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Role of Oxidative Stress and Antioxidants in Male Infertility

Minireview

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"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 (ROS). All living aerobic cells are normally exposed to a background level of oxidative stress. Various stress-related conditions, such as chronic disease states, aging, toxin exposure, physical injury, and exposure to many types of foods, can enhance this oxidative process and cause cell damage. Recently, a plethora of knowledge on free-radical biology has provoked our collective research interests, with emphasis on clinical conditions such as aging, dietary intake, health awareness, cosmetic use, the pathobiology of disease, and the perceived beneficial effects of antioxidants. ROS have been implicated in over a hundred disease states, ranging from arthritis and connective tissue disorders to carcinogenesis, infection, and acquired immunodeficiency syndrome. In the area of reproduction, the role of oxidative stress and its therapy with antioxidants remain in their initial stages and thereby warrant additional exploration. The aim of this minireview is to further stimulate interest in basic and clinical research on the roles of ROS and antioxidants in reproduction, particularly the development of newer modes of diagnosis and therapy for the infertile couple.

What are ROS and How Do They Relate to Oxidative Stress?

ROS are highly reactive oxidizing agents belonging to the class of free radicals. A free radical is any compound (not necessarily derived from oxygen) containing one or more unpaired electrons. The most common ROS that have potential implications in reproductive biology include superoxide ($O_2^{\cdot -}$) anion, hydrogen peroxide (H_2O_2), peroxyl ($ROO^{\cdot}$) radicals, and the very reactive hydroxyl ($OH^{\cdot}$) radicals.

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The assumption that free radicals can influence male fertility has received substantial scientific support (see Gagnon et al, 1991, for review). The proposed mechanism for loss of sperm function due to oxidative stress has been shown to involve excessive generation of ROS (Aitken and Clarkson, 1987). H_2O_2 has both beneficial and damaging effects on sperm and thus can influence the fertilization process. The nitrogen-derived free radical nitric oxide ($NO^{\cdot}$) also appears to play a significant role in reproduction and fertilization. The ultimate effects of $NO^{\cdot}$ depend upon its concentration and interactions with H_2O_2. Hence, free radicals and ROS are associated with oxidative stress and likely play a number of significant and diverse roles in reproduction.

Spermatozoa and ROS

There had been much speculation as to whether the origin of ROS in semen is from spermatozoa or from infiltrating leukocytes (Kessopoulou et al, 1992; Krausz et al, 1994). Iwasaki and Gagnon (1992) reported that the leukocyte-free Percoll fractions of semen samples obtained from non-azoospermic infertile men generate detectable levels of ROS when compared to the semen of normal and azoospermic men, suggesting that damaged spermatozoa are likely to be the source of ROS. Also, higher levels of ROS were correlated with a decreased number of motile sperm; conversely, greater sperm motility was observed in samples with lesser amounts of detectable ROS.

It is important for the clinician to recognize that assisted reproductive techniques (Percoll gradients/sperm washing/centrifugation) may induce damage to spermatozoa by either inadvertently removing the scavenging capability of seminal plasma or by increasing ROS generation by spermatozoa (Aitken and Clarkson, 1987). Cellular damage is theoretically the result of an improper balance between ROS generation and scavenging activities. The scavenging potential of spermatozoa is normally maintained by adequate levels of superoxide dismutase (SOD), catalase, and probably glutathione (GSH) peroxidase and reductase in the seminal plasma.

Leukocytospermia and ROS

The presence of leukocytes (predominantly granulocytes) in semen has been associated with severe male factor infertility cases. The exact site of origin of these leukocytes in semen, their mode of action, and the roles that bacteria, viruses, and subsequent genitourinary inflammation might

have on sperm function are not clear. Experimentally, ROS production by human spermatozoa and contaminating leukocytes can be stimulated by phorbol esters and certain formyl peptides with deleterious effects on sperm motility and fertilization (Krausz et al, 1994). Although the presence of leukocytes in semen did not diminish the *in vitro* fertilizing capacity of spermatozoa, the introduction of leukocytes into washed sperm preparations did reduce sperm function by the production of ROS (Aitken et al, 1994). This finding seems paradoxical but does indicate that seminal plasma has significant antioxidant or ROS scavenging capacity that may prevent sperm damage by leukocytes.

An association between leukocytospermia and ROS has been recently found to correlate with increased chemokine (interleukin 8; IL-8), and decreased SOD activity (Rajasekaran et al, 1995). This demonstrates that increased oxidative stress during leukocytospermia is caused by a defective ROS scavenging system that can in turn be modulated by certain proinflammatory cytokines. Based upon these observations, it may be useful to assess the oxidative stress status (OSS) in infertile or subfertile patients, particularly those with chronic genitourinary inflammation. In this context it would also be worthwhile to explore ROS production and the presence of potential scavengers in the vaginal secretions and cervical mucus of the female partner of infertile couples.

Detection of ROS

In many complex biological systems, including semen, the true ROS status reflects a relative balance between ROS generated and ROS scavenged. The measurement of the rate of ROS generation by luminol-induced chemiluminescence has been the most common method to quantitate ROS. Although this rate measurement is a dynamic system, it may not accurately represent the potential sperm-damaging ROS status. For such evaluations, the amount of ROS detected instead of ROS generated will represent a more physiological assessment of oxidative stress (Gagnon, 1992). The methods commonly used for measuring ROS can be categorized into 1) reactions involving nitroblue tetrazolium (NBT) or cytochrome c-Fe^{3+} complexes that measure ROS on the cell membrane surface; 2) those that measure ROS (generated inside or outside the cell) utilizing luminol-dependent chemiluminescence; and 3) electron spin resonance methods, which are more sensitive and can identify the type of ROS generated inside the cell but require skillful operation, accurate interpretations, and expensive instrumentation.

Artificially Induced ROS and Sperm Function

ROS can be artificially generated under defined experimental conditions in order to further study their mode of action on human spermatozoa. The reaction between xanthine and xanthine oxidase results in the univalent and divalent reduction of dioxygen to generate superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), respectively. In the presence of ferric ions, these radicals further generate the highly reactive hydroxyl radical ($OH^{\cdot}$), which is especially deleterious to spermatozoa.

$$\text{xanthine-hypoxanthine} \xrightarrow{\text{xanthine oxidase}} \text{uric acid} + O_2^{\cdot -} + H_2O_2$$

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{Fe^{3+}} OH^{\cdot} + OH^{-} + O_2.$$

Electrolysis of physiological buffer under defined conditions also generates ROS, which can damage sperm motion (Rajasekaran et al, 1994). Selective modifications of these defined conditions can identify 1) the free radicals involved, 2) their mode of action on spermatozoa, and 3) the evaluation of selective protective mechanisms.

Mode of Action of ROS

Mammalian spermatozoa are rich in polyunsaturated fatty acids and are thus very susceptible to ROS attack, resulting in a decrease in sperm motility, presumably by a rapid loss of intracellular ATP, causing axonemal damage (de Lamirande and Gagnon, 1992), a decrease in sperm viability, and an increase in midpiece morphology defects, with deleterious effects on sperm capacitation and the acrosome reaction. Lipid peroxidation of sperm membrane is considered to be the key mechanism of this ROS-induced sperm damage (Alvarez et al, 1987) leading to infertility (Fig. 1).

Lipid peroxidation of Spermatozoa—Lipid peroxidation (LPO) is the most extensively studied manifestation of oxygen activation in biology. The most common types of LPO are 1) nonenzymatic membrane LPO and 2) enzymatic (NADPH- and ADP-dependent) LPO. The enzymatic reaction involves NADPH–cytochrome P-450

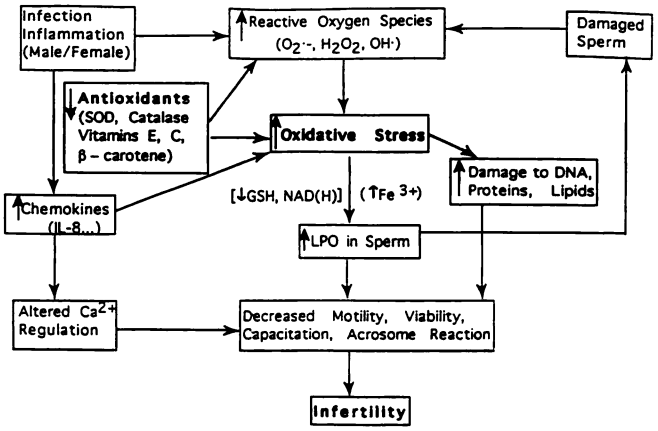

FIG. 1. Scheme suggesting, interacting mechanisms in the role of oxidative stress and antioxidants in male infertility.

reductase and proceeds via an ADP–$Fe^{3+}O_2^{\cdot -}$ (perferryl) complex. In spermatozoa, production of malondialdehyde (MDA)—an end product of LPO induced by ferrous ion promoters—has been reported by Alvarez et al (1987). This MDA formation can be assayed by the thiobarbituric acid (TBA) reaction, which is a simple and useful diagnostic tool for the measurement of LPO for *in vitro* and *in vivo* systems.

Biological Implications of Spermatozoa LPO and Oxidative Stress

The spermatozoa, unlike other cells, are unique in structure, function, and susceptibility to damage by LPO. In order to understand the biological mechanisms of LPO in infertility, three important questions need to be addressed: 1) What are the mechanisms of LPO of sperm *in vivo*? 2) What are the consequences of damage to sperm membranes, proteins, and nucleic acids? 3) What regulates the antioxidant defense mechanisms in seminal plasma?

In general, the most significant effect of LPO in all cells is the perturbation of membrane (cellular and organellar) structure and function (transport processes, maintenance of ion and metabolite gradients, receptor-mediated signal transduction, etc.). Low levels of NADH and glutathione, as a result of the increased activity of glutathione peroxidase to remove metabolites of LPO, will further affect cellular Ca_2^+ homeostasis. Minor alterations in sperm membranes in selected cases of dyspermia can be reversed by GSH therapy (Lenzie et al, 1994). Studies on how these cellular changes caused by LPO affect seminal parameters and sperm function, and the reversal of these effects, are open to further investigations.

Besides membrane effects, LPO can damage DNA and proteins, either through oxidation of DNA bases (primarily guanine via lipid peroxyl or alkoxyl radicals) or through covalent binding to MDA, resulting in strand breaks and cross-linking. ROS can also induce oxidation of critical-SH groups in proteins and DNA that will alter spermatozoal structure and function, with an increased susceptibility to attack by macrophages. The oxidative damage to mitochondrial DNA is well known to occur in all aerobic cells that are rich in mitochondria; this may include spermatozoa. In addition, the redox status of human spermatozoa is likely to affect phosphorylation and ATP generation, with a profound influence on its fertilizing potential. Aitken et al (1995) recently showed that stimulation of endogenous NADPH-dependent ROS generation in human sperm appears to regulate the acrosome reaction via tyrosine phosphorylation. In general, oxidizing conditions increase tyrosine phosphorylation, with enhanced sperm function, whereas reducing conditions have the opposite effect. However, this has been debated for a long time, and it is still not clear whether sperm have an NADPH-dependent oxygenase system. Nonetheless, how these mitochondrial DNA or membrane changes regulate specific sperm functions in association with altered tyrosine phosphorylation is an area of interest. These studies may open a new series of diagnostic tools to assess sperm function and damage in clinical infertility.

Antioxidants (Potential Scavengers of ROS)

Antioxidants, in general, are compounds and reactions that dispose, scavenge, suppress the formation of ROS, or oppose their actions. A variety of biological and chemical antioxidants that attack ROS and LPO are presently under investigation. Among the well-known biological antioxidants, superoxide dismutase (SOD) and its two isozymes and catalase have a significant role. SOD spontaneously dismutates $O_2^{\cdot -}$ to form O_2 and H_2O_2, whereas catalase converts H_2O_2 to O_2 and H_2O.

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2.$$

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). SOD and catalase also remove $O_2^{\cdot -}$ generated by NADPH oxidase in neutrophils and may play an important role in decreasing LPO and protecting spermatozoa during genitourinary inflammation.

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. GSH reductase then regenerates reduced GSH from oxidized GSH (GSSG), as shown in the following equation:

$$2GSH + H_2O_2 \xrightarrow{\text{peroxidase-(Se)}} GSSG + 2H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{reductase}} 2GSH + NADP^+.$$

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 would be interesting to explore this antioxidant mechanism in human spermatozoa. In addition, GSH peroxidase and GSH reductase may directly act as antioxidant enzymes involved in the inhibition of sperm LPO. GSH has a likely role in sperm nucleus decondensation and may alter spindle microtubule formation in the ova, thus affecting pregnancy outcome. In this context, the γ-glutamyl transpeptidase (γGGT), considered to be present in the midpiece and acrosomal regions of spermatozoa of certain mammalian species (e.g., the boar) may further affect oocyte GSH

content at the time of sperm penetration. Thus, because of the great number of mitochondria present in spermatozoa, these antioxidant mechanisms are important in the maintenance of sperm motility, the rate of hyperactivation, and the ability to undergo the acrosome reaction during sperm preparation techniques, especially in the absence of seminal plasma. Albumin, used in sperm-washing procedures, is likely to serve as an antioxidant by providing thiol groups required for "chain breaking" antioxidant activity. A high GSH/GSSG ratio will help spermatozoa to combat oxidative insults. 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.

Within the category of chemical antioxidants, both natural and synthetic products have garnered attention by the cosmetic, nutrition, and pharmaceutical industries. Their usefulness in reproduction and management of infertility has not yet been developed. Vitamins E and C have shown minimal effects on the improvement in post-thaw sperm parameters, although they may protect spermatozoa against endogenous oxidative DNA and membrane damage. In this regard, carotenoids (beta-carotene) and ubiquinols 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. Pentoxifylline, a sperm motility stimulator, can also act as a suppressor or scavenger of ROS (Sikka et al, 1993). 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). The effect of vitamin E supplementation in combination with *in vitro* fertilization (IVF) techniques is a worthy notion. Further controlled clinical studies will determine if many of these putative antioxidants can improve infertility in subgroups of patients.

The Concept of Oxidative Stress Status

The balance of ROS can be termed as the "balance of creation and destruction." Under normal circumstances, there is an appropriate balance between pro-oxidants and antioxidants. A shift in the levels of ROS towards pro-oxidants in semen and vaginal secretions can induce an oxidative stress on spermatozoa. Concomitantly, a decrease in antioxidant activities (e.g., SOD, catalase, GSH peroxidase and reductase, GSH) in semen will correlate with idiopathic infertility. 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. This will result in increased LPO, decreased sperm motility, viability, and function, and, ultimately, infertility (see Fig. 1).

A situation in which there is a shift in this ROS balance towards pro-oxidants, because of either excess ROS or diminished antioxidants, can be classified in terms of positive oxidative stress status (OSS). There is at present no true ROS detection method available that will evaluate this balance. However, assessment of OSS, or a similar paradigm when monitored more objectively, would be a good indicator of sperm damage caused by oxidative stress. Chronic asymptomatic genitourinary inflammation can be regarded as a condition with positive OSS that may be the real cause of idiopathic infertility in such patients.

Measurement of OSS

Assessment of the rate of ROS production/generation using luminol as a probe can be a dynamic measure of oxidative stress. However, the clinical evaluation of this ROS generation is limited by a very short half-life of these free radicals. The potential methods that can be developed for evaluation of OSS may utilize measurement of an oxidized component that remains in the body fluids (e.g., TBA-reactive substances, GSH/GSSG balance, the levels of unaltered tocopherol or ascorbate). Although there have been concerns about the specificity, interference, and reliability of measuring TBA–MDA activity as an indicator of LPO, this test remains one of the most efficacious methods for assessing oxidative damage to sperm (Alvarez et al, 1987). Eventually, this TBA–MDA measurement will need to be combined with other assays that suggest ROS production and antioxidant protection for the overall assessment of OSS in infertility. Measurement of IL-8, e.g., when combined with SOD or other antioxidants in infertile patients with leukocytospermia, will indicate a positive OSS in this population (Rajasekaran et al, 1995) and should be treated accordingly. Thus, it would be important to assess OSS either in the semen in the male or the vaginal fluids in the female before, during, and after clinical trials. This would be indicative of whether an individual with low OSS is at a reduced risk of infertility. If a positive correlation is observed between OSS and the outcome of the trial, a predictive value could be determined.

Summary

Oxygen toxicity is an inherent challenge to aerobic life, including spermatozoa, the cells responsible for propagation of the species. How this toxicity affects the spermatozoan in its interactions with the ovum is still unknown. An increase in oxidative damage to sperm membranes, proteins, and DNA is associated with alterations in signal transduction mechanisms that affect fertility. Recent evidence suggests that spermatozoa and oocytes pos-

sess an inherent but limited capacity to generate ROS to aid in the fertilization process. Though a variety of defense mechanisms encompassing antioxidant enzymes (SOD, catalase, and GSH peroxidase and reductase), vitamins (E, C, and carotenoids), and biomolecules (GSH and ubiquinol) are available, a balance of the benefits and risks from ROS and antioxidants appears to be necessary for the survival and functioning of spermatozoa. An assay system for the evaluation of OSS needs to be developed. Such an assay will assist the clinician in the assessment of fertility status of both male and female partners. The determination of this OSS value will also theoretically identify the subgroups of responders and nonresponders to any putative antioxidant therapy. Though the therapeutic use of antioxidants appears attractive, clinicians need to be aware of exaggerated claims of antioxidant benefits by various commercial supplements for fertility purposes until proper multicenter clinical trials have been completed.

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