

Review Article

The Enzymatic Antioxidant System of Human Spermatozoa

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The ejaculated spermatozoon, as an aerobic cell, must fight against toxic levels of reactive oxygen species (ROS) generated by its own metabolism but also by other sources such as abnormal spermatozoa, chemicals and toxicants, or the presence of leukocytes in semen. Mammalian spermatozoa are extremely sensitive to oxidative stress, a condition occurring when there is a net increase in ROS levels within the cell. Opportunely, this specialized cell has a battery of antioxidant enzymes (superoxide dismutase, peroxiredoxins, thioredoxins, thioredoxins reductases, and glutathione *s*-transferases) working in concert to assure normal sperm function. Any impairment of the antioxidant enzymatic activities will promote severe oxidative damage which is observed as plasma membrane lipid peroxidation, oxidation of structural proteins and enzymes, and oxidation of DNA bases that lead to abnormal sperm function. Altogether, these damages occurring in spermatozoa are associated with male infertility. The present review contains a description of the enzymatic antioxidant system of the human spermatozoon and a reevaluation of the role of its different components and highlights the necessity of sufficient supply of reducing agents (NADPH and reduced glutathione) to guarantee normal sperm function.

1. Introduction

Mammalian and particularly human spermatozoa are sensitive to high levels of reactive oxygen species (ROS) [1, 2]. In the 40s, this toxic effect was first observed independently by different investigators: McLeod working with human and Tomic and Walton working with bull sperm samples; they found that the spermatozoon is very sensitive to high concentrations of hydrogen peroxide (H₂O₂) [3–5]. These pioneer works opened a new era of research in the biology of reproduction field to understand the mechanisms and players affected by toxic levels of ROS of sperm physiology. The oxidative stress is produced by a net increase of ROS levels because of an increase of their production and/or a decrease of antioxidant defences [6, 7]. It generates substantial damage to all components of the sperm cell; thus, significant levels of lipid peroxidation, protein, and DNA oxidation are seen in this situation [7, 8] and are often associated with

infertility [9–12]. This damage is translated to changes in the plasma membrane fluidity, inactivation of key enzymes, and damage of the paternal DNA leading to impairment of sperm motility, mutations in the genomic message, and a variety of reproductive outcomes including, fertilization and embryo development failure, miscarriages, and abnormal offspring [7, 8, 13–21]. Many pathological conditions such as infections of the male reproductive tract, cryptorchidism, varicocele, exposition to drugs (e.g., chemotherapeutics agents), environmental factors (e.g., plasticisers, dioxins), and aging have oxidative stress as a common component of their pathophysiological mechanisms [22–25]. Therefore, the control of exogenous and/or endogenous ROS production and action is of paramount importance to assure maintaining normal sperm function.

On the other hand, low levels of ROS are essential for the spermatozoon to achieve fertilizing ability [26–29]. Superoxide (O₂^{•-}), hydrogen peroxide (H₂O₂), and nitric

oxide (NO^*) are produced by mammalian spermatozoa under capacitating conditions and triggered phosphorylation events in time dependent manners that culminate with the ability to induce the acrosome reaction upon specific physiological stimuli [27, 30–38].

The purpose of this review is to update the knowledge on the antioxidant defences in the human spermatozoa to fight against oxidative stress and in their role as regulators of the redox signaling.

2. Oxidative Stress and Male Infertility

The oxidative damage due to high levels of ROS has been associated with men infertility in 30–80% of cases [9–12, 39]. It is intriguing why the spermatozoon, a highly specialized cell, shows high sensitivity to ROS; it is suggested that this is due to a large surface of plasma membrane with high quantities of polyunsaturated fatty acids susceptible to lipid peroxidation [17, 40]. What is even more intriguing is the fact that low amounts of ROS are essential for sperm activation to allow this cell to acquire fertilizing ability [28, 29, 41]. In light of this evidence, it is obvious that a well-regulated production and action of ROS must take place in the ejaculated spermatozoon to assure normal performance. Some protection against oxidative stress remains within the spermatozoa; the proper functioning of the antioxidant system, composed of non-enzymatic and enzymatic players, assures the health of the spermatozoon. Although the contribution of vitamins E and C, ubiquinol, and other antioxidant molecules is important for the spermatozoon protection, this review will be focused on the enzymatic antioxidant system.

3. Antioxidant Enzymes in Human Spermatozoa

Aerobic cells must fight a battle against ROS, the very reactive molecules that are end products of the oxidative phosphorylation using oxygen to obtain energy. The ROS that can be produced and act on spermatozoa are $\text{O}_2^{\bullet-}$, H_2O_2 , NO^* , and peroxynitrite (ONOO^-) which is the product of the combination of $\text{O}_2^{\bullet-}$ with NO^* . These molecules can react directly with lipids, proteins, and nucleic acid or can be combined with metals and trigger, for instance, lipid peroxidation [6, 42, 43].

ROS are produced exogenously by leukocytes present in the ejaculate or by the spermatozoa themselves. Particularly, defective spermatozoa produce significant levels of ROS that can be toxic for them and for healthy spermatozoa present in semen [44, 45].

The formation of the enzymatic antioxidant system, whose components vary among species, is a major achievement during spermatogenesis to guarantee the protection of spermatozoa against oxidative stress. Below there is a detailed description of each antioxidant enzyme and their relevance in human spermatozoa.

3.1. Superoxide Dismutase. Superoxide anion is a moderate reactive ROS with a short half-life (1 millisecond). In the

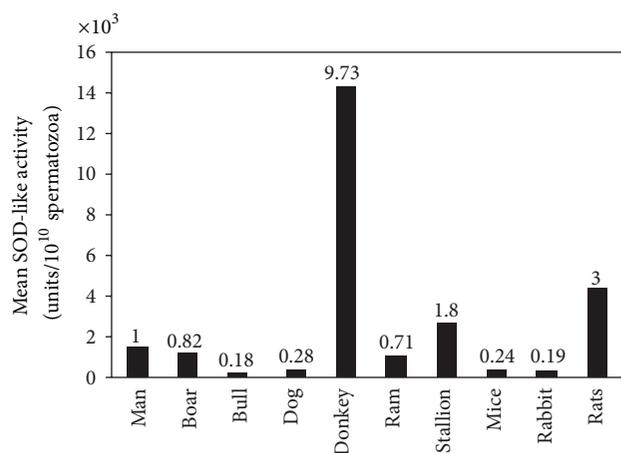
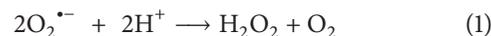


FIGURE 1: Comparison of SOD-like activity in spermatozoa from several mammals. The relative SOD-like activity in relation to that of human spermatozoa is present on the top of each bar. Considering SOD-like activity of human spermatozoa equal to 1, donkeys, rats, and stallions are those with the highest enzymatic activity. Data obtained from studies [46–49].

cell, $\text{O}_2^{\bullet-}$ is converted into a strong oxidant ROS, H_2O_2 , either spontaneously or by an enzymatic reaction catalyzed by superoxide dismutase (SOD) (see the following):

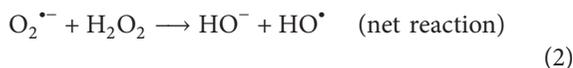


There is a wide difference in SOD activity among mammalian spermatozoa varying from ~10 times more than humans in donkey spermatozoa to ~0.2 times in bull or rabbit spermatozoa (Figure 1) [46–49]. These different SOD activities are one of the causes of the variability in sensitivity to ROS that can be encountered in mammalian spermatozoa. Three SOD isoforms are present in aerobic cells: the cooperzinc SOD (Cu-ZnSOD or SOD1) present in their cytosol, the manganese SOD (MnSOD or SOD2) present in mitochondria, and the secreted SOD (SOD3). It is important to clarify that, up to now, there is no study showing the presence of SOD isoforms in mammalian spermatozoa by either immunoblotting or immunocytochemistry. Thus, it is correct to define the $\text{O}_2^{\bullet-}$ scavenging capacity of spermatozoa as SOD-like activity [49, 50]. From studies measuring SOD-like activity, it was concluded that the protection by CuZn-SOD is limited in normal spermatozoa as they contain very little cytoplasm [45, 51]. The amounts of MnSOD and of SOD3 in human spermatozoa are negligible [52]. Interestingly, the seminal plasma is well equipped with SOD isoforms; the CuZn-SOD accounts for the 75% of enzymatic activity and the SOD3 for the other 25% [52] that can compensate for the limited SOD activity in the spermatozoon.

It seems then that the protection against $\text{O}_2^{\bullet-}$ depends on active CuZn-SOD present in the spermatozoon; however, spermatozoa from infertile men have no variation in SOD-like activity regardless of whether their samples produce significant amount of ROS or not [50]. In other studies, it was found that infertile men have increased SOD-like activity in their spermatozoa, indicating that the infertility is

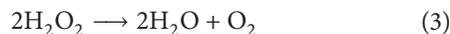
associated with an increase of H_2O_2 production rather than a decrease in SOD-like activity [45, 53]. Particularly in this case, the increased SOD-like activity is a marker for abnormal spermatogenesis and/or epididymal maturation since more residual cytoplasm, containing this enzymatic activity, is present in the abnormal spermatozoa from infertile men [45]. In these abnormal spermatozoa, there is a net increase in $O_2^{\bullet-}$ as well due to the presence of higher amounts of glucose-6-phosphate dehydrogenase (G6PDH), the first enzyme of the pentose phosphate pathway that produces NADPH which is used for the sperm oxidase to generate $O_2^{\bullet-}$ [45]. Then, $O_2^{\bullet-}$ dismutates (spontaneously or by enzymatic activity of SOD) to H_2O_2 which is the major culprit for damaging the spermatozoa [54].

An important role of SOD is to prevent the formation of hydroxyl radical (HO^\bullet) that occurs when $O_2^{\bullet-}$ and H_2O_2 react with ferric ion (Fe^{3+}) by the Haber-Weiss reaction [55]:



The HO^\bullet is highly reactive, especially with lipids, promoting lipid peroxidation in human sperm membranes [47, 56]. Another important player in the protection against HO^\bullet effects is the α -tocopherol or vitamin E that inhibits the propagation of lipid peroxidation cascades promoting a net decrease in the levels of oxidized lipids [56, 57].

3.2. Catalase. According to reaction (1), the spontaneous or enzymatic dismutation of $O_2^{\bullet-}$ promotes the formation of H_2O_2 . This ROS is more stable than $O_2^{\bullet-}$ with a half-life of minutes to hours and it was shown to be highly toxic for spermatozoa from many species including humans [3–5]. It is then necessary that the spermatozoon has a way to remove H_2O_2 . The first candidate to consider is catalase (CAT), an enzyme that converts H_2O_2 into oxygen and water according to the following reaction:



In somatic cells, CAT is abundant in the peroxisomes [58, 59]. The CAT activity is revealed when H_2O_2 diffuses to the peroxisomes, an event that is happening only when high concentrations of this ROS are present in the cell. During spermatogenesis, there is a removal of cytoplasm from the spermatids forming the residual body containing peroxisomes among other cytoplasmic structures [60, 61]. Immunoblotting studies demonstrated that bull spermatozoa do not contain CAT [62] and so far no similar studies have been conducted in human spermatozoa. Recently, it was demonstrated that the H_2O_2 scavenging capacity of human spermatozoa is not altered by the presence of sodium azide, a known inhibitor of CAT activity [63]. Based on the above, it is possible to conclude that CAT is absent or it is present in negligible amounts in human spermatozoa and therefore

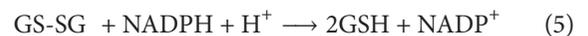
it is not a significant player in the antioxidant protection of the spermatozoon against high levels of H_2O_2 .

Other enzymes capable of removing H_2O_2 are the peroxidases, represented by the glutathione peroxidase and peroxiredoxin family of antioxidant enzymes. These enzymes are also present in spermatozoa and they are considered candidates for the antioxidant protection against oxidative stress driven by H_2O_2 and other ROS.

3.3. Glutathione Peroxidases. The glutathione peroxidase (GPX) family is composed of 8 members that are distributed in different tissues but with differences among species [64]. They catalyze the reaction needed to remove H_2O_2 and other hydroperoxides using reduced glutathione (GSH):



In order to keep removing hydroperoxides, the oxidized glutathione (GS-SG) must be reduced back to GSH by the enzyme glutathione reductase (GRD) using NADPH as reducing agent:



There are selenium- (Se-) dependent and selenium-independent GPXs: the first group is represented by GPX1 to 4 and the second group by GPX5 to 8 [65–69]. Glutathione peroxidases can also reduce $ONOO^-$ [64], a very reactive ROS capable of harming cells promoting tyrosine nitration in proteins involved in motility and sperm capacitation [70, 71]. Of great importance for spermatozoa is the presence of the selenoprotein phospholipid hydroperoxide GPX4 (also known as PHGPX), a structural protein which is essential for normal formation of the mitochondrial sheath and constitutes approximately 50% of the sperm midpiece protein content localized in the mitochondrial helix [72]. Male mice lacking the mitochondrial PHGPX (mGPX4) are infertile with abnormal and less motile spermatozoa than wild type animals [73, 74]. The need for mGPX4 to assure normal sperm function has been also demonstrated in humans since infertile men have shown low sperm motility with abnormal morphology [75]. It is important to highlight that what is relevant for fertility is the ability of mGPX4 to interact with hydroperoxides to form the mitochondrial sheath during spermiogenesis and not its antioxidant activity which is less than 3% of the total PHGPX protein content in ejaculated spermatozoa and can be only obtained after in vitro solubilisation with high concentrations of dithiothreitol (0.1 M) in the presence of guanidine [72]. Selenium is essential to assure normal GPX4 function during spermiogenesis as it was confirmed by the presence of abnormal spermatozoa with poor motility observed in selenium-deficient mice [76] or large domestic animals living in Se-deficient areas (for review see [77]).

The sperm chromatin formation during spermiogenesis is accomplished in part by the nuclear isoform of GPX4 (snGPX4); this enzyme mediates the oxidation of thiols groups of protamines by hydroperoxides. However, other enzymes may play significant role in the formation of sperm

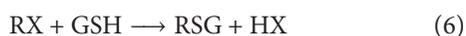
TABLE 1: Characterization of peroxiredoxins.

Class	2-Cys	Atypical 2-Cys	1-Cys
Isoform	PRDX1 to PRDX4	PRDX5	PRDX6
Substrates	H ₂ O ₂ , organic hydroperoxides	H ₂ O ₂ , organic hydroperoxides, ONOO ⁻	H ₂ O ₂ , organic hydroperoxides, phospholipid hydroperoxides ONOO ⁻
PLA ₂ activity	No	No	Yes
Sulfonated form	Yes	No	Yes
Reductant system			
Oxidized form	TRX/TRD	TRX/TRD	GSH/GSTs
Sulfonated form	Sulfiredoxin/sestrin 1	No	No

chromatin because the nGPX4^{-/-} mice are fertile [78]. It is possible then that other proteins are involved in the sperm chromatin remodelling and potential candidates are peroxiredoxins (see Section 3.5).

The contribution of GPXs to the protection against ROS is limited in human spermatozoa since, contrary to rodents, human spermatozoa, testes, or seminal plasma lacks GPX2, GPX3, and GPX5 [79, 80] and GPX4 is insoluble and enzymatically inactive in mature ejaculated spermatozoa [72, 78, 81]. The role of GPX1 in human sperm is controversial because in many studies GPX1 activity was measured using cumene hydroperoxide and NADPH [82]; substrates are also used by other enzymes such as peroxiredoxins (see Section 3.5). Up to now, there is no report demonstrating the presence of GPX1 in spermatozoa by either immunocytochemistry or immunoblotting. It seems that the role of GPX1 as important antioxidant enzyme is questionable because *Gpx1*^{-/-} males are fertile and they are not susceptible to oxidative stress [83] and lipid peroxidation does not increase in human spermatozoa incubated with H₂O₂ in the presence of carmustine (inhibitor of glutathione reductase (GRD)) or diethyl maleate (binds to GSH making it nonaccessible for GPX/GRD system) that affects the GPX/GRD system activity [63].

3.4. Glutathione Transferases. Glutathione S-transferases are antioxidant enzymes participating in the detoxification of cells and organs by conjugating the xenobiotics and other toxic compounds with GSH (see reaction below):



It has been suggested that GSTs play a role as proteins participating in the sperm-zona binding in caprine and humans [84, 85]. The GSTs participate in the antioxidant protection since infertile men with a null genotype for GST-Mul have spermatozoa with significant oxidative damage [86]. It is interesting to note that not all the GST isoforms may play a significant role in the protection of spermatozoa against oxidative stress; for example, the levels of GST A1-1 and P1-1 in seminal plasma are similar in fertile or infertile men [87]. Recently, it was reported that GSTM1, GSTT1, and GSTZ1 polymorphisms seem not to be associated with sperm quality

in humans, but only GSTT1 was associated with reduced sperm concentration [88]. Based on all these studies, it is evident that GSTs are playing different roles depending on the isoform considered and more research is necessary to have a complete picture of the participation of this large family of antioxidant enzymes in male reproduction.

3.5. Peroxiredoxins. Peroxiredoxins (PRDXs) are thiol-dependent peroxidases highly expressed from yeast to humans that do not require selenium or heme group to have enzymatic activity [89–98]. These acidic proteins contain one or two Cys residues at the active site which are required for their activity [95] and are used to classify them in three groups: 2-Cys PRDXs (isoforms 1 to 4), atypical 2-Cys PRDX (isoform 5), and 1-Cys PRDX (isoform 6) (Table 1). They can reduce a variety of reactive oxygen species such as organic and inorganic hydroperoxides and ONOO⁻ [99–101]. Although ONOO⁻ can be scavenged by GPXs and PRDXs, the latter preferentially catalyze its fast reduction [102]. After H₂O₂ (or other ROS) are bound to the Cys residues in the active site, the enzyme becomes inactive; it is then necessary for the activity of the thioredoxin- (TRX-) thioredoxin reductase (TRD) system [96, 97, 103] in the case of PRDX1 to 5 or the GSTpi/GSH for PRDX6 [104, 105] to activate PRDXs again. As an example, the reaction of PRDX with H₂O₂ is presented in the following reaction:



Hydrogen peroxide rapidly reacts with PRDXs oxidizing their SH at the active site even at low levels [106–110] (Figure 3). Particularly in human spermatozoa, PRDX6 is able to react with H₂O₂ concentrations as low as 50 μM that is able to induce capacitation [29, 37, 110]. These biochemical characteristics allow PRDXs to participate in the cellular redox signaling to promote physiological events and they have a major role as H₂O₂ scavengers and sensors [6, 111, 112]. This role is emphasized by their wide subcellular distribution (cytosol, nucleus, mitochondria, endoplasmic reticulum, and plasma membrane [96, 97, 113–117]). Studies in human spermatozoa revealed that the 6 isoforms are differentially localized in the subcellular compartments (Figure 2) that contain at least two members of the PRDX family [110].

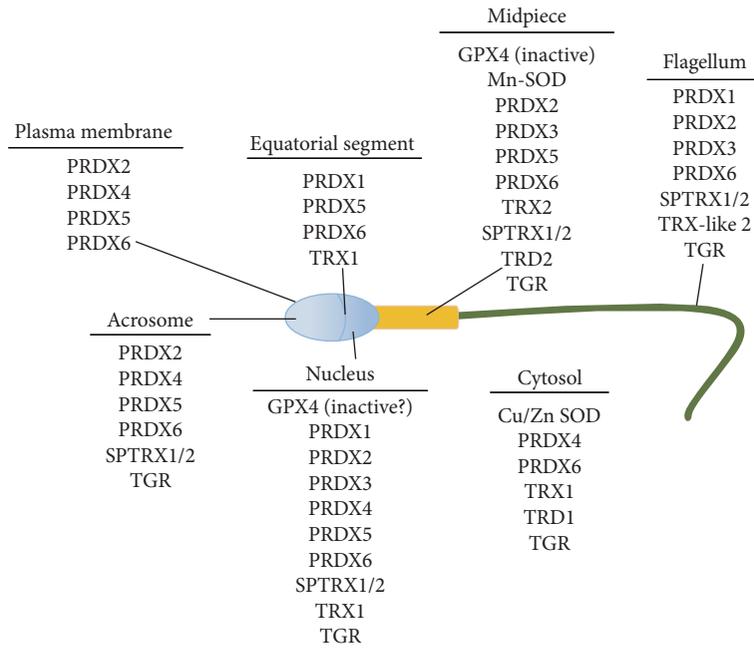


FIGURE 2: Subcellular localization of antioxidant enzymes in the human spermatozoon. See description in the text.

PRDX1, mainly a cytosolic enzyme in somatic cells, is located in the Triton-insoluble; immunocytochemistry studies revealed its presence in the equatorial region, nucleus, and flagellum of human spermatozoa [63, 110]. Three distinctive bands (23, 42, and 54 kDa), detected with the anti-PRDX1 antibody, are present in the Triton-X100 insoluble fraction, with the exception of p54 that is found only in the Triton-soluble fraction. PRDX2 is present in the head (acrosome, nucleus, and equatorial region), plasma membrane, and flagellum. Specific signal of PRDX3 (mostly restricted in mitochondria of somatic cells) is found in the nucleus, flagellum, and mitochondria. PRDX4 is present as two isoforms of 27 and 31 kDa (p27 and p31, resp.) and it is located in the plasma membrane (p27), acrosome (p27 and p31), and cytosol (p27). Another isoform present in the mitochondria is PRDX5 which is located also in the plasma membrane, equatorial region, and acrosome. Noteworthy, plasma membrane and acrosome of boar spermatozoa contain PRDX5 and its involvement in sperm-egg interaction was suggested [118, 119].

PRDX6 is the isoform most abundant and widely distributed in all subcellular compartments of human spermatozoon [110]; infertile men have spermatozoa with low amounts of PRDX6 which is highly oxidized and therefore inactive [120]. Moreover, H₂O₂, organic hydroperoxides, and peroxyxynitrite are substrates of human sperm PRDX6; H₂O₂ is the only ROS able to form high molecular mass complexes (Figure 3) [110], denoting a wide protection capability against ROS of sperm by PRDX6 in humans. Altogether, these findings suggest a key role of this enzyme in the defence against oxidative stress.

The participation of PRDXs in the maintenance of sperm quality has been studied using knockout models

Prdx4^{-/-} males which showed reduced testis weight due to an increased apoptosis during spermatogenesis and their spermatozoa display high levels of DNA damage [121]. We recently communicated that males lacking PRDX6 gene show significant higher levels of DNA and protein oxidation and impaired motility compared to wild type controls [122]. Other knockout models for PRDXs have been developed, although the information regarding the reproductive phenotype is limited. It is important to highlight that animals lacking PRDX1 are tumor prone, their life span is reduced [123], and their tissues contain elevated levels of damaged DNA [124]. Additionally, cellular senescence is accelerated in PRDX2^{-/-} mouse embryonic fibroblasts [125]. In these two knockout animals, severe anaemia is seen as part of the phenotype [108, 123]. Altogether, these animal models demonstrated the central role of PRDXs to fight against oxidative stress.

Studies on boar and mouse spermatozoa revealed that PRDX2 follows a similar path as GPX4; it is a soluble enzyme in spermatids but turns into an insoluble structural protein that colocalizes with the GPX isoform, becoming part of the mitochondrial sheath [126]. Immunocytochemistry studies revealed a broader localization of PRDX2 in the plasma membrane, mitochondrial sheath, flagellum, and head of human spermatozoa [63]. More studies are in the way to determine whether PRDX2 has similarities in solubility as was observed in mouse or boar [126].

In somatic cells, GPX1, GPX4, PRDX3, and PRDX5 are responsible for scavenging 99.9% of H₂O₂ consumption in mitochondria [127]. Sperm mitochondrion is the main source of the high levels of ROS associated with male infertility [21]. PRDXs are the major defence against increased levels of ROS in sperm mitochondria because GPX4, a structural protein associated with the mitochondrial sheath, does not

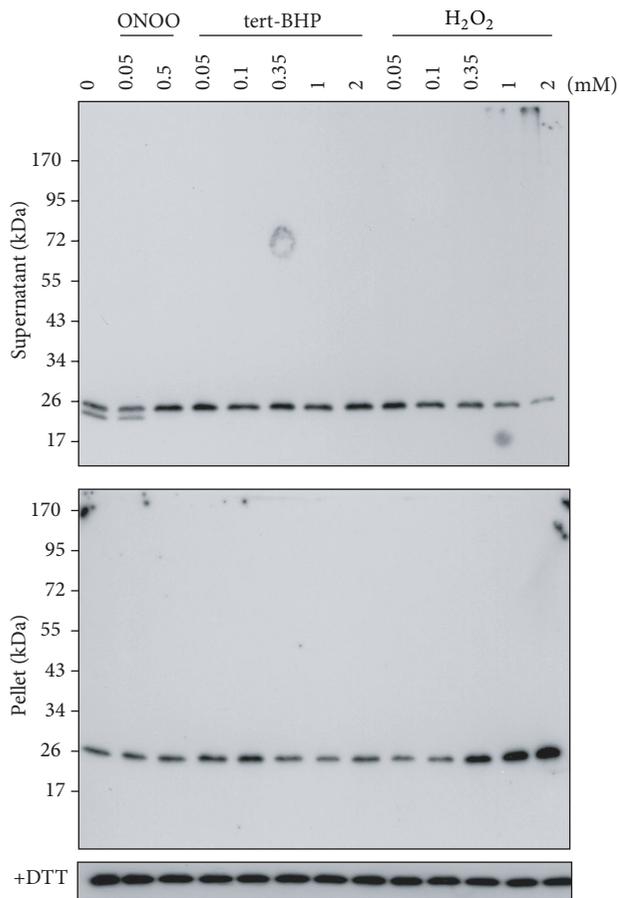


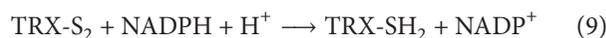
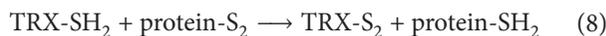
FIGURE 3: PRDX6 differentially reacts with different ROS in human spermatozoa. Percoll-washed spermatozoa were incubated with different concentrations of H_2O_2 , $ONOO^-$, or tert-butyl hydroperoxide (tert-BHP) for 30 min at $37^\circ C$ and then sperm proteins were electrophoresed and immunoblotted with anti-PRDX6 antibody as previously described [110]. PRDX6 is present as a doublet observed in absence of H_2O_2 or 0.05 mM $ONOO^-$ that becomes a single band with high intensity upon increased concentrations of ROS. High levels of H_2O_2 (but not of $ONOO^-$ or tert-BHP) generate the formation of high molecular mass complexes. Representative blot from other four experiments performed with different healthy donors.

have antioxidant activity and GPX1 activity is absent or present in negligible amounts. It is known that abnormal spermatozoa have significant high amount of unsaturated, unesterified fatty acid that promotes ROS generation by sperm mitochondria, generating an oxidative stress causing impairment of sperm function [21, 128]. Failure of the PRDXs system in mitochondria to remove H_2O_2 leads to an increase of toxic levels of this ROS that will compromise normal sperm function and evolve into male infertility [129].

Recently, it was reported that PRDXs play a significant role in the protection of human spermatozoa against oxidative stress [120]. In this study, the levels and the thiol oxidation status of PRDXs from spermatozoa of infertile men (with clinical varicocele or idiopathic infertility) were compared with those from healthy donors. Only the total

amounts of PRDX1 and PRDX6 were lower in spermatozoa from infertile men compared to those from healthy donors. Moreover, it was observed that there is a great variability in the quantities of PRDX4 and PRDX5. The thiol oxidation status, an indication of inactive PRDXs, was higher in infertile men for PRDX1, PRDX5, and PRDX6 compared to healthy donors. Those samples with low amounts and highly thiol-oxidized PRDXs showed high levels of lipid peroxidation and DNA damage along with low motility [120]. Noteworthy, regression analyses revealed that these damages depend on the levels of thiol oxidation of PRDXs. Based on these data, it can be concluded that sufficient quantities of active PRDXs are needed to assure sperm competence.

3.6. Thioredoxins. The thioredoxins are small proteins widely distributed in both the plant and the animal kingdom. They are important reducers of disulfide groups in several proteins including PRDXs (see reaction (8)) [130–132]. They work together with the thioredoxin reductases (see reaction (9)) forming the TRX/TRD system which requires reducing equivalents in the form of NADPH to accomplish its biological role as disulfide reducing and redox signaling regulators [130, 132–134].



In humans, and rodents at least, there are sperm specific isoforms called spermatid-specific TRX (SPTRX), which are present in the postmeiotic phase of the spermatogenesis and they are associated with the formation of the sperm tail [135, 136]. It is suggested that human SPTRX and human SPTRX are involved in stabilization by disulfide cross-linking of different tail structures during spermiogenesis [137]. A differential expression of these isoforms has been described during spermatogenesis; SPTRX expression peaks at steps 14–16 of the rat spermiogenesis whereas SPTRX is found in the fiber sheath at stages 15–19 [136, 138]. SPTRX appears to be required for the formation of fiber sheath but not in the fully differentiated mature spermatozoa; however, SPTRX remains associated with the fiber sheath in cauda epididymal and ejaculated spermatozoa [139]. These studies highlight the potential requirement of SPTRX in posttesticular events necessary for sperm maturation and activation required for fertilization [139]. The isoforms SPTRX and SPTRX present in normal spermatozoa were described [136, 138, 139]. Immunocytochemistry approaches denoted the presence of SPTRXs not only in the sperm flagellum but also in the head and midpiece, suggesting other functions for this enzyme in the spermatozoon [135].

The third isoform, SPTRX, is expressed at the spermatid level and probably required at later stages of spermiogenesis [139]. SPTRX is associated with the Golgi and the perinuclear region in spermatids and it was found only in abnormal human spermatozoa from infertile men [140], suggesting an incomplete spermiogenesis with retention of residual bodies and cytoplasmic droplets.

It was also reported in human spermatozoa the presence of TRX1, TRX2, and TRX-like-2 [139, 141] and the

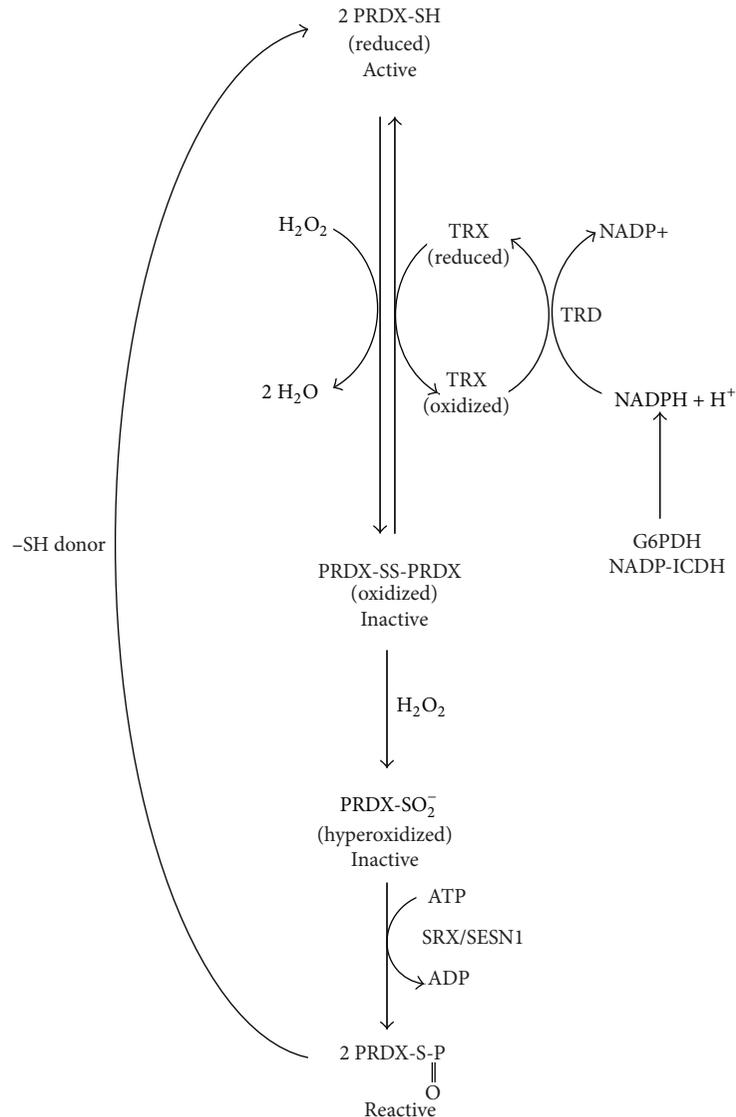


FIGURE 4: Fate of 2-Cys PRDXs when scavenging ROS. 2-Cys PRDXs react with H_2O_2 and become oxidized and therefore inactive. The TRX/TRD can reduce PRDXs in order to allow another cycle of scavenging of ROS. To assure proper function of this system, glucose-6-P dehydrogenase (G6PDH) and/or NADP-dependent isocitrate dehydrogenase (NADP-ICDH) supply enough reducing equivalent in the form of NADPH. If there is a strong oxidative stress (with high amounts of intracellular H_2O_2 , for example), 2-Cys PRDXs are sulfonated and inactive. This inactivation cannot be reversed by the TRX/TRD system and 2-Cys-PRDXs can only be reactivated by sulfiredoxin (SRX) or sestrin 1 (SESNI) and donors of thiol groups (-SH) like reduced glutathione.

TRD1, TRD2, and thioredoxin glutathione reductase (TGR) [135, 139], necessary enzymes to reduce the oxidized TRXs isoforms (Figure 4). Thus, human spermatozoa have a TRX/TRD system that plays a role supporting antioxidant capabilities, for instance, by reducing PRDXs to protect the spermatozoon against oxidative stress.

4. Antioxidant Enzymes Working Together to Generate Healthy Spermatozoa and Assure Normal Sperm Function

Above, it was described in detail the function of each antioxidant enzyme present in human spermatozoa. It is

important to highlight the interrelationships among certain isoforms that are working together to achieve a specific goal, such as supporting and assuring normal function in ejaculated spermatozoa. In light of the studies performed using knockout models and the evidence in infertile men [73–75], mGPX plays an essential role during spermiogenesis in the generation of a normal mitochondrial sheath [72]. However, it is imperative to stress that its participation as an antioxidant enzyme in ejaculated spermatozoa is very limited if not completely absent [72]. In the case of snGPX, the exclusive role of this isoform in the formation and maintenance of the sperm chromatin structure is still controversial; it is known that snGPX participates in protamine thiol oxidation, but it was demonstrated that it is not essential as knockout mice

lacking this enzyme are fertile [78]. Perhaps other proteins are participating in modeling the sperm chromatin since the animals lacking PRDX6 or TRX1 and TRX2 show abnormal sperm chromatin [122, 142].

An important group of antioxidant enzymes is the PRDX family in human spermatozoa and from other species; it is striking that the presence of all isoforms is differentially distributed in the sperm subcellular compartments [110] (Figure 2). The specific location of PRDXs and their behaviour in the presence of ROS suggest different roles for each isoform that surpass their traditional function as ROS scavengers. In this regard it is interesting to note the formation of high molecular mass complexes by PRDX1 and PRDX6 (but not for other PRDXs) when the human spermatozoa are exposed to a strong oxidative stress [110] similar to that seen in infertile men [120]. These complexes are formed by sulfonated PRDXs that were highly oxidized in order to protect other sperm proteins from being affected by high levels of ROS [110]. The addition to the sulfonic group promotes the change from antioxidant to chaperone activity in 2-Cys PRDXs [143–147]. Sulfonated 2-Cys PRDXs can be reactivated to PRDX with antioxidant capacity again by either sulfiredoxin (SRX) or sestrin 1 (SESN1) [148–152] (Figure 4). Studies are on the way to elucidate which proteins are present in these complexes and whether spermatozoa have SRX and/or SESN1 to reactivate thiol-oxidized 2-Cys PRDXs to better understand the role of PRDXs in the protection of human spermatozoa. A particular case is the hyperoxidation of PRDX6 that occurs in human spermatozoa as response to oxidative stress [110]. The sulfonated form of PRDX6 present in the high molecular mass complexes observed in spermatozoa treated with high concentrations of H_2O_2 or in infertile men [110, 120] cannot be reduced to its active form [153]; thus it is then probable that the sulfonated form of the sperm PRDX6 becomes irreversibly inactive and associated with impaired function.

A prerequisite for full PRDX activity is a functional TRX/TRD system, sufficient NADPH and/or GSH availability [63] (Figure 4). The presence of TRX or specific TRX isoforms and their respective reductive enzymes TRD and TGR in the human spermatozoon accounts for the maintenance of reduced PRDX to guarantee full ROS scavenging capacity. In order to have this system working properly, it is essential to assure sufficient levels of NADPH, usually generated by glucose-6-phosphate dehydrogenase (G6PDH) of the Pentose Phosphate pathway [54, 154] and by the NADP-dependent isocitrate dehydrogenase (NADP-ICDH) [155]. The TRX/TRD system is useful to reduce 2-Cys PRDXs (PRDX1 to 4) and possibly atypical 2-Cys PRDX (PRDX5), but it is not clear how PRDX6 is reduced in the case of spermatozoa. The amount of GSH is very limited in mammalian spermatozoa [156, 157], thus the reduction of PRDX6 by GSH may not be possible after facing an oxidative stress. The limited PRDX6 reduction capabilities could be one of the causes of the sensitivity of spermatozoa from humans and other species to high concentrations of ROS. A mechanism for reduction of PRDX6 in somatic cells involves GSTpi [104, 105, 158]; although a potential candidate, it is still elusive whether GSTpi is present in the human spermatozoon. The finding

that oxidative stress promotes the sulfonation of PRDX6 (an indication of protein hyperoxidation) present in the high molecular mass complexes of sperm under oxidative stress [110] and the presence of those complexes in sperm from infertile men [120] demand an answer as to whether there is a mechanism capable of reactivating sulfonated PRDX6 to its reduced form. It is known that SRX cannot reactivate the sulfonated PRDX6 [148], thus discarding the possibility of SRX as reducer for PRDX6 in spermatozoa. It is rather possible that the sulfonation of PRDX6 is irreversible and may be a cause of sperm impairment in infertile men [120]. Further research must be done to elucidate the mechanism that reduces PRDX6 in spermatozoa.

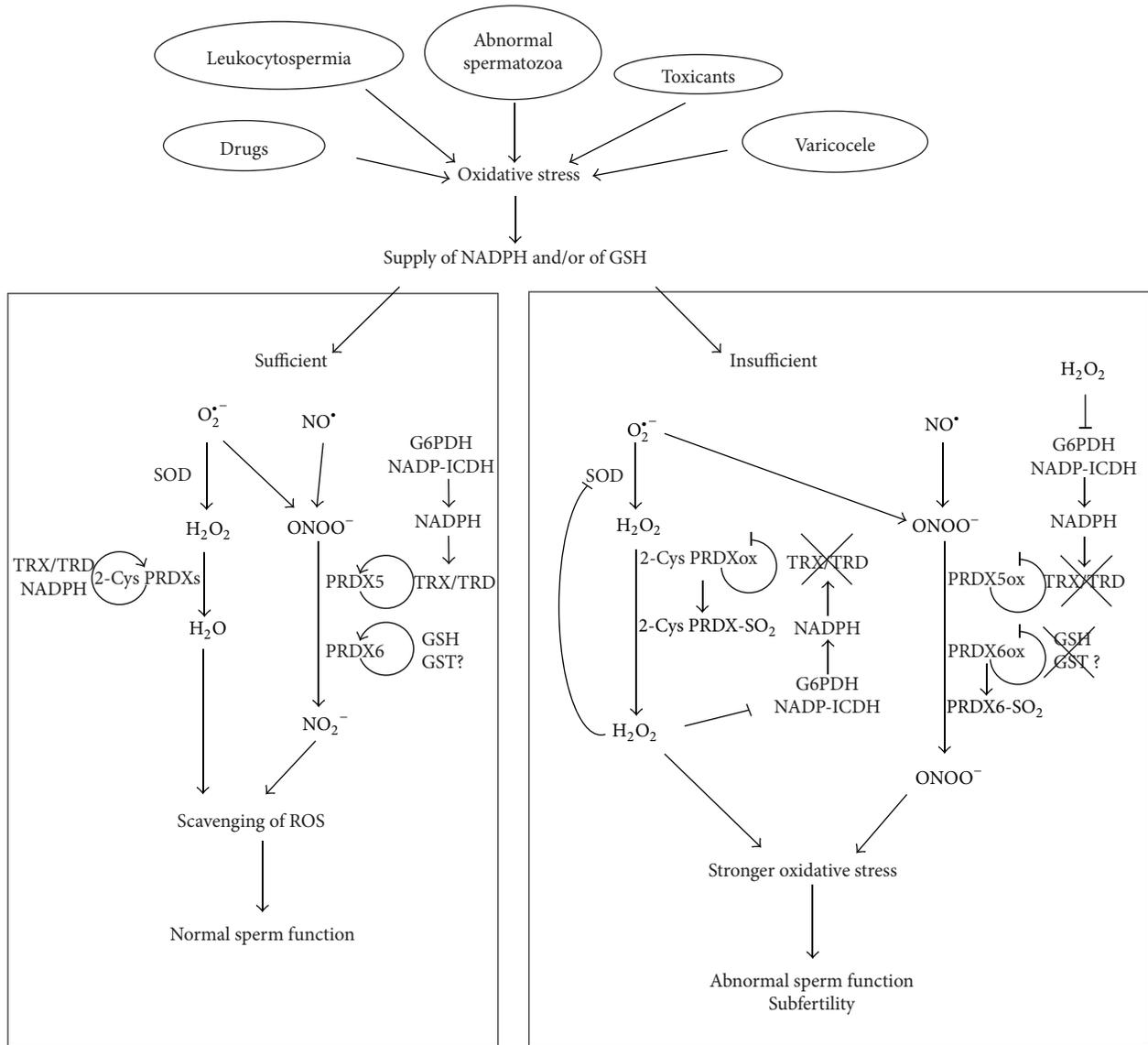
As it was mentioned at the beginning of this review, many conditions are associated with the generation of oxidative stress which promote abnormal sperm function and infertility [22–25]. In healthy spermatozoa, high levels of $O_2^{\cdot-}$, H_2O_2 , NO^{\cdot} , and $ONOO^-$ are scavenged by the collaborative work among SOD, PRDXs, and the TRX/TRD system (Figure 5). In order to assure a full capacity of PRDX enzymatic activity a sufficient supply of NADPH is needed to allow the reduction of TRX by TRD after the former reduces the 2-Cys or atypical 2-Cys PRDXs (PRDX5). Moreover, enough concentration of GSH and probably GST activity are necessary to assure the reduction of 1-Cys PRDX (PRDX6). In the case of sufficient supply of NADPH by G6PDH and/or NADP-ICDH and enough GSH in the presence of GSTs, the PRDXs along with the TRX/TRD system scavenge ROS reducing their concentrations to nontoxic levels (Figure 5, panel on the left).

However, this mechanism of protection is delicate and if the oxidative stress persists it can be affected by inactivation of its components, such as oxidation of PRDXs, as seen in infertile men [120]. The oxidation of PRDXs could be the result of direct effect of ROS on these enzymes or the inactivation of the TRX/TRD system when there is not enough NADPH as reducing equivalent. The lack of sufficient NADPH could be the consequence of G6PDH inactivation by high levels of H_2O_2 [54]. Similar fate of inactivation could have the NADP-ICDH [159], thus eliminating the possibility of assuring enough reducing equivalent to recycle the oxidized TRX required to reduce the thiol-oxidized 2-Cys PRDXs and PRDX5 (Figure 5, panel on the right).

The GSH reserved are very limited in spermatozoa [156, 157]; therefore, a strong oxidative stress may deplete these reserves and thus impact negatively on the reduction of thiol-oxidized PRDX6, with the consequence of having this enzyme completely inactive and impossible to scavenge any more ROS. Noteworthy is that hyperoxidation of PRDX6 will produce the sulfonated form that seems to be an irreversible state for the 1-Cys PRDX [148].

5. Conclusions

The enzymatic antioxidant system in human spermatozoon is very delicate and susceptible of inactivation by high levels of ROS. Because spermatozoa cannot respond to an oxidative stress with the synthesis of more antioxidant enzymes, it is imperative that the spermatozoon has enough amounts



2-Cys PRDX: reduced 2-Cys PRDXs
 2-Cys PRDXox: oxidized 2-Cys PRDXs
 PRDX5ox: oxidized PRDX5
 PRDX6ox: oxidized PRDX6
 2-Cys PRDX-SO₂: sulfonated form of 2-Cys PRDXs

PRDX6-SO₂: sulfonated form of PRDX6
 × : inhibition due to insufficient NADPH or GSH
 —| : inhibition of enzymatic activity
 ⤷ : inhibition of reactivation of PRDXs

FIGURE 5: Antioxidant defence against oxidative stress in human spermatozoa and effects of availability of intracellular concentrations of NADPH and/or GSH on the antioxidant protection in human spermatozoa. The insufficient supply of NADPH and/or GSH, due to inactivation of G6PDH and probably NADP-ICDH by H₂O₂, promotes the establishment of a stronger oxidative stress that produces enzymatic inactivation of SOD and the formation of sulfonated form of 2-Cys PRDXs and of PRDX6, thus becoming impossible to reactivate their activity to fight against high levels of ROS and conducting impairment of sperm function and infertility.

of these proteins to fight against the high level of ROS and assure normal sperm function. When thiol-oxidized PRDXs are present in high amounts and the TRX/TRD system cannot reduce them, there is a permanent oxidative damage that it is associated with impaired sperm function. More studies are needed to better understand the antioxidant system and how ROS are regulated in both healthy and

pathological conditions to seek new therapeutic interventions for infertile men.

Abbreviations

- ATP: Adenosine triphosphate
- Cu/ZnSOD: Cooper/zinc superoxide dismutase (SOD1)

Cys:	Cysteine residue
DEM:	Diethyl maleate
DTT:	Dithiothreitol
G6PDH:	Glucose-6-phosphate dehydrogenase
GPX:	Glutathione peroxidase
GRD:	Glutathione reductase
GSH:	Glutathione, reduced form
H ₂ O ₂ :	Hydrogen peroxide
MnSOD:	Manganese superoxide dismutase (SOD2)
NADP-ICDH:	NADP-dependent isocitrate dehydrogenase
NADP ⁺ :	Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH:	Nicotinamide adenine dinucleotide phosphate, reduced form
NaN ₃ :	Sodium azide
O ₂ ^{•-} :	Superoxide anion
ONOO ⁻ :	Peroxynitrite
PRDX:	Peroxiredoxin
ROS:	Reactive oxygen species
SH:	Sulfhydryl (thiol) group
SO ₂ :	Sulfinic acid group
SESNI:	Sestrin 1
SPTRX:	Sperm specific thioredoxin
SRX:	Sulfiredoxin
SS:	Disulfide
TGR:	Thioredoxin glutathione reductase
TRD:	Thioredoxin reductase
TRX:	Thioredoxin.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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