Journal of Andrology, Vol. 26, No. 6, November/December 2005

DOI: 10.2164/jandrol.05016

Minireview

Prevention of Oxidative Stress Injury to Sperm

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Received for publication January 25, 2005; accepted for publication June 9, 2005.

The greatest paradox of aerobic respiration is that oxygen, which is essential for energy production, may also be detrimental because it leads to the production of reactive oxygen species (ROS) (Saleh and Agarwal, 2002). When levels of reactive oxygen species (ROS) overwhelm the body's antioxidant defense system, oxidative stress (OS) occurs. OS is a condition in which the elevated levels of ROS damage cells, tissues, or organs (Moller et al, 1996; Sharma and Agarwal, 1996; Saleh et al, 2003).

ROS are free radicals that play a significant role in many of the sperm physiological processes such as capacitation, hyperactivation, and sperm-oocyte fusion (Aitken et al, 2004; Allamaneni et al, 2004; de Lamirande et al, 1998). However, they also trigger many pathological processes in the male reproductive system, and these processes have been implicated in cancers of the bladder and prostate, as well as in male infertility (Bankson et al, 1993; Hietanen et al, 1994; Agarwal and Saleh, 2002).

Spermatozoa are sensitive to OS because they lack cytoplasmic defenses (Donnelly et al, 1999; Saleh and Agarwal, 2002). Moreover, the sperm plasma membrane contains lipids in the form of polyunsaturated fatty acids, which are vulnerable to attack by ROS. ROS, in the presence of polyunsaturated fatty acids, triggers a chain of chemical reactions called lipid peroxidation (Agarwal et al, 1994; Kobayashi et al, 2001; Zalata et al, 2004). ROS can also damage DNA by causing deletions, mutations, and other lethal genetic effects (Moustafa et al, 2004; Tominaga et al, 2004).

It is difficult to block the OS-induced injury to cells or tissues because ROS are continuously produced by cellular aerobic metabolism (Davies, 2000). Several clinical trials are currently attempting to minimize the toxic effects of OS on human spermatozoa (Agarwal et al, 2004). In this review, we highlight the various protective measures available to minimize OS-induced injury to spermatozoa.

Sources of ROS in Semen

There are two main sources of ROS in semen: leukocytes and immature spermatozoa (Garrido et al, 2004). Of these, leukocytes are considered to be the primary source (Aitken et al, 1992). Leukocytes, particularly neutrophils and macrophages, have been associated with excessive ROS production that ultimately leads to sperm dysfunction (Aitken and Baker, 1995; Aitken et al, 1997; Hendin et al, 1999; Ochsendorf, 1999; Pasqualotto et al, 2000; Saleh et al, 2002; Shalika et al, 1996; Sharma et al, 2001).

Spermatozoa produce ROS mainly when a defect occurs during spermiogenesis that results in retention of cytoplasmic droplets (Gomez et al, 1996; Zini et al, 1993). A strong positive correlation exists between immature spermatozoa and ROS production, which in turn negatively affects the sperm quality (Gil-Guzman et al, 2001; Said et al, 2004).

The two main sites of ROS production are the mitochondrion and the sperm plasma membrane. The mitochondrion is the powerhouse of respiration. Hence, it is the major site of ROS generation, which is produced through the nicotinamide adenine dinucleotide-dependent oxido-reductase pathway (Gavella and Lipovac, 1992). In contrast, the sperm plasma membrane produces ROS through the nicotinamide adenine dinucleotide phosphate-dependent oxidase system (Aitken et al, 1992; Agarwal et al, 2003). Xanthine oxidase—a key enzyme in purine catabolism—is also involved in the production of ROS in spermatozoa (Aitken et al, 1993; Sanocka et al, 1996).

Oxidative Damage to Spermatozoa by Life-Style Behaviors

Damage Caused by Pollution— Environmental pollution and radiation can generate various ROS such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), and hydroxyl radical ($^{\cdot}OH$) (Gate et al, 1999). According to an Italian study on tollgate workers, traffic pollution damages sperm and may reduce fertility in young and middle-aged men (De Rosa et al, 2003). The workers in that study showed poorer sperm quality than age-matched men living in the same area who were not exposed to the same level of automobile pollution. In addition, sperm function tests revealed that the workers had less active spermatozoa. In the subset of workers with abnormal sperm parameters, sperm count and viability were inversely correlated with lead (Pb) levels, whereas sperm motility, viability, membrane function, nuclear DNA integrity, linearity, and amplitude of sperm lateral movement were inversely correlated with methemoglobin levels—a marker for nitrogen oxide (De Rosa et al, 2003).

Damage Caused by Smoking— Smoking induces OS either by increasing levels of oxidants originating from the smoke or by decreasing levels of antioxidants in the seminal plasma (Fraga et al, 1996). Therefore, smokers may need to increase their intake of antioxidants to compensate for this deficiency (Bui et al, 1992). According to a study conducted by our group, smoking greatly increases levels of leukocyte and ROS (Saleh et al, 2002). Other studies have reported that tobacco adversely affects sperm quality (ie, concentration, motility, and morphology) (Kunzle et al, 2003, 2004). Smoking also predominately affects sperm DNA, as evidenced from the increased level of 8-deoxygunosine (8-oxodG)—a marker for oxidative DNA damage (Potts et al, 1999). Therefore, smoking may not affect the fertilization rate per se, but it will increase the risk of heritable mutations (Kunzle et al, 2003; Lewin et al, 1991; Rubes et al, 1998; Vine et al, 1996).

Oxidative Damage to Spermatozoa by Infection

Sperm damage can be caused either by the invading pathogens or by the defense mechanisms that are employed against them (Ochsendorf, 1999). For example, when microorganisms invade the human body, it produces polymorphonuclear leukocytes and macrophages, which are the major sources of ROS production (Ochsendorf, 1999; Saran et al, 1999; Zalata et al, 2004). Prostatitis and accessory gland infection increase OS, which severely damages spermatozoa (Potts and Pasqualotto, 2003). In addition, a past infection by the sexually transmitted *Neisseria gonorrhea* is associated with leukocytospermia (Trum et al, 1998). Although there is no direct evidence that *Neisseria gonorrhea* directly increases ROS production, the associated leukocytospermia is well known to produce ROS (Trum et al, 1998). According to a study by Depuydt et al, leukocytospermia and male accessory glands infection reduce a man's fertilizing potential by affecting sperm parameters both in vitro and in vivo (Depuydt et al, 1998).

Iatrogenic Oxidative Damage to Spermatozoa

Prolonged in vitro incubation of semen samples that contain high levels of immature spermatozoa before sperm processing increases the risk of OS damage to mature spermatozoa (Gil-Guzman et al, 2001). In a study by Aitken and Clarkson, it was reported that repeated centrifugation mechanically injures spermatozoa and increases ROS production (Aitken and Clarkson, 1988). OS may also damage sperm during cryopreservation. A study by Bilodeau et al revealed that ROS generated during freeze-thaw cycles are detrimental to sperm function and that levels of antioxidants were diminished during each cycle (Bilodeau et al, 2000).

Strategies for OS Prevention

Modifying Life-Style Habits— As reported earlier, because smoking is known to increase OS in seminal plasma, patients should be advised to abstain from it. It is also of extreme importance to avoid excessive exposure to environmental pollution in working and living conditions, as environmental pollution increases the OS-related sperm damage.

Antioxidants— Antioxidants are the main defense against OS induced by free radicals. There are prevention antioxidants and scavenger antioxidants. Prevention antioxidants such as metal chelators and metal-binding proteins block the formation of new ROS, whereas scavenger antioxidants remove the ROS that have already formed.

Prevention Antioxidants— Transition metal ions, mainly iron, are involved in the generation of the highly reactive $^{\bullet}OH$ by Fenton's reaction (Biemond et al, 1984; Ochsendorf, 1999). They stimulate lipid peroxidation by decomposing the peroxides into peroxyl and alkoxyl radicals, which in turn causes the chain reaction of lipid peroxidation. Metal chelators such as transferrin, lactoferrin, and ceruloplasmin that are present in human semen control lipid peroxidation of the sperm plasma membrane, protecting its integrity (Sanocka and Kurpisz, 2004). Other metal chelators such as ethylene diamine tetraacetic acid, 1,10-phenanthrolin, and neocuproine have been shown to reduce sperm DNA damage in fishes (Bruskov et al, 2002). However, similar data are lacking in human.

In vitro supplementation of metal chelators such as DL-penicillamine, 2,3-dimercaptopropan-1 sulphonate and meso-2,3-dimercapto-succinimic acid showed enhancement of sperm quality during assisted reproductive technique (Henkel and Schill, 2003). In a study by Wroblewski et al, incubating sperm with D-penicillamine significantly increased sperm motility (Wroblewski et al, 2003).

Cadmium (Cd), a class B element, is capable of replacing zinc, thereby exerting its toxic effect in

spermatogenesis. Metallothionein removes Cd from the body by binding to it, which results in improved spermatogenesis, maturation, and capacitation of spermatozoa (Omu and Fernandes, 2001).

Scavenger Antioxidants— Dietary antioxidants form an essential part of the human antioxidant defense system. Fruits and vegetables as well as daily dietary supplements constitute the potential sources of various antioxidants. The National Academy of Sciences has recommended 60 mg vitamin C per day for an adult male. The daily requirement of vitamin E varies from 50 to 800 mg, depending on the intake of fruits, vegetables, tea, or wine. Carotenoids and selenium work synergistically with vitamin E and have a recommended dietary allowances value of 1000 and 70 micrograms per day, respectively (National Academy of Sciences, 1989).

Oxidative stress may also be limited by using chain-breaking antioxidants such as vitamin E and vitamin C as drug supplements. Vitamin C is a major chain-breaking antioxidant and is present in the extracellular fluid. It neutralizes hydroxyl, superoxide, and hydrogen peroxide radicals and prevents sperm agglutination (Agarwal et al, 2004). In addition, it also helps recycle vitamin E (Sies et al, 1992). Vitamin C is found in reduced quantity in the seminal plasma of infertile men (Lewis et al, 1997). Vitamin C increases sperm counts in vivo in infertile male patients with oral doses ranging from 200-1000 mg/day (Dawson et al, 1987). The combination of flavonoids and vitamin C increases the effectiveness of both of these compounds (Mathiesen et al, 1996). They conserve the alpha-tocopherol content of low-density lipoprotein and delay the onset of lipid peroxidation (de Whalley et al, 1990). Quercetin and xanthohumol constitute some of the important dietary flavonoids.

The principal chain-breaking antioxidant vitamin E is present within the cell membrane. It neutralizes H_2O_2 and protects the plasma membrane from lipid peroxidation. In a randomized cross-over study, oral administration of 600 mg/day of vitamin E improved sperm function as assessed by the zona binding test (Kessopoulou et al, 1995).

Selenium is a necessary component for the synthesis of glutathione peroxidase. It works synergistically with vitamin E (Bartfay et al, 1998). According to a study conducted by Keskes-Ammar et al, 225 µg/day of selenium and 400 mg/day of vitamin E oral supplementation for 3 months significantly decreased malondialdehyde concentrations in seminal plasma and improved sperm motility (Keskes-Ammar et al, 2003). However these findings were not confirmed in another study (Iwanier and Zachara, 1995).

Carotenoids such as beta-carotene and lycopene also form an important component of the antioxidant defense (Gupta and Kumar, 2002). Beta-carotenes protect the plasma membrane against lipid peroxidation. Lycopene is found in abundance in tomatoes (Agarwal and Rao, 2000) and has a suggested daily intake of 5-10 mg/day. Lycopene has been shown to be twice as potent as beta-carotene and 10 times more potent than vitamin E in scavenging singlet oxygen and inhibiting lipid peroxidation in serum plasma (Di Mascio et al, 1989).

Glutathione is the most abundant antioxidant found in the body. It plays an important role in protecting lipids, proteins, and nucleic acids against oxidative damage. It combines with vitamin E and selenium to form glutathione peroxidase. In a placebo-controlled, double-blind, cross-over trial, 600 mg glutathione was administered for 2 months by intramuscular injection in 20 infertile men. Glutathione therapy significantly increased sperm motility, particularly forward progression (Lenzi et al, 1993).

Coenzyme Q-10 (CoQ-10) found in the sperm mid-piece (Lewin and Lavon, 1997) recycles vitamin E, prevents its prooxidant activity (Thomas et al, 1996; Thomas et al, 1997), and is also involved in

energy production. In a study conducted on 32 infertile males, CoQ-10 inhibited H_2O_2 formation in seminal fluid and in seminal plasma (Alleva et al, 1997). In an in vitro study on 22 semen samples of asthenozoospermic men, incubation with 50 µM of CoQ-10 significantly increased sperm motility. In the same study, in vivo supplementation of 60 mg of CoQ-10 in 17 infertile men improved their fertilization rate without changing their semen parameters (Lewin and Lavon, 1997).

Zinc and copper are trace metals that constitute a part of superoxide dismutase—a key enzymatic antioxidant. Their adequate intake is necessary to maintain the optimal functioning level of these enzymes. At present, it is estimated that the average daily intake in United States is 12.3 mg of zinc and 900 µg of copper per person. Caution should be exercised during intake of these trace metals because some of them can catalyze the reactions that lead to the formation of ROS. An in vitro study by Lloyd et al on salmon sperm DNA determined that at concentrations of 20-50 µM and above, these metal ions caused a maximum DNA strand breaks (Lloyd et al, 1998). No such studies are available in humans, and the in vivo dosage required for these concentrations in seminal plasma is still unknown.

Other antioxidants may also protect against OS, such as alpha lipoic acid and carnitines. Alpha lipoic acid (thiols) undergoes reduction to form dihydrolipoic acid, thereby regenerating other antioxidants such as vitamins C and E and reduced glutathione through redox cycling (Biewenga et al, 1997). In contrast, carnitines are dietary antioxidants that decrease ROS by removing excess intracellular toxic acetyl-CoA that are responsible for mitochondrial ROS production. Carnitines also improve sperm motility (Agarwal and Said, 2004; Vicari and Calogero, 2001).

However, the positive effects of antioxidants are still debatable. Multiple studies have found no improvement following antioxidant intake (Agarwal et al, 2004; Ford and Whittington, 1998; Martin-Du Pan and Sakkas, 1998; Moilanen and Hovatta, 1995; Rolf et al, 1999).

Prevention of Iatrogenic Oxidative Damage— The use of specific sperm preparation techniques has greatly reduced the OS associated with sperm handling and cryopreservation. Sperm separation techniques such as migration-sedimentation, density centrifugation gradient, and glass-wool filtration significantly reduce the level of ROS by removing leukocytes, which are the major source of ROS (Henkel and Schill, 2003).

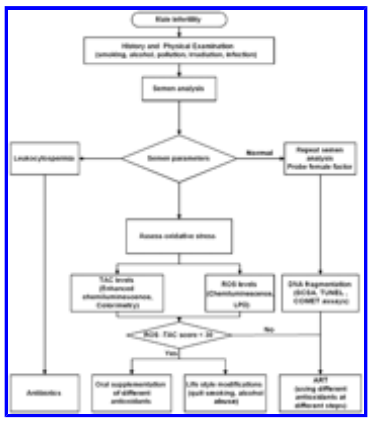
In vitro supplements used during sperm preparation and assisted reproductive technique also help to protect spermatozoa against ROS. Moreover, adding antioxidants to the culture media neutralizes ROS produced by the leukocytes and immature spermatozoa and improves spermoocyte fusion (Irvine, 1996). In an in vitro study, rebamipide effectively scavenged ROS during sperm processing and cryopreservation (Park et al, 2003). In another study performed on samples from 25 male partners of infertile couples, in vitro supplementation with superoxide dismutase and catalase prevented lipid peroxidation of the sperm plasma membrane by ROS and contributed to the recovery of high-quality spermatozoa after freezing-thawing procedures (Rossi et al, 2001). Similarly, it has been found that adding glutathione and hypotaurine protects spermatozoa against oxidative damage induced by H_2O_2 (Donnelly et al, 2000).

Pentoxifylline—a methylxanthine derivative that inhibits phosphodiesterase—has been approved by the US Food and Drug Administration for use in humans. It has a beneficial effect on sperm motility and acrosome reaction and reduces the O_2^- release by the human spermatozoa (Agarwal et al, 2004; Henkel and Schill, 2003; McKinney et al, 1996). N-acetyl-L-cysteine—a precursor of glutathione—reduces the ROS production in human ejaculate (Agarwal et al, 2004; Oeda et al, 1997), as well as ROS-induced DNA damage (Lopes et al, 1998). The use of vitamin E in vitro has been also documented

to improve sperm motility and viability (Verma and Kanwar, 1999). Hughes et al (1998) has determined that in vitro supplementation of vitamins C, E, and urate separately has protective effects on sperm DNA integrity on irradiation (Hughes et al, 1998).

Conclusions

Spermatozoa are under a continuous influence of OS because of excessive generation of ROS. Although spermatozoa are affected in different ways by OS, there are sufficient antioxidant protections that can decrease the progression of the damage. However, when an imbalance exists between levels of ROS and the natural antioxidant defenses, various measures can be used to protect spermatozoa against the OS-induced injury (Figure). Diet forms an important component of the antioxidant protection system; it supplies the major antioxidants such vitamin C, vitamin E, and carotenoids. Therefore, food rich in these elements should form a part of the daily diet. For those patients who are suspected to have high levels of ROS, antioxidant supplements can be considered. Nevertheless, further studies are required to validate their use in this group of patients. In certain cases, it is also essential to modify certain lifestyle behaviors because many habits and environmental factors increase the production of ROS and affect fertility.



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Suggested algorithm for evaluation and management of infertile patients with increased oxidative stress in their seminal plasma. Oxidative stress should be assessed as a part of the male infertility evaluation. ROS-TAC score was calculated by applying principal component analysis to normalized values of ROS and TAC in semen specimens from a population of proven fertile donors; scores below 30 indicate the presence of oxidative stress. TAC indicates total antioxidant capacity; ROS, reactive oxygen species; COMET, single-cell microgel electrophoresis; TUNEL, terminal deoxynuceotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling; SCSA, sperm chromatin structure assay; and ART, assisted reproductive technology.

Another important method for decreasing OS is the use of antioxidants during various sperm processing techniques. Antioxidants decrease the oxidative damage to spermatozoa induced during these techniques. There are many controversies regarding the doses, types, and combinations that could be used in different sperm-handling techniques. Future research should address these issues to develop standard and reliable protocols.

Acknowledgments

We thank the Glickman Urological Institute at the Cleveland Clinic Foundation for research support, and Mrs Robin K. Verdi for secretarial support.

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