Oxidative stress and male infertility: role of antioxidants and their possible applications in assisted reproduction

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Summary

Low levels of reactive oxygen species (ROS) are essential for the regulation of sperm functions such as capacitation, acrosome reaction, and sperm-oocyte fusion. However, oxidative stress (OS), caused by the imbalance between ROS and antioxidant scavenging systems of the male reproductive tract, can impair semen quality, leading to infertility. Spermatozoa are highly vulnerable to ROS, that are generated from both endogenous and exogenous sources. Pathological conditions, inducing supra-physiological levels of ROS, can damage sperm DNA and membrane, altering viability, motility and genomic integrity. Hence, many studies have been carried out to understand the impact of OS on spermatogenesis, during the past decades. Although, antioxidant oral administration has been demonstrated to enhance semen quality in subfertile men, it is still a matter of debate if it can positively influence fertilization and embryo developmental competence. This paper will review the physiological and the pathological roles of ROS, the sources of OS *in vivo* and *in vitro*, as well as the usefulness of antioxidants in male infertility and during sperm handling in ART.

KEY WORDS: oxidative stress, antioxidants, male infertility, ART.

Introduction

Infertility, defined by the World Health Organization (WHO), as the inability to achieve pregnancy within 12 months of regular sexual intercourse, affects 15% of all couples of which the male factor accounts for 25 to 50% (1). Oxidative stress (OS), caused by the imbalance between the production of reactive oxygen species (ROS) and the protective action of antioxidant system, is one of the major causes of male infertility. High ROS levels are shown to be detrimental to spermatozoa by damaging the membrane via lipid peroxidation, and the DNA by strand breakage. Furthermore, oxidative stress has been related to reduced sperm count and activity, decreased motility and abnormal morphology (2). It is estimated that about 30-40% of infertile men present elevated levels of ROS in semen associated with lower antioxidant capacity (3). Henceforth, oral antioxidant administration has been reported to enhance semen quality in subfertile men and suggested to improve pregnancy rates (2).

Assisted Reproductive Techniques (ART) outcome, including fertilization and clinical pregnancy rates, are influenced by a multitude of factors among which OS plays a significant role, causing production of defective gametes or poorly-developing embryos (4). Therefore, the tight control of ROS levels within physiological concentration, during *in vitro* fertilization process, could represent a crucial factor to optimize ART clinical efficiency.

These encouraging evidences, have led to an exponential growth of studies on the effective

role of antioxidants in male infertility and ART outcome. Thus, this paper will review the physiological and the pathological roles of ROS, the sources of OS and their impact on spermatogenesis, as well as the use of antioxidants in male infertility and clinical ART.

ROS production in seminal plasma

Low levels of ROS are essential for the regulation of spermatogenesis, being involved in sperm chromatin condensation and control of germ cell number by regulating apoptosis and proliferation of spermatogonia (5). In mature spermatozoa, ROS play an important role in the capacitation, acrosome reaction, mitochondrial sheath stability, sperm motility and can also function as signalling molecules (5). ROS found in seminal plasma originates from various endogenous and exogenous sources. The principal sources of ROS in spermatozoa are represented by NADPH oxidase in the cell membrane, leakage of electrons from the respiratory chain in the mitochondria and NADH oxido-reductase located in sperm midpiece (6). Immature spermatozoa retaining residual cytoplasm, which can activate the NADPH system, macrophages and leukocytes derived from the prostate gland and seminal vesicles, more concentrated during infection or inflammation, are the main exogenous sources responsible for ROS production (7). Pathological conditions such as varicocele, an abnormal dilatation of veins in the pampiniform plexus around the spermatic cord, have also been associated with OS, increasing in correspondence to the grade of varicocele (8).

Among exogenous causes, several lifestyle factors such as excessive smoking, alcohol consumption, and environmental factors such as radiation, i.e. emitted from mobile phones, and toxins released from structural materials or industrial products could contribute to ROS production. All of these exogenous elements have been reported to be related with impaired semen quality (9-12).

Physiological roles of ROS in seminal plasma

Different studies have demonstrated that low and controlled concentrations of ROS play an important role in sperm function. ROS induce an increase in cyclic adenosine 3',5'-monophosphate (cAMP), responsible for spermatozoa hyperactivation during capacitation, a fundamental step for acrosome reaction and attaining fertilization competence (13). In fact, *in vitro* experiments demonstrated that spermatozoa undergo hyperactivation when incubated with low concentrations of OH- (14) and AR when physiological concentrations of O2-, H2O2, and NO were added to the seminal plasma (15). Finally, ROS have been shown to be involved in sperm-oocyte fusion increasing the membrane's fluidity through inhibition of protein tyrosine phosphatase activity and prevention of dephosphorylation and deactivation of phospholipase A2 (PLA2), in turn responsible for the cleavage of secondary fatty acid from the triglycerol backbone of the membrane phospholipid (16, 17).

Pathological roles of ROS

It has been shown that ROS can damage spermatozoa by sperm membrane lipid peroxidation (LPO), DNA damage and unsuccessful sperm apoptosis. The sperm membrane contains high levels of polyunsaturated fatty acid (PUFAs), highly vulnerable to ROS. Approximately 50% of PUFAs are composed of Docosahexaenoic Acid (DHA), involved in regulating spermatogenesis and membrane fluidity (18). LPO causes the loss of 60% of PUFAs from the membrane, decreasing its fluidity, increasing non-specific permeability to ions, and inactivating membrane-bound receptors and enzymes (2). Malondialdehyde and 4-hydroxynonenal, the final products of LPO, can cause severe cell dysfunctions at both genomic and proteomic levels (19).

Although, sperm DNA is highly condensed and organized by protamination and packaging into toroids (20), when these modifications are rendered poor and incomplete, DNA prevails more vulnerable to OS that can cause base-free sites, deletions, frame-shift mutations, DNA cross-links, and chromosomal rearrangements. Although it has been clearly established that most of the DNA damage in spermatozoa is oxidative (21), the exact mechanisms through which this can cause DNA fragmentation are still a matter of debate.

Moreover, ROS are able to induce overproduction of germ cells from seminiferous tubules, bypassing the essential and selective apoptosis of pre-meiotic spermatogonia, during the first round of spermatogenesis (22, 23).

The antioxidant system in seminal plasma

In order to protect spermatozoa against ROS, semen is equipped with several enzymatic/non-enzymatic factors, and low molecular weight compounds possessing potent antioxidant capacity. Main antioxidant enzymes present are superoxide dismutase, catalase, glutathione peroxidase and reductase.

Superoxide dismutases (SOD) catalyze the conversion of superoxide into oxygen and H2O2, thereby preventing LPO and improving motility (23). They exist in both intracellular (copper-zinc SOD and manganese SOD, located in the cytoplasm and mitochondrial matrix respectively) and extracellular forms (EC-SOD, SOD-3, related to the surface polysaccharides) (24). SOD-1 accounts for 75% of the SOD activity in seminal plasma and the remaining 25% is held by SOD-3. Catalase (CT), present both in human and rat spermatozoa and in the seminal plasma of several animals, catalyzes the decomposition of H2O2 to molecular oxygen and water (24) and activates the sperm cell capacitation induced by nitric oxide (25).

Glutathione peroxidase (GPX), which catalyzes the reduction of H2O2 and organic peroxides, including the peroxides of phospholipids, act as scavenging antioxidants in the epididymis and testes (26). In sperm GPX is primarily located in the mitochondrial matrix, but a nuclear form that protects sperm DNA from oxidative damage and participates in the process of chromatin condensation has also been found (27).

Finally, as mentioned above, low molecular weight molecules, which include glutathione, pantothenic acid, coenzyme Q10, carnitine, vitamins A, E, C, B complex and minerals, such as zinc, selenium, and copper, play an important role in neutralizing over-production of ROS in semen (28).

Among non-enzymatic antioxidants, the most abundant ones in seminal plasma are reported below. Firstly, carotenoids (precursors of vitamin A), including β-carotene and lycopene, responsible for the integrity of cell membranes and regulating epithelial cell proliferation, are involved in the regulation of spermatogenesis (29).

Furthermore, vitamin E (α-tocopherol) is a chain-breaking antioxidant, that acts by neutralizing H2O2 and quenching free radicals. Its main role is halting chain reactions that produce lipid peroxides and protecting the membrane from the damage induced by ROS.

Vitamin C (ascorbic acid) is another chain-breaking antioxidant, the concentration of which in the seminal plasma, is about 10 times higher than in blood serum. It reacts with OH, O2-, and H2O2 in the extracellular fluid, thus protecting sperm viability and motility (30).

Carnitine is a water-soluble antioxidant, that acts by assisting free fatty acid utilization and preventing LPO (22), thus protecting the sperm DNA and membrane from oxidative damage.

Glutathione that protects the cell membranes and DNA, is the most abundant intracellular thiol and exerts anti-oxidant properties by reconstruction of thiol groups (-SH) in proteins, that are eliminated during oxidative stress. Glutathione deficit has been reported to lead to instability of the spermatozoa midpiece, and cause disorders in motility (31). N-acetyl-cysteine (NAC) is a glutathione precursor that works by boosting the amount of reducing agent produced and ridding the spermatozoa of free radicals (22). It has been reported that GPX maintains sperm viability and motility (3), preserving the tail-beat frequency, reducing LPO, and improving the sperm capacitation (32).

Minor antioxidants are represented by albumin, taurine/hypotaurine, inositol, and some metals. Albumin, interacts with peroxyl radicals and prevents chain reactions that further generates other free radicals. Taurine scavenges ROS, while inositol is known to enhance GSH activity. Furthermore, selenium contributes to the protection of sperm DNA and cell membranes, particularly in combination with vitamin E. Zinc acts as a chelator and binds ROS (33), while manganese enhances sperm motility and viability.

Antioxidant effects on male infertility

Several *in vivo* and *in vitro* studies addressed the effects of exogenous antioxidants on sper-

matogenesis and semen quality, studying the effects of single and/or combined treatment of antioxidants on semen parameters, including concentration, motility and morphology. However few studies have investigated the effects of sperm treatment with antioxidants on fertilization, embryo quality, and pregnancy rates.

In vivo *studies*

Although, different clinical studies have been carried out to understand the effects of oral antioxidant therapy in subfertile men, currently it is difficult to compare their results and reach a consensus (34). Most trials are small in size (usually less than 100 men), with no homogenous target population. The study exclusions criteria or outcomes analyzed are often different and, moreover, the type, dose, and duration of the antioxidant therapy also differ substantially. However, herein we try to summarize main results obtained till date, regarding most commonly used antioxidants.

Vitamin C and E have been largely investigated. Administration of hydrophilic vitamin C and lipophilic vitamin E seems to dramatically reduce the OS on spermatozoa. A dose-dependent improvement in sperm motility has been found to be associated with vitamin C level in seminal plasma, especially in smokers (35). Vitamin E has been reported to increase sperm motility and function in two randomized trials (36, 37). Its oral administration significantly increased sperm motility by decreasing sperm production of MDA (38) and was also associated with a statistically significant increase in live birth rate (39).

Two randomized, placebo-controlled trials found that selenium, an essential micronutrient for normal testicular development, spermatogenesis, sperm motility and function, significantly improves sperm motility, concentration, morphology and pregnancy rates in men with idiopathic infertility when compared to placebo (40, 41). These effects are seen to be enhanced, when its administration is combined with vitamin E (42). Although, zinc therapy did not significantly raise its level in semen, improvements in sperm concentration, motility and morphology were seen, together with a reduction in anti-sperm antibody (ASA) and tumor necrosis factor-alpha (TNF-α) levels (43-45).

Randomized, placebo controlled trials have found that coenzyme Q10 can significantly improve sperm motility (46, 47). Although, treatment with coenzyme Q10 was reported to result in a two-fold increase in pregnancy rates (47), a recent meta-analysis revealed no effects of coQ10 on pregnancy rates, but only on sperm parameters (48).

Although, several studies in men with idiopathic infertility suggested beneficial effects of carnitines on sperm motility, concentration (49-52) and pregnancy rates (52); it should be noted that these trials were not homogeneous as different forms of carnitine were used.

Among carotenoids, lycopene treatment in idiopathic infertility patients led to a significant improvement in sperm concentration, motility and morphology, particularly in patients with baseline sperm concentrations higher than 5 million/mL (53).

Numerous studies have been carried out to investigate the effects of combined antioxidant therapies for the treatment of idiopathic male infertility. Combination of vitamins A, C and E has been reported to improve only sperm concentration (54, 55). A randomized controlled trial with vitamins A, C, E plus NAC and zinc found a significant increase in sperm concentration, without any influence on pregnancy rates (55). Interestingly, combined oral administration of vitamin E and selenium significantly improved sperm morphology and motility and decreased MDA concentrations (56-58). Moreover, supplementation of selenium plus NAC significantly increased sperm motility, concentration, normal morphology, and ejaculate volume (41). Tremellen et al., (57) in a randomised control trial, reported that a commercial combination of anti-oxidants (Menevit), administered to IVF-ICSI patients significantly improved pregnancy rates and viable pregnancies at 13 weeks of gestation.

The positive impact of sperm antioxidant treatments on pregnancy rates have been confirmed in a review (34) and underlined by Showell et al. in a recent meta-analysis (39).

In vitro *studies*

Despite numerous clinical reports about *in vivo* effects of antioxidants on sperm parameters and fertility outcomes, only a few studies have investigated their effects *in vitro*, both in animals and in human. Herein, we try to summarize the different results from studies evaluating the role of *in vitro* supplementation of var-

ious antioxidants in protecting spermatozoa from the loss of motility and DNA damage due to: 1) exogenous ROS, 2) semen handling, or 3) cryopreservation and thawing.

In vitro studies have shown that vitamin E prevents the loss of sperm motility and also improves the score of hamster egg penetration test (15). Supplementation of freezing media with vitamin E improved the viability, motility, DNA integrity (58-61) and zona-binding index (36) of thawed spermatozoa. Vitamin C treatment has been demonstrated to have a positive role in increase of sperm viability, progressive motility and in decrease of DNA damage and MDA levels (35, 61).

It has been reported that 50 μM CoQ10 increases sperm motility of asthenozoospermic men *in vitro* (62). Melatonin treatment has been shown to improve sperm viability, motility and morphology (63, 64).

We recently demonstrated that CoQ10, in combination with the antioxidant zinc and the micronutrient D-aspartate, contained in the dietary supplement Genadis (Merck Serono), has protective effects on human sperm motility, DNA fragmentation, and lipid peroxidation (65). Moreover, zinc, D-Asp, and CoQ10, at the concentrations previously found to exert protective effects on human spermatozoa, were also effective on bull spermatozoa (66). Furthermore, in bovine, we found that treated spermatozoa exhibited an improved ability to promote the development of 8-cell embryos at Day 3, associated with an increase of blastocyst rate, presenting a lower percentage of TUNEL-positive blastomeres, at Day 8 (66).

Oxidative stress during assisted reproductive techniques

In a typical ART setting, the potential sources of oxidative stress *in vitro* include endogenous and exogenous factors. Although, gametes and pre-implantation embryos themselves have the potential to generate ROS, OS during ART could also arise from several exogenous factors such as exposure to visible light, centrifugation, cryopreservation, culture media, oxygen concentration, pH and temperature (Table 1).

During *in vitro* handling, gametes and embryos are exposed to visible light (400-700 nm), both from the microscope and ambient lighting. The oxidative stress induced by light is highly dependent on the duration of exposure, intensity and spectral composition (67). Blue light, for its intrinsic capacity to generate H2O2 and alter enzymes of respiratory chain, is more dangerous than visible light. In mouse, exposure to blue light caused decreased blastocyst rates, higher blastomeric apoptosis rates and higher ROS production in morula (68).

In porcine parthenogenetically-activated embryos, the exposure of culture medium alone to ambient light yielded a higher percentage of blastocysts with poor morphology in a time dependent manner (69). Moreover, human spermatozoa capacitated in media exposed to visible light, showed an increased hyper-activated motility causing a premature loss of energy needed for fertilization (70-71).

Thus, the use of light filters on inspection microscopes (which cuts off light <500 nm) (72), illumination levels kept at a minimum without compromising visual inspection and shorter inspection time could help curb these effects (67).

Although, controversial data exists on the developmental competence of embryos cultured in 5 or 20% O2, it is well established that atmospheric oxygen concentration could enhance the activity of oxygen-dependent oxidase enzymes (73), increasing the OS (74) and negatively impacting the embryo quality.

To avoid freeze/thaw induced decrease in motility and viability of spermatozoa (75-78) antioxidant supplementation of cryopreservation media has been largely used. Quercetin (77) and catalase (78) have been reported to protect spermatozoa from oxidative stress-induced damage, improving sperm motility, viability and DNA integrity. Addition of vitamin E (58) pentoxifylline (79-82) and biotin (59) to cryopreservation medium improved post-thaw motility and survival.

Other procedures

Among the other procedures routinely used in ART, centrifugation, long incubations and type of culture medium could further generate OS (65, 66, 83). Centrifugation produces ROS in a manner dependent on the duration and the g-force applied (84, 85). Thus addition of antioxidants such as pentoxifylline (86) in culture media, could reduce centrifugation-induced sperm oxidative damage (87).

Table 1 - Effects of *in vitro* handling on sperm oxidative stress.

Oxidative Stress Source	Species	Effect	References
Exposure to blue light	mouse	Reduced blastocyst rates, increased apoptosis	(68)
Light exposure	pig	Poor blastocyst morphology	(69)
	human	Increased sperm hyperactivation	(70, 71)
Cryopreservation	human	Decreased sperm motility and viability	(58, 59, 75-78)
Long incubation	human	Decreased sperm motility, increased lipid peroxidation and DNA fragmentation	(65)
	bovine	Decreased sperm motility, increased DNA fragmentation, impaired embryo development	(66)
Sperm preparation media	human	Oxidative DNA damage and DNA fragmentation	(89)
Centrifugation	human	Increased ROS production and lipid peroxidation	(84, 87)

Incubation promotes ROS generation in a time dependent manner. Thus, ICSI carries a lower risk of ROS production compared to IVF that requires a long co-incubation of oocytes with a high sperm concentration in fertilization media (88).

The rate of ROS generated in culture media varies with their composition and specifically with the presence of metallic ions (iron, Fe2+ and copper, Cu2+) (73, 89). Addition of metal chelators (e.g. EDTA) or antioxidants may reduce ROS formation (90, 91).

Conclusions

OS represents a major contributory factor to male infertility and there is growing evidence that an increase in OS significantly impairs the spermatozoa and possibly the embryo development. Although antioxidant administration appears to be an appropriate treatment for male infertility most studies were restricted to the effects of antioxidant therapy on semen parameters and seldom evaluate its influence on live birth rates. To this end, a greater number of adequately powered randomized, controlled trials are needed to determine most effective antioxidants, dosing, and combination treatments. Moreover, supplementation of culture media with antioxidants during sperm *in vitro* manipulation in ART could also improve gamete quality and the developmental competence of resulting embryos.

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