involved in many pathological processes and aging [17-19]. Subsequently, their signaling function was evaluated, and then it was found that they were regulated by hormones like insulin and were regulators of metabolic pathways [20-22].

They are short-lived reactive chemical intermediates, which contain one or more electrons with unpaired spin. Free radicals are highly reactive and oxide lipids in membranes, carbohydrates and amino acids in proteins, and damage nucleic acids. Free radicals are active participants in different processes, and they cannot be considered only damaging agents, but real players in many normal functions of living organisms. They are normal by-products in various metabolic and physiological processes, whereas excessive production of them results in the oxidative stress.

The dioxygen molecule (O_2) is a biradical, because it contains two electrons with the same spin in an external antibonding molecular orbital. Molecular oxygen can be reduced via a four-electron mechanism with acceptance of four protons yielding two water molecules. In this case, the free biradical is simply converted to a non-radical species due to acceptance of the four electrons and four protons. However, there is another way to reduce molecular oxygen; this is one-electron successive reduction. Receiving one electron, O_2 is converted to the superoxide anion radical ($O_2^{\cdot -}$), containing one unpaired electron in an external antibonding orbital. Accepting a second electron and two protons converts the superoxide anion radical into hydrogen peroxide (H_2O_2); H_2O_2 has a non-radical nature and is chemically more active than molecular oxygen but less active than $O_2^{\cdot -}$.

The formation of the most reactive of oxygen species, the hydroxyl radical ($HO^{\cdot}$), results from the further reduction of H_2O_2 leading to its dismutation. Finally, acceptance of a fourth (final) electron and one more proton $HO^{\cdot}$ forms a water molecule. Since $O_2^{\cdot -}$, H_2O_2, and $HO^{\cdot}$ are chemically more reactive than molecular oxygen, they are collectively called ROS, but only $O_2^{\cdot -}$ and $HO^{\cdot}$ are actually free radicals, whereas H_2O_2 is not. Therefore, in biological research, the term "free radicals" is frequently replaced by "reactive oxygen species" (ROS), which is a more general term and includes both free radical and non-radical species.



ROS formation and redox signaling play a role in physiology and in a variety of pathologies, including inflammatory, infectious, and degenerative disorders, either in humans or in animals [23-25]. ROS are involved in a variety of pathophysiological conditions of the testis,

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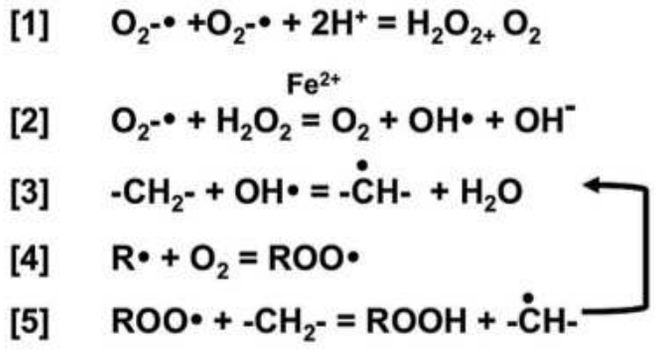
and oxidative stress is known to inhibit ovarian and testicular steroidogenesis. The disruption of redox signaling and control and imbalance in favor of prooxidant species define oxidative stress [26, 27].

Oxidative stress is a state in which an oxidant-generating system overcomes an antioxidant defense system, a process that is involved in many diseases including male factor infertility and/or subfertility. ROS are products of normal cellular metabolism and are formed during the normal enzymatic reactions of intercellular and intracellular signaling [28]. ROS overproduction can be induced through physiological or pathological mechanisms, including ROS generation by leukocytes as a cytotoxic mechanism of host defense, during hypoxic states leading to high levels of ROS, as well as by drugs with oxidizing effects on cells. Then, when mitochondria become a target of elevated levels of ROS, the process of oxidative phosphorylation might be affected because of a possible damage of proteins and membrane lipids. Lipids are present in the sperm plasma membrane in the form of polyunsaturated fatty acids (PUFAs) that contain more than two carbon-carbon double bonds. ROS attacks PUFA in the cell membrane, leading to a cascade of chemical reactions called lipid peroxidation.



At low concentrations, ROS are metabolic intermediates in the metabolism of prostanoids, in gene regulation and cellular growth and in signal transduction [29, 30]. At high concentrations, ROS exert bionegative effects and damage all major classes of biomolecules.

During reproduction, ROS are involved in many important mechanisms of sperm physiology. An increase in ROS generation at the beginning of capacitation is followed by an increase in tyrosine phosphorylation [31]. The motility was associated with the generation of superoxide anion and a phosphorylation of tyrosine residues.

Furthermore, the acrosome reaction was associated with an extracellular superoxide anion of spermatozoa [32]. In the male genital tract, ROS are generated by spermatozoa and leukocytes including neutrophils and macrophages. In the semen, sperm cells are one of the major cellular sources of ROS. The male germ cells produced a small amount of ROS from the earliest stages of the development [33]. They are involved in the sperm chromatin condensation, regulating the number of germ cells by induction of apoptosis or proliferation of spermatogonia [34]. In the mature sperm, ROS play an important role in the capacitation, acrosome reaction and sperm motility, and they can also function as signaling molecules. There are at least two mechanisms of their production: the membrane nicotinamide adenine dinucleotide phosphate

(NADPH) oxidase, an enzyme complex that is contained in the cell membrane, and the mitochondria.

Furthermore, many studies have demonstrated that low and physiological levels of ROS play an important role in processes such as capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion in order to ensure appropriate fertilization, whereas high levels of ROS cause sperm pathologies such as ATP depletion and loss of sperm motility and viability [35]. When the ROS overcomes the antioxidant defense systems and disrupts the intricate balance between ROS and antioxidants, pathological defects occur that causes significant damage to biomolecules such as lipids, proteins, nucleic acids, and carbohydrates [36]. The ROS found in the seminal plasma originates from various endogenous and exogenous sources; there are many endogenous sources of ROS in the seminal plasma such as peroxidase-positive leukocytes including polymorphonuclear leukocytes and macrophages [37]. Most of these peroxidase-positive leukocytes derive from the prostate and seminal vesicles; if these sources of ROS are triggered by many intracellular or extracellular stimuli, as inflammation or infection, they can increase ROS and the NADPH production via the hexose monophosphate shunt [38, 39]. An increase in proinflammatory cytokines, such as interleukin (IL)-8, and a decrease in the antioxidant superoxide dismutase (SOD) can result in a respiratory burst, production of high levels of ROS, and oxidative stress. Between exogenous sources of ROS, there are toxins, phthalates, and others [40]. Infections lead to an excessive ROS production, resulting in an oxidative burst from neutrophils/macrophages as a first-line defense mechanism. When there is an infection, an imbalance of prooxidants and antioxidants favors the oxidative stress that damages the sperm functions such as motility and fertilization. In the testis and epididymis infections, the ROS produced are very detrimental to the spermatozoa because of the long contact time and the loss of antioxidant protection.

During the final phase of the ejaculation, only high numbers of ROS-producing leukocytes are harmful to sperm functions. An infection which involves ROS in the epididymis, prostate gland, and/or seminal vesicles could indirectly damage sperm functions [41].

In 1943, a paper was published showing the effect of high oxygen tensions on motility and prevention of this phenomenon by adding catalase, which suggested the involvement of oxygen overload in motility of spermatozoa [42]. Indeed, ROS generation was dependent on the oxygen tension; higher oxygen tensions increased ROS generation, mainly from leukocytes, whereas low oxygen tensions improved the survival rate and penetration capacity [43].

Oxidative stress has been considered a main cause to male infertility, but studies have showed that low and verified concentrations of ROS play a pivotal role in sperm physiological processes such as capacitation, hyperactivation, acrosome reactions, and signaling processes to provide a suitable fertilization, but an increase in oxidative stress leads to male infertility by the induction of peroxidative damage to the sperm plasma membrane, DNA damage, and apoptosis. ROS must be maintained at appropriate levels to ensure appropriate physiological function while preventing pathological damage to the spermatozoa. ROS is thought to influence fertility by affecting sperm membranes and sperm DNA. They reduce sperm motility and its ability to fuse with the oocyte and compromise paternal genomic contribution to the embryo; in fact, sperm are vulnerable to oxidative stress-induced damage due to the high

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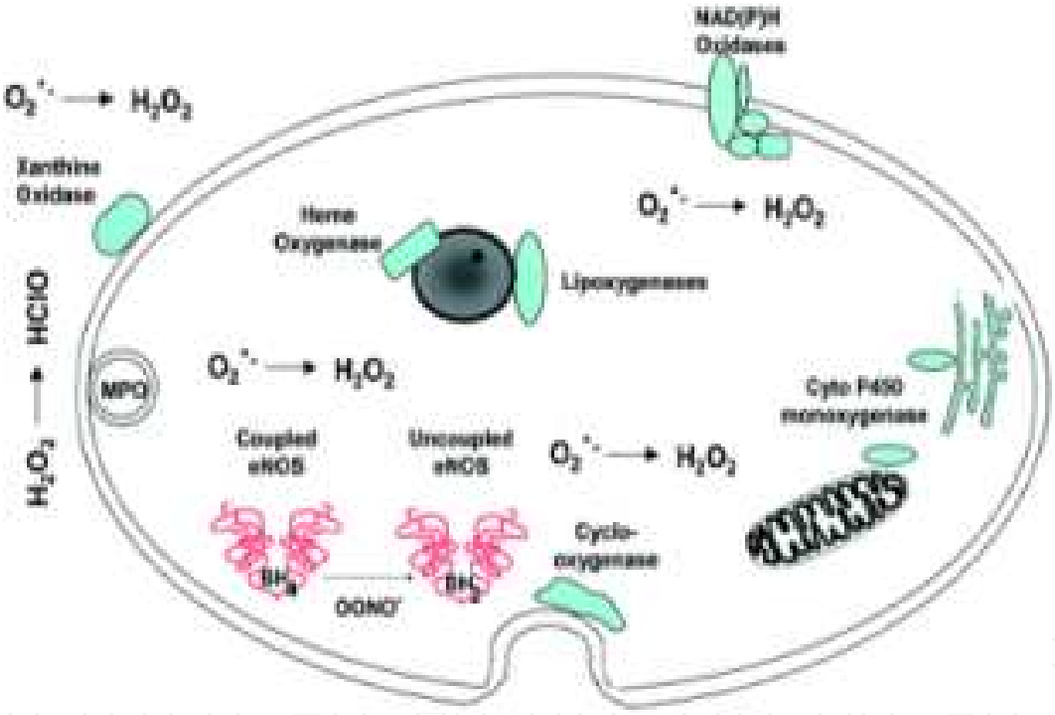
portion of PUFA and also due to the low concentrations of scavenging enzymes in their cytoplasm, both contributing to the defective sperm function observed in a high percentage of infertility.

There are many agents that cause an increase in testicular oxidative stress, such as environmental toxins or conditions such as varicocele, orchitis, cryptorchidism, and aging, all of which leads to an increase in germ cell apoptosis and hypospermatogenesis. ROS-induced DNA damage may also potentiate germ cell apoptosis, leading to a decrease in sperm count and thus to the decline of semen quality, both of which are associated with male infertility [39]. Large amounts of pathogenic mutant mtDNA accumulate in the testis; the resulting mitochondrial respiratory dysfunction in spermatogenic cells leads to a decrease in energy production that ultimately induces meiotic arrest and abnormalities in sperm morphology, stressing the importance of mitochondrial respiratory function in mammalian spermatogenesis [44].



2. Apoptosis and oxidative stress

Oxidative stress is implicated between causes of male infertility. ROS production and its effects on semen quality have been widely clarified. Oxygen is essential to sustain life, and physiological levels of ROS are necessary to maintain normal cell functions. However, products of oxygen such as ROS can be detrimental to cell function and survival [45].

ROS are detrimental to sperm survival and function due to its adverse effects on sperm membrane and genetic material. High frequency of single- and double-stranded DNA breaks

due to oxidative stress activates apoptosis by inducing cytochrome c and caspases 9 and 3 [46]. Disruption of inner and outer mitochondrial membranes results in release of cytochrome c, a protein which activates caspases and induces apoptosis. Mitochondrial exposure to ROS results in the release of apoptosis-inducing factor, which directly interacts with the DNA and leads to DNA fragmentation [46]. Seminal oxidative stress, sperm DNA damage, and apoptosis constitute a unified pathogenic molecular mechanism in infertility. Therefore, apoptosis in semen could be a useful indicator of semen quality.

3. Antioxidants in male fertility

Antioxidants are substances, enzymatic and nonenzymatic, which serve to eliminate ROS. Enzymatic oxidants, or natural oxidants, include glutathione reductase (GSH), superoxide dismutase (SOD), and catalase, while some non-enzymatic oxidants include vitamins (C, E, and B), carotenoids, carnitines, cysteines, pentoxifylline, metals, taurine, and albumin [47]. Glutathione reductase and peroxidase are the principal reducing agents in the body and behave as antioxidant scavengers in the epididymis and testes [48]. Their action on sperm membranes confers protection on to the lipid components, preserving the sperm viability and motility [49]. Preceding in vitro studies have demonstrated that GSH reduces lipid peroxidation and improves the sperm membrane characteristics [50]. The main antioxidant enzyme system in the semen includes SOD, catalase, and glutathione peroxidase.

SODs are metalloenzymes that catalyze the dismutation reactions of the superoxide anion and are present in intracellular and extracellular forms; two of the intracellular forms are copper-zinc SOD, which is localized in the cytoplasm and contains copper and zinc (Cu, ZnSOD, SOD1) in the active site, and manganese SOD, which is located primarily in the mitochondrial matrix and contains manganese in the active site (MnSOD, SOD2). The extracellular form of SOD (EC-SOD, SOD3) acts in the extracellular space and it is related to the surface polysaccharides though it may also be present in a free form [51]. SOD presents high activity in the seminal plasma with 75% of its activity connected to the activity of SOD1 and the remaining 25% to SOD3; these isoenzymes are maybe derived from the prostate [52]. SOD and catalase protect sperm from superoxide anions catalyzing the conversion of superoxide into oxygen and H_2O_2, thereby preventing lipid peroxidation and enhancing motility [53].

$$\cdot O_{2^-} + \cdot O_{2^-} \times + 2H^+ \rightarrow H_2O_2 + O_2$$

SOD and catalase assist in removing ROS that has the potential to damage sperm. Catalase catalyzes the conversion of H_2O_2 to O_2 and H_2O and presents a heme group with a central iron atom. It acts mainly in the endoplasmic reticulum, peroxisomes, mitochondria, and cytosol in many cell types [54]. Catalase was found in the human and rat sperm cells and in the seminal plasma; the prostate seems to be its source [55]. The sperm cell capacitation induced by nitric oxide is activated by catalase [56].

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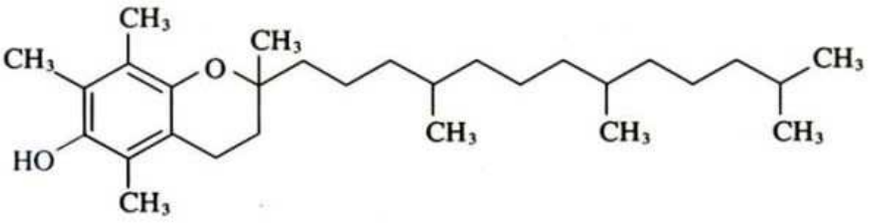
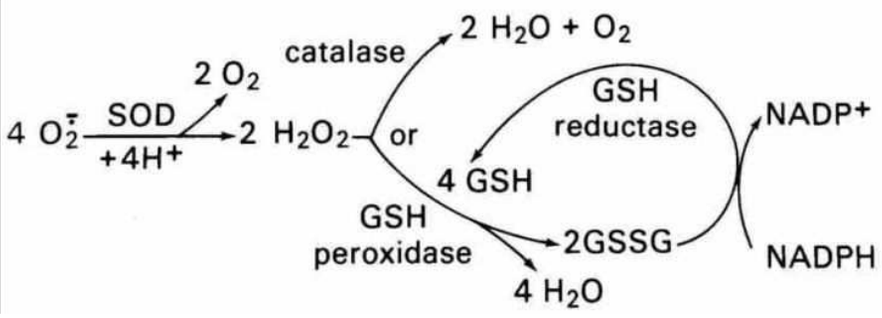


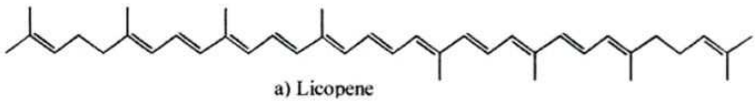
$$2H_2O_2 \quad O_2 + 2H_2O$$

Glutathione peroxidase (GPX), another antioxidant enzyme in the semen, catalyzes the reduction of H_2O_2 and organic peroxides [51]. GPX contains selenium in the form of selenocysteine in its active site. It is located in the sperm in the mitochondrial matrix [52] but has also been found to have a nuclear form that preserves sperm DNA from oxidative damage and enters in the process of chromatin condensation. It was found in the seminal plasma; therefore, it could originate from the prostate [57, 58].

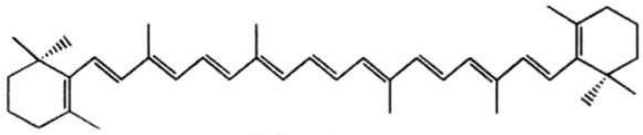


Between nonenzymatic antioxidants, there are vitamin E which encompass a group of potent, lipid-soluble, chain-breaking antioxidants. Structural analyses have revealed that molecules having vitamin E antioxidant activity include four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ). Vitamin E (α-tocopherol), a chain-breaking antioxidant in the sperm's cell membrane, neutralizes H_2O_2 and quenches free radicals, therefore stopping chain reactions that develop lipid peroxides and protecting the membrane from the oxidative damage [48]. Vitamin E improves the activity of other scavenging oxidants and helps to keep motility and morphology of the sperm [54]. It preserves the spermatogenesis in male rats and fails to conserve zygotes in female rats. Selenium deficiency can induce male infertility and could thus support an antioxidant function of vitamin E in the reproductive system. Therefore, vitamin E and selenium can act in synergy in membrane protection from oxidative stress. Vitamin E is known to readily reduce alkyl peroxy radicals of unsaturated lipids, thereby generating hydroperoxides that are reduced by the selenoperoxidases, in particular by phospholipid-hydroperoxide glutathione peroxidase.

Vitamin C or L-ascorbic acid, or ascorbate (the anion of ascorbic acid), is an essential nutrient for humans and many animals. Vitamin C is a major chain-breaking antioxidant and is present in the extracellular fluid. It neutralizes hydroxyl, superoxide, and hydrogen peroxide radicals

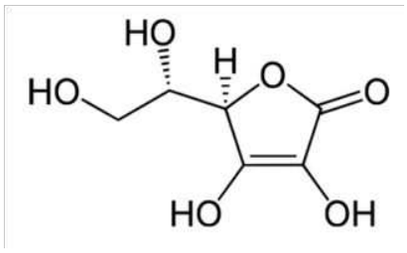


a) Licopene

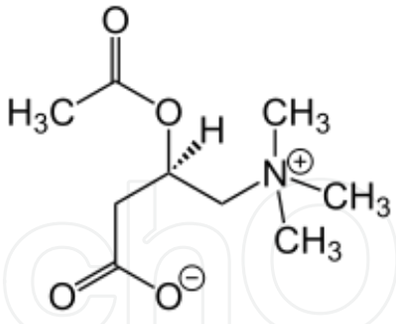


b) β-carotene

and prevents sperm agglutination [53]. It also helps to recycle vitamin E. It plays a significant role in removing oxidative stress in the seminal plasma. It reacts with OH^-, O_2^-, and H_2O_2 in the extracellular fluid, thus protecting sperm viability and motility [59].



Carnitine, a water-soluble antioxidant, participates in sperm motility and prevents lipid oxidation; it protects the sperm DNA and membranes from oxidative damage and maintains sperm viability and motility [60].



Carotenoids are a family of pigmented compounds that are synthesized by plants and microorganisms, but not animals. They are present as micro-components in fruits and vegetables and are responsible for their yellow, orange, and red colors. Carotenoids are thought to be responsible for the beneficial properties of fruits and vegetables in preventing diseases including cardiovascular diseases, cancer, and other chronic diseases. Carotenoids (β-carotene and lycopene) are very efficient singlet molecular oxygen quenchers; they prevent peroxidation in the seminal plasma [59].

Cysteines, precursors of intracellular GSH, increase the amount of GSH synthesized that prevents oxidative damage to the cell membrane and DNA. There are a few other minor

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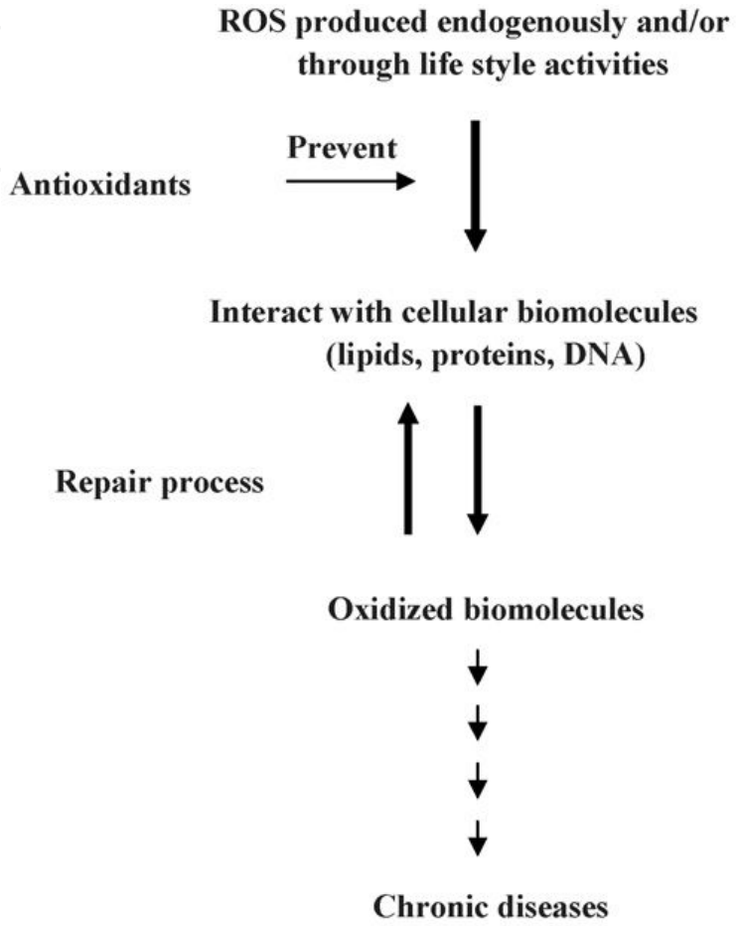
antioxidants that contribute to relieving oxidative stress, such as albumin, taurine/hypotaurine, inositol, and some metals. Albumin, a plasma protein, interacts with peroxyl radicals and inhibits the chain reactions that generate ROS production and preserve motility and viability of sperm.



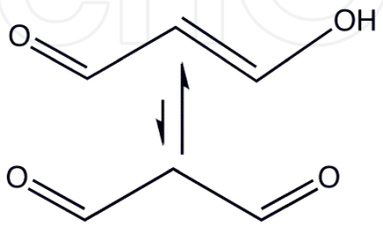
Taurine, a non-enzymatic antioxidant, scavenges ROS; inositol enhances GSH activity and preserves normal sperm morphology.

Selenium is an important component in the regular development and maturation of the testes and contributes to the protection of sperm DNA and cell membranes, particularly when used as an adjunct to vitamin E. The specific role of selenium in spermatogenesis appears to be related to phospholipid hydroperoxide glutathione peroxidase, which is expressed depending on the developmental state of spermatids.

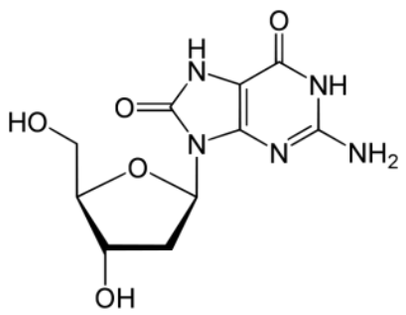
Zinc acts as a chelator and binds ROS; manganese enhances sperm motility and viability [61, 62]. Chrome, another essential micronutrient, is a component of enzymes involved in carbohydrate metabolism. Its supplementation reduces fat deposition in rats, preventing obesity, the initial phase of inflammation, and oxidative stress [63]. Although seminal plasma contains a range of protective antioxidants such as SOD, catalase, and glutathione peroxidase, these defenses are less abundant in the sperm and seem to be impaired in cases of male infertility [64].

4. Measurement of ROS

Oxidative stress results from an imbalance between ROS production and the intracellular and extracellular antioxidants that scavenge ROS. There are many direct assays that measure the oxidation of the sperm cell membrane. The most used assay measures malondialdehyde (MDA), one of the final products of sperm cell membrane lipid peroxidation [65, 66]. Increased levels of MDA correlate with decreased sperm parameters.



Quantification of sperm DNA damage has also been used as assay for intracellular ROS-induced oxidant injury by measuring a specific product of oxidant-induced DNA damage, 8-oxo-7, 8, -dihydro 2′ deoxyguanoside (8-OHdG), used as a specific marker of oxidative injury to sperm DNA [67].



The most used method for measurement of seminal ROS is the indirect chemiluminescence assay. Luminol (5-amino-2, 3, dihydro 1, 4, phthalazinedione), or lucigen, can be used for quantification of redox activities of spermatozoa [68]. Lucigen measures only extracellular superoxide radicals, while luminol is used to measure extracellular and intracellular levels of ROS.

The nitroblue tetrazolium assay requires a light microscope and allows differentiation of spermatic and leukocytic ROS without the steps required in chemiluminescence assays. Nitroblue tetrazolium interacts with superoxide radicals in the sperm and leukocytes by changing to diformazan, a blue pigment. The concentration of diformazan correlates with the concentration of intracellular ROS [68].

The antioxidant levels of the semen can also be determined by chemiluminescence assay or by a colorimetric assay. Antioxidant levels are measured through the addition of a known concentration of ROS to the semen, leading to the development of the chemiluminescence

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signal or a color change. This assay allows the antioxidants in the semen to scavenge the known ROS and then the measurement of residual ROS level. The intensity of the signal produced is inversely correlated with the total antioxidant capacity of the sample [69].

Another method for measuring oxidative stress can be carried through the measurement of lipid peroxidation in the whole sperm by a commercially assay kit (LP Sperm Test, Diacron International, Grosseto, Italy). The assay is based on the ability of peroxides to promote the oxidation of Fe^{2+} to Fe^{3+}; the product of peroxidation (Fe^{3+}) binds to the thiocyanate, developing a colored complex measured photometrically [70].

5. ROS In Vitro Fertilization (IVF) or artificial insemination

New studies are underway to find new methods for supporting longer storage of cooled animal semen. All aerobic organisms require oxygen for life; although it is an essential element, oxygen is responsible for ROS production. It is known that high concentrations of ROS cause sperm pathology. Low concentrations of ROS play an important role in sperm physiology, while higher concentrations are detrimental. A study showed the influence of ROS on capacitation and the acrosome reaction in frozen-thawed bull spermatozoa; they concluded that ROS is required in the capacitation process and that hydrogen peroxide may participate as an inducer of the acrosome reaction [71, 72].

ROS act as second messengers and are involved in the sperm capacitation, acrosome reaction, and oocyte fertilization. They regulate the increase of cyclic adenosine monophosphate (cAMP), protein kinase A (PKA) activation, and phosphorylation of PKA substrates (arginine-X-X-(serine/threonine) motif), phosphorylation of extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase kinase (MEK) proteins and threonine-glutamate-tyrosine motif, and tyrosine phosphorylation of fibrous sheath proteins [73]. When ROS increase, the endogenous antioxidant defenses of gametes decrease and oxidative stress is induced [74]. High concentrations of ROS induce changes in sperm cell functions, altering fluidity and integrity of sperm membranes due to lipid peroxidation. Furthermore, ROS can damage DNA in the sperm nucleus, deplete ATP in mitochondria, and cause loss of sperm motility, viability, and capacity for fertilization [75]. Oxidative stress may be a cause of male infertility and contribute to DNA fragmentation in spermatozoa. There are few studies on the effects of antioxidant addition to extenders during cooling and/or freezing mammalian spermatozoa. Spermatozoa are subjected to peroxidative damage due to an excess of ROS because of the high presence of polyunsaturated fatty acids in membrane phospholipids. The antioxidant systems control the balance between production and neutralization of ROS and protect spermatozoa against peroxidative damage [76]. Recent studies moreover show a physiological SOD activity in human seminal plasma [77, 78]. SOD is an important antioxidant defense in all cells exposed to oxygen. Their use as additives in semen extenders has had controversial effects [79, 80]. SOD is responsible for H_2O_2 and O_2 production, by dismutation of superoxide radicals. The addition of SOD to semen improves the quality of semen and reduces ERK activation [81, 82]. The addition of SOD to the semen extender could prolong

storage of stallion semen, allowing longer distance shipments and a more precise timing of insemination, increasing the high rates of fertility. Furthermore, the antioxidant addition might also bring benefits to spermatozoa in the female reproductive tract [83]. ROS are responsible for the deterioration in quality of semen stored at 5°C, and the addition of SOD to the semen extender improves the quality of cold-stored semen.

Another work evaluated the effect of SOD supplementation in ovary transport media during 4°C storage of cat ovaries at different time intervals on the occurrence of ovarian apoptosis and on the ability to undergo in vitro oocyte development. The authors by immunohistochemical analysis, reverse transcriptase polymerase chain reaction (RT-PCR) analysis, and viability test analysis have demonstrated that SOD supplementation in transport media of domestic cat ovaries reduces cellular apoptosis and enhances COC survival and in vitro embryo production (IVEP) [84].

6. Conclusion

Oxidative stress has been extensively studied for about four decades. Substantial progress has been achieved to date from descriptive characterization of this process to delineation of molecular mechanisms underlining adaptive responses and targeted manipulations of expected responses. Oxygen toxicity is an inherent challenge to aerobic life, including spermatozoa, the cells responsible for propagation of the species. The oxidative damage to sperm membranes, proteins, and DNA is connected with changes in signal transduction mechanisms that affect fertility.

Spermatozoa and oocytes possess an inherent but limited capacity to generate ROS to aid in the fertilization process. Although a variety of defense mechanisms including antioxidant enzymes, vitamins, and biomolecules are available, a balance of the benefits and risks from ROS and antioxidants appears to be necessary for the survival and function of spermatozoa.

The antioxidants α-tocopherol (Vitamin A), ascorbic acid (Vitamin C), and retinoids (Vitamin A) are all potent scavengers of reactive oxygen species. Many studies have investigated the role of these and other antioxidants in improving sperm parameters.

The origin and the etiologies of increased ROS in males with suboptimal sperm quality are increasingly clear, presenting many pathways for a potential therapy. However, well-designed randomized controlled trials will be required to evaluate the potential of antioxidant systems. Furthermore, prooxidative and antioxidative properties of therapeutics are currently receiving more attention as part of anti-infectious therapies too.

ROS production might be beneficial or harmful for living organisms; this also applies in spermatozoa, which require low levels of ROS to show their full capacity in fertilizing. Conversely, oxidative stress is damaging for spermatozoa and many other cellular types; an excess of ROS has been associated with many diseases including diabetes, cancer, atherosclerosis, and Parkinson disease.

Oxidative stress might also be a consequence of unhealthy lifestyles such as smoking, alcohol abuse, or exposure to chemical or electromagnetic pollution. ROS are important contributors to the regulation of sperm function in both a positive and a negative sense. Thus, these cells generate low levels of ROS in order to promote capacitation and the functional evolution of sperm behaviors needed for fertilization, including hyperactivation and the presentation of zona recognition molecules on their surface. If fertilization does not occur, the continued generation of ROS activates the intrinsic apoptotic cascade.

Future progress in the field needs identification of the most crucial cellular targets for ROS action as well as discovery of the underlying mechanisms and consequences of the interaction between ROS and cellular components.

The mechanisms responsible for removing ROS and their regulation would be the second hot topic for ongoing studies of ROS metabolism.

In recent years, it was discovered that ROS and ROS-regulated pathways are actively involved in modification of diverse cellular processes starting from core metabolism and hormonal signaling through to complicated processes such as fertilization and development. The latter along with some biotechnological avenues would also extend ROS-related studies in practical directions. Therefore, much remains to be learned about the effects of ROS on biological systems, the adaptive strategies that overcome ROS attack, and the natural use of ROS in the signaling and regulation of metabolism.

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