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WHAT AN ANDROLOGIST/UROLOGIST SHOULD KNOW ABOUT FREE RADICALS AND WHY

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Free radicals are a group of highly reactive chemical molecules that have one or more unpaired electrons and can oxidatively modify biomolecules that they encounter.[1] Reactive oxygen species (ROS) are free radicals that are derived from the metabolism of oxygen. They are present in all aerobic organisms. ROS play an important role in many physiologic functions such as phagocytosis, which is mediated by neutrophils and macrophages. They also act as a cell messenger, taking part in many signaling processes, mainly by oxidatively modifying the compounds.[2] However, ROS are highly reactive, and when their levels are high, they can react with any nearby molecules and cause defects in the functioning of these molecules. Oxidative stress is a condition that occurs as a result of excessive generation of ROS and diminished capacity of free radical scavenging by antioxidants.[3] Many diseases, including cancer, are thought to be the consequence of reactions between free radicals and DNA, resulting in germ line mutations that are transmitted to offspring.[4] Some pathologic processes such as aging, ischemic heart disease, sickle cell anemia, and rheumatoid arthritis are also attributed to free radical-induced oxidation of various biologic components.[5]

ROS include the various radicals such as superoxide, hydroxyl, peroxyl, and hydrogen peroxide. In addition, another class of free radicals that are nitrogen derived is called reactive nitrogen species. They are considered a subclass of ROS.[6] Nitrous oxide, peroxynitrite, nitroxyl anion, and peroxynitrous acid are some of the most common reactive nitrogen species.

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Even though free radicals were first described in spermatozoa by MacLeod in 1943,[4] their role in male infertility was not studied extensively until the last decade. Research in the last two decades has confirmed the physiologic and pathologic significance of free radicals in human reproduction.[7–9] Studies have shown that 30% to 80% of unselected infertile patients have high levels of seminal ROS. It is important for andrologists to understand free radicals because of their effect in decreasing male fertility potential in the general population and increasing the risk of testicular cancer by altering the sperm DNA.[10,11]

Although almost all cellular and metabolic processes generate free radicals, an andrologist should be more concerned with the sources of ROS in the male reproductive system. Leukocytes, particularly neutrophils and macrophages, have been associated with excessive ROS production, and they ultimately cause sperm dysfunction.[12] Another important source of ROS is immature and morphologically abnormal spermatozoa.[13] The production of ROS is also increased by lifestyle factors such as smoking and pollutions. Smoking increases ROS production, causing sperm DNA damage, and suppresses antioxidants in both semen and serum.[14] A study from our group has demonstrated that a significant negative correlation exists between ROS production and semen quality.[15] Furthermore, as the concentration of immature spermatozoa in the human ejaculate increases, the concentration of mature spermatozoa with damaged DNA also increases.[15] In assisted reproductive techniques, mechanical injury to spermatozoa (eg, repeat centrifugation during sperm preparation) and external conditions such as high oxygen tension can increase ROS production.[16]

PHYSIOLOGIC ROLE OF FREE RADICALS

Free radicals play a vital role in the physiologic functioning of the spermatozoa. Research has shown that spermatozoa require a small amount of ROS for various reactions to achieve fertilizing capacity.[17] Hydrogen peroxide stimulates sperm capaci-

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tation by hyperactivation and acrosome reaction.[18] According to Zini *et al.*,[19] superoxide and nitric oxide also take part in these processes. Free radicals are also involved in the fusion of spermatozoa with the oocyte.[18] Nitric oxide plays a role in the sperm's ability to fuse with oocyte, but it has no action in zona pellucida binding.[20] Low concentrations of hydrogen peroxide cause tyrosine phosphorylation, which in turn results in the binding of the spermatozoal membrane proteins with ZP-3 proteins on the zona pellucida and ultimately, spermatozoa-ooctye fusion.[21]

However, many questions regarding the physiologic action of ROS remain unanswered. One question is whether the inability of spermatozoa to produce free radicals results in a decreased fertilizing capacity of spermatozoa. Another is whether increased levels of antioxidants cause scavenging of physiologic levels of free radicals and thus sperm dysfunction.

SPERM DYSFUNCTION AND FREE RADICALS IN SEMEN

Excessive levels of free radicals alter normal physiologic functioning of spermatozoa. ROS appears to have a spectrum of variable effects on spermatozoa depending on the extent of oxidative stress.[22] For example, a study by Aitken *et al.*[23] reported that a low concentration of hydrogen peroxide did not have any effect on sperm motility but did suppress sperm-egg fusion. This may also explain why patients with normal semen parameters can still experience infertility. In such patients, the ROS levels are not high enough to impair basic semen analysis parameters but can cause defects in other processes that are required for fertilization, such as sperm-oocyte interaction.

Excessive ROS levels are related to an increase in lipid peroxidation of the sperm plasma membrane.[24] Lipid peroxidation results in loss of membrane fluidity, which is essential for sperm motility and sperm-oocyte fusion. The acrosome reaction is defective in spermatozoa with a high concentration of ROS. Griveau and Le Lannou[25] reported that the acrosome reaction in human spermatozoa appeared susceptible to ROS and that hydrogen peroxide inactivates several enzymes, including glutathione peroxidase, superoxide dismutase, and glucose-6-phosphate. Several studies have noted that levels of ROS correlate with motility. Motility is impaired either because of adenosine triphosphate depletion in axons or lipid peroxidation of the sperm plasma membrane.[26] In inflammation and infection, ROS generation by leukocytes becomes uncontrolled and leads to excessive ROS levels in tissues. Even when the initially stimulated leukocytes become burnt out, the process of ROS generation continues by the stimulation of surrounding leukocytes and spermatozoa by mediation of cytokines (interleukin-6 and interleukin-8).

FREE RADICALS AND PREGNANCY OUTCOME

Clinicians and patients alike want to know whether levels of ROS in semen can directly predict fertility. Very few studies have analyzed such a direct relationship between ROS levels and pregnancy outcome. In a prospective study, ROS generation was shown to be negatively associated with fertility in vivo.[27] By using the ROS-total antioxidant capacity (TAC) score, another study found that seminal oxidative stress levels were lower in male patients whose partner achieved pregnancy than in those patients whose partner did not (mean ROS-TAC score 47.7 ± 13.2 versus 35.8 ± 15.0; $P < 0.01$).[28]

ROS may also play a role in the outcome of pregnancy as evidenced by the ability of antioxidants to increase the pregnancy rate in infertile patients. Some studies have demonstrated an improvement in the pregnancy rate after antioxidant supplementation. Oral administration of vitamin E (300 mg) twice a day in a randomized, double-blind, placebo-controlled trial showed significant improvement in pregnancy rates (21%; 11 of 52) in infertile patients, but not in the placebo group.[29] Combination therapy of *N*-acetyl-cysteine or vitamin A and E and essential fatty acids significantly reduced ROS and improved pregnancy rates.[30] Several studies have demonstrated the ability of carnitines (L-carnitine 1 g and acetyl-carnitine 0.5 g twice a day) to reduce ROS production significantly and increase the chances of pregnancy.[31]

FREE RADICALS AND ASSISTED REPRODUCTIVE TECHNIQUES

Various methods of assisted reproductive techniques (ARTs) have recently been developed. They were originally designed for treatment of female factor infertility, but are now used extensively for the treatment of male factor infertility. Intrauterine insemination, in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI) are some of the important ARTs. The levels of ROS in mature spermatozoa correlate significantly with the fertilizing potential of spermatozoa.[32] High ROS levels are associated with a reduced pregnancy rate after IVF or ICSI and arrested embryo growth.[32] A recent meta-analysis by our group found that ROS levels correlated significantly with the IVF fertilization rate (estimated overall correlation -0.374, 95% CI -0.520 to -0.205).[33] Measuring ROS levels in semen specimens before IVF may be useful in predicting IVF outcome and in counseling selected patients with male factor or idiopathic infertility.

One of the main disadvantages of ARTs is the absence of a natural selection process. It is possible that sperm damaged by free radicals may occasionally be used for fertilization, resulting in early embryo death when the paternal gene is switched on. ICSI is the most promising ART, but it is associated with the greatest number of miscarriages. This may be because embryologists are unable to choose sperm without DNA damage selectively and as a result, damaged sperm get injected into an oocyte.[17]

Sperm separation techniques such as density gradient centrifugation, migration-sedimentation, glass wool filtration, and conventional swim-up method play a critical role in separating out spermatozoa with high motility. The first three methods (density gradient centrifugation, migration-sedimentation, and glass wool filtration) are more effective in significantly reducing levels of free radicals than the conventional swim-up technique.[34] However, repeated centrifugation causes mechanical injury to spermatozoa and increases ROS production.[16] Researchers are currently studying the use of antioxidants and other substances to prevent ROS generation during sperm preparation processes.

FREE RADICALS IN SPERM DNA DAMAGE AND APOPTOSIS

Free radicals can cause changes in genetic information by oxidatively modifying the constituents of DNA. A study by Shen *et al.* has demonstrated that smoking increases the DNA damage mediated by oxidative stress.[35] The extent of oxidative damage to DNA can be estimated by measuring 8-hydroxy-2-deoxyguanosine.[10] Normally, when free radicals damage the sperm plasma membrane and DNA, the damaged spermatozoa lose their ability to fertilize an oocyte. However, this may not be the case with ARTs in which fertilization occurs despite dysfunctional plasma membrane; thus, the chance that DNA-damaged sperm will fertilize an oocyte is increased. The percentage of sperm with DNA damage correlated negatively with the fertilization rate in a study by Sun *et al.*[36] in 298 semen samples from couples undergoing IVF. In a similar study on patients undergoing ICSI, Lopes *et al.*[37] observed that the DNA damage measured by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay correlated negatively with the fertilization rate, which was as low as 20%. The damaged DNA in spermatozoa may also affect embryo development.[38] Oocytes can repair DNA damage to some extent, but when the damage is severe, embryo death, miscarriage, and congenital malformations occur.

Apoptosis is a physiologic phenomenon in the body that helps to discard abnormal spermatozoa. ROS generated from abnormal spermatozoa may stimulate the process of apoptosis, resulting in the death of spermatozoa. ROS may initiate a chain of reactions by activating caspases that ultimately lead to apoptosis.[39] When ROS levels are raised pathologically, the process of apoptosis may also be initiated in mature spermatozoa. The process of apoptosis may be accelerated by ROS-induced DNA damage, which ultimately leads to a decline in the sperm count. Oxidative stress due to excessive generation of ROS is presumed to cause spermatozoa DNA damage and has correlated positively with apoptosis.[40] According to a retrospective study by Host *et al.*,[41] apoptosis correlated negatively with the fertilization rate.

FREE RADICALS AND CLINICAL DIAGNOSES OF INFERTILITY

Ongoing research has expanded the list of clinical diagnoses in patients with infertility in which ROS are implicated as executors of the pathologic findings. Up to 95% of patients with spinal cord injury are known to have elevated seminal levels of ROS.[42] The cause may be a result of prolonged stasis of semen in the reproductive tract and inflammation. The role of ROS in varicocele has also been extensively investigated. Elevated ROS levels and decreased TAC levels have been noted in patients with varicocele.[43] Excessive release of nitric oxide within dilated spermatic veins has been observed in adults with varicocele, which suggests that high levels of oxidative stress are present.[44] Formation of peroxynitrite from the reaction of nitric oxide with superoxide could be a causative factor for impaired sperm function in patients with varicocele.[45] One study found reduced ROS levels and increased antioxidant activity in seminal plasma after varicocelectomy in infertile men with varicocele.[46] A positive correlation was noted between ROS levels and the varicocele grade.[22]

When infection of the male reproductive tract occurs, one can obviously expect levels of ROS to be elevated owing to infiltration by white blood cells. In patients with prostate-vesiculo-epididymitis, the ROS levels were raised compared with patients without prostate-vesiculo-epididymitis.[47] In the former group of patients, the pregnancy rate improved after treatment with nonsteroidal anti-inflammatory drugs and antioxidants.[48] Idiopathic infertility in which routine semen parameters are normal and no other cause of infertility is present may be a result of ROS-mediated damage.[49] Patients with such a form of infertility with elevated ROS are expected to show a good response to antioxidant treatment.

INFERTILITY DUE TO ELEVATED FREE RADICALS IN SYSTEMIC DISEASES

Excessive generation of ROS may be an important cause of many diseases such as diabetes mellitus, cardiovascular diseases, systemic infection, and cancer.[4,5,50] These diseases are known to affect fertility in different ways. In diabetes mellitus, increased production of ROS is a result of resultant hyperglycemia, which in turn affects the blood vessels and nerve endings and can cause erectile dysfunction.[50] Many drugs, such as quinines and nitroso-aromatic compounds, generate free radicals.[5] Moreover, detoxification of even commonly used drugs such as aspirin and acetaminophen can increase ROS production by increasing cytochrome P450 activity.[51] Environmental conditions such as radiation and pollution also increase levels of oxidative stress.[52] An andrologist should be aware of the above systemic conditions and external factors that can cause a generalized increase in oxidative stress status in the human body and perhaps also in the reproductive tract. By identifying high oxidative stress as a cause of infertility in such conditions, an andrologist can initiate appropriate therapeutic interventions directed against oxidative stress.

ANTIOXIDANTS

The human body has a number of mechanisms to minimize free radical-induced damage and to repair damage that has already occurred. However, spermatozoa are unique in human cells in that they are unable to repair oxidative damage because of the lack of cytoplasmic enzymes. Three different antioxidant protection systems provide the necessary protection against the damaging effect of free radicals: dietary antioxidants that are obtained as part of our regular diet; endogenous antioxidants that are the part of body's own defense mechanism; and the metal-binding proteins that combine with the metals and prevent their catalytic effect.[53]

Endogenous antioxidants are the group of antioxidants consisting of low-molecular-weight compounds, such as bilirubin, thiols, uric acid, and coenzyme Q10, and larger molecular enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase. According to a study conducted by Michiels *et al.*,[54] all three of these enzymes are essential for adequate antioxidant defense and all of them act in a synergistic manner. In a study of 38 asthenospermic patients, in vivo administration of coenzyme Q10 increased the motility of the spermatozoa.[55]

Diet should consist of an adequate amount of chain-breaking antioxidants such as vitamin C or vitamin E, in addition to beta-carotenes, carotenoids, and flavonoids. In a study conducted by Kessopoulou *et al.*,[56] oral administration of vitamin E improved sperm function. Similar studies have been conducted on other dietary antioxidants.

Metal-binding proteins, such as albumin, ceruloplasmin, metallothionein, transferrin, ferritin, and myoglobulin, form a valuable additive to the antioxidant defense system by inactivating the transition metal ions, which would otherwise catalyze the production of free radicals.[1] A study conducted by Twigg *et al.*[57] on healthy normozoospermic donors demonstrated the protective effect of albumin either by binding to lipid peroxides or binding to ferrous ion promoters. However, it should be noted that in some circumstances antioxidants can act as pro-oxidants. Thus, additional research is required in terms of the dose and combination of antioxidants to be used clinically.

EVALUATION OF PATIENTS FOR OXIDATIVE STRESS

Numerous methods are available to determine ROS levels in semen. Of these, chemiluminescence is the most widely used method. It can discriminate between the production of superoxide and hydrogen peroxide by spermatozoa depending on the probes used.[58] Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multiparameter data measures. It measures the fluorescent intensity of different dyes such as 2,7 dichlorofluorescin diacetate (DCFH-DA), hydroethidine after oxidation by ROS and thus quantifies the amount of ROS present in the semen.

Many indirect methods are available for the detection of ROS-induced damage to lipid membranes. The thiobarbituric acid assay is commonly used. Thiobarbituric acid-reactive substances such as malondialdehyde are measured by spectrophotometry analysis.[59] The TAC of the semen is determined by either the enhanced chemiluminescence assay or the calorimetric assay. To measure the overall oxidative stress, the ROS-TAC score can be used, which was developed by principal component analysis. A low ROS-TAC score indicates the presence of high oxidative stress and thus sperm dysfunction.[28]

Currently, no standard and clinically useful methods are available for estimating oxidative stress. Many innovative methods have been used in other fields of medicine such as electron spin resonance and aromatic trapping for the determination of free radicals. These methods can be used, along with other currently accepted methods, to evaluate and standardize the accurate measurement of free radicals in andrology. Recently, we attempted to define the basal levels of ROS in normal donors in neat (whole unprocessed) semen specimens and in mature and immature spermatozoa.[60] These basal val-

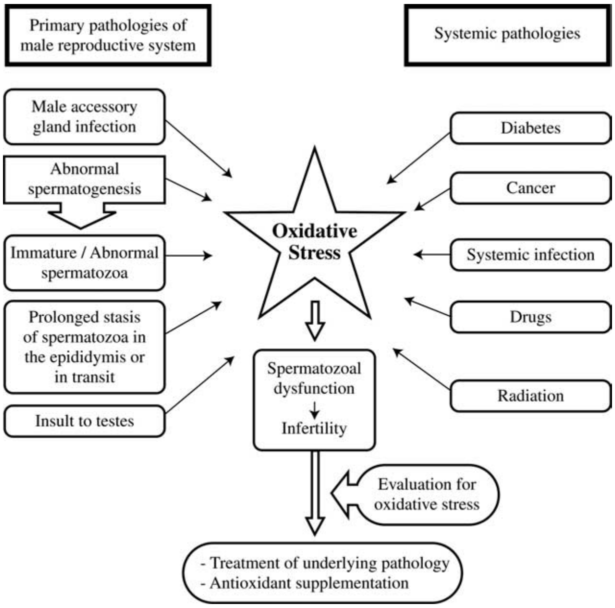

FIGURE 1. *Origin of oxidative stress in the etiopathogenesis of male infertility and its management.*

ues can be used to identify the pathologic levels of ROS in infertile men and may guide therapeutic interventions. We have attempted to provide a schematic representation of the various factors influencing oxidative stress and its management (Fig. 1).

OXIDATIVE STRESS: CAUSE OR EFFECT

Do excessive free radicals cause male infertility or are they a consequence of a pathologic process in the male reproductive tract (ie, are they byproducts without any significance)? This is a major question that has not been clearly answered and has major therapeutic implications. If the second situation is true, no scientific rationale exists for the use of antioxidants. However, the current evidence indicates that the first situation is correct. The body's defense mechanism produces leukocytes against infections such as prostatitis and urinary tract infection. Leukocytes are the major producers of free radicals.[61] They are required for the effective defense against the invading pathogens. Varicocele has also been documented to increase the free radical generation in seminal plasma. Lifestyle behaviors such as smoking, alcohol abuse, and exposure to radiation and pollution increase ROS production. Hence, production of free radicals can be considered as secondary to the primary disease process. However, they themselves cause the damage to the spermatozoa resulting in the infertility. It affects the sperm plasma membrane integrity and damages DNA. Thus, they are the cause of the clinical effects of the primary pathologic processes; therefore, methods to decrease their actions have therapeutic implications.

CONCLUSIONS

It is crucial for andrologists to understand free radicals—their sources, mechanism of generation, and the damage they can cause to the male reproductive system. In addition, it is also essential to be aware of the various diseases that increase ROS generation in the blood, plasma, and seminal fluids. A multifaceted approach is required for the treatment of male infertility induced by free radicals. Methods can be used to decrease ROS production (eg, the addition of antioxidants during sperm preparation techniques). With the abundance of many synthetic and natural antioxidants, it is critical to use them judiciously. The critical mechanism of pathologic free radical generation and crucial antioxidant protective systems in the male reproductive system are beginning to be elucidated for therapeutic targeting. Additional research is needed in this direction before they can be used clinically.

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