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INVITED REVIEW

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Reactive oxygen species in male reproduction: A boon or a bane?

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Abstract

Reactive oxygen species (ROS) are free radicals derived from oxygen during normal cellular metabolism. ROS play a crucial role in the physiological processes and signalling pathways associated with male fertility. At physiological concentrations, ROS act as molecular mediators of signal transduction pathways involved in the regulation of the hypothalamic–pituitary–gonadal axis, spermatogenesis and steroidogenesis. They also trigger the morphological changes required for sperm maturation, such as DNA compaction and flagellar modification. Furthermore, ROS modulate crucial processes involved in the attainment of sperm fertilising ability such as capacitation, hyperactivation, acrosome reaction and sperm–oocyte fusion. Conversely, oxidative stress prevails when the concentration of ROS overwhelms the body's antioxidant defence. Various endogenous and exogenous factors enhance the synthesis of ROS resulting in the disruption of structural and functional integrity of spermatozoa through the induction of apoptotic pathway and oxidation of molecules, such as lipids, proteins and DNA. Therefore, maintenance of a balanced redox state is critical for normal male reproductive functions. This article discusses the dual role of ROS in male reproduction, highlighting the physiological role as well as their pathological implications on male fertility.

KEYWORDS

free radicals, male infertility, oxidative stress, reactive oxygen species, spermatozoa

1 | OVERVIEW

Reactive oxygen species (ROS) are short-lived (10^{-9} s) reactive molecules belonging to the class of free radicals, which are derived from oxygen and characterised by the presence of one or more unpaired electrons in their outer shell. Due to their unstable chemical structure, they attack nearby organic molecules, such as lipids, proteins and DNA, in order to reach a balanced state. The most important ROS include superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), peroxyl radicals ($ROO^{\cdot}$), alkoxyl radicals ($RO^{\cdot}$), organic hydroperoxides (ROOH) and hydrogen peroxide (H_2O_2) (Aitken, 2017). Although the latter could be considered as a nonradical oxidant, H_2O_2 can react with ferrous ions and enhance the synthesis of $OH^{\cdot}$ through the Fenton and the Haber-Weiss reactions. Additionally, nitrogen-based free radicals, such as peroxynitrite ($ONOO^{-}$) and nitric oxide ($NO^{\cdot}$), are also a subclass of ROS.

The most important source of endogenous free radicals is mitochondria, the organelles responsible for cellular energy production in the form of adenosine triphosphate (ATP). In the inner mitochondrial membrane, different substrates are oxidised and reduced through the electron transport chain complex, generating an electron flux which terminates with ATP synthesis and the reduction of molecular oxygen to water. Although this process is highly efficient, about 1%–2% of oxygen is reduced to superoxide by complex I- and III-mediated single electron transfer (Fukai & Ushio-Fukai, 2011). Non-mitochondrial sources of ROS include peroxisomal β-oxidation,

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microsomal enzymes of cytochrome P450 family and respiratory burst of phagocytic cells generated by NADPH oxidase (Brochier-Armanet, Talla, & Gribaldo, 2009).

At physiological levels, ROS function as signalling molecules and are crucial for normal cell functions (Boveris & Chance, 1973; Du Plessis, Agarwal, Halabi, & Tvrda, 2015). However, excessive levels of ROS push the cellular reductive–oxidative (redox) balance towards an oxidative state, which impairs the physiological functions of proteins, lipids and DNA leading to cellular damage (Bardaweel et al., 2018). Cells are deployed with an antioxidant defence system that regulates the levels of pro-oxidants and protects the cells from the deleterious effects of free radicals. These defences are classified into enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). SOD neutralises O_2^-, converting it to H_2O_2, which is then decomposed to O_2 or alcohols by CAT and GPx, respectively, with the synthesis of water (Kehrer, 2000; Valko, Izakovic, Mazur, Rhodes, & Telser, 2004). Non-enzymatic antioxidants include vitamins (A, C and E), coenzymes Q10, glutathione, β-carotene, selenium and zinc which act as cofactors for many antioxidant enzymes (Hanukoglu, 2006).

Redox proteins, on the other hand, are structurally characterised by the presence of catalytic sites which can accept or donate electrons (Zhou, Cheng, Yin, & He, 2011). The ability to mediate the electron transfer makes them key proteins in redox reactions occurring in several biological processes. A classic example of a redox protein is thioredoxin (Trx), which takes advantage of the disulphide in its active-site and acts as a scavenger in association with another flavoenzyme, thioredoxin reductase (TrxR), and NADPH (He et al., 2017).

A balance between the generation and elimination of ROS is critical for maintaining normal cellular functions. Oxidative stress prevails when ROS generation overwhelms the antioxidant defence mechanisms (Fridovich, 1989). Oxidative stress has been implicated in the pathogenesis of several diseases such as obesity, metabolic syndrome, diabetes, cardiovascular disease, neurodegenerative diseases, malignancies and infertility (Brigelius-Flohé & Maiorino, 2013; Niedzielska et al., 2016; Nohl & Jordan, 1980; Pizzino et al., 2017). Over the past few decades, the role of ROS and associated oxidative stress in male infertility has received great attention in reproductive research. Although the ability of mammalian spermatozoa to generate ROS was first documented in 1943 (MacLeod, 1943), the landmark article in this field was published by Jones et al. in 1979, which highlighted the susceptibility of human spermatozoa to oxidative stress (Jones, Mann, & Sherins, 1979). Subsequently, in the mid-1980s, Aitken and his group demonstrated the association between defective sperm function and generation of ROS by human spermatozoa (Aitken & Clarkson, 1987; Aitken, Clarkson, Hargreave, Irvine, & Wu, 1989). Although earlier studies were mostly on the deleterious effects of ROS on sperm functions, since 1990, substantial evidence has accumulated on the physiological role of ROS and controlled redox balance in male reproduction (Aitken, Paterson, Fisher, Buckingham, & Van Duin, 1995; Lamirande & Gagnon, 1993b).

AUTHOR'S PERSPECTIVE

Key points

- A physiological redox balance regulates sperm production and maturation in the testes and epididymis, respectively, as well as fertilization.
- ROS are essential mediators of signal transduction pathways involved in the regulation of spermatogenesis, steroidogenesis and sperm functions.
- Spermatozoa and leukocytes are the most important sources of ROS production in seminal fluid.
- An imbalance between oxidants and antioxidants leads to oxidative stress, with detrimental effects on male fertility.

Potential areas of research

- Do Sertoli cells play a role in ROS formation in the testicular milieu?
- What is the role of ROS in the regulation of the blood–testis barrier?
- A redox balance is necessary for the regulation of male reproduction at several steps. What is the tipping point for ROS?

Studies indicate that low levels of ROS are crucial for spermatozoa to acquire their fertilising ability (Du Plessis et al., 2015; O'Flaherty, de Lamirande, & Gagnon, 2006; Rivlin, Mendel, Rubinstein, Etkovitz, & Breitbart, 2004). In fact, testicular and epididymal functions have been reported to be sensitive to the levels of ROS (Fujii & Imai, 2014; Maiorino & Ursini, 2002; Vernet, Aitken, & Drevet, 2004). Therefore, maintaining a balanced redox state is critical for normal male reproductive functions (Figure 1). This review aims to provide an in-depth discussion on the dual role of ROS in male reproduction with a balanced outlook on the physiological role of ROS in male reproduction and its pathological implications on male fertility.

2 | ROS IN THE TESTIS

2.1 | Spermatogenesis as a source and target of ROS

Spermatogenesis is a physiological process where diploid progenitors, called spermatogonial stem cells (SSCs), go through mitotic and meiotic divisions, leading to the final synthesis of haploid gametes (Clermont, 1963). Differential production of ROS by subsets of human spermatozoa at different stages of maturation has been

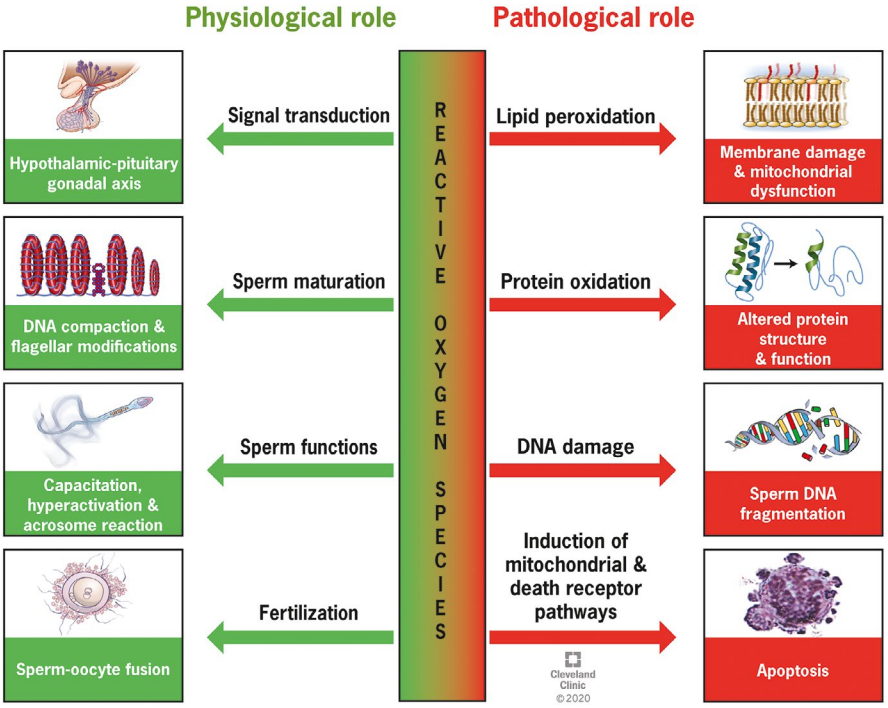

FIGURE 1 Physiological role of ROS in male reproduction and their pathological implications on male fertility

reported (Gil-Guzman et al., 2001). ROS production has been noted to be highest in immature spermatozoa with abnormal head morphology and cytoplasmic retention, whereas it is lowest in mature spermatozoa and immature germ cells. During spermiogenesis, spermatids undergo membrane remodeling and reduction in cytoplasmic volume up to 70%, while in the process of spermiation, the residual body is released and phagocytosed by the Sertoli cells. Therefore, dysregulation of spermiogenesis or spermiation results in the release of abnormal spermatozoa retaining cytoplasmic droplets, which represent an important source of ROS.

On the other hand, as spermiation is accompanied by a significant decline in cytoplasmic defensive enzymes, late stages of spermatids and spermatozoa are easy targets of free radicals. Furthermore, high levels of polyunsaturated fatty acids (PUFA) in the plasma membrane of spermatozoa increase their susceptibility to ROS (Aitken, 1999). The differentiating spermatogonia and spermatocytes are also vulnerable to deleterious effects of ROS due to their high mitotic and meiotic activity, respectively (Oldereid, Thomassen, & Purvis, 1998). A study published by Aguilar-Mahecha, Hales, and Robaire (2001) revealed differential expression pattern of stress response genes and antioxidant enzymes in isolated murine germ cells (pachytene spermatocytes, round and elongated spermatids) (Aguilar-Mahecha et al., 2001). Low and constant expression of glutathione-S-transferase in contrast to increasing levels of copper–zinc SOD1 and heme oxygenase 2 has been reported alongside the germinal cycle (Aguilar-Mahecha et al., 2001), suggesting differential susceptibility of germ cells to oxidative stress.

ROS act as cellular mediators and are required for a normal spermatogenesis. Ideally, the SSCs must self-renew to maintain the stem cell population as well as generate progenitor cells that progress through the spermatogenic cycle to form spermatozoa. Morimoto et al. (2013) reported the involvement of ROS, generated by NADPH oxidase 1 (NOX1), in maintaining the stem cell pool using murine model (Morimoto et al., 2013). Although high concentrations of H_2O_2 suppressed the growth of cultured SSCs, a moderate increase induced cellular proliferation. Furthermore, ROS depletion *in vivo* decreased SCC in the testis, and NOX1-deficit SSCs displayed reduced self-renewal division upon transplantation. Morimoto et al. (2013) suggested that ROS generated by NOX1 play a crucial role in SSC self-renewal via mediating the phosphorylation of c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) (Morimoto et al., 2013).

A balance between pro-oxidants and antioxidants is essential for testicular sperm maturation and DNA compaction (Cabrillana et al., 2017; Du Plessis et al., 2015; Su et al., 2005). Phospholipid hydroperoxide glutathione peroxidase (PHGPx or GPx4), a member of the GPx superfamily, catalyses the oxidation of protein sulfhydryl groups during the final phase of germ cell maturation (Maiorino & Ursini, 2002). The expression pattern of PHGPx in male germ cells is quite specific with peak levels in elongated spermatids (Mori et al., 2001). During the late stages of spermatogenesis, PHGPx facilitates the testicular phase of chromatin condensation in the nucleus as well as the stabilisation of mitochondrial capsule in the mid-piece of spermatozoa by catalysing the reduction in H_2O_2 coupled with oxidation of protein sulfhydryl groups and formation of cross-links (Maiorino & Ursini, 2002). Peroxiredoxins (PRDXs) catalyse the reductive removal of H_2O_2 using Trx as an electron donor. The testis-specific PRDX4 is involved in oxidative protein folding and thus functions as a molecular chaperone in the replacement of histones with protamine during spermatogenesis (Fujii & Imai, 2014). Structural features

of sperm flagella are stabilised by disulphide bonds, which are known to be regulated by testis-specific Trxs (Miranda-Vizuete et al., 2004). The redox state of this protein is controlled by the regulatory enzyme, TrxR (Su et al., 2005). Another selenoprotein, thioredoxin-glutathione reductase (TGR), is highly expressed in elongating spermatids. Along with PHGPx, TGR serves as a disulphide bond formation system and plays an important role in arranging a complex network of spermatid proteins including mitochondrial sheath assembly (Su et al., 2005).

This highlights the role of H_2O_2 in spermatogenesis as well as the significance of physiologically controlled oxidative stress in male fertility. Furthermore, stage-specific expression of antioxidant enzymes along with cell to cell variation in ROS production clearly indicates that spermatogenesis is a source as well as target of ROS, and therefore maintaining a balance is crucial for normal spermatogenesis.

2.2 | Steroidogenesis as a source and target of ROS

Androgens are produced through steroidogenesis, a multi-step process catalysed by a series of enzymes with cholesterol as the basic substrate. The binding of the luteinizing hormone (LH) to G protein-coupled receptors expressed on Leydig cells stimulates the transport of cholesterol by steroidogenic acute regulatory protein (StAR) into the mitochondria, where enzymes belonging to the cytochrome P450 family (cyt P450) as well as several hydroxysteroid dehydrogenases mediate the conversion of this organic molecule into androgenic hormones (i.e. testosterone, dihydrotestosterone, androstenedione, androsterone, dehydroepiandrosterone—DHEA) (Flück & Pandey, 2014). The cyt P450 enzymes of the steroidogenic pathway contribute to the generation of free radicals (Hanukoglu, 2006). These enzymes can catalyse monooxygenase reactions using steroidogenic or alternative products as pseudosubstrates, leading to the formation of CYP–pseudosubstrate–O_2 complex. The inability of the resulting complex to be hydrolysed by the enzyme leads to the oxidation of NADPH and the production of oxygen-derived free radicals (Hanukoglu, 2006). Therefore, ROS (H_2O_2 and O_2^-) are produced during normal steroidogenesis and, within critical levels, play an important role in regulating the steroidogenic activity of the Leydig cell (Tai & Ascoli, 2011).

A physiological amount of ROS is essential for the activation of the steroidogenic pathway (Tai & Ascoli, 2011). Binding of LH to the LH receptor in Leydig cells results in the phosphorylation of ERK1/2 cascade, which has been implicated as a modulator of steroidogenesis, and as a critical component of proliferation and survival of Leydig cells. Using MA-10 Leydig cells, it has been demonstrated that the cAMP-induced activation of Ras and phosphorylation of ERK1/2 is mediated by mitochondrial ROS (Tai & Ascoli, 2011). Furthermore, pro-oxidants have been reported to increase the phosphorylation of the three major MAPK families (ERK1/2, JNK and p38) in GSH-depleted MA-10 cells, which signifies the central role of redox balance in signalling pathways associated with steroidogenesis (Chen et al., 2010).

Besides being a well-known source of ROS, steroidogenesis is also a direct target of free radicals. The H_2O_2 and other organic peroxides react directly with the heme catalytic group of cyt P450 and inactivate the enzyme. Furthermore, H_2O_2 is converted to the highly reactive OH, which could attack the heme of cyt P450 directly or by inducing lipid peroxidation of the membrane lipids in which it is embedded (Peltola, Huhtaniemi, Metsa-Ketela, & Ahotupa, 1996; Quinn & Payne, 1985). Studies have revealed that ROS can negatively influence the hormonal LH signalling by modulating oxidant-sensitive MAPK pathways (Abidi, Leers-Sucheta, Cortez, Han, & Azhar, 2008; Abidi, Zhang, et al., 2008) as well as inhibiting the mitochondrial cholesterol transport (Diemer, Allen, Hales, & Hales, 2003). Zaidi et al. (2014) demonstrated that oxidative stress-induced activation of p38 MAPK protein negatively impacts the gene transcription of StAR protein in murine Leydig tumour cells (MLTC-1), resulting in impaired mitochondrial transport of cholesterol and reduced synthesis of androgens (Zaidi et al., 2014). Moreover, ROS have been reported to inhibit the expression of steroidogenic enzyme genes via c-Jun-mediated suppression of transactivation of Nur77 (Lee et al., 2009), one of the major transcriptional factors that regulate the expression of steroidogenic enzyme genes. Apart from increased levels of free radicals, diminution of functional antioxidant systems also results in the establishment of a high oxidative environment, leading to reduced Leydig cell steroidogenesis (Cao, Leers-Sucheta, & Azhar, 2004; Chen et al., 2008). Therefore, an appropriate testicular redox state is vital for maintaining the intratesticular levels of androgen as well as for the normal progression of testosterone-dependent spermatogenesis.

2.3 | ROS as a regulator of germ cell apoptosis

Apoptosis plays a crucial role in maintaining germ cell population in compliance with the nurturing capacity of the Sertoli cells and in preventing the defective germ cells from undergoing subsequent maturation within the testicular milieu (Shaha, Tripathi, & Prasad Mishra, 2010). The two major pathways of testicular apoptosis are the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. In rodents, both the intrinsic and extrinsic pathways of apoptosis have been reported to be involved in the first wave of spermatogenesis (Lee, Richburg, Shipp, Meistrich, & Boekelheide, 1999; Shaha et al., 2010). Caspases, a family of cysteine proteases, play a central role in execution of both these pathways and are regulated by several pro-/anti-apoptotic proteins. Maintaining a subtle balance between spermatogonial proliferation and apoptosis is essential for normal spermatogenesis.

ROS are known to modulate both mitochondrial and death receptor-apoptotic pathways (Chandra, Samali, & Orrenius, 2000; Redza-Dutordoir & Averill-Bates, 2016; Rincheval et al., 2012). In fact, oxidative and mitochondrial changes have been reported to regulate germ cell apoptosis in the human testis. Erkkilä, Pentikäinen, Wikström, Parvinen, and Dunkel (1999) induced testicular germ cell death by incubating the segments of seminiferous

tubules without survival factors and investigated the effect of different oxygen tensions on germ cell apoptosis (Erkkilä et al., 1999). The study revealed that suppression of apoptosis is inversely correlated with the partial oxygen pressure, which suggests a role of ROS in apoptosis. Furthermore, incubation with immunosuppressive agents, such as cyclosporin A, prevented apoptosis, indicating the role of mitochondrial permeability transition in triggering germ cell apoptosis (Erkkilä et al., 1999). High levels of ROS disrupt the mitochondrial membrane permeability and induce the release of cytochrome C and activation of the caspase cascade, resulting in apoptosis.

The Fas/FasL, which belongs to the tumour necrosis factor/nerve growth factor (TNF/NGF) receptor family, has also been recognised as a key regulator of germ cell apoptosis (Pentikäinen, Erkkilä, & Dunkel, 1999). Germ cells tagged with Fas activate the apoptotic signal when bound to its cognate ligand (FasL) expressed in the Sertoli cells. Around 50%–60% of all germ cells that enter meiosis-I are marked with Fas and efficiently eliminated by the Sertoli cells (Rodriguez, Ody, Araki, Garcia, & Vassalli, 1997). The Fas/FasL system serves as a checkpoint to expel excessive and defective germ cells, thereby regulating the quality as well as the quantity of the spermatozoa produced in the mammalian testis. An oxidative insult to testis and the associated increase in ROS have been reported to induce massive germ cell death via both the Fas/FasL and mitochondrial pathways (Vaithinathan, Saradha, & Mathur, 2010). Conversely, Bcl-2, an apoptosis regulator gene, has been reported to inhibit apoptosis by decreasing intracellular ROS levels (Kane et al., 1993).

2.4 | ROS as a modulator of signal transduction pathways

Testicular functions are regulated by the hypothalamic–pituitary–gonadal axis. The binding of GnRH to the G protein-coupled receptors on the anterior pituitary gland induces the release of Ca^{2+} from endoplasmic reticulum and activation of protein kinase C (PKC), which in turn activates the MAPK cascades (ERK, JNK and p38) in gonadotropes (Stamatiades & Kaiser, 2018). Stimulation of these pathways results in the activation of intermediate early genes (*Egr1*, *Atf3*, *c-Jun* and *c-Fos*) which are implicated in regulating *Fshb* and *Lhb* promoter activity in response to GnRH (Stamatiades & Kaiser, 2018). ROS are potent signalling intermediates produced in response to GnRH stimulation and influence the gonadotrope response by targeting the MAPK cascade (Kim & Lawson, 2019). Recently, Kim and Lawson (2019) demonstrated ROS generation in response to GnRH stimulation of mouse primary pituitary cells and the LβT2 gonadotrope cell line through Ca^{2+}- and PKC-mediated activation of NOX/dual oxidase (DUOX), a major enzymatic source of ROS (Kim et al., 2015). Furthermore, pharmacological inhibition of NOX/DOUX enzymes resulted in the inhibition of mediators such as *Egr1*, *Atf3*, *c-Fos* and *Jun*, leading to the blockade of pulsatile secretion of LH and FSH (Kim et al., 2015). These findings indicate the central role of ROS in GnRH signalling mediated by MAPK pathways.

Although ROS generated by receptor-activated oxidases exhibit signalling properties at physiological levels, excess ROS exert cytotoxic effects and can disrupt signalling pathways. The physiological levels of ROS are accomplished by a complex reduction system involving PRDXs, which reduces cellular peroxides and shuttles the ROS to cellular reducing machinery. PRDXs are themselves susceptible to hyperoxidation and inactivated by excess ROS. Kim et al., (2019) investigated the GnRH-induced hyperoxidation of PRDX in LβT2 cell line and reported that sulfiredoxin (SRXN1), an ATP-dependent reductase, plays a crucial role in the resolution of GnRH-induced ROS by reducing the hyperoxidised PRDX species and recycling them to the redox protein pool (Kim et al., 2019). Therefore, redox signalling plays a central role in GnRH stimulation of gonadotropes and regulation of gonadotropin gene expression.

3 | ROS IN THE EPIDIDYMIS

Spermatozoa emerging from the testis are not fully mature and, hence, unable to fertilise an oocyte. The epididymis plays a key role in spermatozoa acquiring the ability to fertilise in addition to storage and transportation (Sullivan & Mieusset, 2016). Modifications to spermatozoa in the epididymal environment include development of progressive motility, chromatin condensation and plasma membrane remodelling, which are essential for capacitation and fertilisation in the female reproductive tract (Aveldano, Rotstein, & Vermouth, 1992; Sullivan & Mieusset, 2016). Although spermatozoa are highly susceptible to oxidative stress-mediated damage, physiological levels of ROS are crucial for epididymal sperm maturation.

3.1 | Source of ROS in the epididymal environment

Spermatozoa represent the main source of ROS in the epididymis. In 1997, Fisher and Aitken detected a spontaneous synthesis of $O_2^{-\cdot}$ by epididymal spermatozoa of rats, hamsters, guinea pigs and mice recovered from the caput, corpus and cauda regions of the epididymis (Fisher & Aitken, 1997). Epididymal spermatozoa also synthesise H_2O_2, particularly at the most advanced stage of maturation in the caudal section. A tissue-specific SOD isoform is secreted by the cauda epididymal epithelium that binds to the sperm membrane and mediates the dismutation of O_2^- to H_2O_2. This association explains the variation in H_2O_2 generation along the epididymal tract in agreement with the observation that after a simple sperm wash such difference in H_2O_2 synthesis is not detectable (Fisher & Aitken, 1997).

3.2 | Role of ROS in epididymal sperm maturation

Redox status influences the sulfhydryl oxidation of sperm proteins, regulating DNA compaction and the acquisition of progressive motility in the epididymis (Aitken & Vernet, 1998; Cabrillana et al., 2017).

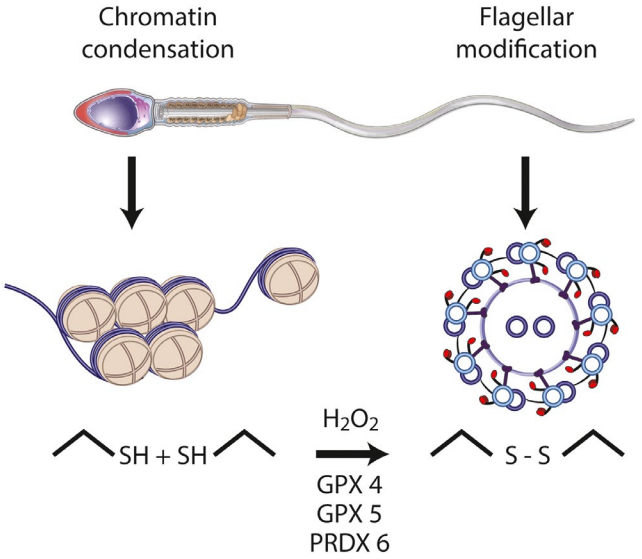

FIGURE 2 Role of sulfhydryl oxidation in epididymal sperm maturation. H_2O_2 and antioxidants, such as glutathione peroxidase (GPX) 4, GPX5 and peroxiredoxin (PRDX) 6, contribute to the formation of sulfhydryl bridges between cysteine residues of protamines and flagellar proteins, resulting in chromatin condensation and the acquisition of progressive motility, respectively

The formation of disulphide bridges between cysteine residues of proteins belonging to the outer dense fibres (ODF) family and cysteine-rich secretory proteins (CRISPs) family contributes to the maturation of flagella towards a more rigid structure and ensures the development of progressive motility (Cabrillana et al., 2017; Hu et al., 2018) (Figure 2).

Several antioxidants contribute to the maintenance of a physiological redox balance in the epididymis (O'Flaherty, 2019). Therefore, besides acting as ROS scavengers, antioxidants regulate the bioavailability of oxidants, which in turn are used to catalyse protamines and flagellar sulfoxidation. While the disulphide formation on cysteine sulfhydryls of sperm protamines is partially complete in the testis, the majority of the sulfoxidation takes place in the epididymal environment. The nuclear form of GPX4 has been suggested to be involved in ROS-mediated protamine cross-linking during epididymal maturation (O'Flaherty, 2019). In fact, spermatozoa of nuclear GPX4(−/−) knockout mice showed higher levels of free thiols and, consequently, poor DNA compaction (Conrad et al., 2005). A low ratio of -SH/(-SH + S-S) in spermatozoa indicates over-oxidation and reflects altered maturation, while a high ratio is attributed to inefficient protamine oxidation and correlates with DNA denaturation in infertile subjects (Seligman, Kosower, Weissenberg, & Shalgi, 1994; Zini, Kamal, & Phang, 2001).

The epididymal proteins are secreted in small vesicles of about 50–500 nm, called epididymosomes, which mediate the transfer of proteins to the sperm's surface or sub-cellular domains (Sullivan, 2015). The selenium-independent GPX5 is secreted in the caput epididymal region in association with epididymosomes and is transferred to the acrosomal region of spermatozoa during epididymal transit, where it is primarily involved in preventing premature acrosome reaction (Schwaab et al., 1998; Vernet et al., 2004). Furthermore, epididymal GPX5 also plays an important role in flagellar modification and sperm chromatin compaction (Fujii & Imai, 2014).

Another epididymosome-associated protein, the epididymal sperm binding protein 1 (ELSPBP1), acts as a scavenger of ROS generated by the dying spermatozoa (D'Amours et al., 2012). In association with the biliverdin reductase (BLVRA), ELSPBP1 catalyses the reduction of biliverdin to bilirubin, which in turn is re-oxidised by ROS (Sullivan, 2015). Through this enzymatic loop, ROS generated by the dying spermatozoa are restrained and the live spermatozoa are protected from oxidative stress during the epididymal transit. Other antioxidants in the epididymal environment include glutathione-S-transferase, a specific epididymal SOD isoform, indoleamine dioxygenase and PRDX6 (Vernet et al., 2004). PRDX6 (−/−) knockout mice showed higher age-dependent increase in oxidative stress along with reduced motility, poor DNA compaction and protamination, which further underlines the importance of redox balance in epididymal sperm maturation (Ozkosem, Feinstein, Fisher, & O'Flaherty, 2015).

Although ROS are vital for epididymal sperm maturation, a shift to oxidative stress can induce lipid peroxidation, trigger premature capacitation and reduce the fertilising potential of spermatozoa. Furthermore, the DNA of spermatozoa entering the epididymis has not achieved full compaction and hence exhibit higher susceptibility to the oxidative stress-mediated damage. Therefore, a redox balance must be achieved for an optimal epididymal sperm maturation.

4 | ROS IN SEMEN

Seminal fluid serves as a nutritive medium for the spermatozoa during their transit from the epididymis to the female reproductive tract. The human ejaculate contains a variety of cells including mature and immature spermatozoa, round cells, leukocytes, macrophages and epithelial cells. Among these cells, leukocytes and immature spermatozoa are the main endogenous sources of seminal ROS, while several exogenous factors are also known to induce the generation of ROS (Agarwal, Virk, Ong, & du Plessis, 2014) (Figure 3).

4.1 | Endogenous and exogenous sources of ROS

Activated leukocytes are potent ROS generators in semen. The prostate and seminal vesicles release peroxidase-positive leukocytes, which include macrophages (20%–30%) and polymorphonuclear leukocytes (50%–60%), into the seminal fluid through their secretions (Agarwal et al., 2014). In case of infection or inflammation, these peroxidase-positive leukocytes discharge 100-folds more ROS than normal physiological condition, mediated by increased production of NADPH through the hexose monophosphate (HMP) shunt (Henkel, 2011). NADPH oxidase, a membrane

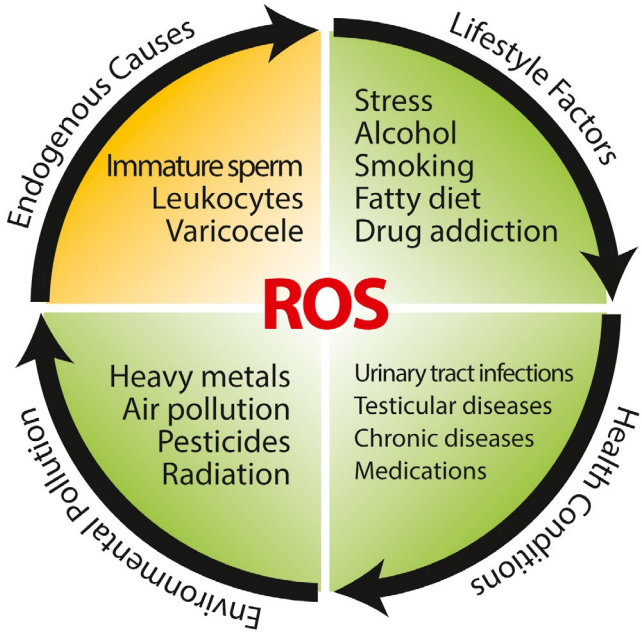

FIGURE 3 Endogenous and exogenous sources of ROS. ROS are synthesised endogenously (in orange) by immature spermatozoa and leukocytes, while exogenous factors can induce oxidative stress (in green)

bound enzyme, catalyses the transfer of electrons from NADPH to oxygen resulting in the generation of reactive oxygen metabolites. Activated leukocytes also release high levels of pro-inflammatory cytokines and trigger the myeloperoxidase system leading to a respiratory burst and increased generation of ROS (Blake, Allen, & Lunec, 1987).

Aberrant or immature spermatozoa are another main source of ROS in the semen (Agarwal et al., 2014). Defective spermatogenesis results in the retention of excess residual cytoplasm in the mid-piece of aberrant spermatozoa. The resultant increase in the cytosolic enzyme glucose-6-phosphate dehydrogenase (G6PDH) regulates the flux of glucose through the HMP shunt and enhances the availability of NADPH. The latter is used as a source of electrons by the NADPH oxidase system of spermatozoa leading to excessive generation of ROS (Aitken, 2017).

Varicocele is one of the leading causes of male infertility and is characterised by abnormal dilation of the pampiniform plexus in the scrotum. Testicular hyperthermia due to retrograde blood flow as well as hypoxia contributes to increased generation of ROS. Seminal levels of ROS have been found to be directly correlated with the clinical grade of the varicocele (Alkan et al., 2018).

Various exogenous factors that induce the generation of ROS include a fatty diet, smoking, alcohol or drug abuse, radiation, psychological stress, consumption of medications (cyclophosphamide, opioids, etc.), exposure to environmental and air pollution (Agarwal et al., 2014). Furthermore, chronic diseases (such as diabetes, chronic kidney disease, β-thalassaemia, thyroid dysfunction, hyperhomocysteinaemia), urogenital/systemic infections, and testicular diseases (such as cryptorchidism, testicular torsion) have been reported to be associated with seminal oxidative stress (Valko et al., 2007).

4.2 | ROS and sperm functions

Ejaculated spermatozoa move along the female reproductive tract in order to fertilise the oocyte, a process which involves several physiological processes such as capacitation, hyperactivation, acrosome reaction and the membrane fusion between the spermatozoa and the oocyte. ROS regulate these processes, which impart the functional competence in the spermatozoa ensuring successful fertilisation.

4.2.1 | Capacitation

During capacitation, spermatozoa undergo molecular modifications that ensure the proceeding of acrosome reaction and the fusion with oocyte membrane (Du Plessis et al., 2015). These include (a) basification of intracellular pH, (b) activation of cAMP-dependent pathways, (c) removal of cholesterol from sperm membrane and (d) protein phosphorylation at serine, threonine and tyrosine residues by cAMP-dependent kinases. ROS play an important role in regulating these processes (Aitken, 2017; Du Plessis et al., 2015). Suppression of capacitation has been reported in epididymal spermatozoa incubated with catalase, which inhibits H_2O_2 synthesis (Griveau, Renard, & Lannou, 1994). Moreover, incubation with low concentrations of H_2O_2 resulted in a higher rate of capacitated spermatozoa, highlighting the importance of redox balance in the regulation of capacitation (Aitken et al., 1995; Bize, Santander, Cabello, Driscoll, & Sharpe, 1991; Griveau et al., 1994). Several studies have stressed the role of ROS in regulating the cAMP pathway which involves activation of PKA and phosphorylation of PKA substrates. In fact, a high protein phosphorylation rate and increased levels of the second messengers cAMP and Ca^{2+} have been associated with ROS synthesis during the capacitation process (Aitken, 2017).

ROS generation is particularly sensitive to intracellular pH (Aitken, 2017). Removal of bicarbonate from the medium affected the synthesis of ROS and the protein phosphorylation rate, resulting in the inability of spermatozoa to undergo capacitation (Aitken & Vernet, 1998). In addition to mediating kinase activation, ROS also suppress phosphatases by oxidising cysteine residues, which are essential for their activation. Hence, ROS enhance the rate of protein phosphorylation by modulating kinase activation and phosphatase inhibition (Aitken & Vernet, 1998).

The oxidation of cholesterol and its subsequent efflux from plasma membrane is essential in the preparation of spermatozoa for the acrosome reaction. It results in a higher permeability to HCO_3^- and Ca^{2+}, through the activation of transporters (Na^+/HCO_3^- cotransporter—NBC) and ion channels (e.g. CatSper) (Suzuki & Yanagimachi, 1989).

4.2.2 | Hyperactivation

Once the spermatozoa reach the oocyte in the female reproductive tract, they lose the ability to move progressively but instead exhibit hyperactivated motility. At this stage, spermatozoa display a nonlinear high amplitude flagellar movement, with significantly increased lateral displacement of the sperm head. Hyperactivation aids the propulsion of spermatozoa in penetrating through the cumulus cells surrounding the oocyte and thus facilitates fertilisation. The role of O_2^- in modulating hyperactivation was demonstrated by an *in vitro* study that revealed the inhibition of sperm hyperactivation upon incubating the spermatozoa with SOD (de Lamirande & Gagnon, 1993a). Capacitation triggers phosphorylation of proteins located in the flagellar fibrous sheath, such as calcium-binding and tyrosine phosphorylation-regulated protein (CABYR) and protein A-kinase anchoring proteins (AKAPs), suggesting the role of cAMP-PKA pathway in the regulation of hyperactivation (Carrera et al., 1996; Naaby-Hansen et al., 2002). Calcium regulates sperm motility through Ca^{2+} permeable cation channels of spermatozoa (CatSper) localised in the flagellar membrane, which in turn regulate the extracellular Ca^{2+} uptake and mobilisation of stored Ca^{2+} from the sperm neck region. An increase in intracellular Ca^{2+} triggers the switching of sperm motility from the progressive to hyperactivated state and enhances its ability to penetrate through cumulus cells (Alasmari et al., 2013).

4.2.3 | Acrosome reaction

The acrosome reaction (AR) involves exocytosis of the acrosomal matrix rich in digestive enzymes (e.g. acrosin and hyaluronidase) which allows sperm penetration across the cumulus cells and zona pellucida. To clarify the role of ROS in capacitation, de Lamirande, Tsai, Harakat, and Gagnon (1998) incubated the capacitated spermatozoa with several AR inducers, such as a calcium ionophore (A23187), biological ultrafiltrates and lysophosphatidylcholine (De Lamirande et al., 1998). They demonstrated the role of Ca^{2+} and low concentrations of H_2O_2 and O_2^- in the induction of tyrosine phosphorylation and AR, which was further confirmed by additional studies (De Lamirande et al., 1998; Du Plessis et al., 2015). Later on, NO was reported to be involved in AR through the synthesis of the second messenger cGMP and the activation of kinases (PKC and protein kinase G — PKG) (Revelli et al., 2001). Although high concentrations of ROS affect sperm fertilisation at this stage, a physiological amount of H_2O_2, O_2^- and NO is essential for AR (Du Plessis et al., 2015).

Acrosomal enzymes are released subsequent to capacitation, and only capacitated spermatozoa can undergo AR, explaining why the molecular mechanisms are shared by these two processes. Protein phosphorylation associated with capacitation is extended to AR-related proteins, such as PKC and phospholipase A2 (PLA2) (Du Plessis et al., 2015). Initiation of this process is mediated by the release of Ca^{2+} during capacitation, which causes the cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). The IP_3, via activation of actin-severing proteins, induces the fusion of acrosomal and plasma membranes and eventually results in acrosomal exocytosis. Subsequently, DAG activates PKC resulting in further influx of Ca^{2+} and activation of PLA2. The activated PLA2 catalyses the cleavage of secondary fatty acids from the triglycerol skeleton of membrane phospholipids and thereby increases the sperm plasma membrane fluidity essential for the fusion with oocyte.

4.2.4 | Sperm–oocyte fusion

The fusion between spermatozoa and oocyte, the final step of fertilisation, occurs in the perivitelline space, and sperm membrane fluidity is the key determining factor for successful fertilisation. The high content of PUFA, particularly docosahexaenoic acid (22:6), and activated PLA2 play a major role in regulating membrane fluidity (Goldman, Ferber, & Zort, 1992). H_2O_2 and O_2^- trigger the activation of kinases (such as PKC) and the inactivation of phosphatases, resulting in higher enzymatic PLA2 activity. The release of a larger number of fatty acids catalysed by PLA2 contributes to the increased fluidity of plasma membrane. Thus, ROS increase the sperm–oocyte fusion rate by enhancing the membrane fluidity of the spermatozoa (Flesch & Gadella, 2000).

5 | ROS AND MALE INFERTILITY

About 30%–80% of infertile men have been reported to have elevated seminal ROS levels (Agarwal et al., 2019). Several studies have reported the adverse effects of oxidative stress on male fertility, and recently, the term MOSI (Male Oxidative Stress Infertility) has been suggested to describe infertile patients with abnormal semen characteristics and oxidative stress (Agarwal et al., 2019). High levels of ROS, upon overcoming the scavenging action of antioxidants, have pathological consequences on male reproduction mediating the oxidation of molecular components such as lipids, proteins, sugars and DNA (Figure 1).

The sperm membrane has a high amount of PUFA, which contributes to the membrane fluidity that is essential for sperm–oocyte fusion. On the other hand, the presence of carbon–carbon double bonds makes such lipids highly susceptible to oxidative attacks. In fact, ROS induces the formation of lipid peroxide radicals which in turn react with other molecules, triggering a self-propagating chain reaction leading to the amplification of lipid peroxidation (Aitken, 2019). The peroxidative damage to membrane lipids results in the impairment of membrane structure and fluidity, dysregulation of membrane-associated processes, including enzymatic activities, membrane receptor-associated signalling and dysregulation of ionic flow through the membrane (Barati, Nikzad, & Karimian, 2020; Gao, Li, Chen, & Zhou, 2017). Since the mitochondrial membrane is also damaged by ROS, motility is the first to be affected by lipid peroxidation, causing a reduction in mitochondrial membrane potential and defects in the sperm mid-piece and axonemal region (Bardaweel

et al., 2018). Degradation of PUFAs (particularly linoleic and arachidonic acids) leads to the formation of sub-products, such as malondialdehyde or 4-hydroxy-2-nonenal (HNE), which reflect the lipid peroxidative status (Gao et al., 2017).

Protein oxidation of the α-central carbon generates radical amino acids and induces the cleavage of peptide skeletons (Gao et al., 2017). Furthermore, the SH-rich side chains of cysteine and methionine are prone to be oxidised with generation of disulphides and methionine sulphoxide, respectively. In the same way, proline, arginine, lysine and threonine are oxidised, resulting in the formation of aldehydes and ketones, which indicate the protein oxidation status (Gao et al., 2017). These modifications alter the protein structure and their function, with repercussion on spermatogenesis and fertility.

Oxidative stress can have deleterious effects on DNA integrity and an increased rate of sperm DNA fragmentation has been reported in infertile patients having high levels of ROS (Santi, Spaggiari, & Simoni, 2018). During spermatogenesis, genomic DNA is folded around protamines, which enforce a compact state through the formation of disulphide bonds. Therefore, protamine oxidation leads to a lesser degree of compaction, making sperm DNA more susceptible to ROS-mediated oxidation. In addition, reaction between deoxyribose sugars and ROS disrupts the DNA strands, while oxidation of purine and pyrimidine bases alters the normal DNA reading, leading to a higher mutation rate. The oxidation of guanine into 8-hydroxydeoxyguanosine can be easily detected using specific antibodies, and it represents the most sensitive marker of oxidative stress-related sperm DNA damage (De Iuliis et al., 2009). Since spermatozoa lack base repair mechanisms, activation of the apoptotic cascade occurs in case of excessive DNA damage, leading to reduced sperm concentration and male infertility.

6 | CONCLUSION

ROS have dual effects, both physiological and pathological, on male reproduction. Within physiological levels, ROS are actively involved in the regulation of spermatogenesis and fertilisation. From the testis to the female reproductive tract, ROS regulate sperm production as well as maturation and thus contribute to the development of motile sperm that have the ability to fertilise an oocyte. Conversely, high levels of ROS induce oxidative stress and impair testicular spermatogenesis and steroidogenesis, epididymal sperm maturation and fertilisation. Therefore, maintaining a subtle balance between the levels of ROS and antioxidants is crucial for male fertility. A better understanding of this balance will aid in the diagnosis and treatment of infertile men.

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