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Role of Oxidative Stress, Reactive Oxygen Species & Antioxidants in Male Reproductive Functions

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ABSTRACT

Gametes are susceptible to reactive oxygen species (ROS) attack. When manipulated *in vitro* during assisted reproductive techniques, these cells have the risk of generating and being exposed to supra-physiological level of ROS. Defective sperm functions are the most prevalent causes of male infertility and a difficult condition to treat. Male infertility is a major problem of mammalian reproduction. Numerous factors influence male infertility and oxidative stress is one of them. The term oxidative stress is generally applied when oxidants outnumber the antioxidants. The imbalance between the production of ROS and a biological systems ability to readily detoxify the reactive intermediates or easily repair the resulting damage is known as oxidative stress. The main destructive aspects of oxidative stress are the production of ROS, which include free radicals and peroxides. In this article we discuss the role of oxidative stress in different sperm functions, means of ROS generation and their physiological implications in semen and how antioxidants act as defense in protecting sperm from oxidative /ROS attack.

Keywords: Antioxidants, LPO, oxidative stress, ROS, Semen

The ultimate goal of a spermatozoon is the successful fertilization of ovum resulting in normal conception (Sikka *et al.* 1995). In order to achieve this, the spermatozoa after spermiation must mature within the male genital tract, travel through the female reproductive system, undergo capacitation and acrosome reaction, bind to and penetrate the zona pellucidae of the ova as well as the oolemma, and finally fuse with the female pronucleus. But during these processes, sperm get exposed to various ROS and which interfere with its normal functions. Since, integrity and functional activity of sperm membrane is of fundamental importance in the fertilization process; assessment of membrane functions may be useful indicator of the fertilizing ability of spermatozoa (Kaushik *et al.*, 2015a). Defective sperm functions are the most common causes of infertility, which are difficult to evaluate and treat. Part of this difficulty are due to our incomplete understanding of the factors contributing to normal and abnormal sperm functions leading to male infertility (Kefer *et al.* 2009).

Indeed, mammalian spermatozoa have the distinction of being the first cell type in which the cellular generation of this powerful oxidant was demonstrated. Mammalian spermatozoa membranes are rich in poly-unsaturated fatty acids (PUFAs) and are sensitive to oxygen induced damages mediated by lipid peroxidation lead to oxidative stress (Agarwal *et al.* 2008). Therefore, the routine semen analysis (measurement of seminal volume, spermatozoal motility, density, viability and morphology) does not necessarily provide complete diagnostic information of the factors responsible for

propagation of the reactive oxygen species and status of oxidative stress (Sikka, 1996). How the oxidative stress/reactive oxygen species (ROS) affects the spermatozoan is still unknown. Therefore, various tests are being employed for the analysis of oxidative stress such as enzymatic, biochemical and immunological assays (Bar-Chama and Lamb, 1994). Though a variety of defense mechanisms encompassing antioxidant enzymes {superoxide- dismutase (SOD), catalase, and glutathione peroxidase (GSHPx) and reductase (GR)}, vitamins (E, C, and carotenoids), and biomolecules (ubiquinol) are available, but still a balance of the benefits and risks from ROS and antioxidants appears to be necessary for the survival and functioning of spermatozoa. Keeping in mind, the need of the development of appropriate assays for evaluation and prevention of oxidative stress (OS), the authors provide an overview that highlights the factors/conditions which affect normal sperm functions and treatment strategies to overcome this stress in human ejaculated spermatozoa.

Oxidative Stress and its Effects on Sperm Functions

Oxidative stress is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants commonly known as reactive oxygen species (Sikka *et al.* 1995). It is a manifestation of excess oxidants or reactive oxygen species (ROS) against a deleted antioxidant defense mechanism in cell (Adewoyin *et al.* 2017). The role of oxidative stress in infertility and methods for counteracting its impact on reproductive tissues with antioxidants is still in its infancy (Joyce, 1987). Limited endogenous mechanisms exist in sperm to reverse the damages caused by oxidative stress. A shift in the levels of ROS towards pro-oxidants in semen can induce an oxidative stress on spermatozoa, because of either excess ROS or diminished antioxidants can be classified in terms of positive oxidative stress (Sikka *et al.* 1995). There is at present no true ROS detection method available that will evaluate this balance. However, assessment of oxidative stress, or a similar paradigm when monitored more objectively, would be a good indicator of sperm damage caused by oxidative stress (Sikka *et al.* 1995).

Under normal circumstances, there is an appropriate balance between pro-oxidants and anti-oxidants. Concomitantly, a decrease in antioxidant activities (*e.g.*, SOD, catalase, GSHPx and GR) in semen correlates with idiopathic infertility (Gagnon *et al.* 1991). It is possible that an increased rate of ROS production (suggesting high oxidative stress) may inhibit the action of these antioxidant enzymes, or alternatively the inherent decreased expression of these antioxidant enzymes may cause increased oxidative stress (Sikka *et al.* 1995). This will result in increased lipid peroxidation (LPO), decreased sperm motility, viability and function, and ultimately leads to infertility. Measurement of such oxidative stress may help in the medical treatment of male factor infertility by suitable antioxidants (Sikka *et al.* 1995).

All cellular components including lipids, proteins, nucleic acids, and sugars are potential targets of oxidative stress (Agarwal *et al.* 2008). The extent of OS-induced damage depends on the nature and amount of ROS involved and also on the duration of ROS exposure and extra-cellular factors such as temperature, oxygen tension and the composition of the surrounding environment (e.g. ions, proteins, and ROS scavengers) (Agarwal *et al.* 2008).

Tools for Evaluation of Oxidative Stress

Assessing seminal oxidative stress over time would help in monitoring antioxidant therapies and define effective doses and duration. Many different assays are available; they are defined into direct assays (chemi-luminescence and flow cytometry assay) that measures the degree of oxidation within sperm cell membrane or indirect assays (e.g., LPO levels and measures of redox potential) that measures detrimental effects of oxidative stress and tissue (Agarwal

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et al. 2004).Each test has advantage and disadvantage. Direct assays can provide accurate measures of oxidative stress, but expensive, but indirect assays are simple and less expensive.

Tests of sperm functions, such as sperm DNA fragmentation (SDF) and measures of oxidative stress have been found to better understanding of the true male fertility potential (Agarwal *et al.* 2016). Assessment of the rate of ROS production/generation using luminol as a probe can be a dynamic measure of oxidative stress (Aitken and Clarkson, 1987). The potential methods that can be developed for evaluation of oxidative stress may utilize measurement of an oxidized component that remains in the body fluids (*e.g.*, *thiobarbituric* acid (TBA) reactive substances; reduced glutathione (GSH)/ oxidized glutathione (GSSG) balance; the levels of unaltered tocopherol or ascorbate). Although there have been concerns about the specificity, interference, and reliability of measuring *thiobarbituric* acid- *malondialdehyde* (TBA-MDA) activity as an indicator of LPO, this test remains one of the most efficacious methods for assessing the oxidative damage to sperm (Alvarez *et al.* 1987). Eventually, this TBA-MDA measurement will need to be combined with other assays which would be able to measure the rate of ROS production and antioxidant protection for the overall assessment of oxidative stress in infertility (Rajasekaran *et al.* 1995).

Reactive Oxygen Species (ROS)

The proposed mechanism for loss of sperm function upon oxidative stress has been shown to involve excessive generation of ROS (Aitken, 1995). The ROS has both beneficial and damaging effects on sperm and thus can influence the fertilization process (Aitken and Clarkson, 1987). One of the major cellular sources of reactive oxygen species (ROS) in the semen is sperm cells. From the earliest stages of development of germ cells,they are able to produce small amount of ROS (Jedrezejowska *et al.* 2012). Virtually every human ejaculate is considered to be contaminated with potential sources of ROS. Human semen is known to contain different types of cells such as mature and immature spermatozoa, round cells from different stages of spermatogenesis, leukocytes, and epithelial cells. It is observed that beside these different cell types, leukocytes and spermatozoa have been shown to be the two main sources of ROS in semen (Agarwal *et al.* 2008). Reactive oxygen species are generated by exogenous agents (e.g.,radiations, chemicals and hypoxia) or via endogenous processes such as in normal cellular metabolism. Other exogenous and endogenous sources of ROS are given in table 1 (Kova *et al.* 2012).

Table 1: Exogenous and endogenous sources of reactive oxygen species (ROS)

Endogenous Sources of ROS	Exogenous Sources of ROS
Mitochondria	Cigarette smoke
Peroxisomes	UV light
Phagocytes	Ionizing radiations
Cytochrome P450	Pollutants
NADPH Oxidase	Ozone
Nitrogen oxide synthase	Chemotherapeutic agent
Xanthine oxidase	Pesticide
Endoplsamic reticulum	Organic solvent
Heme protein	Alcohol
Excessive physical exercise	Metals
Reaction of metal ions (Fe, Cu, Ti, Ni, Co, Pb, Mo, As)	

Reactive oxygen species (ROS) can be detected by various means, such as spectrophotometric assay, enzyme-linked immunosorbent assay (ELISA), and one-dimensional or two-dimensional electrophoresis followed by western blot immunoassay (Rossi *et al.,* 2003). The most common method of measuring ROS is a chemiluminescence assay. Under immunological probes, luminol (5 –amino 2,3 dihydro 1,4 phthalazinedione) and lucigen probes can be used for quantification of redox activities of spermatozoa. Luminol can

measure both intracellular and extracellular ROS, whereas, lucigen can measure only the superoxide radical released extracellularly (Aitken *et al.* 2004).

Reactive oxygen species represent a broad category of molecules including radical (hydroxyl ion, superoxide, NO, peroxyl etc.) and non-radical (ozone, singlet oxygen, lipid peroxide, hydrogen peroxide) and oxygen derivatives (Agarwal and Prabakaran, 2005). The most common ROS that play important role in reproductive biology include superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals and the very reactive hydroxyl (OH^-) radicals (Sikka *et al.* 1995). Reactive oxygen species are highly reactive oxidizing agents belonging to the class of free-radicals. A free radical is any compound (not necessarily derived from oxygen) which contains one or more unpaired electrons. The assumption that free radicals can influence male fertility has been proved (Gagnon *et al.* 1991). Beside ROS, it is observed that reactive nitrogen species also play a very significant role in sperm dysfunction. Reactive nitrogen species (nitrous oxide, peroxynitrite, nitroxyl ion etc.) are free nitrogen radicals and considered a subclass of ROS (Agarwal *et al.* 2008). Reactive nitrogen species (RNS); the nitrogen-derived free radical nitric oxide ($NO\cdot$) and peroxynitrite anion ($ONOO^-$) also appear to play a significant role in the reproduction and fertilization. NO has been shown to have detrimental effects on normal sperm function inhibiting both motility and sperm competence for zona binding. The ultimate effects of $NO\cdot$depend upon its concentration and interactions with hydrogen peroxide (Sikka *et al.* 1995).

Role of ROS on Sperm Function

The assumption that ROS can influence male fertility has received substantial scientific support (Agarwal *et al.* 2008). ROS are double edge sword; they have role in pathological processes but play important roles in physiological processes. When ROS are present at certain levels, they greatly overwhelm the capacity of endogenous cellular antioxidants defense system that causes oxidative stress (Merve and Elmas, 2016). Controlled generation of ROS may function as signaling molecules (second messengers) in many different cell types, they are important mediators of sperm functions. Evidences have been reported to especially superoxide anion ($\cdot O_2$) are required for the late stage of embryo development such as two germ cell layers and egg cylinder (Kodama *et al.* 1996). Although a significant negative correlation between ROS and *in-vitro* fertilization (IVF) rate has been found (Agarwal and Prabakaran, 2005). Substantial evidence exists to suggest that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities (Gagnon *et al.* 1991; Aitken, 1997; Aitken, 1999) Low levels of ROS have been shown to be essential for fertilization, acrosome reaction, hyperactivation, motility, and capacitation (Agarwal *et al.* 2004; Griveau and Le Lannou, 1997). Co-incubation of spermatozoa with low concentrations of hydrogen peroxide has been shown to stimulate sperm capacitation, hyperactivation, acrosome reaction, and oocyte fusion (Griveau *et al.* 1994; Aitken, 1995; Kodama *et al.* 1996). ROS can have beneficial or detrimental effects on sperm functions depending on the nature and the concentration of the ROS as well as the location and length of exposure to ROS (Sies, 1993). During epididymal transit, sperm acquire the ability to move progressively; however, they acquire the ability to fertilize in the female tract through a series of physiological changes called 'capacitation' (Sies, 1993). Under physiological conditions, spermatozoa produce small amounts of ROS, which are needed for capacitation and acrosomal reaction. Studies have indicated that male germ cells at various stages of differentiation have the potential to generate ROS. *In vivo* physiological concentrations of ROS are involved in providing membrane fluidity, maintaining the fertilizing ability, mitochondrial sheath stability and acrosome reaction of sperm (Bucak *et al.* 2010). Excessive ROS

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impairs motility and capacity of fertilization. ROS cause adverse effects on the sperm plasma membrane, DNA and physiological processes, thereby, affecting the quality of spermatozoa. The sperm plasma membrane is particularly vulnerable to oxidative stress because it is richly endowed with polyunsaturated fatty acids such as docosaheaxaenoic acid that redirects it highly susceptible to oxidation and other chemical and structural modifications (Jones *et al.* 1979; Aitken, 1994). ROS generated *in vitro* decreases motility as well as inducing sperm DNA damage. ROS are known to cause significant DNA damage to both mitochondrial and nuclear genome of human spermatozoa. Reactive oxygen metabolites attack DNA bases (particularly guanine) and phosphodiester backbones, destabilizing this molecule and creating the cellular conditions that ultimately result in DNA fragmentation (Duru *et al.* 2000; Wong *et al.* 2002; Villegas *et al.* 2005). Numerous studies have demonstrated a strong association between ROS and sperm DNA damage by using techniques such as terminal deoxynucleotidyltransferase –mediated dUTP nick end labeling (TUNEL), sperm chromatin structure assay (SCSA) and measurement of DNA oxidation adduct (Aitken *et al.* 1998; Oger *et al.* 2003; Henkal *et al.* 2005; Kao *et al.* 2008; DeLulis *et al.* 2009).

Additionally cold shock arising from cryopreservation also plays an important role in the molding of membranes by determining their sol gel balance and the dynamic status the affects the fusion of the plasma membrane of the male and female gametes (Bucak *et al.* 2007). The proportion of fully functional spermatozoa in a freeze thawed sample is considerably reduced. Motility is one of the parameter most seriously affected by freezing. It is also a strong predictor of the ability of a given sample to achieve fertilization *in vitro*. It has been suggested that motility, membrane integrity and mitochondrial functions are similarly affected by cryopreservation (Watson, 2000).

Lipid Peroxiation of Spermatozoa

Lipid peroxidation is the most extensively studied manifestation of oxygen activation in biology (Darley-Usmar *et al.* 1995). Lipids are considered to be the most susceptible macromolecules and are present in sperm plasma membrane in the form of PUFA; fatty acids that contain more than two carbon–carbon double bonds. ROS attacks PUFA in the cell membrane leading to a cascade of chemical reactions called lipid peroxidation. One of the by-products of lipid peroxidation is malondialdehyde (MDA), which has been used in various biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa (Aitken *et al.* 1994). In spermatozoa, production of MDA, an end product of LPO induced by ferrous ion promoters, has been reported (Darley-Usmar *et al.* 1995; Bansal and Bilaspuri, 2009). Formation of MDA can be assayed by the TBA activity, which is a simple and useful diagnostic tool for the measurement of LPO for *in vitro* and *in vivo* systems (Taourel *et al.* 1992).

Creatininekinase and lipid peroxidation

It has been established that the concentration of creatinine kinase (CK) which reflects the degree of cytoplasmic extrusion during the last phase of spermatogenesis; is an objective measure of the cellular maturity and fertilizing potential of human spermatozoa (Huszar and Vigue, 1990, 1993, 1994). It has been suggested that increased ROS production and CK activity are related to increased cytoplasmic content of sperm due to retarded extrusion and both biochemical parameters reflect diminished cellular maturity of spermatozoa. It has been found a close relationship between increased rate of lipid peroxidation and increased cytoplasmic content of sperm. It has been established that high CK content of spermatozoa is associated with an increase in size and roundedness of sperm head and increase incidence of amorphous sperm forms. There found a close correlation between MDA production and CK activity that indicated

both parameters reflect sperm biochemical maturity independently from sperm conc. in specimens (Huszar and Vigue, 1994).

Life Style and Oxidative Stress

The decreasing trend in sperm count has led to speculation that recent environmental dietary and/or lifestyle changes are interfering with man's ability to produce spermatozoa. It is believed that these factors exert their detrimental effects through oxidative stress. The lifestyle choices, including smoking, obesity, poor nutrition, and exposure to environmental toxins all lead to increased systemic or seminal oxidative stress. When considering therapeutic measures to decrease oxidative stress, counseling toward lifestyle modifications must be considered first and foremost. An important challenge to proper counseling is the paucity of evidence supporting objective improvement in infertility following lifestyle modification (Kefer *et al.* 2009).

Cases of male infertility also reported in the people who are working in the area where environment temperature is comparatively higher than the normal temperature such as mines, brick clines, thermal power plants etc. It is proved that oxidative stress also induced by hyperthermia and which is one of the major cause of sub-fertility in males (Paul *et al.* 2009; Zhang *et al.* 2012; Zhaojian *et al.* 2018; Kaushik *et al.* 2018).

Antioxidant Defence Mechanism Against ROS

Studies have shown that antioxidants protect spermatozoa from ROS producing abnormal spermatozoa, scavenge ROS produced by leukocytes, improve semen quality reduce cryodamage to spermatozoa, block premature sperm maturation and stimulate spermatozoa and improve assistant reproductive technique outcome (Agarwal *et al.* 2008).

Antioxidants, in general, are compounds and reactions that dispose, scavenge, suppress the formation of ROS and/or free radicals, oppose their actions and halt the chain reaction leading to oxidative stress in body tissue (Singh *et al.* 2015). Since ROS has both physiological and pathological roles, an array of antioxidants maintains a steady state of ROS in the seminal plasma. Antioxidants act as free radical scavengers to protect spermatozoa against ROS. A variety of biological and chemical antioxidants that attack ROS and LPO are presently under investigation (Agarwal *et al.* 2008). Seminal fluid is rich in antioxidants that nourish and protect sperm. They exist in two forms; an enzymatic and non enzymatic antioxidant. Antioxidant enzymes are naturally occurring in sperm cell or seminal plasma and are thought to originate from prostrate (Majzoub and Agarwal, 2018). Seminal plasma contains superoxide dismutase, catalase, and glutathione peroxidase / glutathione reductase in addition to non-enzymatic antioxidants such as ascorbate, urate, vitamin E, pyruvate, glutathione, albumin, vitamin A, ubiquitol, taurine, and hypotaurine. These antioxidants compensate for the loss of sperm cytoplasmic enzymes as the cytoplasm is extruded during spermiogenesis, which, in turn, diminishes endogenous repair mechanisms and enzymatic defenses (Kerr, 1992).

Glutathione is the most abundant intracellular thiol that is a sulfur containing components and hence is present in large amount. It has an antioxidant properties by reconstruction of thiol groups (-SH) in protein, which can be eliminated during oxidative stress (Jedrezejowska *et al.* 2012). Glutathione has a number of physiological and pharmacological qualities that act against lipid peroxidation of the cell membrane (Bansal and Bilaspuri, 2008). Glutathione therapy has been proposed in various pathological situations in which ROS could be involved in idiopathic infertility. Marked improvement in total sperm motility and morphology occurring during glutathione therapy suggests that glutathione could act indirectly on spermatozoa by improving the

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metabolic conditions of the epididymal and testicular structures (Frei *et al.* 1990). These conditions persisted even after the therapy was stopped. Glutathione is known to act as a free radical scavenger resulting in improved semen quality. It is demonstrated that demonstrated the effectiveness of glutathione, alone or in combination with hypotaurine, to react directly with cytotoxic produced during lipid peroxidation and thus protecting the thiol groups on the sperm plasma membrane (Baker *et al.* 1996; Seligman *et al.* 1994). Reduced glutathione is also known to neutralize hydroxyl radicals and function in the detoxification of peroxides through its interaction with sperm glutathione peroxidase. It may also facilitate the antioxidant action of alphatocopherol in the seminal plasma by participating in the regeneration of this vitamin from tocopheryl radicals. Parenteral administration of glutathione has been shown to improve sperm motility and morphology in infertile men with abnormal semen quality associated with varicoceles or genital tract inflammation (Lenzi *et al.* 1994).

GSH peroxidase, a selenium-containing antioxidant enzyme with GSH as the electron donor, removes peroxyl($ROO^{\cdot}$) radicals from various peroxides including H_2O_2 (Calvin *et al.* 1981).

GSH reductase, a potent enzyme that regenerates reduced GSH from oxidized GSH (Lenzi *et al.* 1994). A selenium-associated polypeptide, presumably GSH peroxidase, has been demonstrated in rat sperm mitochondria; it plays a significant role in this peroxyl scavenging mechanism and, ultimately, in maintaining sperm motility (Calvin *et al.* 1981). It seems that the role of these GSH enzymes and their associated mechanisms as related to biological antioxidants in infertility is an important area for further development.

It would be interesting to explore this antioxidant mechanism in human spermatozoa. These antioxidant mechanisms are important in the maintenance of sperm motility, the rate of hyper activation, and the ability to undergo the acrosome reaction during sperm preparation techniques, especially in the absence of seminal plasma. A high GSH/GSSG ratio will help spermatozoa to combat oxidative insults (Sikka *et al.* 1995; Bansal and Bilaspuri, 2008).

Superoxide dismutase, among the well-known biological antioxidants, SOD and its two isozymes and catalase have a significant role (Agarwal *et al.* 2008). SOD spontaneously dismutates -O_2 to form O_2 and H_2O_2, whereas catalase converts H_2O_2 to O_2 and H_2O. Many studies have been reported in the literature on the role of SOD as an antioxidant in reproductive biology. SOD protects spermatozoa against spontaneous O_2 toxicity and LPO (Alvarez *et al.* 1987).

Antioxidant Additives

Albumin, used in sperm washing procedures, is likely to serve as an antioxidant by providing thiol compound soluble in fat, mainly present in cell membrane groups required for “chain breaking” antioxidant activity.

Within the category of chemical antioxidants, both natural and synthetic products have gained attention by the cosmetic, nutrition, and pharmaceutical industries. Their usefulness in reproduction and management of infertility has not yet been developed.

Vitamin E (a–tocopherol) is organic chemical compound soluble in fat mainly present in cell membrane. Vitamin E protects the sperm cell membrane components from damage and to a lesser extent, decreases the production of ROS (Kaushik *et al.* 2015b). It also prevents reduced motility of sperm and improves their ability to fertilize in the hamster egg penetration test. Antioxidant activity of vitamin E is mainly based on quenching lipid peroxidation initiated by ROS (Huszar and Vigue, 1990). Vitamin C (ascorbic acid) is a water soluble substance. This vitamin has high antioxidant potency, which is valuable in protecting sperm DNA from harmful effects of ROS (Jedrezejowska *et al.* 2012). Both

vitamins may protect spermatozoa against endogenous oxidative DNA and membrane damage. The effect of vitamin supplementation in combination with IVF techniques is a worthy notion (Watson, 2000).

Carotenoids (beta-carotene) and ubiquinols are a group of fat soluble organic compound found mainly in yellow, red, orange and pink vegetables dyes. These are precursor of vitamin A. They are natural antioxidant, responsible for integrity of cell membrane and regulating epithelial cell proliferation and are involved in regulation of spermatogenesis. Their deficiency in diet can lead to reduction in sperm motility (Jedrezejowska *et al.* 2012). They may also play a role in quenching singlet oxygen and reducing lipid derived free radicals with detrimental effects on sperm LPO, hence, the application of ROS scavengers (e.g., SOD, catalase) is likely to improve sperm motility and function (Agarwal *et al.* 2014).

Zinc supplementation orally, successfully restored seminal catalase activity and improved sperm concentration and progressive motility in the asthenozospemric men (Hadwa *et al.* 2015).

Pentoxifylline, a sperm motility stimulator, can also act as a suppressor or scavenger of ROS (Kerr, 1992). High levels of alpha-tocopherols in serum achieved upon oral administration of vitamin E have been shown to improve the performance of spermatozoa in the zona binding test (Kessopoulou *et al.* 1994). Antioxidants have been demonstrated to decrease DNA fragmentation induced by OS.

Future Prospectives

Evaluation of OS status and use of antioxidants is not routine in clinical practice. The immediate need is to simplify and validate the evaluation of ROS and OS status so that it can be performed routinely without the use of sophisticated equipment. Also, it is important to establish reference values for ROS above which antioxidants could be used for male infertility treatment. The dose and duration of these antioxidants should also be determined and standardized. With the increase in the use of ART procedures there should be an effort to develop optimum combinations of antioxidants to supplement sperm preparation media.

Conflict of interest

The authors declare that the research paper has been written in the absence of any commercial or financial relationships that could be construed as real or potential conflict of interest.

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