

ROLE OF REACTIVE OXYGEN SPECIES AND ANTIOXIDANTS ON PATHOPHYSIOLOGY OF MALE REPRODUCTION

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ABSTRACT

The excessive generation of reactive oxygen species (ROS) by abnormal spermatozoa and contaminating leukocytes has been defined as one of the few etiologies for male infertility. Administration of antioxidants in patients with 'male factor' infertility has begun to attract considerable interest. The main difficulty of such an approach is our incomplete understanding of the role of free radicals in normal and abnormal sperm function leading to male infertility. Mammalian spermatozoa membranes are very sensitive to free radical induced damage mediated by lipid peroxidation, as they are rich in polyunsaturated fatty acids. Limited endogenous mechanisms exist to reverse these damages. ROS attacks the fluidity of the sperm plasma membrane and the integrity of DNA in the sperm nucleus. ROS induced DNA damage accelerate the germ cell apoptosis. Unfortunately spermatozoa are unable to repair the damage induced by excessive ROS as they lack the cytoplasmic enzymes required to accomplish the repair. Assessment of such oxidative stress status (OSS) may help in the medical treatment. Treatment strategies must be directed toward lowering of ROS levels to keep only a small amount necessary to maintain normal cell function.

KEY WORDS

Oxidative stress, Reactive oxygen species, Male infertility, Oxidative stress status

INTRODUCTION

Infertility has been a major medical and social preoccupation. Despite the enormous progress in research and reasoning, most of the blame for infertility, until recently, was placed on the female. Only during the last 15 years, advances in understanding of gonadal/sperm physiology led to a dramatic increase in our knowledge of male infertility. Defective sperm function is the most prevalent cause of male infertility and is difficult to treat (1). Many environmental, physiological, and genetic factors have been implicated in the poor sperm function and infertility. Although techniques like intracytoplasmic sperm injection (ICSI) offer considerable promise to such male factor patients, the indiscriminate use of such assisted fertility treatments, especially when the etiology of sperm dysfunction is poorly understood is not warranted. Thus, it is very important to identify the factors/

conditions, which affect normal sperm function.

Free radical-induced oxidative damage to spermatozoa is one condition, which is recently gaining a considerable attention even in alcoholic (2) and diabetic men (3) for its role in inducing poor sperm function. Understanding of how such conditions affect sperm function will help in designing new and effective treatment strategies.

ROS, antioxidants and oxidative stress

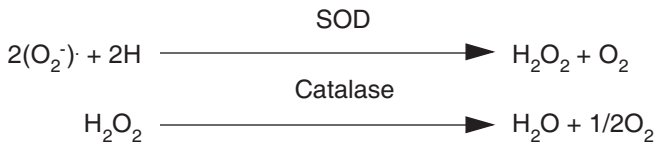
ROS are highly reactive oxidizing agents, with one or more unpaired electrons belongs to the group of free radicals. ROS have a tendency toward chain reaction, in such a manner that "radical begets radical". Most common of those having potential implications in reproductive biology include superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2), peroxy (ROO^-) radical and the very reactive hydroxyl (OH^-) radical. The nitrogen derived free radical nitric oxide (NO^-) and peroxynitrite anion ($ONOO^-$) also appear to play a significant role in the reproduction and fertilization. The ultimate effects of (NO^-) depend upon its concentration and interactions with hydrogen peroxide. Peroxynitrite (oxoperoxonitrate) anion may

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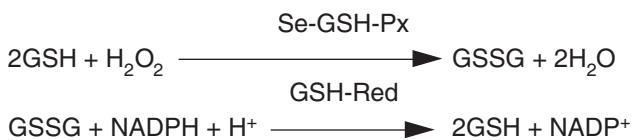
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be formed *in vivo* from superoxide and nitric oxide and actively reacts with glutathione, cysteine, deoxyribose, and other thiols/thioethers (4). This can form a strongly nitrating species in the presence of metal ions or complexes.

ROS must be continuously inactivated to keep only a small amount necessary to maintain normal cell function. Seminal plasma is endowed with an array of antioxidants to protect spermatozoa against oxidants (5-7). Antioxidants, in general, are compounds and reactions, which dispose, scavenge, and suppress the formation of ROS, or oppose their actions. Among the well-known biological antioxidants, SOD and its two isozymes, and catalase have a significant role. SOD spontaneously dismutates (O_2^-) anion to form O_2 and H_2O_2 , while catalase converts H_2O_2 to O_2 and H_2O .



SOD protects spermatozoa against spontaneous O_2 toxicity and lipid peroxidation (LPO) (8). SOD and catalase also remove (O_2^-) generated by NADPH-oxidase in neutrophils and may play an important role in decreasing LPO and protecting spermatozoa during genitourinary inflammation (9). Glutathione peroxidase (Se-GSH-Px) with glutathione (GSH) as the electron donor removes peroxy ($ROO\cdot$) radicals from various peroxides including H_2O_2 (8). Glutathione reductase (GSH-Red) then regenerates reduced GSH from GSSG as shown in the following equation:



A selenium-associated polypeptide, presumably Se-GSH-Px, has been demonstrated in rat sperm mitochondria, which plays a significant role in this peroxy scavenging mechanism and in maintaining sperm motility (10). In addition, Se-GSH-Px and GSH-Red directly act as antioxidant enzymes involved in the inhibition of sperm LPO (11). GSH has a likely role in sperm nucleus decondensation and may alter spindle microtubule formation in the ovum, thus affecting the outcome of pregnancy. In this context, the γ -glutamyl transpeptidase (γ GT), considered being present in the midpiece and acrosomal regions of spermatozoa of certain mammalian species (e.g., the boar) may further affect GSH content of oocyte at the time of sperm penetration (11, 12). A high GSH/GSSG ratio will

help spermatozoa to combat oxidative insult (5).

Chain Breaking antioxidants such as α -tocopherol inhibit LPO in membranes by scavenging peroxy ($RO\cdot$) and alkoxy ($ROO\cdot$) radicals. The ability of α -tocopherol to maintain a steady state rate of peroxy radical reduction in the plasma membrane depends on the recycling of α -tocopherol by external reducing agents such as ascorbate or thiols (13). In this way, α -tocopherol is able to function again as a free radical chain breaking antioxidant, even though its concentration is low (14). Ascorbic acid co administration has a protective effect on testicular oxidative stress and steroidogenic dysfunction in ethanol treated rats (15). For efficient interception, the radical to be interrupted must have a relatively long half-life (16). The peroxy radicals are major reaction partners because their half-life extends into the range of seconds (17).

“Oxidative stress” (OS) is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants commonly known as ROS (18). OS precipitates the range of pathologies that currently are thought to afflict the reproductive function (19, 20, 2). Oxidative stress down regulates the steroidogenic activity leading to altered testicular function (2). 25% to 40% of infertile men had high levels of ROS in semen samples. The generation of ROS has become a real concern because of their potential toxic effects at high levels on sperm quality and function (5).

Leukocytes and spermatozoa as sites of ROS production

Leukocytes are present throughout the male reproductive tract and are found in almost every human ejaculate (20). Leukocytospermia is the presence of peroxidase-positive leukocytes in concentrations of $>1 \times 10^6$ per milliliter of semen (21). Its clinical significance is currently a subject of controversy (22). On one hand leukocytospermia has been linked with poor sperm quality, reduced sperm hyperactivation and defective sperm function (23). On other hand, no correlation was found between seminal leukocyte concentrations and impaired sperm quality (24).

There has been much speculation as to whether the origin of ROS in semen is from spermatozoa or from infiltrating leukocytes (25, 26). The leukocyte free percoll fractions of semen samples obtained from nonazoospermic 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 (27). 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 (27). 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 (28).

Studies by Ollero et al (29) and Gil Guzman et al (30) shown that levels of ROS production in semen were negatively correlated with the percentage of normal sperm forms as determined by the WHO (31) classification and by the strict criteria of Kruger et al (32). There was significant variation in levels of ROS production in subsets of spermatozoa at different stages of development. ROS production was found to be highest in the immature sperm fraction (containing sperm with abnormal head morphology and cytoplasmic retention) and lowest in the mature sperm fraction (containing normal-looking motile sperm) and in immature germ cells.

Male germ cells at various stages of differentiation from pachytene spermatocytes to mature caudal epididymal spermatozoa have the potential to generate ROS (33, 34). Spermatozoa may generate ROS in two ways: 1) the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the level of the sperm plasma membrane (35) and 2) the NADPH- dependent oxido-reductase (diphorase) at the level of mitochondria (36). The mitochondrial system is the main source of ROS in spermatozoa from infertile men (37). Levels of ROS production by pure sperm populations were negatively correlated with the quality of sperm in the original semen. High levels of ROS production in human ejaculates may originate from morphologically abnormal spermatozoa and/or seminal leukocytes (20).

When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective, and spermatozoa are released from the germinal epithelium carrying surplus residual cytoplasm. Under these circumstances, the spermatozoa that are released during spermiation are thought to be immature and functionally defective (22). Retention of residual cytoplasm by spermatozoa is positively correlated with ROS generation via mechanisms that may be mediated by the cytoplasmic enzyme glucose-6-phosphate dehydrogenase (G6PD) (23). Huszar and Vigue (24) have found that sperm morphological irregularities are significantly correlated with high creatine kinase (CK) activity in human spermatozoa. Similarly, recent studies have found an inverse relationship between CK levels and sperm morphological forms and have suggested that CK

levels can be used as a reliable marker for sperm quality and fertilizing potential in subfertile men (38, 39). A positive relationship was found between CK activity and the rate of lipid peroxidation measured by malondialdehyde (MDA) formation, in sperm fractions separated by percoll as per Huszar and Vigue (40).

The exact site of origin of leukocytes in semen, their mode of action, and the role that bacteria, viruses and subsequent genitourinary-inflammation on sperm function are not clear. Experimental ROS production by human spermatozoa and contaminating leukocytes stimulated by phorbol esters and certain formyl peptides results in deleterious effects on sperm motility and fertilization (41). 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 (42). This finding seems paradoxical but does indicate that seminal plasma has significant antioxidant or ROS scavenging capacity, which may prevent sperm damage by leukocytes. An association between leukocytospermia and ROS has been recently found to correlate with increased chemokine (IL-8), and decreased SOD activity of the semen (43). This demonstrates that increased oxidative stress during leukocytospermia is caused by a defective ROS scavenging system, which, in turn, can be modulated by certain proinflammatory cytokines. A significant shift towards increased production of proinflammatory chemokine (GRO- α) compared to anti-inflammatory cytokine (IL-10) during leukocytospermia suggests an active chemotactic pro-inflammatory response (44). This shift may be responsible for a significant oxidative stress to spermatozoa due to leukocytes in the semen of the infertile patient (5). Based upon these observations, it may be useful to assess the oxidative stress status (OSS) of semen in infertile or subfertile patients, particularly those with chronic genitourinary inflammation.

ROS and sperm function

Until recently, ROS were exclusively considered toxic to the human spermatozoa. However a strong body of evidence suggests that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities (45-48). The ultimate goal of spermatozoa is the successful fertilization of ovum resulting in normal conception. 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 pellucida of the ova as well as the

oolemma, and finally fuse with the female pronucleus. Low levels of ROS can enhance the ability of human spermatozoa to bind with zona pellucida. Other studies have found that incubating spermatozoa with low concentrations of hydrogen peroxide stimulates sperm capacitation, hyper activation, acrosome reaction and oocytes fusion (49). Reactive oxygen species other than hydrogen peroxide such as nitric oxide and superoxide anion have also been shown to promote sperm capacitation and acrosome reaction (50).

Theoretically, cellular damage in the semen is the result of an improper balance between ROS generation and scavenging activities i.e. positive oxidative stress status (OSS), a situation in which there is a shift towards pro-oxidants, because of either excess ROS or diminished antioxidants. Levels of antioxidants in seminal plasma from infertile men are significantly low (51). However pathological levels of ROS detected in semen from infertile men are more likely a result of increased ROS production rather than reduced antioxidant capacity of the seminal plasma (52). Mammalian spermatozoa are rich in polyunsaturated fatty acids and, thus, are very susceptible to ROS attack which results in a decreased sperm motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability, and increased midpiece morphology defects with deleterious effects on sperm capacitation and acrosome reaction (53). Lipid peroxidation of sperm membrane is considered to be the key mechanism of this ROS-induced sperm damage leading to infertility (54).

Biological implications of LPO and oxidative stress to spermatozoa

Spermatozoa, unlike other cells, are unique in structure, function, and susceptibility to damage by LPO (8). Spermatozoa are unable to repair the damage induced by excessive ROS because they lack the cytoplasmic enzyme systems that are required to accomplish this repair. This is one of the features that make spermatozoa unique in their susceptibility to oxidative insult (26). In order to understand the biological mechanisms of LPO in infertility, three important questions need to be addressed: (a) What are the mechanisms of LPO of sperm *in vivo*? (b) What are the consequences of damage to sperm membrane, proteins, and nucleic acids? (c) What regulates the antioxidant defense mechanisms in seminal plasma?

Lipid peroxidation of spermatozoa

Lipid peroxidation (LPO) is the most extensively studied

manifestation of oxygen activation in biology. LPO is broadly defined as “oxidative deterioration of PUFA” which are fatty acids that contain more than two carbon carbon double bonds (51). The most common types of LPO are: (a) nonenzymatic membrane LPO, and (b) enzymatic (NADPH and ADP dependent) LPO. The enzymatic reaction involves NADPH-cytochrome P-450 reductase and proceeds via an ADP-Fe³⁺ O₂⁻ (perferryl) complex (55). In spermatozoa, production of malondialdehyde (MDA), an end product of LPO induced by ferrous ion promoters, has been reported (56) which 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 (56).

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 Ca²⁺ homeostasis. Minor alterations in sperm membranes in selected cases of dyspermia can be reversed by GSH therapy (57). Studies on how these cellular changes caused by LPO affect seminal parameters and sperm function and reversal of these effects are open to further investigations

Impairment of sperm motility

The increased formation of ROS has been correlated with a reduction of sperm motility (53, 58). The link between ROS and reduced motility may be due to a cascade of events that results in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with a reduction in membrane fluidity that is necessary for sperm oocyte fusion (59). Another hypothesis is that H₂O₂ can diffuse across the membranes into the cells and inhibit the activity of some enzymes such as glucose 6-phosphate dehydrogenase (G6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which in turn controls the intracellular availability of NADPH. This, in turn, is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase (60). Inhibition of glucose-6 phosphate dehydrogenase (G6PD) leads to a decrease in the availability of NADPH and a concomitant accumulation of oxidized glutathione, which in turn can reduce the antioxidant defenses of the spermatozoa and peroxidation of membrane lipids (61).

Oxidative stress induced DNA damage

The oxidative damage to mitochondrial DNA is well known to occur in all aerobic cells, which are rich in mitochondria and this, may include spermatozoa. Two factors protect the sperm deoxy ribonucleic acid (DNA) from oxidative insult: the characteristic tight packing of the DNA and the antioxidants present in the seminal plasma (62). ROS induces DNA damage in the form of modification of all bases (primarily guanine via lipid peroxy or alkoxy radicals), production of base-free sites, deletions, frameshifts, DNA cross-links through covalent binding to MDA, and chromosomal rearrangements (63). Oxidative stress has also been associated with high frequencies of single and double DNA strand breaks (62, 64). ROS can also induce oxidation of critical -SH groups in proteins and DNA, which will alter structure and function of spermatozoa with an increased susceptibility to attack by macrophages (65). Strong evidence suggests that high levels of ROS mediate the DNA fragmentation commonly observed in spermatozoa of infertile men (66, 67).

In addition, the redox status of human spermatozoa is likely to affect phosphorylation and ATP generation with a profound influence on its fertilizing potential (68). Aitken *et al.* recently showed that stimulation of endogenous NADPH-dependent ROS generation in human sperm appears to regulate acrosome reaction (9) via cAMP mediated, tyrosine phosphorylation (69). In general, the oxidizing conditions increase tyrosine phosphorylation with enhanced sperm function while reducing conditions have the opposite effect. However, this has been debated for a long time, and it is still not clear whether sperm have a NADPH-dependent oxygenase system. Nonetheless, how these mitochondrial DNA or membrane changes regulate specific sperm functions in association with altered tyrosine phosphorylation is an interesting area for further investigation. These studies may open a new series of diagnostic tool in clinical infertility to assess sperm function and damage.

Oxidative stress and sperm suicide (apoptosis)

High levels of ROS disrupt the inner and outer mitochondrial membranes resulting in release of cytochrome-C protein from the mitochondria that activates the caspases and induces apoptosis (70, 71). A ROS dependent pathway for apoptosis was suggested based on the finding that H_2O_2 induces apoptosis in cell cultures (70). Apoptosis in sperm may also be initiated by ROS-independent pathways involving the cell surface protein Fas (Fas is a member of the tumour necrosis factor (TNF) receptor family) (72). It is also reported that, B-

cell lymphoma/leukaemia-2 (Bcl-2), the inhibitor gene of programmed cell death, protects the cells from apoptosis, probably by mechanisms that reduce ROS production (73).

The relative proportion of ROS-producing immature sperm was directly correlated nuclear DNA damage values in mature sperm and inversely correlated with recovery of motile, mature sperm. These interesting findings led to the hypothesis that oxidative damage of mature sperm by ROS producing immature sperm during their comigration from seminiferous tubules to the epididymis may be an important cause of male infertility.

Assessment of oxidative stress

However, many men who demonstrate normal parameters on standard semen analysis remain infertile suggesting the routine semen analysis (measurement of seminal volume, spermatozoal motility, density, viability and morphology) does not necessarily provide complete diagnostic information (19). As a result of active research in the area of evaluation of human semen, a series of sperm function assays have been developed. However, no single test is capable of evaluating all of the steps involved in fertilization. At present only a combination of assays complementing each other can provide a comprehensive evaluation of sperm function (74). Although, ideal tests of sperm function will markedly improve the clinician's ability to diagnose male factor infertility and help in its management, evaluation of the potential causes of sperm damage leading to abnormal sperm function and infertility is an important area of investigation.

In many complex biological systems including semen, the true ROS status leading to oxidative stress reflects a relative balance between the ROS-generated and ROS-scavenged. The measurement of the rate of ROS generation by luminol induced chemiluminescence has been the most common method for quantitating ROS. Although this rate measurement is dynamic, it may not accurately reflect the status of potential sperm damaging ROS. For such evaluations, the amount of ROS-detected, rather than the ROS-generated will represent a more physiological assessment of oxidative stress (17). The methods commonly used for measuring ROS can be categorized into: (a) reactions involving nitroblue tetrazolium (NBT) or cytochrome C- Fe^{3+} complexes which measure ROS on the cell membrane surface, (b) reactions that measure ROS (generated inside or outside the cell) utilizing luminol-dependent chemiluminescence, and (c) the 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.

Oxidative stress status (OSS) evaluation

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 anti-oxidants. 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, Se-GSH-Px, GSH-Red, GSH) in semen correlates with idiopathic infertility (75). 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 (17). This will result in increased LPO, decreased sperm motility, viability and function, and ultimately leads to infertility. Direct detection of free radicals is only possible by electron spin resonance (ESR or EPR for electron paramagnetic resonance). Unfortunately this method is restricted to expensive laboratory equipment and – even more limiting – to cell free systems, tissue culture and small organisms. Peroxides are among the most important ROS generated by free radical action. There are several different methods for their detection. The most important ones are luminometric and colorimetric methods, which are based on the peroxide-peroxidase reaction, which leads to a light emission or colour production. Assessment of the rate of ROS production/generation using luminol as a probe can be a dynamic measure of oxidative stress (28). Due to their simplicity, these methods are frequently used. However, clinically the evaluation of this ROS generation is limited by a very short half life of these free radicals (25).

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). MDA measurements are only sufficiently specific if the detection is carried out by HPLC. In principle, aldehydes react with thiobarbituric acid to form a reddish colour complex. (Thiobarbituric acid reactive substances, TBARS). In the colorimetric version all side reaction products, which usually also have reddish colours, contribute to the reaction unless they are separated by HPLC. 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

the oxidative damage to sperm (8). 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 OSS in infertility. Measurement of IL-8, for example, when combined with assessment of SOD or other antioxidants in infertile patients with leukocytospermia will indicate a positive OSS in this population and can be treated accordingly (76).

Specific detection methods for single antioxidants (e.g. vitamin C or E) are usually based on HPLC methods requiring special equipment, but are very exact. On the other hand there are several colorimetric methods, which give a less specific summary of actions of all antioxidants present in samples. Their main disadvantage is the fact that actions of unwanted antioxidants, such as uric acid or bilirubin, contribute to the signal, thus giving results which indicate a sufficient antioxidant defense. A third set of methods is based on the so called lag phase measurement assuming that LDL particles remain unchanged as long as they are protected by antioxidants. These methods reflect the protective effect of antioxidants quite realistically, but require specific equipment (ultracentrifuge, UV-photometer, fluorimeter) and therefore are restricted to specialized laboratories. Finally peroxidases, which also have peroxide detoxifying properties and therefore antioxidant like functions can be determined photometrically, but care must be taken that no haemolytic samples are assayed.

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 any clinical studies. This would be indicative that an individual with low OSS does not contribute to the infertility. If a positive correlation is observed between OSS and the outcome of the trial, a predictive value could be determined.

CONCLUSIONS

Oxygen toxicity is an inherent challenge to aerobic life forms, including the spermatozoa. How this toxicity affects sperm function is still unknown. Increased oxidative damage to sperm membranes (indicated by increased LPO), proteins, and DNA is associated with alterations in signal transduction mechanisms that affect fertility. Spermatozoa possess an inherent but limited capacity to generate ROS which may help the fertilization process. A variety of defense mechanisms encompassing antioxidant enzymes (SOD, catalase, glutathione peroxidase and reductase), vitamins (E, C, and carotenoids), and biomolecules (glutathione and ubiquinol) are involved in biological systems. A balance between the benefits

and risks from ROS and antioxidants appears to be necessary for the survival and normal functioning of spermatozoa. An assay system for the evaluation of oxidative stress status (OSS) may aid the clinician in the assessment of fertility status of both male and female partners. Determination of this OSS value will also theoretically identify the subgroups of responders and non-responders to any putative antioxidant therapy.

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