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Physiological and Pathological Roles of Free Radicals in Male Reproduction

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Abstract

Oxidative stress (OS) is a condition caused by an imbalance between reactive oxygen species (ROS) overgeneration and decreased antioxidant defense mechanisms in the cell. OS has become a prominent factor in male reproductive dysfunction as ROS cause damage to sperm DNA, lipids and proteins, alterations to critical sperm structures and signaling pathways, leading to a decreased sperm activity and fertilizing capacity. At the same time, small amounts of ROS play vital roles in events leading to sperm maturation and acquisition of functional activity, which is why a proper oxidative balance is of paramount importance for a proper male fertility. Understanding the physiological and pathological roles of ROS in male reproduction has become an essential pillar of modern andrology; however, numerous questions related to the controversial behavior of ROS in male reproductive cells and tissues still remain unanswered. This chapter aims to summarize current evidence available on the relationships between free radicals, antioxidants and male reproduction and to trigger more scientific interest, particularly with respect to the design of efficient strategies to diagnose or treat male sub- or infertility associated with OS.

Keywords: free radicals, reactive oxygen species, oxidative stress, antioxidants, spermatozoa, male infertility

1. Introduction

Aerobic life inherently depends on oxygen, which is essential for a controlled oxidation of molecules containing carbon, subsequently leading to the release of energy. Nevertheless, aerobic cells, including spermatozoa, are persistently counteracting the so-called Oxygen Paradox: while oxygen is crucial to sustain aerobic life, it is simultaneously toxic to the cell survival [1]. Normal aerobic metabolism leads to the generation of by-products called free

radicals (FR) [2, 3], which, under physiological conditions, are necessary for a normal cell function [4]. On the other hand, if FR concentrations become too high, either because of their overgeneration or due to low levels of antioxidant defense mechanisms, oxidative stress (OS) emerges with unpredictable consequences on the cell behavior and survival [5].

Oxidative stress has been implicated in the pathogenesis of a variety of human diseases such as atherosclerosis, cancer, diabetes, liver damage, AIDS, Parkinson's disease and health complications associated with premature birth [6]. In the meantime, seminal OS is believed to be one of the main factors in the pathogenesis of sperm dysfunction in male sub- or infertility [7–9]. Several intrinsic and extrinsic factors have the ability to promote reactive oxygen species (ROS) generation in the testicular as well as post-testicular (e.g. epididymal) environment, resulting in defective spermatogenesis and altered sperm function [9]. As expected, approximately 25% of infertile patients exhibit higher ROS levels in semen as opposed to fertile men [7, 10–12].

Although the origin of ROS generation in semen and their roles in male reproduction have only recently been uncovered, numerous questions still remain unanswered, thus offering multiple strategies for future research. As such, the role of free radicals and oxidative stress in fertility and subfertility is an area requiring continuous scientific attention.

2. Free radicals: general characteristics

A free radical (FR) is defined as any atom, molecule or a fragment of atoms and molecules with one or more unpaired electrons, capable of short independent existence. The abstraction or gain of one electron by a nonradical molecule may (or may not) convert it to a radical species [13]. Free radicals may have a positive, negative or a neutral charge [14]:

A → minus one electron → $A^{+\bullet}$.

B → plus one electron → $B^{-\bullet}$.

It is precisely the presence of an unpaired electron that results in certain common properties shared by most radicals. Free radicals are generally unstable and highly reactive. They can either donate an electron to or accept an electron from other molecules, thus behaving as oxidants or reductants [13].

In cells, one-electron modification of molecules can yield sulfur-, oxygen-, carbon- and nitrogen-derived free radicals [14]. Furthermore, ions of transition metals have a radical nature [13].

The most common and important free radicals related to biological systems are oxygen-derived radicals called reactive oxygen species (ROS) and nitrogen-derived molecules, defined as reactive nitrogen species (RNS) [15]. ROS represent a broad category of molecules including radical and non-radical oxygen derivatives [16]. Reactive nitrogen species are nitrogen-free radicals and commonly accepted as a subclass of ROS [13, 15]. A summary of the most common oxygen- and nitrogen-derived free radicals is provided in **Table 1**.

Radicals		Non-radicals	
Reactive oxygen species (ROS)			
Superoxide	$O_2^{\bullet-}$	Hydrogen peroxide	H_2O_2
Hydroxyl radical	$OH^{\bullet}$	Hypochlorous acid	HOCl
Peroxyl radical	$ROO^{\bullet}$	Hypobromous acid	HOBr
Alkoxyl radical	$RO^{\bullet}$	Ozone	O_3
Hydroperoxyl radical	$HO_2^{\bullet}$	Singlet oxygen	$^1\Delta_g$
Lipid peroxyl radical	$LOO^{\bullet}$	Lipid peroxide	LOOH
Reactive nitrogen species (RNS)			
Nitric oxide	$NO^{\bullet}$	Nitrous acid	HNO_2
Nitrogen dioxide	$NO_2^{\bullet}$	Nitrosyl cation	NO^+
		Nitroxyl anion	NO^-
		Dinitrogen tetroxide	N_2O_4
		Dinitrogen trioxide	N_2O_3
		Peroxynitrite	$ONOO^-$
		Peroxynitrous acid	ONOOH
		Nitronium (nitryl) cation	NO_2^+
		Nitryl chloride	NO_2Cl
		Alkyl peroxynitrite	ROONO

Table 1. Overview of reactive oxygen and nitrogen species.

3. Sources of ROS in semen

Virtually every ejaculate may contain potential sources of ROS. Leukocytes activated by multiple factors, especially inflammation and infection, are among significant ROS producers in semen [17]. Subpopulations of leukocytes, which may be found in semen, mainly consist of polymorphonuclear (PMN) leukocytes (50–60%) and macrophages (20–30%) [18]. PMN leukocytes represent an important source of ROS due to their abundant presence in semen. Furthermore, external stimuli induce the activation of macrophages, leading to an oxidative burst and ROS overgeneration. Under normal circumstances, these monocytes are of paramount importance in defending male reproductive structures against nearby cells and pathogens [19].

The Endz test based on myeloperoxidase staining is an efficient technique to quantify seminal leukocytes during semen quality assessment [20]. According to the World Health Organization (WHO), if the leukocyte concentration in the ejaculate exceeds 1×10^6/mL, leukocytospermia is present [21].

Numerous reports have studied possible relationships between seminal leukocytes and male reproductive dysfunction, resulting in two different directions. On the one hand, some studies failed to reveal any correlation between leukocytospermia and sperm damage [22], whereas inversely, other studies emphasized on a strong link between the presence of seminal leukocytes and abnormal sperm quality [23]. In particular, Sharma et al. [24] observed that even small numbers of white blood cells may be responsible for seminal OS, and hence sub-threshold levels of leukocytes, as seen in ejaculates collected from otherwise healthy subjects, may not be considered safe as previously believed. Moreover, activated leukocytes may be responsible for a 100-fold increase in ROS production in comparison to non-activated white blood cells [25].

Leukocytospermia has been furthermore associated with increased ROS production by spermatozoa, most likely triggered by a direct cell-to-cell contact of the leukocyte with the sperm cell or by the release of soluble products acting on the spermatozoon [23, 24].

Spermatozoa have also been reported to generate ROS independently of leukocytes, and this ability primarily depends on the maturation level of the sperm cell. During the epididymal transit, the main morphological change that takes place in the spermatozoon is the migration of the cytoplasmic droplet, a remnant of the cytoplasm associated with testicular sperm. The droplet migrates from the proximal to the distal position during maturation and is normally shed from spermatozoa during or shortly after ejaculation [26]. Failure to extrude excess cytoplasm during sperm differentiation and maturation traps a number of enzymes, including glucose-6-phosphate dehydrogenase (G6PD) and ß-nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which have been associated with ROS generation through the formation of the NADPH intermediate [27]. As such, immature and functionally defective spermatozoa with abnormal head morphology and cytoplasmic retention are another important source of ROS in semen [12]. According to Gil-Guzman et al. [28] there is a strong positive correlation between immature spermatozoa and ROS production, which in turn is negatively correlated with semen quality. The study revealed that after a density gradient separation of human ejaculates, the layer of immature spermatozoa produced the highest levels of ROS. Furthermore, elevated concentrations of immature spermatozoa were accompanied by increased amounts of mature spermatozoa with damaged DNA [28].

Sertoli cells have also been revealed to have the ability to generate ROS, which may be inhibited by the addition of scavestrogens (J811 and J861). Scavestrogens are derivates of 17alpha-estradiol and serve as effective FR-quenching molecules that able to inhibit iron-catalyzed cell damage *in vitro*. As such, Sertoli cells may play a vital role in ROS-mediated spermatogenesis. Due to currently limited evidence, there is a need to further understand the function of Sertoli cells in the process of ROS generation [29, 30].

Varicocele is defined as the excessive dilation of the *pampiniform venous plexus* around the spermatic cord and this endogenous condition is highly linked to testicular and seminal OS. While its role in male infertility is well researched, recent studies have linked higher grades of varicocele with higher ROS levels [29]. In addition, research has shown that spermatozoa from varicocele patients tend to have high levels of oxidative DNA damage [31]. The most common management option is varicocelectomy, which has been effective in the reduction of ROS levels in affected patients [29, 31].

3.1. Endogenous ROS production by sperm

Superoxide ($O_2^{\bullet-}$) is considered to be the primary ROS produced by respiring cells, including spermatozoa [32]. It is a regular by-product of oxidative phosphorylation, created between complex I and III of the electron transport chain as a result of a monovalent reduction of oxygen and the addition of a single electron [33].

In the male gamete, $O_2^{\bullet-}$ is predominantly generated through two reduced forms of ß-nicotinamide adenine dinucleotide phosphate (NADPH) oxidases that are similar to those found in phagocytic leukocytes: the NADH-dependent oxidoreductase located in the inner mitochondrial membrane and the NAD(P)H-oxidase found in the plasma membrane [34]. The hypothesis that these enzymes are primarily responsible for low-level generation of $O_2^{\bullet-}$ important in cell signaling events in spermatozoa is based essentially on two observations. Firstly, adding pharmacological doses of NADPH to purified sperm suspensions has led to an increase in $O_2^{\bullet-}$ production, subsequently leading to a decline in the sperm function [34, 35]. Secondly, such increased $O_2^{\bullet-}$ production could be inhibited by superoxide dismutase (SOD), which protects male reproductive cells against the toxic effects of NADPH [34]. Additionally, the cytoplasmic enzyme G6PD controls the rate of glucose flux and intracellular availability of NADPH through the hexose monophosphate shunt. This in turn serves as a source of electrons by spermatozoa to fuel $O_2^{\bullet-}$ generation through the NADPH oxidase [35, 36]. Lastly, another relevant source of $O_2^{\bullet-}$ in spermatozoa is electron leakage from the mitochondrial electron transport [34].

Although $O_2^{\bullet-}$ is relatively unreactive, in the presence of hydrogen (H^+) it undergoes either a spontaneous or SOD-catalyzed dismutation into hydrogen peroxide (H_2O_2)—a membrane permeable molecule [15], which is considered to be the major initiator of peroxidative damage in the plasma membrane of spermatozoa [27]. H_2O_2 can be either scavenged by glutathione peroxidase (GPx) or catalase, catalyzing its dismutation into water and oxygen.

Moreover, $O_2^{\bullet-}$ as well as H_2O_2 can undergo a series of cellular transformations to generate the highly reactive hydroxyl radical ($OH^\bullet$) through the Fenton and Haber-Weiss reactions, comprising a reduction of ferric (Fe^{3+}) to ferrous ion (Fe^{2+}) in the presence of $O_2^{\bullet-}$, followed by the H_2O_2 conversion to $OH^\bullet$. Furthermore, $O_2^{\bullet-}$ has the ability to interact with nitric oxide ($NO^\bullet$) to generate peroxynitrite ($ONOO^-$), subsequent reactions of which may lead to either apoptosis or necrosis [30].

3.2. Endogenous RNS production by sperm

The primary RNS species produced by male gametes is nitric oxide ($NO^\bullet$). Its production is catalyzed by nitric oxide synthase (NOS) in a redox reaction between L-arginine and oxygen, initiated by NADPH, and with L-citrulline as a byproduct. $NO^\bullet$ interacts with $O_2^{\bullet-}$ to create peroxynitrite ($ONOO^-$), a highly toxic-free radical [13]. Interestingly, both high and low concentrations of $NO^\bullet$ may result in significant alterations of the sperm function as a result of the production of $ONOO^-$ [30].

Inversely, physiological $NO^\bullet$ levels are reported to have beneficial effects, acting in signal transduction pathways involved in spermatozoa motility, capacitation and acrosome reaction [37].

3.3. External sources of ROS

ROS generation can be exacerbated by a multitude of environmental, infectious and lifestyle-related etiologies.

A wide range of industrial by-products and waste chemicals (e.g. polychlorinated biphenyls, nonylphenol or dioxins) have been associated with several adverse health effects, many of which are related to male infertility. These chemicals have been shown to increase the production of reactive species such as $O_2^{\bullet-}$ and H_2O_2 in the testes, damage sperm DNA and impair spermatogenesis [38]. Persistent environmental contaminants, such as heavy metals and pesticides, may also lead to OS, particularly among workers exposed to such pollutants. These individuals often present with a decreased semen volume and density, accompanied by increased oxidative damage to the sperm lipids, proteins and DNA [39].

Radiation is a natural source of energy with significant effects on living organisms. Mobile devices are becoming more accessible to the general population, particularly to adolescent males and men of reproductive age. Cell phones release radiofrequency electromagnetic radiation, exposure to which has shown to increase the risk of oligo-, astheno- or teratozoospermia. Furthermore, *in vitro* studies have demonstrated that EMR induces ROS generation and DNA fragmentation in human spermatozoa, alongside a decreased sperm concentration, motility and vitality depending on the duration of exposure to radiation [40].

Various components of cigarette smoke have been associated with OS exacerbation. Cigarettes contain a broad array of free radical-inducing agents such as nicotine, cotinine, hydroxycotinine, alkaloids and nitrosamines [41, 42]. The prime component of tobacco is nicotine, which is a well-known ROS producer in spermatozoa with detrimental effects on the sperm count, motility and morphology. Moreover, smokers exhibited a lower hypo-osmotic swelling test percentage, indicating a weaker plasma membrane integrity when compared to non-smokers [41]. Smoking increases ROS production by causing leukocytospermia as shown by Saleh et al. [42], who also demonstrated that in smokers, the seminal ROS and total antioxidant capacity score was increased—a direct indication of oxidative imbalance in affected ejaculates. A different study showed that levels of seminal plasma antioxidants were diminished in smokers. This was furthermore confirmed by the presence of increased levels of 8-hydroxy-2'-deoxyguanosine [43].

By directly affecting the liver, alcohol intake increases ROS production while simultaneously decreasing the antioxidant capacity of the body. Although alcohol consumption has been repeatedly associated with systemic OS, its effect on semen parameters has not been explored to a larger extent. In a study comprising 8344 subjects, moderate alcohol consumption did not negatively affect semen parameters [44]. Nevertheless, it was revealed that chronic drinkers had reduced levels of testosterone, possibly due to an impaired hypothalamic-pituitary axis and damage to the Leydig cells [45]. Increased alcohol levels block gonadotropin-releasing hormone, leading to reduced luteinizing hormone and testosterone levels. Furthermore, alcohol has been shown to increase ROS generation when consumed by malnourished individuals [44].

Lastly, diet may affect semen parameters. In a Danish study, men with the highest saturated fat intake presented with a significantly lower total sperm count and concentration in

comparison to those with the lowest saturated fat intake [46]. These observations were supported by a later report focused on studying the link between dairy food intake and male fertility and revealing that a low-fat dairy diet may lead to a higher spermatogenesis [47]. On the other hand, omega-3 fatty acids and omega-6 fatty acids were shown to improve sperm count, motility and morphology [48]. With regard to obesity and its relation to semen parameters, currently available data are conflicting. In a study on Iranian men, it was found that overweight men tend to have lower sperm counts [49]. Inversely, a different study reported that underweight subjects had lower sperm counts than normal and overweight men [48]. Moreover, a study comprising Tunisian men revealed that sperm concentration, motility and morphology did not vary across different BMI values [50].

4. Physiological roles of ROS

Aerobic metabolism utilizing oxygen is essential for energy requirements of reproductive cells, and free radicals do play a significant role in physiological processes occurring within the male reproductive tract. Spermatozoa themselves produce small amounts of ROS that are essential for a variety of physiological processes such as capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion [30].

4.1. Sperm maturation

During transit and storage in the epididymis, spermatozoa undergo membrane, nuclear and enzymatic remodeling, involving the release, attachment and rearrangement of surface proteins [6, 30, 51]. Such changes are based on the assembly of several signal transduction pathways necessary for the subsequent ability of spermatozoa to undergo hyperactivation and capacitation.

ROS are essential for a proper chromatin packing during the maturation of mammalian spermatozoa, leading to a characteristic chromatin stability. This unique chromatin architecture results from an extensive inter- and intra-molecular disulfide bond stabilization between the cysteine residues of protamines—small nuclear proteins that replace histones during spermatogenesis. Oxidation of the thiol groups in protamines takes place during the transport of spermatozoa from the caput to the cauda epididymis [52]. As demonstrated by Aitken et al. [53], a spontaneous luminol peroxidase signal indicating the presence of ROS was exclusive to mature spermatozoa collected from the cauda region. ROS may act as oxidizing agents in this process, hence facilitating the formation of disulfide bonds, increasing chromatin stability and protecting DNA from possible damage [30, 52]. As spermatozoa possess minimal to none repair mechanisms [9], chromatin condensation is a crucial protective mechanism, in which ROS actually protect male gametes against future oxidative insults.

Likewise, peroxides have been associated with formation of the mitochondrial capsule—a coat surrounding sperm mitochondria providing protection against possible proteolytic degradation [54]. It is suggested that during spermatogenesis peroxides may oxidize the active form of phospholipid hydroperoxide glutathione peroxidase (PHGPx), creating an

intermediate that subsequently interacts with thiol groups to form a seleno-disulfide bond. The resulting mitochondrial capsule is made out of a complex protein network rich in disulfide bonds. Mitochondria require such protection as their proper function is crucial for metabolism, cell cycle control and oxidative balance [51, 53, 54].

Although several studies have reported improved sperm DNA integrity and reduced ROS production as a result of daily antioxidant consumption [55], an unusual decondensation of sperm DNA has been revealed as well [56]. Hence it may be hypothesized that high antioxidant levels may alter the oxidative conditions necessary for a proper formation of the inter- and intra-molecular disulfide bonds, leading to a lower DNA compaction.

4.2. Capacitation

Capacitation is a prominent process of final maturation that spermatozoa undergo in the female reproductive tract, during which sperm motility changes from a progressive state to a highly energetic one. It is hypothesized that capacitation occurs exclusively in mature spermatozoa in order to reach the oocyte taking advantage of hyperactive motility and an increased responsiveness to chemotactic agents. Numerous receptors on the sperm head become activated, providing energy to the sperm to penetrate the zona pellucida. As such, capacitation sets up the path necessary for subsequent hyperactivation and acrosome reaction [57]. Most prominent molecular processes associated with capacitation include Ca^{2+} and HCO_3^- influx, cholesterol efflux, increased cAMP activity, ROS generation, pH, protein phosphorylation and membrane hyperpolarization [32, 58].

Numerous of studies on both human and animal spermatozoa indicate that H_2O_2 is the primary ROS responsible for capacitation to occur. This process is associated with an increase in tyrosine phosphorylation, and it has been shown that the amount and banding pattern of tyrosine phosphorylation by adding exogenous H_2O_2 was similar to that observed during endogenous ROS production, providing evidence that H_2O_2 may be responsible for the enhancement of capacitation [32, 57, 58]. This hypothesis was further confirmed by Rivlin et al. [59] who showed that catalase decreased, while H_2O_2 increased the tyrosine phosphorylation in a dose-dependent manner, thereby solidifying the involvement of H_2O_2 in the process of capacitation.

At the same time, de Lamirande and Gagnon [58] indicated that $O_2^{\bullet-}$ may be also involved in this process. Of note is also the role of $NO^\bullet$, which is present in the female genital tract. $NO^\bullet$ may initiate the acrosome reaction, the effects of which are likely achieved through a complex mechanism involving H_2O_2 [59, 60].

Finally, a combination of $O_2^{\bullet-}$ and $NO^\bullet$ forms $ONOO^-$, which allows oxysterol to be produced. Oxysterol, which removes cholesterol from the lipid bilayer, inhibits tyrosine phosphate and promotes cyclic adenosine 3′,5′-monophosphate (cAMP) production [60]. This process is vital as cAMP must increase in concentration for capacitation to occur. cAMP and its subsequent pathways involve protein kinase A, which phosphorylates MEK (extracellular signal-regulated kinase)-like proteins as well as tyrosine present in fibrous sheath proteins [57, 58].

The results of the above studies show that ROS can positively enhance sperm capacitation, but diverge over the specific ROS involved. Both $O_2^{\bullet-}$ and H_2O_2 may stimulate different molecules in the biochemical pathways, and depending on the *in vitro* method used to induce capacitation, specific ROS involved may therefore differ. Several studies have confirmed the lack of molecular specificity in the activation of capacitation and tyrosine phosphorylation, as both SOD and catalase have been shown to negate the positive effect exogenously induced capacitation and hyperactivation [59].

Although physiological ROS levels are necessary for capacitation, their overgeneration may trigger apoptosis. When the levels of oxysterols and lipid aldehydes increase, cell-mediated suicide may occur accompanied by an enhanced mitochondrial $O_2^{\bullet-}$ production, lipid peroxidation (LPO), cytochrome c release and subsequent caspase activation [53, 61].

4.3. Motility and hyperactivation

Hyperactivation is an incompletely understood process to be observed in the final maturation stage of spermatozoa and is considered a subcategory of capacitation. Normally spermatozoa exhibit a low amplitude flagellar movement accompanied by low, linear velocity. In the hyperactivated state, spermatozoa movement is of high amplitude, asymmetric flagellar movement, pronounced lateral head displacement and non-linear trajectory, allowing the sperm to penetrate the *cumulus oophorus* and zona pellucida surrounding the oocyte. Furthermore, hyperactive motility may enable the progressive movement through the oviduct by preventing stagnation, adding yet another benefit to the sperm function [62]. The biochemistry of hyperactivation is poorly understood, but it is known to involve a rise in cAMP activity and pH [58], increased generation of ROS, an initial influx of bicarbonate ions and an increase in intracellular Ca^{2+} concentrations [62].

Extracellular $O_2^{\bullet-}$ is considered nearly essential for hyperactivation in mammalian spermatozoa, as the presence of SOD, but not catalase, reduced the percentage of spermatozoa exhibiting hyperactivity in a variety of culture media [58]. *In vitro* experiments have also revealed that $O_2^{\bullet-}$ is a vital trigger of sperm motility hyperactivation [30]. At the same time, $NO^{\bullet}$ plays important roles in regulating hyperactivation in mammalian epididymis. H_2O_2 may interact with this process as well; however, its activity may be dependent upon $NO^{\bullet}$ regulation. This hypothesis was confirmed by *in vitro* experiments, according to which catalase vital against H_2O_2 toxicity prevents $NO^{\bullet}$-induced capacitation and hyperactivation [60].

4.4. Acrosome reaction

Acrosome reaction (AR) is related to the release of proteolytic enzymes, primarily acrosin and hyaluronidase, in order to degrade the zona pellucida of the oocyte. Once degraded, hyperactive motility propels the spermatozoa into the perivitelline space, at which point the spermatozoa may eventually fuse with the oocyte [63]. Compared to the slow, reversible process of capacitation, this is a permanent, fast-acting step associated with a respiratory burst (rapid extracellular $O_2^{\bullet-}$ production) increasing the tyrosine phosphorylation of specific proteins [57, 64]. $O_2^{\bullet-}$ produced *via* NADPH oxidase may dismutate into H_2O_2, and these two molecules may have a positive effect

on the AR [6, 32, 64]. •NO has also been reported to increase the percentage of sperm undergoing the AR [37]. At the same time, results regarding the specific ROS are conflicting. The majority of studies note positive effects of H_2O_2 and negative effects of catalase, thus suggesting that H_2O_2 is the major species responsible for a proper AR [58, 64].

Moreover, ROS act as signal transducers in the AR. Elevated ROS production may occur upon interaction with the *cumulus oophorus*, thereby enhancing the signal for exocytosis initiated by either progesterone or the zona pellucida. *In vivo*, binding of the zona pellucida and a certain stimulus *via* progesterone on capacitated spermatozoa initiates this process and is associated with an influx of extracellular Ca^{2+} into the cytosol [6]. *In vitro* studies indicate that ROS can induce the Ca^{2+} influx and initiates the biochemical cascade associated with the AR [53, 64].

4.5. Sperm-oocyte fusion

A link exists between enhanced ROS levels and increased sperm-oocyte fusion. High rates of sperm-oocyte fusion are correlated with increased expression of phosphorylated tyrosine proteins [6], suggesting that sperm-oocyte fusion is related to the events of capacitation and AR. Both H_2O_2 and $O_2^{•-}$ contribute to the increase in fertilization rates as revealed by the fact that the addition of catalase or SOD significantly decreased the fusogenicity, whereas the addition of H_2O_2 or $O_2^{•-}$ significantly increased the fusogenicity [53, 64].

Ultimately, ROS are thought to increase membrane fluidity using two mechanisms: (1) de-esterification of membrane phospholipids and (2) activation of phospholipase A2 (PLA2) [65].

Once the zona pellucida and corona radiata are penetrated by the sperm cell, the oocyte prevents eventual polyspermy by turning the vitelline layer into a hard envelope. o,o-Dityrosine crosslinks catalyzed by ovoperoxidase lead to the formation of a single macromolecular structure acting as the envelope [66]. H_2O_2 serves as the substrate to ovoperoxidase to provide for the envelope formation. With our understanding of ROS and their spermicidal effect, H_2O_2 proves to be an effective spermicide agent against polyspermy [66, 67].

5. Oxidative stress (OS)

The term oxidative stress refers to a critical imbalance between ROS production and antioxidant defense mechanisms available to the biological system [15]. According to Sies [5], it is a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential cellular damage.

Essentially, OS may result from:

1. Diminished antioxidants, e.g. mutations affecting antioxidant defense enzymes or toxic agents that deplete such mechanisms [5].

2. Increased ROS or RNS generation either by exposure to increased levels of toxins that act as reactive species themselves or are metabolized to induce further biological oxidation or

by excessive activation of 'natural' FR-generating systems (e.g. phagocytic oxidative outburst during chronic inflammatory diseases) [5, 15]. This mechanism is normally thought to be more relevant to mammalian diseases and is frequently the target of attempted therapeutic intervention.

OS can result in:

1. Adaptation: Usually by upregulation of antioxidant defense systems.

2. Cell and tissue injury: OS can cause damage to all molecular targets: DNA, proteins and lipids. Often it is not clear which is the first point of attack, since injury mechanisms may overlap [5].

3. Cell death: This process may occur by two mechanisms, necrosis or apoptosis. During necrotic cell death, the cell swells and ruptures, releasing its contents into surrounding areas and affecting adjacent cells. The intracellular content can include antioxidants such as catalase or glutathione (GSH) as well as prooxidants such as copper and iron. As such, necrosis may lead to further oxidative insults in the internal milieu [3–5, 15]. During apoptosis, the cell's own "suicide mechanism" gets activated. As such, apoptotic cells do not release their content into surrounding environment and apoptosis does not cause damage to the neighboring cells [5].

An intricate cellular architecture of spermatozoa renders them to be particularly sensitive to OS. Sperm plasma membranes contain large quantities of polyunsaturated fatty acids (PUFAs). On the other hand, their cytoplasm contains low concentrations of scavenging enzymes [68]. OS usually results in a decreased sperm motion and viability, accompanied by a rapid loss of ATP, axonemal damage, increased midpiece morphology defects, followed by alterations in the sperm capacitation and acrosome reaction [32]. Lipid peroxidation has been repeatedly postulated to be the key mechanism of ROS-induced sperm damage, possibly leading to male reproductive dysfunction [68].

5.1. Lipid peroxidation (LPO)

Sperm plasma membranes are largely composed of PUFAs, which are exceptionally susceptible to oxidative damage due to the presence of more than two carbon–carbon double bonds [68]. These fatty acids maintain the fluidity of membranes [69]. ROS attack PUFAs, leading to a cascade of chemical reactions called lipid peroxidation (LPO). As the LPO proceeds, more than 60% of PUFAs may be lost. LPO affects most prominent structural and functional characteristics of the membrane, including fluidity, ion gradients, receptor transduction, transport processes as well as enzymatic activities. As a result, properties that are crucial for a normal fertilization are impaired [68, 69].

LPO is a self-propagating process that may be divided into three phases: the initiation phase, the propagation phase and the termination phase. Before any of these processes takes place, $O_2^{\bullet-}$ is generated either intracellularly through the NADPH system or through

leukocytes as an extracellular source. $O_2^{\bullet-}$ can be directly protonated to create the hydroperoxyl radical ($HO_2^{\bullet}$) or it can be converted into H_2O_2 *via* SOD. H_2O_2 may be subsequently converted into $OH^{\bullet}$ *via* the Fenton reaction involving ferrous iron. Generation of $OH^{\bullet}$ and $HO_2^{\bullet}$ mark the beginning of the initiation stage, as neither $O_2^{\bullet-}$ nor H_2O_2 is not energetically rich enough to initiate LPO directly [70]. During the initiation phase, one hydrogen is taken from unsaturated lipids to form lipid radicals. These radicals subsequently interact with oxygen to generate lipid $HO_2^{\bullet}$, which may be transformed into lipid peroxides through available antioxidants, stabilizing the sperm plasma membrane. Nevertheless, during the propagation stage, in the presence of a transition metal ion, lipid peroxides will be transformed into alkoxyl radical and $HO_2^{\bullet}$ through the Fenton and Haber-Weiss reaction, subsequently acting upon additional lipids until the damage is widespread and irreversible [68–70]. During the termination phase, two radicals react with each other to form a stable product and LPO finally ceases [70].

Numerous pathological effects of LPO on the sperm function are currently known. Overall, LPO causes DNA and protein damage through oxidation of lipid peroxyl or alkoxyl radicals. DNA fragmentation by LPO can occur *via* base modifications, strand breaks or crosslinks [71]. LPO generally results in loss of membrane fluidity and subsequently a decreased sperm motility and sperm-oocyte fusion [68–71].

Furthermore, during LPO, ROS initiate a cascade of events involving the xanthine and xanthine oxidase system and deplete the ATP production which may ultimately lead to sperm death [68].

5.2. DNA damage

The unique sperm chromatin packing alongside antioxidant molecules present in the seminal plasma provide notable protection to sperm DNA against oxidative damage. Nevertheless, spermatozoa lack any specific DNA repair mechanisms and hence depend on the oocyte for eventual DNA repair following fertilization. ROS-associated catalysis and apoptosis are considered to be the primary mechanisms that induce DNA fragmentation in spermatozoa [72].

DNA bases and phosphodiester backbones are believed to be most susceptible to ROS-associated peroxidative damage. At the same time, sperm mitochondrial DNA is more vulnerable to oxidative insults when compared to the nuclear genome [73]. Furthermore, because of the structure of the Y chromosome as well as its inability to repair double strand breaks, Y-bearing spermatozoa are more susceptible to DNA damage than X-carrying counterparts [74]. Y-bearing spermatogonia can be a target of mutations in the euchromatic Y region (Yq11), known as the azoospermia factor, resulting in infertility [75].

Various types of DNA abnormalities may occur in sperm that have been exposed to ROS artificially. These include base modifications, production of base-free sites, deletions, frame shifts, DNA crosslinks and chromosomal rearrangements. OS has also been associated with high frequencies of single- and double-strand DNA breaks. ROS can also cause gene mutations, such

as point mutation and polymorphism, resulting in decreased semen quality. These changes may be observed especially during the prolonged meiotic prophase, when the spermatocytes are particularly sensitive to damage and widespread degeneration can occur [72–74]. Also, mutations in the mitochondrial DNA (mtDNA) may cause a defect of mitochondrial energy metabolism and therefore lower levels of mutant mtDNA may compromise sperm motility *in vivo* [76]. Other mechanisms such as denaturation and DNA base-pair oxidation may also be involved [74].

Increased DNA damage has become a serious issue during artificial reproduction techniques (ARTs), as it has been correlated with decreased fertilization rates *in vitro* and increased early embryo death. Unfortunately, no successful method to prevent or treat sperm DNA damage is currently available [77].

5.3. Protein oxidation

Proteins are a critical target for oxidation because of their abundance and high rate constants for interactions with diverse ROS. As such, protein damage is a major consequence of both intracellular and extracellular oxidative insults. ROS may attack both the side chains and backbone, and the extent of the insult depends on multiple factors. In some cases, the damage is limited to specific residues, whereas in case of other ROS, the damage is widespread and nonspecific [78].

Oxidative attacks on proteins generally result in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electric charge and increased susceptibility or extreme tolerance to proteolysis [79].

The resulting products of protein oxidation include reactive hydroperoxides, which may be employed as biomarkers for protein oxidation *in vitro* and *in vivo*. As protein damage is usually non-repairable, oxidation may have deleterious consequences, including the loss (or sometimes gain) of enzymatic, structural or signaling function, fragmentation, unfolding, altered interactions with other proteins and modified turnovers. Generally, oxidized proteins are degraded by proteasomal and lysosomal pathways; however, in some cases, such altered material is poorly degraded and may accumulate within cells contributing to multiple mammalian pathologies [78, 79].

The amino acids in a peptide differ in their susceptibility to oxidative insults, while various ROS differ in their potential reactivity. Primary, secondary and tertiary protein structures alter the relative susceptibility of certain amino acids. Sulfur-containing amino acids and particularly thiol (−SH) groups are very susceptible to ROS-associated damage [79, 80].

According to Mammoto et al. [81], protein oxidation in spermatozoa leads to a blocked sperm-egg fusion, the capacity to penetrate the zona pellucida, as well as sperm-egg binding. Sinha et al. [80] showed that oligospermia is linked to a quantitative reduction in the SH-groups in spermatozoa. Thus, oxidation of the sperm SH-proteins may be a notable mechanism responsible for the suppressive effects of ROS on sperm functions.

5.4. Apoptosis

Usually, when cellular components undergo serious damage, apoptosis or programmed cell death is initiated. During spermatogenesis, abnormal spermatozoa are eliminated primarily through apoptosis. The exact mechanism of action is not fully understood yet; however, previous studies have speculated that ROS serve as an activator of the mitochondria to release the signaling cytochrome c [82, 83]. This molecule initiates a cascade of events involving caspases 3 and 9, eventually leading to sperm apoptosis. The Fas-protein may be also an integral component in the apoptotic pathway. When Fas-ligand or anti-Fas antibody binds to Fas, apoptosis is initiated [83]. An additional mechanism involves the inflammatory production of ROS, primarily hypochlorous acid (HOCl), which is a product of H_2O_2 and chloride ion. This molecule oxidizes a variety of cellular components, thus causing apoptosis [84]. Said et al. [85] emphasized that HOCl is associated with elevated levels of apoptotic markers in spermatozoa.

Numerous studies have focused to study apoptosis in spermatozoa. Various authors [35, 86] have reported increased ROS levels and apoptotic markers measured by fluorescence in samples of infertile subjects. In deer spermatozoa, it was demonstrated that H_2O_2 addition stimulates apoptosis, whereas $O_2^{\bullet-}$ and $OH^\bullet$ do not have this ability [86]. Meanwhile studies in primate, murine and boar spermatozoa indicated that $NO^\bullet$ was correlated with apoptosis possibly through caspase activation [87, 88].

On the other hand, in certain males, abortive apoptosis appears to fail in the clearance of spermatozoa that are marked for elimination by apoptosis. As such, the subsequent population of ejaculated spermatozoa may exhibit an array of anomalies consistent with characteristics typical for cells that are in the process of apoptosis. Apoptotic failures may lead to a decreased sperm count resulting in subfertility [82, 83].

5.5. Effects on sperm motility

Spermatozoa motility is an important prerequisite to secure their distribution in the female sexual system, followed by an effective passage through the cervical mucus and penetration into the egg [89]. Increased ROS levels have been repeatedly correlated with a decreased sperm motility [10–12, 90], although the exact mechanism involved is still not completely understood. One hypothesis suggests that H_2O_2 diffuses across the membranes into the cells and inhibits the activity of vital enzymes such as NADPH oxidase [6]. At the same time, a decreased G6PDH leads to a reduced availability of NADPH accompanied by a build-up of oxidized glutathione. Such changes may lead to a decline in the intracellular antioxidant levels and a subsequent peroxidation of membrane phospholipids [65].

Another hypothesis presents a series of interrelated events leading to a decreased phosphorylation of axonemal proteins, followed by sperm immobilization, both of which are linked to a reduced membrane fluidity crucial for sperm-oocyte fusion [10, 32]. When spermatozoa are incubated with selected ROS overnight, loss of motion characteristics

observed is highly correlated with sperm LPO. Furthermore, the ability of antioxidants to revive sperm motility is evidence that LPO is a major cause for motility loss in spermatozoa [68, 69].

6. The role of antioxidants in male reproduction

Because ROS have both physiological and pathological functions, biological systems have developed defense systems to maintain ROS levels within a certain range. Whenever ROS levels become pathologically elevated, antioxidants scavenge them to minimize any potential oxidative damage [1].

Antioxidants are defined as molecules that dispose, scavenge and inhibit the formation of ROS or oppose their actions. According to Ďuračková [13], antioxidants can protect cells against OS *via* three mechanisms: prevention, interception and repair.

Antioxidants may be divided into two dominant categories:

- Enzymatic (e.g. superoxide dismutases, catalase and glutathione peroxidases).

- Non-enzymatic (e.g. vitamin C, vitamin E, vitamin A, carotenoids, albumin, glutathione, uric acid, pyruvate, etc.) [13].

Due to the size and small volume of cytoplasm, as well as the low concentrations of scavenging enzymes, spermatozoa have limited antioxidant defense possibilities. Mammalian spermatozoa predominantly contain enzymatic antioxidants, including SOD and glutathione peroxidases (GPx), which are mainly located in the midpiece. A few non-enzymatic antioxidants, such as vitamins C and E, transferrin and ceruloplasmin, are present in the plasma membrane of spermatozoa and act as preventive antioxidants [16].

Under normal circumstances, the seminal plasma is an important protectant of spermatozoa against any possible ROS formation and distribution. Seminal plasma contains both enzymatic antioxidants, as well as an array of non-enzymatic antioxidants (e.g. ascorbate, urate, vitamin E, pyruvate, glutathione, albumin, taurine and hypotaurine) [9].

Studies have shown that antioxidants protect spermatozoa from ROS generating abnormal spermatozoa, scavenge ROS produced by leukocytes, prevent DNA fragmentation, improve semen quality, reduce cryodamage to spermatozoa, block premature sperm maturation and generally stimulate sperm vitality [91, 92].

6.1. Superoxide dismutases (SOD)

Superoxide dismutases are metal-containing enzymes that catalyze the conversion of two superoxides into oxygen and hydrogen peroxide, which is less toxic than superoxide [1, 13]:

$$M^{(n+1)+} - SOD + O_2^{\bullet -} \; \rightarrow \; M^{n+} - SOD + O_2 \tag{1}$$

$$Mn^+ - SOD + O_2^{\bullet-} + 2H^+ \; \rightarrow \; M^{(n+1)+} - SOD + H_2O_2 \tag{2}$$
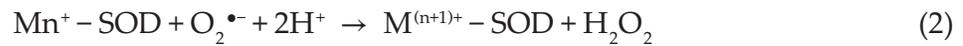
where M = Cu (n = 1); Mn (n = 2); Fe (n = 2); Ni (n = 2).

The enzymes are present in both intracellular and extracellular forms. The first intracellular form is the dimeric copper-zinc SOD, localized primarily in the cytosol and/or intermembrane space and containing copper and zinc (Cu/ZnSOD, SOD-1) in its active center. The second form is manganese SOD, which is found predominantly in the mitochondrial matrix and has manganese in its active center (MnSOD, SOD-2) [93].

The secretory tetrameric SOD (EC-SOD, SOD-3) may be detected in the extracellular space. The enzyme is associated with surface polysaccharides although it may also be found as a free molecule. Structurally, SOD-3 is similar to SOD-2; however, it has zinc and copper in its active center instead of manganese [1, 5, 15]. The cytosolic Cu/Zn-SOD is the dominant SOD isoenzyme found in the seminal plasma and spermatozoa [93].

SOD protects spermatozoa against spontaneous O_2 toxicity and lipid peroxidation [69]. The enzyme also prevents premature hyperactivation and capacitation induced by $O_2^{\bullet-}$ before ejaculating [10, 32].

Numerous studies have suggested a significant role for SOD in sperm motility both *in vivo* and *in vitro*. The addition of SOD to human and animal semen [94–96] has been shown to protect spermatozoa against the harmful effects of ROS and improve sperm motility and membrane integrity during liquid storage or cryopreservation. As such, it may be concluded that the SOD content in mature spermatozoa may be a good predictor of post-thaw motility recovery following sperm preservation.

6.2. Catalase (CAT)

Catalase catalyzes the decomposition of hydrogen peroxide to molecular oxygen and water, thereby completing the detoxifying reaction started by SOD. A characteristic feature of its structure is a heme system with centrally located iron [1, 13]:

$$H_2O_2 + Fe(III) - E \; \rightarrow \; H_2O + O = Fe(IV) - E(.+) \tag{3}$$

$$H_2O_2 + O = Fe(IV) - E(.+) \; \rightarrow \; H_2O + Fe(III) - E + O_2 \tag{4}$$
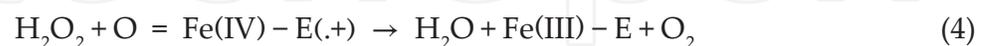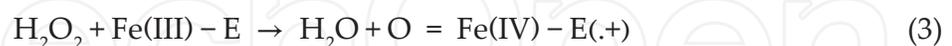
Fe()-E represents the iron center of the heme group attached to the enzyme.

CAT has been found in peroxisomes, mitochondria, endoplasmic reticulum and the cytosol in a variety of cells [93]. In semen, the enzyme was detected in human, bovine and rat spermatozoa, as well as seminal plasma, with the prostate as its source [97, 98].

Catalase activates sperm capacitation induced by nitric oxide [59, 60]. Furthermore, it plays an important role in decreasing lipid peroxidation and protecting spermatozoa during genitourinary inflammation [25].

Numerous studies have revealed a positive relationship between sperm motility and the presence of CAT in mammalian ejaculates. Also, positive correlations were observed between sperm morphology and protein expression of CAT in seminal plasma [98, 99]. Furthermore, CAT supplementation to fresh, processed and cryopreserved semen resulted in a higher sperm vitality, progressive motility and DNA integrity [100].

6.3. Glutathione peroxidase (GPx)

Glutathione peroxidases are a family of selenium-containing enzymes, which catalyze the reduction of H_2O_2 and organic peroxides, including phospholipid peroxides [93]. In their active site, the enzymes contain selenium in the form of selenocysteine.

The net reaction catalyzed by glutathione peroxidase may be represented as:

$$2GSH + H_2O_2 \rightarrow GS-SG + 2H_2O \tag{5}$$
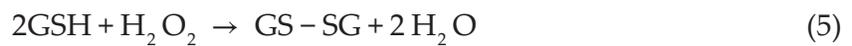
where GSH symbolizes reduced glutathione and GS-SG represents glutathione disulfide. The reaction is based on the oxidation of selenol of a selenocysteine residue by H_2O_2. This process leads to its derivation with selenic acid (RSeOH). This by-product is subsequently converted back to selenol through a two-step process that starts with a reaction comprising GSH to generate GS-SeR and water. A second GSH molecule then reduces the GS-SeR intermediate back to selenol, releasing GS-SG as a by-product [1, 5, 13]:

$$\begin{aligned} RSeH + H_2O_2 &\rightarrow RSeOH + H_2O \; RSeOH + GSH \\ &\rightarrow GS\text{-}SeR + H_2O \; GS\text{-}SeR + GSH \rightarrow GS\text{-}SG + RSeH \end{aligned} \tag{6}$$
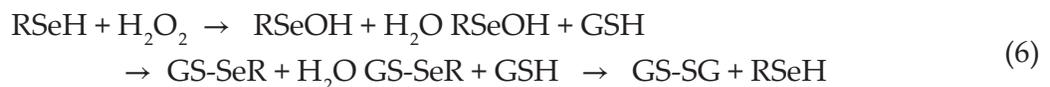
Glutathione reductase then reduces the oxidized glutathione to complete the cycle:

$$GS-SG + NADPH + H^+ \rightarrow 2\,GSH + NADP^+ \tag{7}$$
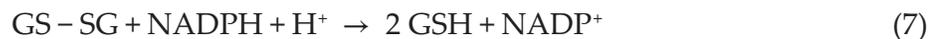
The classic intracellular GPx1 is expressed in sperm nucleus, mitochondria and cytosol, as well as in the testes, prostate, seminal vesicles, vas deferens, epididymis, and has a significant relationship with sperm motility [101, 102].

More importantly, a direct relationship has been reported between male fertility and phospholipid hydroperoxide glutathione peroxidase (PHGPx; GPx4), a selenoprotein that is highly expressed in testicular tissue and has a prominent role in the formation of the mitochondrial capsule [51, 53, 54]. Glutathione peroxidases remove peroxyl (ROO•) radicals from various peroxides, including hydrogen peroxide [13].

6.4. Other enzymes

Other enzymes, such as glutathione reductase, ceruloplasmin or heme oxygenases, may also participate in the enzymatic control of oxygen radicals and their products. A short overview of minor antioxidant enzymes is provided in **Table 2**.

Glutathione reductase (GR)	• Location: Found in the epididymis, sertoli cells, vas deferens, seminal vesicles, epithelium and prostate gland [103, 104]. • Roles: Catalyzes reduction of oxidized glutathione. Maintains glutathione homeostasis. Altered in infertile men, and these alterations seem to be linked to sperm morphology [103–105].
Glutathione S-transferase (GST)	• Location: Most abundant in the seminiferous tubular fluid of mammalian testes, sperm acrosomes, human sperm and mouse spermatogenic cells [106–108]. • Roles: Detoxification enzymes, intracellular-binding proteins [106]. Involved in epididymal maturation, capacitation and sperm-oocyte interactions [107, 108].
Ceruloplasmin	• Location: Semen, probably of testicular origin [109]. • Roles: Cu-dependent ferroxidase, a fundamental bridge between Fe utilization and Cu status. Associated with the oxidation of ferrous ion into ferric [110]. Prevents non-enzymatic generation of superoxide and scavenges superoxide, hydroxyl and singlet oxygen [110, 111]. Has positive impact on sperm parameters and male fertility [112]. Serves as a marker of a proper seminiferous tubule function [109].
Transferrin	• Location: Seminal plasma [111, 113]. • Roles: Primary binding and transport protein for iron and regulates iron transport and storage [110]. Serves as a reliable index of seminiferous tubular function [111].
Heme oxygenase (HO)	• Location: Two forms of heme oxygenase, HO-1 and HO-2, were identified in human testis and seminal plasma [114, 115]. • Roles: HO is strongly induced by oxidant stress and protects against oxidative insults. Increases reduced glutathione levels, degrades heme and intervenes with the metabolism of biliverdin and bilirubin, which have potent antioxidant properties [116]. HO is highly expressed in fertile normozoospermic subjects with positive correlations to sperm concentration, motility and morphology. HO enzyme activity is related to spermatogenesis and sperm motility processes [114, 115].

Table 2. Overview of minor antioxidant enzymes.

6.5. Non-enzymatic antioxidants

Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplements. The body's complex antioxidant system is affected by dietary intake of antioxidants, vitamins and minerals, such as vitamin C, vitamin E, zinc, selenium, taurine and glutathione.

6.5.1. Glutathione (GSH)

Glutathione is the most abundant thiol protein in mammalian cells [117]. Being an endogenous source, it is synthesized by the liver but it can also be derived from dietary sources such as fresh meat, fruits and vegetables. This molecule has three precursors: cysteine, glutamic acid and glycine. Its cysteine subunit provides and exposes -SH that directly scavenges free radicals. Once oxidized, GS-SG is then regenerated/reduced by glutathione reductase to complete the cycle [13].

High levels are found especially in the testis of rats [118] and the reproductive tract fluids and epididymal sperm of bulls [98]. GSH protects the cell membranes from lipid oxidation and prevents further formation of free radicals. Its deficit leads to instability of the sperm midpiece, which results in motility disorders [118]. Glutathione supplementation in infertile

subjects has led to a significant improvement in sperm parameters and prevents oxidative damage to sperm DNA. A factor increasing the level of GSH is pantothenic acid, which by doing so also protects tissues against oxidative stress [117, 118].

6.5.2. Vitamin C

Vitamin C or ascorbic acid (AA) may be found in its reduced (ascorbate) as well as oxidized form (dehydroascorbic acid), both of which are easily interconvertible and biologically active. Vitamin C is found in citrus fruits, peppers, strawberries, tomatoes, broccoli, brussels sprouts and other leafy vegetables. AA is a water-soluble vitamin, and because of its hydrophilic nature, it has more effective scavenging properties at the plasma level than in the lipid bilayer [119].

Vitamin C has been used in the management of male infertility on empirical grounds, particularly in the presence of non-specific seminal infections [120]. Its presence in the seminal plasma of healthy males has been reported by various authors [121–123]. Chinoy et al. [124] stated that AA was essential for the structural and functional integrity of androgen-dependent reproductive organs. Low concentration of vitamin C showed significant degenerative changes in the testes, epididymis and vas deferens of scorbutic guinea pigs. On the other hand, excessive intake of vitamin C has been reported to cause reproductive failure in the men [125].

AA deficiency may lead to an increase in oxidative damage induced by ROS and a disturbed oxidative balance was observed in ejaculates of 25–45% of infertile men [123]. This was further corroborated by the association of decreased AA followed by an increase in the seminal plasma LPO as observed in a human trial [126, 127]. Moreover, it has been reported that AA supplementation leads to a significant reduction in the ROS concentration, sperm membrane LPO and DNA oxidation together with an increased sperm quality. The results of a recent animal experimental study indicate that vitamin C improves the activity of antioxidant enzymes and significantly reduces malondialdehyde (MDA) concentration in testicular structures [127].

6.5.3. Vitamin E

Vitamin E is a term that encompasses a group of potent, lipid-soluble tocol (tocopherol) and tocotrienol derivatives qualitatively exhibiting the biological activity of RRR-α-tocopherol. Structural analyses have revealed that molecules having vitamin E antioxidant activity include four tocopherols (α-, β-, γ- and δ-) and four tocotrienols (α-, β-, γ- and δ-) with α-tocopherol being the most abundant form in nature and mostly available in food, having the highest biological activity and reversing vitamin E deficiency symptoms. The molecular functions fulfilled specifically by α-tocopherol have yet to be fully described; however, the antioxidant feature is the flagship of the biological activity related to vitamin E [128].

Vitamin E is present within the seminal plasma and plasma membrane. It is a lipid soluble, chain-breaking antioxidant that able to terminate free radical chain reactions, particularly the peroxidation of PUFAs [129, 130].

Numerous reports emphasize on the role of α-tocopherol in the management of male infertility. A positive association was found between α-tocopherol in sperm plasma membranes and the percentage of motile, living and morphologically intact spermatozoa [129]. At the same

time, α-tocopherol levels were decreased significantly in oligo- and azoospermic patients in comparison to normospermic controls [130].

A significant improvement in the *in vitro* ability of spermatozoa to bind the zona pellucida of unfertilized oocytes was found in men with high ROS production supplemented with vitamin E for 3 months [131]. Vitamin E supplementation may also play a role in reducing sperm DNA fragmentation and morphology defects [132].

6.5.4. Other non-enzymatic antioxidants

There are other substances which may contribute to the maintenance of oxidative homeo-stasis. The prime function of these compounds is not to combat the production or action of ROS; however, their presence may decrease the risk of OS development. Albumin, cysteine, taurine, zinc and selenium are the most known representatives. Furthermore, antioxidant substances isolated from natural resources, such as resveratrol, curcumin or lycopene, have recently emerged as suitable dietary supplements or therapeutics due to their chemical diver-sity, structural complexity, availability, lack of significant toxic effects and intrinsic biologic activity. A short overview of secondary non-enzymatic antioxidants is provided in **Table 3**.

N-acetyl-cysteine (NAC)	• A modified derivate of the sulfur-containing amino acid cysteine • Has the ability to reduce free radicals by acting with thiols and hydroxyl radicals. Plays a role as a precursor to glutathione [133] • Reduces seminal OS and sperm DNA damage [134]. When combined with selenium, NAC has a positive impact on sperm concentration and acrosome reaction [133, 134].
Carnitine	• A quaternary ammonium compound acting as a water-soluble antioxidant • Stimulates mitochondrial metabolism. Has the ability to shuttle long-chain lipids across the mitochondrial bilayer and start the process of ß-oxidation to create NADH and $FADH_2$ along with acetyl-CoA [135]. • Acts primarily in the epididymis. Prevents DNA damage and apoptosis during sperm maturation [136].
Taurine (2-aminoethanesulfonic acid)	• Found abundantly in the mammalian body, including testes and spermatozoa [137]. • Participates in bile salt formation, calcium binding and transport, osmoregulation and stabilization of biological membranes. A component of cellular antioxidant defenses [138]. • Taurine administration to semen prevents the loss of sperm motility and viability, promotion of the activity of reduced glutathione, GPx, SOD and CAT while concomitantly lowering LPO and morphological abnormalities of spermatozoa [137]
Zinc	• A trace element with high concentration in the seminal plasma [139]. • Serves as a cofactor to dihydrofolate reductase and methionine synthase needed for homocysteine recycling, membrane and DNA stabilization [140] • Acts as a cofactor for SOD and metallothioneins, assisting in scavenging superoxide and hydroxyl radicals [141].
Selenium	• A trace element positively correlated with increased levels of sperm concentration, motility and morphology [142]. • Cofactor of phospholipid hydroxyperoxide glutathione peroxidase, important for chromatin condensation and formation of the mitochondrial capsule [52–54]
Albumin	• A highly soluble protein containing 585 amino acids • A key element in the regulation of osmotic pressure and distribution of fluid between different compartments [143] and able to bind metals ions, fatty acids, drugs and hormones. • Stimulates spermatozoa motility, eliminates free radicals and protects membrane integrity from heat shock during semen cryopreservation [144, 145]

Bilirubin	• End product of heme metabolism via heme oxygenase-1, biliverdin and biliverdin reductase [146] • May protect vitamin A and linoleic acid from oxidative destruction due to an extended system of conjugated double bonds and a reactive hydrogen atom [147]
Uric acid	• Final enzymatic product of the degradation of purine nucleosides and free bases • Despite being a major antioxidant in the plasma, both correlates with and predicts OS development. It may function either as an antioxidant (primarily in plasma) or pro-oxidant (primarily within the cell) [148]. • A powerful scavenger of singlet oxygen, peroxyl and hydroxyl radicals in the hydrophilic environment, but loses an ability to scavenge lipophilic radicals and cannot break the radical chain propagation within lipid membranes [149]
Resveratrol (3,5,4′-Trihydroxystilbene)	• A polyphenol that belongs to the stilbene family and is found in grapes, berries, pistachios, plums, peanuts and wines [150]. • A free radical scavenger and a potent antioxidant, promotes the activities of a variety of antioxidant enzymes and increases the antioxidant capacity [150] • Copper and iron chelator preventing the Fenton reaction [151] • Stimulates and protects spermatocytes and spermatozoa against LPO, reduces apoptosis of germinal cells [152] and protects against environmental toxins [153] • Enhances spermatogenesis by stimulating the hypothalamic-pituitary-gonadal axis without adverse effects, triggers penile erection and enhances blood testosterone levels, testicular sperm count and epididymal sperm motility [151, 152]
Lycopene (ψ,ψ-Carotene)	• One of over 600 carotenoids found in nature, present in tomatoes, watermelons and pink grapefruits [154]. • A highly unsaturated straight chain hydrocarbon with a total of 13 double bonds, 11 of which are conjugated making the molecule to be twice as potent singlet oxygen quencher as ß-carotene and 10 times more active in comparison to α-tocopherol [154]. • LYC administration leads to a significant improvement of semen parameters (sperm concentration, motility and morphology) in patients with idiopathic infertility, antibody-mediated infertility as well as with different sperm abnormalities [155, 156] • In vitro LYC supplementation has led to an increased post-thaw spermatozoa survival and DNA stability [157], together with an improved sperm morphology and membrane integrity [158].

Table 3. Overview of minor non-enzymatic antioxidants.

7. Strategies to reduce oxidative stress in male reproduction

Antioxidant supplementation has proven to be effective against male reproductive dysfunction *in vivo*. Recent reports have acclaimed significant attention due to the quality of their study design and demonstrated compelling evidence regarding the efficacy of antioxidants towards improving semen parameters. On the other hand, numerous clinical trials studying the effects of dietary antioxidants on semen parameters are still uncontrolled, focus on rather on healthy individuals or have indirect end-points of success. The dose and duration of antioxidant administration also need to be thoroughly examined and standardized. **Table 4** presents the most effective doses for the treatment of male subfertility based on currently available studies that explored the impact of antioxidant supplementation on sperm parameters.

Vitamin C	• 13 infertile patients received 1000 mg of vitamin C twice daily for a maximum of 2 months. Vitamin C supplementation improved sperm count, motility and morphology [159] • 115 men with clinical varicocele and abnormal semen analyses were recruited. After surgery, the subjects received 250 mg vitamin C for 2 months. Vitamin C supplementation following surgery resulted in a better motility and morphology. Prior to surgery, vitamin C was not effective on the sperm count, but it improved sperm motility and morphology [160]
Vitamin E	• 110 asthenozoospermic patients received 300 mg of vitamin E daily over a period of 26 weeks. At the end of the experiment, sperm motility increased, while LPO decreased in the studied population [161]
Vitamin C and vitamin E	• 1000 mg vitamin C and 800 mg vitamin E were administered to 31 subjects diagnosed with asthenozoospermia and normal or only moderately reduced sperm concentration for a period of 56 days. The treatment did not affect sperm concentration, motility and morphology [162] • 64 men with unexplained infertility and an elevated percentage of DNA-fragmented spermatozoa received 1 g vitamin C and 1 g vitamin E daily for 2 months. No differences in basic sperm parameters were found following antioxidant treatment; however, the percentage of DNA-fragmented spermatozoa was markedly reduced [163].
Vitamins A, C, E, N-acetyl-cysteine and zinc	• 20 post-varicocelectomy oligospermic patients were subjected to a daily administration of 0.06 IU/kg of vitamin A, 3 mg/kg of vitamin C, 0.2 mg/kg of vitamin E, 10 mg/kg of NAC and 0.01 mg/kg of zinc over a period of 13 weeks. Sperm count increased by 20-fold, and of the 20 subjects, 6 of the originally infertile men had sperm counts greater than 20million/mL post-treatment [164]
Glutathione	• 600 mg of GSH per day given to 11 men suffering from dyspermia associated with unilateral varicocele or germ-free genital tract inflammation over 2 months lead to an improvement in sperm kinetics and higher sperm concentration [165].
Carnitine	• 3 g/day of L-carnitine was administered to 100 asthenozoospermic men. After 4 months of treatment, a significant improvement was observed in sperm concentration, motility and morphology [166]. • 2 g/day of carnitine administered for 6 months led to a significant improvement in sperm concentration and motility in 100 patients with oligoasthenoteratozoospermia [167], while the following year an increased sperm count and motility were found in 56 infertile men after a combined daily treatment with 2 g carnitine and 1 g acetyl-L-carnitine supplemented for 6 months [168] • No improvements in semen quality was detected in 26 men diagnosed with asthenozoospermia who underwent 6 months of daily treatment with 2 g L-carnitine and 1 g L-acetyl-carnitine [169]
Selenium	• No positive effects were found in 33 subfertile men following 3 months of treatment with 200 µg/day of selenium [170]
Selenium and Vitamins	• 9 oligoasthenoteratozoospermic men were supplemented for a period of 6 months with selenium and vitamin E, leading to improvements in sperm motility, morphology and viability, although the concentration did not change significantly [171] • 46 oligoasthenoteratozoospermic and 16 subfertile patients received selenium alone or in combination with vitamins A, C and E at daily doses of 100 µg, 1 mg, 10 mg and 15 mg, respectively. No improvement was observed in sperm concentration after 3 months, although the motility was increased in the treated subjects [172] • 28 infertile men were supplemented daily by vitamin E (400 mg) and selenium (225 µg) during 3 months. Following treatment, a significant decrease of LPO was observed together with an improvement of sperm motility [173]
N-acetyl-cysteine	• No improvements in sperm parameters were observed after 3 months of 600 mg/day administration of NAC to 27 infertile men [174]. • Supplementation of 600 mg/day of NAC to 60 patients diagnosed with idiopathic infertility seemed to improve sperm motility, volume, viscosity and seminal oxidative status [175].

| Zinc sulfate (ZnSO₄) | • Administration of 250 mg of $ZnSO_4$ twice daily for 3 months to 50 asthenozoospermic patients resulted in a higher sperm count and membrane integrity. $ZnSO_4$ also played an immunological role as T-helper cytokines and interleukin-4 levels increased in the experimental group and TNF-α and antisperm antibodies decreased [176] |
| Coenzyme Q10 | • Supplementation of 60 mg/day of coenzyme Q10 for 103 days led to an increase in the fertilization rate but had no effect on motility, morphology or concentration in 17 patients with low fertilization rates due to male factor infertility [177]
• 60 patients with idiopathic asthenozoospermia who received 200 mg/day of coenzyme Q10 demonstrated significant improvement in motility after 6 months of treatment [178] |

Table 4. Most pronounced studies on the effects of oral antioxidant supplementation on male infertility.

ROS-induced damage may have significant clinical implications in the context of ARTs. Numerous reports have indicated that significantly increased ROS levels may occur in response to repeated cycles of centrifugation involved in conventional sperm preparation techniques used for ARTs [179]. Spermatozoa selected for ART often face OS and a high risk for DNA damage. When intrauterine insemination or *in vitro* fertilization (IVF) is used, such damage does not represent a cause of concern as damage to the sperm membrane lipids ensures that fertilization will not occur. However, in case of intracytoplasmic sperm injection is used, this natural selection barrier may be overlooked and sperm with DNA damage may be directly injected into the ovum [77].

Selection of an effective sperm preparation technique is important to minimize ROS overgeneration and eventual oxidative insults to the male gamete. The density gradient technique is able to separate leukocytes and immature or damaged spermatozoa from normal spermatozoa, which may be subsequently used in ARTs [77, 179, 180].

Assisted reproduction techniques may benefit from *in vitro* supplementation of antioxidants [180]. Various antioxidants such as vitamin E, vitamin C, cysteine, taurine and hypotaurine present in the culture medium have been shown to improve the developmental ability of the embryos by counteracting the effects of ROS [93, 180].

In cases of IVF, incubation times of more than 16–20 hours have been correlated with increased oxidative damage. Shortening the insemination timeframes (up to 1–2 hours or less) may reduce ROS overgeneration in culture media and possibly improve fertilization, embryogenesis and pregnancy rates [77, 179].

8. Methods for detecting reactive oxygen species

Because high levels of ROS have been associated with a decreased male infertility, measuring ROS levels in semen is an important part of the initial evaluation as well as follow-up of men with reproductive dysfunction [10–12]. Chemiluminescence and flow cytometry are currently the most common techniques in clinical andrology to assess and study seminal OS.

Chemiluminescence measures light emitted following administration of specific reagents to a semen sample. Two major probes currently used to assess ROS generation by spermatozoa

are luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigenin (10,10′-dimethyl-9,9′-biacridinium dinitrate). Lucigenin is membrane-impermeable and responsive to ROS, particularly $O_2^{•-}$, in the extracellular space. Inversely, luminol is relatively membrane-permeable and reacts with a variety of ROS, including $O_2^{•-}$, H_2O_2 and $OH^•$ intracellularly as well as extracellularly. Chemiluminescent assays are sensitive, convenient for diagnostic purposes and have relatively well-established normal ranges [11, 12]. Nevertheless, significant set up costs have to be taken into consideration, and the data generated by chemiluminescence must be interpreted carefully because a variety of factors can affect the signals obtained [181].

A possible solution to the disadvantages associated with the chemiluminescence approach can be found in a variety of redox-sensitive fluorescence probes that can be loaded into spermatozoa and subsequently monitored by flow cytometry [182]. Two probes can be used. Dihydroethidium or hydroethidine is a non-fluorescent probe that is oxidized by the superoxide to become ethidium bromide, which will stain the mitochondrial and nuclear DNA [183, 184]. The other fluorescent probe is 2,7-dichlorofluorescein diacetate, a stable non-fluorescent cell-permeable probe that de-esterifies in the presence of intracellular H_2O_2 to form 2,7-dichlorofluorescein [183]. Other ROS such as peroxynitrite, HOCl, and $OH^•$ can also oxidize this probe [184]. Flow cytometry has a higher specificity, accuracy, sensitivity and reproducibility than fluorescent microscopy or chemiluminescence. A large number of cells can easily be analyzed, leading to high specificity and sensitivity [185]. One major disadvantage is that sophisticated and expensive hardware is needed. Also, the results do not quantify the target ROS but simply indicate the percentage of cells exhibiting a high level of activity [182].

Other methods to assess the oxidative balance in semen include indirect measurements such as the total antioxidant assay. This protocol is based on the ability of all antioxidants present in the sample to cease the oxidation of 2,20-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) to $ABTS^+$ by metmyoglobin. Hence, the antioxidants suppress oxidative processes to a degree that is proportional to their final concentration, which may be detected at 750 nm [186]. Another option is to assess the activity of antioxidant enzymes (SOD, CAT, GPx) or the redox potential defined by the ratio of oxidized and reduced glutathione using commercially available assay kits. A popular option is the measurement of oxidative end-products, including protein carbonyls [187], lipid hydroperoxides [188], MDA [98] and oxidative DNA adduct 8-hydroxy 2-deoxyguanosine [189].

Despite a remarkable progress in the evolution and design of new techniques to evaluate seminal OS, more straight-forward and accessible assays with well-defined and clinically significant physiological ranges reflecting normal sperm functions have yet to be introduced in order for oxidative stress to become a standard sub- or infertility marker in andrology laboratories.

9. Conclusions

Oxygen toxicity is an inherent double-edged sword to aerobic life. Increased oxidative insults to sperm lipids, proteins and DNA are associated with alterations of signal transduction

mechanisms crucial for fertility. The origin of ROS generation and the etiologies of increased ROS in men with low sperm quality are becoming increasingly clear, offering multiple management and/or treatment options. Recent evidence suggests that spermatozoa possess an inherent ability to generate ROS essential for the fertilization process. A variety of defense mechanisms against ROS overproduction encompassing antioxidant enzymes, vitamins and other biologically active molecules are involved in biological systems. A balance of the benefits and risks from free radical production seems to be crucial for the sperm survival and function. As male infertility continues to play an increasing role in contributing to the inability to conceive in couples of reproductive age, it is pivotal for andrologists to fully comprehend the importance of thoroughly evaluating seminal oxidative profiles in order to provide a better care for male patients with reproductive dysfunction. Although the therapeutic use of antioxidants appears attractive, clinicians need to be aware of exaggerated claims of antioxidant benefits by various commercial supplements for fertility purposes until proper multicenter trials have been completed. However, initial data emphasizing on the potential of antioxidant supplementation in improving semen quality and conception rates are indeed encouraging.

Acknowledgements

List of abbreviations

AA	Ascorbic acid
ABTS	2,20-azino-di-3-ethylbenzthiazoline sulfonate
AR	Acrosome reaction
ARTs	Artificial reproduction techniques
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
FR	Free radical
G6PD	Glucose-6-phosphate dehydrogenase

GPx	Glutathione peroxidases
GSH	Glutathione
GS-SG	Glutathione disulfide
H$^+$	Hydrogen
H$_2$O$_2$	Hydrogen peroxide
HCO$_3^-$	Bicarbonate
HO$_2{}^\bullet$	Hydroperoxyl radical
HOCl	Hypochlorous acid
ICSI	Intracytoplasmic sperm injection
IVF	*In vitro* fertilization
LPO	Lipid peroxidation
MDA	Malondialdehyde
mtDNA	Mitochondrial DNA
NADPH	ß-nicotinamide adenine dinucleotide phosphate
NO$^\bullet$	Nitric oxide
O$_2$	Oxygen
O$_2{}^{\bullet-}$	Superoxide
OH$^\bullet$	Hydroxyl radical
ONOO$^-$	Peroxynitrite
OS	Oxidative stress
PHGPx	Phospholipid hydroperoxide glutathione peroxidase
PLA2	Phospholipase A2
PMN	Polymorphonuclear
PUFAs	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROO$^\bullet$	Peroxyl radical
ROS	Reactive oxygen species
-SH	Thiol
SOD	Superoxide dismutase
WHO	World Health Organization

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