Mechanisms underlying human sperm cryodamage: the role of reactive oxygen species (ROS) and antioxidants

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ABSTRACT

Sperm cryopreservation is an efficient procedure for male fertility preservation, although the freeze-thaw procedure causes irreversible structural and functional changes in human spermatozoa. Indeed, the procedure is responsible for harmful changes that may affect sperm biology. In mammalian cells, cryopreservation induces a shift of redox homeostasis towards increasing generation of reactive oxygen species (ROS). The characteristics of ROS and the cellular outcomes depend on the cell type. Supra-physiological ROS levels during cryopreservation severely impact sperm survival, reproductive potential and DNA integrity, the latter a fundamental factor for fertilisation and transmission of paternal genetic information to offspring. The aim of this review is to summarise current knowledge of the main molecular mechanisms underlying ROS generation during sperm cryopreservation and its subsequent effects. In addition, we report current experimental approaches based on the supplementation of cryopreservation media with enzymatic and non-enzymatic antioxidants with the aim of minimising the harmful effects of ROS, and thus improving post-thaw sperm quality. Current data indicate that the potential use of antioxidants as constituents of the sperm freezing solution in clinical settings would require considerable attention.

KEYWORDS

Spermatozoa, cryopreservation, ROS, oxidative stress, antioxidants.

Introduction

Sperm cryopreservation is an effective procedure widely used for male fertility preservation in conditions likely to deteriorate sperm quality and/or testicular function, such as cytotoxic chemo- and radio-therapy, surgical treatments or conditions leading to severe oligozoospermia [1-3]. Moreover, cryopreservation is widely employed in the *in vitro* fertilisation field, being routinely used to store donor semen or to preserve spermatozoa obtained from azoospermic patients who have undergone testicular sperm extraction [4,5]. Although sperm cryopreservation is a widely used technique and has been improved over time, there are still unsolved technical and biological issues. During cryopreservation, the processes of cooling, freezing and thawing are responsible not only for a reduced proportion of surviving cells but also for harmful effects on sperm function, as demonstrated by changes in membrane lipid composition and acrosome status, increased DNA fragmentation and oxidation and decreased sperm motility and viability [6]. The damage caused by cryopreservation can be due to osmotic stress, cold shock, intracellular ice crystal formation, excessive production of reactive oxygen species (ROS), alterations in antioxidant defence system, or combinations of these conditions [4,7]. Indeed, the activation both of apoptotic pathways and of lipid peroxidation has been associated with impairment of sperm velocity, motility, viability, mitochondrial activity and chromatin and membrane integrity [8-11]. Spermatozoa from ejaculates with poor semen quality are more vulnerable to cryopreservation damage; it was recently reported that the recovery of motile and viable spermatozoa after freezing and thawing procedures is reduced in the presence of even just one basal semen parameter below the 5th percentile of the World Health Organisation reference values [6].

In the past, several attempts in different mammalian species have been made to reduce sperm freeze-thaw stress. Membrane fluidity was increased to prevent cold shock, and antifreeze molecules were employed to reduce heterogeneous ice nucleation and ice crystallisation. In order to counteract ROS production during the semen cryopreservation process, it was considered important to reduce ROS sources such as leucocytes, dead and defective spermatozoa and semen radiation exposure. A plethora of studies are available that analyse freeze-thaw stress-activating pathways and propose ROS scavenging

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through enzymatic, non-enzymatic, plant-based antioxidants or reductants [12] . However, most of them were carried out in animals of veterinary interest, and therefore data supporting the manipulation of sperm-freezing medium as a means of minimising oxidative damage are relatively less clear for humans than for other species [13]. Thus, a better understanding of the mechanisms underlying cryodamage is required in order to improve the quality of thawed samples [14,15]. To this end, in the present paper we review the role of ROS in cryopreservation damage and the antioxidant approaches tested so far to counteract ROS effects on human sperm biology.

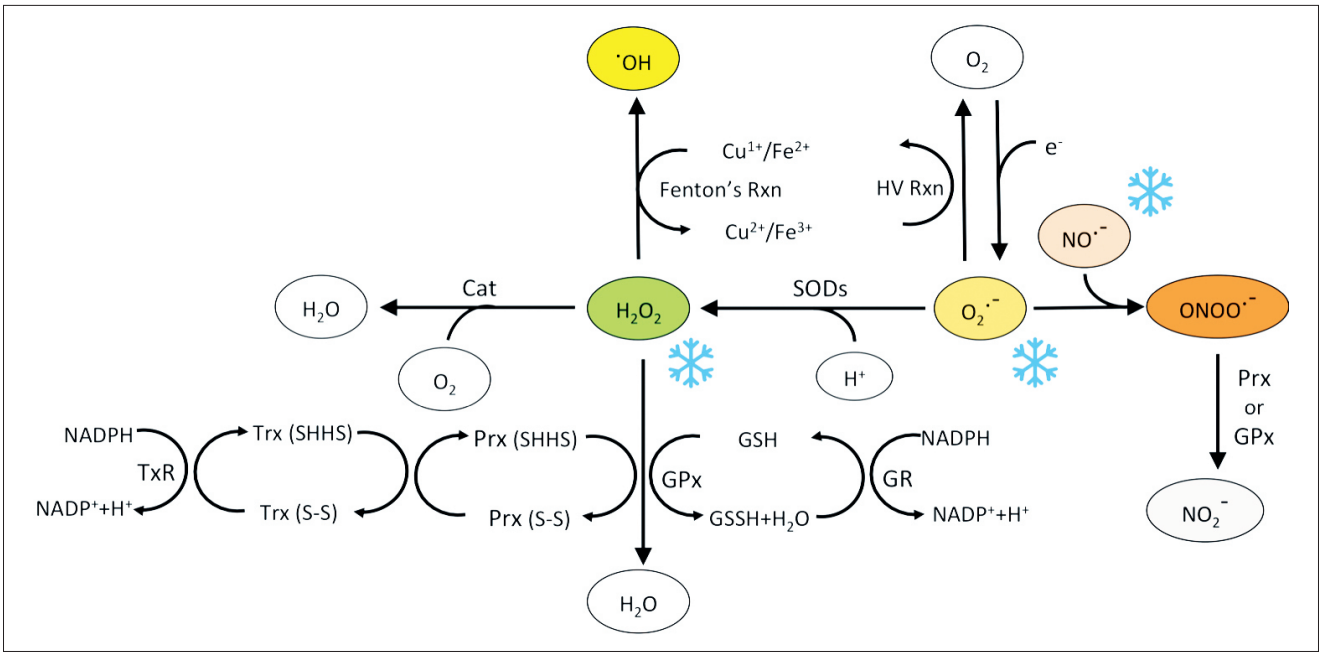
ROS and ROS-scavenging systems in human sperm

Reactive oxygen species (ROS) play a key role in sperm pathophysiology, being required for sperm capacitation, acrosome reaction, and binding to the zona pellucida [16,17]. ROS comprise both free radical and non-free radical oxygen-derived reactive molecules that are constantly generated by the mitochondrial electron transport chain, or during various enzymatic reactions. Common forms of ROS include superoxide anions (O^{2-}), which are capable of dismutating, either spontaneously or enzymatically, to hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hydroxyl (OH^-) radicals. The superoxide anion is extremely harmful because of its high oxidant activity and its ability to cross the biological membranes [18]. H_2O_2 reacts with only certain biomolecules and can cause the oxidation of thiol groups (SH). It is toxic at high concentrations because it can be reduced by ferrous iron, Fe (II), into the more damaging $^{\bullet}OH$ via the Fenton reaction (Figure 1). Due to its long half-life, H_2O_2 can transduce signals at long ranges. Glutathione peroxidase-glutathione reductase and the peroxiredoxin/thioredoxin-thioredoxin reductase pathways utilise NADPH as a reducing equivalent to reduce H_2O_2 to H_2O (Figure 1).

A subset of ROS includes reactive nitrogen species such as the nitric oxide ($NO^{\bullet}$) and the peroxynitrite ($ONOO^-$) anions [19,20]. Under physiological conditions, spermatozoa are known to produce low amounts of ROS, mainly by mitochondrial activity [16,21]. On the contrary, excessive levels of ROS in the semen may be ascribed to the activities of immature germ cells and leukocytes, which negatively influence sperm functions and fertility potential [22,23].

Indeed, spermatozoa are particularly susceptible to oxidative injury due to their low cytoplasmic antioxidant content and the abundance of polyunsaturated fatty acids (PUFAs) in plasma membrane [16,23,24]. PUFAs provide fluidity that is necessary for sperm motility and for membrane fusion events (e.g., the acrosome reaction and sperm-egg fusion), but, unfortunately, they are also prone to free radical attack [21,25,26]. Membrane PUFA peroxidation triggers a cascade of lipid peroxidation on the sperm surface, which disrupts membrane permeability and fluidity, thus leading to irreversible loss of motility and impaired sperm-oocyte fusion [16]. To cope with excessive ROS, sperm and seminal plasma are equipped with a number of enzymatic and non-enzymatic antioxidant systems, especially at the post-testicular level [14,16]. The main antioxidant enzymes contained in seminal plasma include superoxide dismutase, catalase and glutathione peroxidase [27]. The cytosolic copper/

Figure 1 Generation and detoxification of reactive oxygen/nitrogen species. SODs, superoxide dismutases, detoxify $O2^{\bullet-}$ by converting it to H_2O_2 (hydrogen peroxide): SOD1 is localised in both the mitochondria intermembrane space and the cytosol, SOD3 is located extracellularly, and SOD2 is found in the mitochondria matrix. Cat, catalase, reduces H_2O_2 into H_2O and is mostly located in peroxisomes. GPx, glutathione peroxidase, is found in mitochondria and cytosol. GR, glutathione reductase, is an NADPH-dependent flavoprotein that catalyses the reduction of glutathione disulfide (GSSG) to glutathione (GSH). Prx, peroxiredoxins, and Trx, thioredoxins, constitute the Prx/Trx system present in the nucleus, mitochondria, endoplasmic reticulum, peroxisome and extracellular environment. The snowflakes indicate ROS detected during cryopreservation procedures (see the manuscript for further details).



zinc superoxide dismutase (Cu/ZnSOD) and the mitochondrial Mn^{2+}-dependent superoxide dismutase (MnSOD) convert superoxide anions to hydrogen peroxide. This compound is then transformed to water by catalase and glutathione S-peroxidase (GSSPx). In the peroxidase reaction, reduced glutathione (GSH) is oxidized to GSSG (oxidized glutathione). GSSG is reduced by NADPH through the action of glutathione reductase (GSSG-Rx) [19]. Seminal plasma also exhibits non-enzymatic antioxidant activity, mainly relying on ascorbic acid, alpha-tocopherol, pyruvate, GSH, L-carnitine, taurin, hypotaurine, flavonoids, alkaloids, carotenoids, urate, albumin and ubiquinol [17,27].

A poor seminal antioxidant capacity associated with high levels of ROS has been reported in ejaculates from infertile men. Although oxidative stress is regarded as one of the main factors underlying sperm DNA damage and dysfunction in these patients, the question of whether the high ROS levels are due to increased ROS production, decreased ROS scavenging capacity or both is still under debate [17,27].

Cryodamage, ROS and sperm dysfunction

Several studies have pointed out that cryopreservation could exert detrimental effects on sperm survival by affecting sperm ultrastructural and molecular integrity [28,29]. Cold shock, osmotic stress, and ice crystal formation experienced by sperm during cryopreservation may impact sperm physiology with consequences on sperm viability and motility. Similarly, low temperatures and the presence of cryoprotectant may impair membrane cytoskeletal and membrane components, which may affect sperm surface proteins, with consequent alterations in capacitation status and sperm fertilising ability [15,30,31]. Interestingly, metabolomic and proteomic analyses have recently shown that energy pathways including the citrate cycle, glycolysis and pyruvate metabolism play an important role in human sperm cryopreservation [32-34].

Cryodamage is also involved in the downregulation of sperm mRNAs associated with fertilisation, early embryo development, capacitation and successful pregnancy. Some epigenetic-related transcripts are also affected by the cryopreservation process [35].

Oxidative stress is regarded as the main mechanism involved in cryodamage in reproductive and non-reproductive cells [13]. It may arise from osmotic stress and increased oxidative metabolism occurring during the thawing phase. Indeed, during thawing, when oxygen is reintroduced into frozen tissues, there is a burst of glucose-mediated oxidative metabolism with a consequent rise in ROS generation [36,37]. This process is mainly related to dysfunctional mitochondrial electron transport [38,39], as mitochondria represent the main source of ROS in human spermatozoa, especially after cryopreservation [40,41]. ROS in the form of superoxide ($O_2^{\bullet -}$), detected in the cells of various species undergoing cryopreservation, were observed in thawing sperm from some animal species along with elevated mitochondrial H_2O_2 [13].

Thawing-related oxidative stress results in reduced sperm motility and morphology, together with increased DNA fragmentation [15]. Several studies, indeed, showed that ROS are the main effectors of DNA damage during the process of sperm freezing and thawing, and that DNA damage is not linked to caspase-related mechanisms [9,42,43]. Ribas-Maynou *et al*. [15] showed that cryopreservation induces a 10% increase in spermatozoa with single-stranded DNA fragmentation, whereas no effects have been observed on the amount of double-stranded DNA breaks. A significant increase in both sperm DNA fragmentation and oxidation is associated with high levels of ROS after cryostorage of semen from infertile men. Moreover, deficiencies in DNA packaging by protamines have been observed in sperm from subfertile men, making their DNA more susceptible and vulnerable to ROS attack [15]. Interestingly, the extension of sperm DNA damage seems to directly correlate with sperm dysfunction and male infertility [6].

Role of antioxidants in sperm cryopreservation

Although semen displays antioxidant systems, during cryopreservation spermatozoa are more vulnerable to oxidative stress induced by the procedure itself, since the addition of cryoprotectants during sperm preparation prior to freezing dilutes the seminal plasma, thus lowering the efficiency of its antioxidant activity. Therefore, the addition of external antioxidants could be effective in compensating for the lack of natural defences [7,44,45]. Many studies have shown that supplementation of freezing and/or thawing media with antioxidants could protect sperm from the loss of motility that occurs following cryopreservation and thawing [10]. Enzymatic and non-enzymatic antioxidant strategies have been employed, for decreasing oxygen concentration, eliminating the catalytic metal ions, and harvesting a variety of free radicals such as superoxide and hydroxyl anions and hydrogen peroxide, as well as membrane peroxidation.

Several non-enzymatic antioxidants have been shown to counteract the effects of exogenous ROS [46]. Among them, vitamin E, a natural lipid soluble antioxidant, which, in its active form (alpha-tocopherol) removes superoxide anion and hydrogen peroxide, and hydroxylanions, and breaks peroxidation chain reactions [47,48]. Moreover, vitamin E decreases cryopreservation-related ROS generation, thus counteracting membrane lipid peroxidation [49-54]. In domestic animals, *in vitro* exposure to vitamin E improves post-thaw motility and DNA integrity, while its addition during incubation improves motility and viability of normal and abnormal spermatozoa during cryopreservation [26,44,50-54]. Recently, it was observed that gamma-tocopherol, added to cryopreservation medium, induced higher post-thaw human sperm viability and motility than alpha-tocopherol [55]. Melatonin is a powerful antioxidant secreted by the pineal gland [56]. It has been detected in seminal plasma, and sperm membrane displays a melatonin receptor [57,58]. Karimfar *et al*. [29] observed that the addition of 0.01 mM melatonin to the freezing medium improves spermatozoa viability and motility, and lowers intracellular ROS and membrane malondyaldeyde (MDA). In addition, when caffeine was added to melatonin, sperm motility improvement was associated with a healthier mitochondrial status [59]. Trans-resveratrol, the

most common isomer of resveratrol, is one of the most important polyphenols of red wine; it has a powerful antioxidant activity, which it exerts by scavenging free radicals and chelating divalent cations [60]. Besides the ability to inhibit ROS generation by enzymatic and non-enzymatic systems, resveratrol also induces calcium release into cell cytoplasm and increases sperm AMP-activated protein kinase (AMPK) phosphorylation [61]. In particular, trans-resveratrol provides protection against ROS-mediated membrane lipid peroxidation and DNA damage [28], increases mitochondrial membrane potential, and decreases ROS generation [61]. Curiously, in spite of its antioxidant properties against oxidative damage to lipids, resveratrol reduced damage but did not display a protective effect on sperm motility during cryopreservation [62-64]. The addition of ascorbic acid before cryopreservation can reduce DNA damage only in infertile men [63].

Meamar *et al*. [30] observed that the freezing medium supplemented with extract of *Opuntia ficus-indica*, which contains antioxidants and flavanoids, including resveratrol, significantly reduced sperm DNA fragmentation without improving sperm motility or viability. Another polyphenol, quercetin, has been demonstrated to ameliorate sperm motility and viability, and to reduce DNA damage as well as MDA levels [43,65]. In other systems, it has been proposed, but not yet definitively confirmed, that both resveratrol and quercetin may act by activating the NAD-dependent histone deacetylase sirtuin 1 (SIRT1), which triggers deacetylation of liver kinase B1 (LKB1) and phosphorylation of AMPK [66,67]. An interesting compound employed as a cryopreservation extender is the brain-derived neurotrophic factor (BDNF), which is a polypeptide of the neurotrophin family that is mainly expressed in the central nervous system and plays a key role in the differentiation, maturation, survival and regeneration of neuronal cells [68]. BDNF is also expressed in Leydig and Sertoli cells of the human testis, and its receptors (TrkB) have been found in spermatogonia and mature spermatozoa, suggesting a role in the paracrine regulation of spermatogenesis [69,70]. BDNF probably exerts its antioxidant activity by scavenging free radical ions, modulating the activity of antioxidant enzymes and enhancing the expression of sestrin 2, a stress-responsive gene involved in the cellular defence against oxidative damage [71,72]. This factor improves sperm motility and viability and reduces the level of intracellular H_2O_2 and caspase-3 activity [10], therefore, supplementation of sperm freezing and/or thawing media with BDNF could protect sperm cells against ROS- and apoptosis-mediated damage occurring during cryopreservation.

The supplementation of cryopreservation medium with GSH, ascorbate, myo-inositol or the mitochondria-targeted antioxidant MitoTEMPO increases sperm motility and mitochondrial functionality, and potentiates the antioxidant enzymes superoxide dismutase, catalase and GSH-peroxidase in spermatozoa, resulting in decreased activation of oxidative stress markers and a low degree of DNA fragmentation [73-76]. Elamipretide, a novel small mitochondrial targeting peptide, has recently been demonstrated to improve post-thaw motility and viability, and stability of the plasma membrane, mitochondria and chromosomes. Oxidation and acrosome dysfunction were also significantly reduced [77]. Surprisingly, a mitochondriotopic molecule such as L-carnitine, does not protect against DNA oxidation, although it enhances human sperm motility and vitality after cryopreservation [78]. Among the plethora of compounds with antioxidant properties, the efficacy of brown algae Sargassum has been tested in human spermatozoa [79]. Brown Algae Sargassum contains polyphenols, which are known to chelate heavy metals, leading to elimination of free radicals. The addition of Sargassum extract to the cryopreserved medium improves both total and forward sperm motility [79]. Gangliosides are sialic acid-containing glycosphingolipids expressed in the outer leaflet of the plasma membranes of all mammalian cells, including spermatozoa, and they are particularly abundant in neuronal cell membranes.

The protective effect of ganglioside micelles against ROS-induced changes is believed to stem from their ability to scavenge free radicals and prevent their harmful effects [45]. As expected, enrichment of cryopreservation media with gangliosides protected human sperm DNA integrity during the freeze-thaw process, reducing sperm susceptibility to DNA fragmentation. Moreover, gangliosides modulated the generation of superoxide anion, inhibited iron-catalysed hydroxyl radical formation and prevented the hydrogen peroxide diffusion across the sperm membrane [45]. Finally, addition of the enzymatic antioxidant catalase to cryoprotective media reduced the amount of ROS in frozen-thawed spermatozoa and greatly improved sperm motility, viability and mitochondrial function, and inhibited both DNA damage and early apoptotic events. The effects of antioxidants on cryopreserved human sperm have been summarised in Table 1.

Unfortunately, none of approaches reported above was able to abolish the cryodamage. This may be ascribed to i) reduced ability of sperm to use the added antioxidants, ii) the low amount of added antioxidant in comparison with the ROS produced, iii) loss of efficacy of antioxidant compounds during cryopreservation. Recent studies have proposed that the application of stressing conditions prior to cryopreservation would stimulate intrinsic sperm responses against cryodamage. In this context, different stressors such as hydrostatic pressure, osmotic pressure and oxidative agents have been applied at sub-lethal levels and shown to enhance sperm tolerance against stress induced by cryopreservation [80-83].

Conclusions

In this review, we summarised current knowledge about oxidative stress occurring during cryopreservation and the potential for supplementation of cryopreservation extenders with antioxidants as a means of minimising the harmful effects of ROS and improving the post-thaw quality of human sperm.

Cryopreservation leads to high generation of ROS, reducing the fertilising potential of human spermatozoa, which are particularly susceptible to ROS damage. In particular, to overcome oxidative reactions, spermatozoa are dependent on extracellular antioxidant systems contained in seminal plasma, which, however, is separated and discarded during the sperm processing. Therefore, there is need for a source of antioxidants to protect sperm cells during the cryopreservation pro-

cess. Most studies have demonstrated beneficial effects of *in vitro* antioxidant supplements in protecting spermatozoa from exogenous oxidants as well as freezing/thawing procedures. Nevertheless, further studies are warranted to establish the efficacy, suitability and safety of most of these antioxidants as supplements for cryopreservation extenders in clinical practice.

Table 1 Effects of antioxidants on cryopreserved human sperm.

ANTIOXIDANT	POSITIVE EFFECTS
Ascorbic acid [63,73]	ROS (↓) Viability and weak motility (↑) Apoptotic cells (↓) DNA damage (↓)
BDNF [72]	Sperm motility and vitality (↑) H2O2 (↓) Caspase activity (↓)
Brown algae sargassum extracts [79]	ROS (↓) Sperm motility (↑)
Catalase [73]	ROS (↓) Sperm viability and weak motility (↑) MMP (↑) Apoptotic cells (↓) DNA damage (↓)
L-carnitine [78]	Sperm viability and motility (↑)
Elamipretide [77]	Sperm motility and viability (↑) Plasma membrane stability (↑) MMP (↑) Acrosome dysfunction (↓)
Glutathione [74]	DNA damage (↓) MDA (↓) ROS (↓)
Melatonin [29,59,64]	Total antioxidant capacity (↑) GSH concentration (↑) Mitochondrial membrane integrity (↑) MMP (↑) SOD, catalase and GPx activity (↑) BCL-2, SOD2, GSTM1, NRF2, HSP90AA1(↑) Lipid peroxidation and ROS levels (↓) NADPH oxidase activity (↓) Nox5 and Bax expression (↓) Sperm viability and motility (↑)
MitoTEMPO [75]	Sperm motility and vitality (↑) Sperm membrane integrity vitality (↑) MMP (↑) SOD activity, catalase activity, GPx activity (↑) MDA levels (↓)
Myo-inositol [76]	Sperm progressive motility (↑) MDA (↓) Total antioxidant capacity (↑) DNA damage (↓)
Resveratrol [28,61-63]	DNA damage (↓) MDA levels (↓) SOD activity (↑)
Alpha-tocopherol, gamma-tocopherol [55]	Sperm viability and motility (↑)
Opuntia ficus indica extracts [30]	DNA damage (↓)
Quercetin [43,65]	Sperm motility and vitality (↑) DNA damage (↓) MDA levels (↓)

BDNF, brain-derived neurotrophic factor; **MMP**, mitochondrial membrane potential; **MDA**, malondialdhyde. See the manuscript for further details.

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