Clinical Relevance of Oxidative Stress in Patients With Male Factor Infertility: Evidence-Based Analysis

Learning Objective: At the conclusion of this continuing medical education activity, the participant will have a thorough understanding of how oxidative stress affects male infertility as well as how treatment of varicocele decreases oxidative stress and improves fertility.

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INTRODUCTION

Infertility is defined as the inability to conceive after 1 year of unprotected intercourse by couples of reproductive age. According to a national survey of family growth conducted in 1995 by the Centers for Disease Control and the National Institutes of Health, male factor infertility is the causative factor in approximately 40% of the 2.1 million infertile young married couples in the United States. Half of these men experience irreversible infertility and cannot father children. There are a number of common causes of infertility in men including hormonal and gonadal disorders, medical conditions such as cystic fibrosis and sickle cell anemia, renal diseases and urological conditions such as cryptorchidism, varicoceles, testicular cancer, and reproductive tract trauma and/or obstruction. These disorders can temporarily or permanently cause impaired spermatogenesis, diminish sperm migration in the male and female reproductive tracts due to defective motility, result in poor morphology and affect sperm function (acrosome reaction, sperm oocyte fusion), all of which can prevent conception. Many of these conditions increase the production of reactive oxygen/nitrogen species causing oxidative stress, which leads to spermatozoal dysfunction and infertility. This review is designed to assist with the understanding of oxidative stress and its role as related to male factor infertility in clinical practice.

WHAT IS OXIDATIVE STRESS?

Oxidative stress is a cellular condition associated with an imbalance between the production of free radicals, mainly ROS, and their scavenging capacity by antioxidants. When the production of ROS exceeds the available antioxidant defense, significant oxidative damage occurs to many cellular organelles by damaging lipids, proteins, DNA and carbohydrates, thus ultimately leading to cell death. Sperm is particularly susceptible to oxidative damage due to its unique structural composition. In the process of maturation spermatozoa extrude cytoplasm. Since cytoplasm is the major source of antioxidants, lack of cytoplasm causes a deficiency in antioxidant defense. Ironically, when this process is hindered, residual cytoplasm forms a cytoplasmic droplet in the sperm mid region. The residual cytoplasm contains high concentration of some cytoplasmic enzymes (G6PDH, SOD), which are also a source of ROS. In addition, the sperm plasma membrane is rich in polyunsaturated fatty acids that readily undergo lipid peroxidation by ROS, resulting in a loss of membrane integrity.

Immature spermatozoa and seminal leukocytes are the major sources of ROS in semen. In abnormal spermatozoa ROS may be generated at the level of the plasma membrane (nicotinamide adenine dinucleotide phosphate oxidase system) or mitochondria (NADH dependent oxido-reductase). Seminal leukocytes produce H2O2 through the NADPH oxidase system. H2O2 is the most toxic form of ROS for spermatozoa since it is membrane permeable and readily affects the cellular organelles, whereas superoxide and the hydroxyl radical are membrane impermeable and take time to exert their effect. Nitric oxide reacts with the superoxide anion to yield the highly reactive metabolites peroxynitrite and peroxynitrous acid, both of which are strong oxidants. Numerous studies now consider oxidative stress to be a real entity that is likely to have a significant impact on normal sperm function, thus affecting reproduction and fertility (fig. 1).

ROLE OF ROS IN MALE REPRODUCTIVE SYSTEM

Physiological aspects. ROS are required by spermatozoa to attain functional maturity. Low levels of ROS are essential for normal fertilization, capacitation, hyperactivation, motility and acrosome reaction. Spermatozoa acquire fertilizing ability after capacitation occurs in a favorable environment. Capacitation is a process that prepares the spermatozoa for interaction with oocyte leading to fertilization. During this process the concentrations of intracellular calcium, ROS and tyrosine kinase increase, resulting in an increased formation of cyclic adenosine monophosphate. A high concentration of cyclic adenosine monophosphate facilitates the spermatozoa to acquire high motility known as hyperactivation.

Only capacitated sperm exhibit hyperactivated motility and undergo a physiological acrosome reaction thereby acquiring the ability to fertilize. Several studies have reported the role of ROS in promoting sperm capacitation in human and animal models. Sperm capacitation is involved in a number of biochemical events including an increase in redox mediated protein tyrosine phosphorylation. ROS also exert their effect on sperm oocyte interaction. Low levels of lipid peroxidation cause modifications of plasma membranes facilitating sperm adhesion to the oocyte. However, the physiological level of ROS in semen and/or duration of exposure for normal sperm function leading to fertilization is not yet established. Knowing the physiological levels of ROS seems important especially in understanding the etiology of idiopathic male factor infertility.

Pathological aspects. Evidence suggests that high levels of ROS mediate damage to many cellular elements in the testis including DNA of mature spermatozoa. A common by-product of DNA oxidation, 8-hydroxy-2-deoxyguanosine, has been considered a key biomarker of this oxidative DNA damage. Oxidative stress causes abnormal denaturation into single-stranded DNA and double-stranded DNA breaks, DNA base-pair oxidation, chromatin cross-linking, chromosome microdeletions etc. ROS cause various types of gene mutation such as deletion, point mutation or polymorphism, resulting in decreased semen quality.

When the level of DNA damage is less, spermatozoa can undergo self-repair. Oocyte also is capable of repairing damaged...
DNA of spermatozoa. However, if the damage is extensive, apoptosis and embryo fragmentation can occur. Decreased fertilization rate, and poor embryo cleavage and quality have been reported in infertility cases where sperm samples contain a high frequency of damaged DNA. DNA damage in the Y chromosome can cause gene deletion in the Y chromosome of the offspring leading to infertility.

The damage to sperm DNA is critical in the context of assisted reproductive technology, which is being increasingly used to treat infertile couples. The main disadvantage of ART is that it bypasses the natural selection barriers that are present throughout the female reproductive tract before sperm enter the oocyte. With ART, sperm with abnormal genetic material can reach the oocyte with minimal (in vitro fertilization) or no (intracytoplasmic sperm injection) effort. The potential to introduce sperm with damaged DNA by ART has triggered a great deal of scientific interest and debate among clinicians and basic scientists.

Recently some studies have reported a significant disruption of mitochondrial DNA in the testes. Mitochondria are the powerhouse of human cells including spermatozoa. Any disruption to the mitochondrion or its genetic materials causes decreased sperm motility. Some of the proposed mechanisms for oxidative damage to mitochondria are loss of DeltaPsim (mitochondrial electrochemical gradient-dependent membrane potential), low levels of 32 to 30 kDa mature forms of steroidogenic acute regulatory protein and cessation of cholesterol transport. These mechanisms likely contribute to a host of pathophysiological events evident in testicular disorders such as infection, cryptorchidism and varicocele. The 4977 bp deletion of mitochondrial DNA in spermatozoa was detected by polymerase chain reaction, although how it affects fertilizing capacity is not known. ROS can also damage mitochondrial DNA to a higher degree than nuclear DNA. Such damage is believed to be the cause of several human diseases including infertility. Higher amounts of 8-OHdG were detected in mitochondrial DNA than in nuclear DNA. Closer proximity of DNA to ROS generating electron transport chain in mitochondria, inefficient DNA repair mechanisms, and lack of histones leading to less compact chromatin organization and thereby exposed to free radicals were suggested as the reasons for greater susceptibility of mitochondrial DNA.
Apoptosis. Apoptosis (programmed cell death) is a non-inflammatory response to tissue damage characterized by a series of morphological and biochemical changes. It maintains the nursing capacity of Sertoli cells by controlling the over production of male gametes20 and eliminating abnormal spermatozoa. ROS may initiate a chain reaction by activating caspases that ultimately lead to apoptosis.32 Apoptosis is induced by binding of Fas ligand or agonistic anti-Fas antibody to Fas. Some of the Fas labeled cells escape apoptosis, known as abortive apoptosis, failing to clear all of the spermatozoa destined for elimination. This leads to a large population of abnormal spermatozoa in the semen. Several apoptotic markers such as Bcl2, p53 and annexin V were found in human ejaculate.20 Especially, ejaculates of men with abnormal semen parameters (oligozoospermia, azoospermia) bear a large number of spermatozoa that are Fas positive as well as have damaged DNA.32 This relationship among apoptosis, sperm DNA damage and infertility is an interesting area for further scientific exploration (fig. 2).

ROLE OF SYSTEMIC DISEASES AND OXIDATIVE STRESS IN INFERTILITY

Several systemic diseases, such as cancer, cardiovascular problems, infection and diabetes mellitus, are known to increase the production of ROS.15,33,34 Commonly used drugs, such as quinines and nitroso-aromatic compounds also generate free radicals.33 Even the process of hepatic detoxification of the drugs by cytochrome P450 activity increases ROS production.35 In diabetes mellitus increased production of ROS as a result of hyperglycemia affects blood vessels and nerve endings that can cause erectile dysfunction.34 Environmental stressors such as radiation and pollution also increase levels of oxidative stress. By identifying high oxidative stress as a cause of infertility in such conditions, appropriate therapeutic interventions directed against oxidative stress may improve infertility.

INFECTION, OS AND INFERTILITY

Bacteria, such as Shigella,36 Entameoba histolytica,37 Rickettsia rickettsii,38 Salmonella typhi39 etc, have been shown to increase ROS production. Extracellular ROS are produced by leukocytes, mainly neutrophils.40,41 Low levels of leukocytes are normally present in prostatic and seminal vesicle secretions. The invading pathogens or the defense mechanism against them mediated by leukocytes can lead to sperm damage.43,44 The enzymes of leukocytes (NADPH oxidase, etc) are responsible for ROS generation including hydrogen peroxide, singlet oxygen, perichloric acid, and hydroxyl and superoxide anions. These ROS are produced to create a defense against the pathogens.46 Similarly, a past infection by the sexually transmitted Neisseria gonorrhoea has been demonstrated to cause leukocytospermia (defined as >1 million leukocytes in each ml of semen) that in turn increase ROS production.47 When present in high levels

Fig. 2. ROS induced apoptosis. ROS (apoptotic stimulus) trigger mitochondria to release cytochrome C initiating caspase cascade. Interaction between Fas and Fas ligand is also necessary in apoptotic mechanism. DNA fragmentation occurs as result of activation of effector caspases (caspases 3 and 6) eventually causing apoptosis.
activated leukocytes produce ROS that leak out of the cell membrane causing damage to surrounding cellular elements including spermatozoa. The mechanism responsible for diseases such as chronic renal scarring and pyelonephritis can lead to infertility. According to a study by Depuydt et al, leukocytes and infection of the male accessory gland reduced the fertilizing potential by affecting the sperm parameters in vitro and in vivo. Several studies have shown that patients with leukocytospermia have abnormal DNA integrity. Henkel et al demonstrated that many patients who had <1 million seminal leukocytes per ml could sometimes have abnormal sperm motility and poor DNA integrity.

VARICOCELE, OS AND INFERTILITY
Clinical or subclinical varicocele causes male infertility in about 15% of infertile couples. These patients have increased ROS in the serum, testes and seminal samples. ROS in patients with varicocele are formed due to the excessive presence of xanthine oxidase, a source of superoxide anion from the substrate xanthine, and nitric oxide in dilated spermatic veins. Increased NO has been demonstrated in the spermatic veins of patients with varicocele, which could be responsible for the spermatozoal dysfunction.

Serum proteins are prone to oxidative damage as evidenced by the accumulation of protein carbonyls in blood drawn from the spermatic veins of patients with varicocele. Furthermore, increased oxidative products of proteins is correlated with decreased antioxidant capacity in seminal plasma. Varicocelectomy increases the concentrations of antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase and vitamin C, in seminal plasma as well as improves sperm quality. The levels of malondialdehyde, which is a by-product of lipid peroxidation, are increased in testicular tissue of infertile patients with varicocele. It is possible that ROS may have a role in varicocele associated testicular dysfunction. It is also interesting to note that the generation of ROS is proportional to the severity of varicocele. Patients with varicocele have increased 8-OHdG indicating oxidative DNA damage. Thus, increased OS is likely to be one of the important etiological factors in infertile patients with varicocele.

CRYPTORCHIDISM, OS AND INFERTILITY
Cryptorchidism has been associated with increased apoptotic degeneration of the testes resulting in increased ROS production. Xanthine oxidase is believed to be instrumental in ROS formation in cryptorchid testes. Allopurinol, a xanthine oxidase inhibitor, decreases apoptosis and promotes spermatogenesis in testes. Cryptorchidism also induced heat stress causing DNA fragmentation. Evidence from animal models suggests that degenerative testicular response to heat is determined, at least in part, by a genetic component. However, it is not known whether the heat induced ROS formation is responsible for infertility in these patients. It is now evident that an increase in NO and a decrease in total antioxidant capacity occur with cryptorchidism and contribute to accelerated germ cell apoptosis. Administration of a nitric oxide synthase inhibitor, N-omega-nitro-L-arginine methyl ester, attenuated apoptosis and improved spermatogenesis in experimental (murine) models of cryptorchidism.

ROLE OF LIFESTYLE BEHAVIOR AND ENVIRONMENT IN INFERTILITY
Cigarette smoke contains harmful substances such as alkaloids, nitrosamines, nicotine, cotinine and hydroxycotinine, and many of these substances are considered to generate these free radicals. Several studies have reported that cigarette smoking is associated with reduced sperm quality (count and abnormal morphology). Smoking also affects sperm DNA as evidenced by the increased level of 8-oxo-deoxyguanosine in spermatozoa. In addition, infertile smokers showed higher levels of seminal OS than infertile non-smokers. Thus, the decreased sperm quality in smokers could be due to oxidative stress. Since OS has an adverse effect on fertility potential, it is recommended that physicians advise infertile smokers to quit smoking. The OS effect in smokers could also be due to leukocytes in the semen, as the number of leukocytes was significantly higher in smokers.

It has been reported that environmental pollution can induce the production of the ROS (O2− and OH·). In a study of workers exposed to traffic pollution the time of exposure to pollutants was negatively correlated with sperm quality, reducing fertility in young and middle-aged men. Tollgate workers showed poorer sperm quality than age matched men living in the same area who were not exposed to the same level of automobile pollution. Furthermore, sperm function tests revealed that these workers had sperm with lower motility. In the subset of workers abnormal sperm parameters, e.g., motility, viability, membrane function, nuclear DNA integrity, linearity and amplitude of sperm lateral head movement, were inversely correlated with methemoglobin levels, a marker for nitrogen oxide, whereas sperm count and viability were inversely correlated with lead (Pb) levels.

LABORATORY INVESTIGATIONS OF INFERTILITY
The male infertility evaluation starts with a detailed history on general and gonadal growth and development, medications, infection, previous trauma or surgery, exposure to radiation, steroids, harmful chemicals and sexual history. A thorough history often provides clues to the nature of the infertility problem. Followed by history, a complete physical examination of the male reproductive system is crucial for infertility evaluation. Abnormal genitalia and loss of pubic or facial hair may indicate androgen deficiency. A proper physical examination helps to identify varicocele, hernia or epididymal thickening, some of the conditions that affect sperm maturation and transport. Decreased volume density of the seminiferous tubule is reflected by decreased testicular size.

Laboratory evaluation of the infertile male starts with routine semen analysis, which measures semen volume, pH, sperm concentration, motility, morphology and leukocytes. Within 1 month a second semen sample is collected and analyzed to adjust for the frequent variability in semen quality. In the presence of an abnormality in the semen sample serum hormones, especially
testosterone and follicle-stimulating hormone, are measured. In the event of a normal semen analysis no further testing is recommended, including test for antisperm antibodies, semen culture, sperm-cervical mucus interaction, sperm function tests (computer assisted semen analysis, zona-free hamster oocyte penetration test, human zona pellucida binding test etc.), biochemical analysis of fructose, reactive oxygen species, levels of antioxidants and assessment of sperm DNA damage.

Assessment of oxidative stress. To accurately quantify oxidative stress, levels of ROS and antioxidants should be measured in fresh samples. Direct methods such as pulse radiolysis and electron-spin resonance spectroscopy have been useful for other systems of the body. However, the relatively low volume of the seminal plasma, short life span of ROS and need to evaluate in fresh samples have led to non-usage of direct methods for the male reproductive system. Several indirect techniques have been used to measure levels of various ROS and antioxidants.

One of the most widespread methods of measuring ROS is the chemiluminescence assay, which uses sensitive probes such as luminol and lucigenin for quantification of redox activities of the spermatozoa. Although the sensitivity of these probes is high, they are susceptible to interference. Leukocyte contamination is a major confounder. Also, the time of analysis after collection (<1 hour) and the high sperm count (>1 x 10^9/ml) requirement are some of the drawbacks to this technique.

Another method of measuring intracellular ROS inside the cell is by flow cytometry. Different probes such as 2', 7'-dichlorofluorescin-diacetate, hydroethidine are used. Sperm cells are exposed to the probes that react with ROS to emit a red fluorescence. The assay is highly sensitive and requires a relatively low number of cells but is more expensive and requires expertise to handle sophisticated equipment. The colorimetric technique is also widely used for indirectly quantifying ROS. It is based on the principle of spectrophotometry, and measures lipid peroxide end products such as malondialdehyde, lipid hydroperoxides, isoprostanes etc.

Antioxidant measurement. The presence of low antioxidant in the seminal plasma is another important reason for increased OS leading to male infertility. Hence, it is important to measure the total antioxidant capacity of the semen. Different methods such as oxygen radical absorption capacity, ferric reducing ability of plasma and phycoerythin fluorescence based assay are available for measuring TAC. However, the most widely used method for measuring TAC in semen is enhanced chemiluminescence. This method requires expensive instrumentation, and is cumbersome and time-consuming. Another emerging method for measurement of TAC is the colorimetric assay. First described by Miller et al in 1993, this method gained its popularity as the simple, rapid and inexpensive alternative to enhanced chemiluminescence method.

To accommodate for the variations in ROS and TAC values, our group proposed a concept of combined ROS-TAC score. ROS-TAC score was computed using principal component analysis. We calculated ROS-TAC scores from proven fertile men with low levels of ROS. The composite ROS-TAC scores from these men were representative of the fertile group and any score <30 was considered infertile.

Identification of DNA damage and its relevance to infertility evaluation. Multiple techniques are used to measure the DNA defects in human spermatozoa. Some of the currently available tests evaluate the integrity of sperm DNA, including terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; the sperm chromatin structure assay (SCSA) using various dyes such as acridine orange, aniline blue, toluidine blue and chromomycin A3; comet assay; in situ nick translation; and DNA breakage detection-fluorescent in situ hybridization assay. However, all of the major DNA fragmentation tests are similar in their recognition of a threshold that places a man at infertility risk. Quantitative PCR analysis is being used to identify and quantify the amount of DNA damage. With this technique a particular segment of DNA is amplified and the frequency of repetition of the damaged segment is identified. Although sensitive, this technique has limitations associated with selection of appropriate PCR segment and other amplification issues.

It is difficult to determine whether sperm DNA fragmentation can become a useful test as part of routine semen analysis in an infertility clinic. An association among sperm DNA fragmentation (expressed as SCSA DFI), poor embryo quality and failed pregnancy in ICSI has been reported. Miscarriage rates were the highest especially for ICSI procedures in patients whose SCSA-DFI was >30%. The odds of achieving pregnancy with intrauterine insemination is 16 times higher in subjects with SCSA DFI <27% compared to patients with SCSA DFI >27%. Thus, SCSA DFI status has the potential to become a useful tool in infertility evaluation. However, patients must be counseled that a high DFI does not preclude a normal full-term pregnancy and that they should not rush into the idea of using donor sperm if SCSA DFI is >30%.

MANAGEMENT OF MALE INFERTILITY: ROLE OF ANTIOXIDANTS

Lifestyle modification. As reported earlier, infertile couples desiring children should be advised to abstain from smoking as it is known to increase OS in seminal plasma. Also it is important to avoid excessive exposure to environmental pollution in working and living conditions to prevent OS related sperm damage. Antioxidants. Antioxidants are the main defense against OS induced by free radicals. These can be classified as preventative and scavenger antioxidants. Preventative antioxidants, such as metal chelators and metal binding proteins, block the formation of new ROS, whereas scavenger antioxidants, such as vitamins E and C, beta-carotene and other antioxidant dietary supplements, glutathione and enzymes, remove ROS already generated by cellular oxidation. Dietary products such as lycopene (tomato products), vitamin C (citrus fruits and green vegetables) and vitamin E (vegetable oils, nuts and seeds) are some of the excellent source of antioxidants.

Transition metal ions, mainly iron, generate highly reactive hydroxyl radicals by Fenton’s reaction. These radicals stimulate lipid peroxidation by decomposing the peroxides into peroxyl and alkoxyl radicals, and triggering a chain reaction of
lipid peroxidation. Human semen contains metal chelators such as transferrin, lactoferrin and ceruloplasmin that reduce lipid peroxidation of the sperm plasma membrane, protecting its integrity.\(^{88}\)

Metal chelators, such as DL-penicillamine, 2,3-dimercaptopropan-1-sulfonate and meso-2,3-dimercaptopropan-sulfonic acid, showed enhancement of sperm quality.\(^{89}\) Zinc replacement of cadmium, which is a class B element and toxic to spermatogenesis, improves success of assisted reproduction technology. Also, protein metallothionein binds to cadmium and eliminates it from the body, which improves spermatogenesis, maturation and capacitation of spermatozoa.\(^{90}\) Scavenger antioxidants remove ROS and prevent the propagation of lipid peroxidation chain reaction. Dietary antioxidants are an important component of this group of antioxidants. Fruits and vegetables as well as daily dietary supplements constitute the potential sources of various antioxidants.

According to The National Academy of Sciences, 60 mg of vitamin C a day is recommended for an adult male. Vitamin E daily requirement varies from 50 to 800 mg depending on the intake of fruits, vegetables, tea or wine. Selenium and carotenoids work synergistically with vitamin E with recommended dietary allowances of 70 and 1000 mcg a day, respectively.\(^{91}\) Vitamin supplements, mainly vitamins E and C which are chain breaking antioxidants, can reduce oxidative stress in the seminal plasma of infertile men.\(^{68}\) Vitamin C in oral doses of 200 to 1000 mg a day increases sperm counts in infertile males.\(^{92}\) A randomized crossover study revealed that oral administration of 600 mg a day of vitamin E improved sperm function as assessed by the zona binding test although other sperm parameters were not affected.\(^{93}\) Quercetin and xanthohumol are some of the important dietary flavonoids, and they function synergistically with vitamin C.\(^{94}\) Both compounds conserve the alpha-tocopherol content of low density lipids and prevent or delay it from undergoing lipid peroxidation.\(^{95}\)

Coenzyme Q-10 found in the sperm midpiece\(^{96}\) recycles vitamin E and prevents its pro-oxidant activity.\(^{21}\) It has been shown to prevent ROS formation with improvement in fertilization rates.\(^{97,96}\) Glutathione is the most abundant antioxidant found in the body, and it has an important role in protecting lipids, proteins and nucleic acids against oxidative damage. It has been demonstrated that 600 mg glutathione therapy intramuscularly significantly increased sperm motility, particularly forward progression.\(^{96}\) Carotenoids such as beta-carotene and lycopene also form an important component of the antioxidant defense.\(^{96}\) Beta-carotenotes protect the plasma membrane against lipid peroxidation. Lycopene has been shown to be twice as potent as beta-carotene in inhibiting lipid peroxidation in blood plasma.\(^{100}\) Selenium at a dose of 225 mcg a day orally for 3 months significantly decreased malondialdehyde concentrations in seminal plasma and improved sperm motility.\(^{101}\) Carnitines are dietary antioxidants that decrease mitochondrial ROS production and improve sperm motility.\(^{4,102}\) Oral intake of L-carnitines for 6 months improved motility as well as sperm concentration.\(^{98}\)

Apart from the traditional antioxidant therapy other modalities of treatment have been successfully tried. When increased apoptosis is suspected, specific apoptotic inhibitors have prevented cell death and improved survival of the germ cells. Sphin-gosine-1-phosphate, an apoptotic inhibitor, at doses of 1 and 10 mmol/l suppressed apoptosis in germ cells of testis.\(^{103}\) Similarly, N-acetyl cysteine when given in concentrations of 125, 100, 50 and 25 mmol/l suppressed germ cell death in a dose dependent manner.\(^{104}\)

Assisted reproductive technology. The first “test-tube” baby, Louise Brown, was born in 1978. Since then assisted reproduction technologies have helped many infertile couples to have children. In all of these years there has been a phenomenal improvement in every sphere of ART. In vitro fertilization (e.g. gamete intra-fallopian transfer, zygote intra-fallopian transfer) and ICSI form the major components of ART.

ROS is produced in the ART setting in a number of ways. Oocytes and embryos, cumulus cells and spermatozoa are the major sources of ROS. ROS can be decreased using sperm preparation techniques to enhance and maintain sperm quality after ejaculation.\(^{105}\) Several sperm preparation techniques, such as migration-sedimentation, density centrifugation gradient and glass-wool filtration, significantly reduce the level of ROS by removing leukocytes, which are its major source.\(^{89,96}\)

Assisted reproductive techniques may show significant improvement in in vitro supplementation of antioxidants and metal chelators to achieve a better success.\(^{22}\) Excellent results were obtained with the use of many natural and synthetic compounds, such as rebamipide, pentoxyfylline, vitamins E and C, SOD, catalase etc, at different stages of ART procedures. With the abundance of literature supporting the use of antioxidants during ART procedures, their judicial use is recommended.

Identification of appropriate candidates and procedure is a crucial part since multiple factors influence results, and this would prevent the emotional and financial burden associated with ART. Adding testicular sperm extraction and percutaneous epididymal sperm aspiration to the armor of ART, and improvement in cryopreservation techniques help patients with conditions such as azoospermia and cancer.

**CONCLUSION**

In the last 15 years we have witnessed a phenomenal growth in our knowledge of male reproduction, sperm function, and development of diagnostic tools and treatment modalities for male infertility. Presently there are several different techniques available to predict the fertility status of a man. The WHO is frequently updating their training manuals with more focus on quality control and standardization of semen analysis procedures. In addition, with increased understanding of oxidative stress, several new modalities of treatment are now being tried to improve fertility. Many new antioxidants are now available that can decrease oxidative stress and improve sperm quality. However, due to lack of scientific evidence of their effectiveness, these are not yet approved by FDA or other Governing bodies and such an approach raises many concerns. Although current evidence supports the use of systemic antioxidants for the management of select cases of male infertility as well as in vitro supplements during various sperm preparation
techniques, a definitive conclusion cannot be drawn from the available studies, mainly because OS is not the only cause of male infertility. Therefore, antioxidant therapy should be tried only in cases with increased oxidative stress status or established DNA damage. However, evaluation of OS status is still not a routine andrology laboratory procedure in clinical practice mainly due to various technical issues.

Our current focus is to validate and simplify the evaluation of OS status so that it can be performed in a routine andrology clinic and/or laboratory without the need for sophisticated instrumentation. Also, dosage and duration of the antioxidant treatment are still widely debated and should be tailored to the need of each individual. In the future large, placebo controlled multicenter trials will be required to gain a better insight into this important issue. ART procedures should be recommended for carefully chosen infertile couples. This selection process will ensure better success especially in cases of idioopathic infertility, prevent the social and psychological trauma associated with these techniques, and decrease the exorbitant associated costs.

REFERENCES
9. de Lamirande E, Leclerc P and Gagnon C: Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Mol Hum Reprod 1997; 3: 175.
31. Yakes FM and Van Houten B: Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc Natl Acad Sci U S A 1997; 94: 514.


1. What are the major sources of ROS in semen during infection?
   a. Leukocytes
   b. Normal spermatozoa
   c. Morphologically abnormal spermatozoa
   d. RBC
   e. Epithelial cells

2. What is the most common method of measuring ROS at present?
   a. Chemiluminescence
   b. Flow cytometry
   c. Electron spin resonance
   d. Spectrophotometry
   e. HPLC

3. Which of the following is a metal chelator that has proven to decrease the lipid peroxidation of sperm plasma membrane?
   a. D-penicillamine
   b. Transferrin
   c. Beta-carotene
   d. Glutathione
   e. Iron

4. Spermatic veins of patients with varicocele show increased levels of which compound?
   a. H$_2$O$_2$
   b. Superoxide
   c. NO
   d. Hydroxyl radical
   e. Catalase

5. What is the suggested DNA fragmentation index rate above which the probability of spontaneous miscarriage is the highest?
   a. 15%
   b. 35%
   c. 30%
   d. 50%
   e. 90%