Journal of Andrology, Vol. 25, No. 1, January/February 2004

Role of Oxidative Stress and Antioxidants in Andrology and Assisted Reproductive Technology

Andrology Lab Corner*

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Twenty-five years ago the first human, Louise Brown, was conceived outside the body, and this event created a new milestone in the history of mankind and the concept of human in vitro fertilization (IVF). This was a major breakthrough in fertility, and now the whole world is celebrating this event with more than 100 000 children born through this new era of assisted reproductive technology (ART). During the last 25 years, physicians and scientists have improved every aspect of ART, from drawing eggs out of the ovary to improving rate of fertilization, growing embryos in the laboratory, and improving the likelihood of success from about 12% in 1988 to 35% at best at present. During this time many less popular modifications of IVF, eg, gamete intrafallopian transfer (GIFT), zygote intra-fallopian transfer (ZIFT), use of round spermatid nuclear injection (ROSNI), etc were also developed. In 1992 one of the most effective additions to IVF in the form of intracytoplasmic sperm injection (ICSI), in which a single sperm is injected into the center of an egg, revolutionized the world of ART. This made it possible for even so-called azoospermic men to become fathers.

Although efforts to expand these ART procedures for better success rate continue, reports of multiple pregnancies and premature low-birth-weight babies, many of them with serious health problems, are discouraging. On the other hand, simple intrauterine insemination (IUI) using washed spermatozoa is more affordable and can be practiced in many physician office laboratories. IUI generally requires large numbers of forward-progressing motile spermatozoa that are low in number in many male infertility patients. One of the major concerns in using such semen samples is high generation of free radicals (reactive oxygen species; ROS) because of in vitro handling as well as the presence of large number of leukocytes, cellular debris, and/or immature germ cells. The spermatozoa in such semen samples are under high oxidative stress and are susceptible to oxidative damage, especially when level of protective antioxidants in seminal plasma or ART media is low. Such sperm damage can theoretically be prevented by many antioxidants and dietary supplementation. Besides, many sperm stimulation protocols in an ART laboratory using chemical agents, eg, pentoxifylline, platelet-activating factor (PAF), caffeine, 2-deoxy adenosine, L-carnitine and acetyl-carnitine etc have been described to improve the success rate of these procedures. This report reviews the role of oxidative stress and further attempts to highlight certain myths and facts of antioxidants, particularly vitamin E paradox, with a discussion whether such antioxidants when used in vivo or in vitro in ART laboratories will help improve success rate of andrology/ART procedures.

What Is the Role of ART Laboratories in Fertility Practice?

Impairment of normal spermatogenesis and sperm function are the most common causes of male factor infertility. Normal sperm production, maturation, progression (for effective transport through female genital tract), and function (capacitation, acrosome reaction, egg penetration, decondensation of the head) are complex processes but essential to achieve fertilization, pregnancy, and early embryonic development. Abnormal sperm function is difficult to evaluate and treat and thus reflects a major challenge to ART laboratories. There is a lack of consensus and understanding of the factors contributing to normal and abnormal sperm function leading to infertility. Almost 25% of all infertile patients are diagnosed as idiopathic with normal semen parameters. The widespread use of ART has helped many such infertile couples to experience parenthood. Today IVF has become an important tool for alleviating subfertility in the human. Several modifications of IVF (eg, GIFT, ZIFT, ICSI, ROSNI, etc), commonly referred to as medically assisted conception, has been developed. In spite of many advances in the field of ART and the mushrooming of new IVF/ART

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Received for publication September 9, 2003; accepted September 29, 2003.

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centers all around the world, the take-home baby rates have been persistently low and undesirable.

One of the major concerns is that these ARTs bypass natural barriers to fertilization by defective spermatozoa, thus establishing genetic causes of infertility and other health issues in the offspring. Many recent ICSI reports describe fetal malformations and in association with limitations in preimplantation genetic testing are likely to discourage many infertile couples from seeking such help (Palermo et al, 2002). These IVF/ICSI procedures are very expensive with no coverage by most health plans; require sophisticated laboratory facilities with trained certified embryologists and other personnel, and undergo many regulatory restrictions. All these have disheartened many infertile couples as well as fertility service providers with small physician office laboratories and brought about the search for alternate help in the form of less expensive ART facilities. Such ART facilities can provide a simple, less sophisticated sperm wash procedure followed by IUI to achieve normal pregnancy. Thus, IUI for the treatment of infertility has become the most widely used andrology/ART procedure indicated in cases of unexplained infertility, cervical mucus hostility, in men with spinal cord injury undergoing electroejaculation, and/or vibratory stimulation. Many ART laboratories use various additives, sperm stimulants, and sperm enrichment procedures that have helped in the recovery of functional spermatozoa and achieve fertilization. Widespread use of many antioxidants has opened up a debate whether these can improve chances of fertilization by protecting against oxidative stress and sperm damage induced by free radicals (Martin-Du Pan and Sakkas, 1998; Geva et al, 1998; Tarin et al 1998; Donnelly et al, 1999). Understanding the roles and mechanisms of these additives and antioxidants and development of newer modalities to improve pregnancy in subfertile patients is an all-important contribution of an ART laboratory that provides such services.

What Is Oxidative Stress and How Does It Affect Fertility?

Many studies in the area of male infertility have been focused on oxidative stress–related mechanisms of sperm damage (De Lamirande and Gagnon, 1992; Aitken 1994; Sikka et al, 1995; Armstrong et al 1999;; Agarwal and Saleh, 2002). How certain antioxidants will help improve this infertility and understanding the mechanisms of action of oxidative stress and antioxidants are important issues in this direction. Human spermatozoa are capable of generating controlled low amounts of endogenous ROS that play a significant role in inducing sperm capacitation/acrosome reaction and acquisition of sperm-fertilizing ability (Gagnon et al 1991; De Lamirande and Gagnon, 1995).

Normally there exists a balance between free radical generating and scavenging systems (Figure 1). However, high generation of ROS by immature and abnormal spermatozoa, contaminating leukocytes, sperm processing (eg, excessive centrifugation, cryopreservation/thawing), accompanied by low scavenging and antioxidant levels in serum, seminal plasma, and/or sperm-processing media will induce a state of oxidative stress. High levels of ROS (superoxide, hydroxyl, hydrogen peroxide, nitric oxide, peroxynitrile) endanger sperm motility, viability, and function by interacting with membrane lipids, proteins, and nuclear and mitochondrial DNA (Aitken and Clarkson, 1987; Alvarez et al, 1987; Gagnon et al, 1991; Iwasaki and Gagnon, 1992; Hellstrom et al 1994). The possible explanation of this damaging process is based on the fact that mammalian spermatozoa membranes are rich in polyunsaturated fatty acids that make them very fluid but at the same time very susceptible to free radical–induced peroxidative damage. Membrane fluidity is also needed to support the membrane fusion events associated with a variety of secretory episodes (Sikka et al, 1982). In the case of spermatozoa, it leads to acrosome reaction and sperm-egg fusion during fertilization (Aitken and Clarkson 1987). However, to understand the biological mechanisms of sperm oxidative stress and its relation to fertility/infertility, three important questions need to be addressed: 1) what are the mechanisms of oxidative stress–induced damage to these germ cells in vivo; 2) what are the consequences of such damage to sperm membrane lipids, proteins, and nucleic acids; and 3) what regulates the antioxidant defense mechanisms in seminal plasma? An active ART laboratory investigating such events described below may play a critical role in expanding our knowledge in this area.

Peroxidative Damage to Spermatozoa

Peroxidative damage initiated by high ROS generation during oxidative stress as seen in the spermatozoa of infertile men is associated with not only a loss of membrane function, but also the appearance of damage to the DNA located in the sperm head in such patients, leading to a high incidence of DNA strand breaks (Ernster, 1993; Evenson et al, 2002; Agarwal and Said, 2003). Under normal circumstances, spermatozoa with damaged DNA would not participate in the fertilization process because of collateral peroxidative damage to the sperm plasma membrane (Kodama et al, 1996). This peroxidation cascade gets stimulated by the presence of transition metals such as iron to form initiating species (L) that decompose membrane lipid peroxides as shown in Figure 2. In a normal situation, the cellular antioxidant mechanisms present in almost all tissues and their secretions are likely to quench these ROS and protect against oxidative damage (Jones et al, 1979). Antioxidant supplementation can theoretically protect and prevent such peroxidative damage.

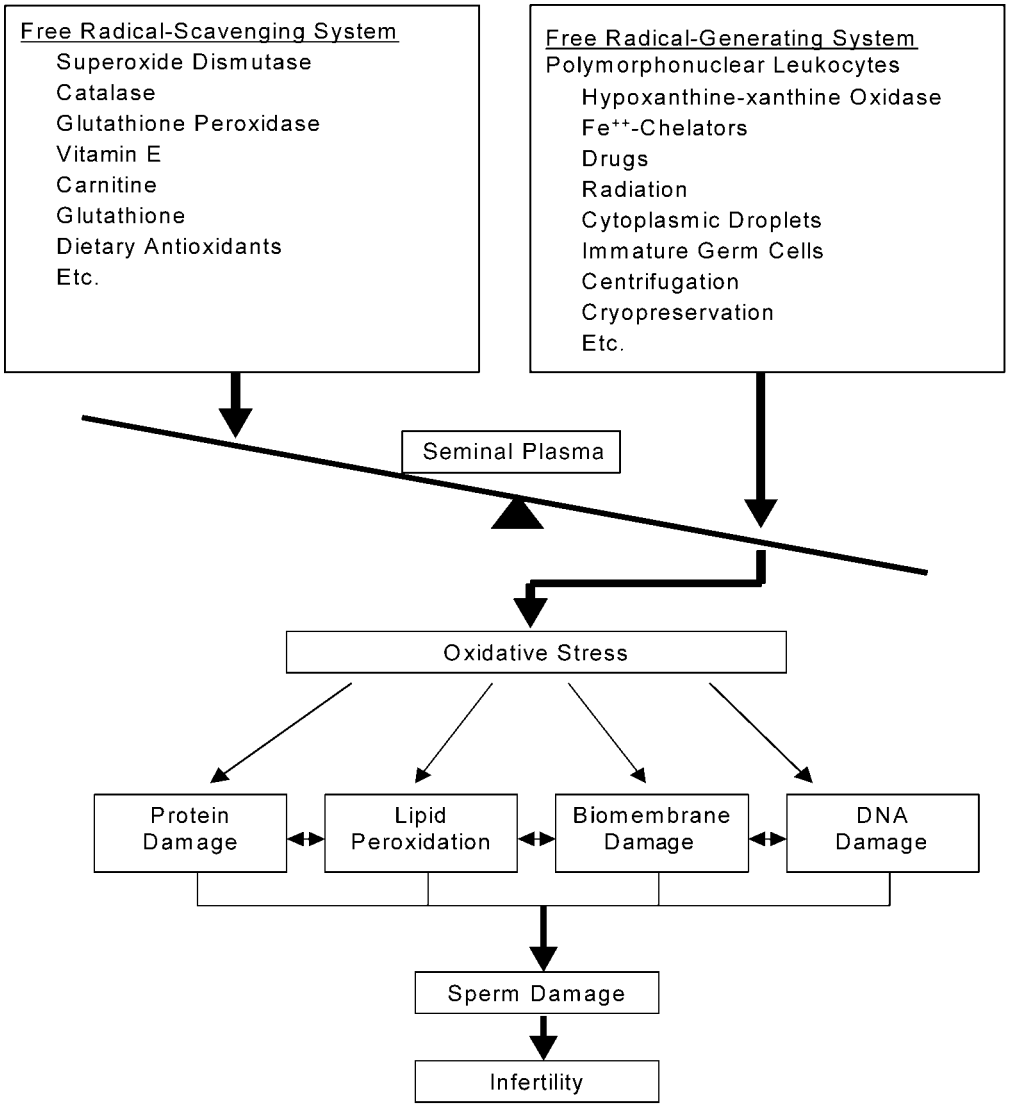

Figure 1. Reactive oxygen species generating and scavenging systems in seminal plasma responsible for oxidative stress and their role in infertility.

This concept of improving fertility potential of such infertile patients under high oxidative stress by use of certain antioxidants has been debated in the past and is again gaining considerable attention in ART and infertility practice (Geva et al, 1998; Martin-Du Pan and Sakkas, 1998; Tarin et al, 1998; Donnelly et al, 1999; Sikka, 2001; Agarwal and Saleh, 2002). Efforts toward the development of new reliable assays in many ART laboratories to evaluate this oxidative stress and determine the role of antioxidants have great potential in such therapeutic practice and have resulted in the establishment of total antioxidant capacity (TAC) measured as ROS-TAC score (Sharma et al, 1999; Said et al, 2003). The concept of overall expression of oxidative stress status (OSS) and its assessment by ROS-TAC score may help improve results of ART because of possible therapeutic interventions by antioxidants and may benefit infertile men in whom oxidative stress has a significant role (Sikka et al, 1995; Saleh and Agarwal, 2002). Also determining the levels and the sources of excessive ROS production in human semen and evaluation of the scavenger system in such laboratories should help design steps to improve success rate of andrology/ART procedures.

Oxidative stress and sperm membrane lipid peroxidation

Spermatozoa, unlike other cells, are unique in structure, function, and very susceptible to damage by ROS (Alvarez et al, 1987; Rao et al, 1989). Orientation of unsaturated fatty acids in the plasma membrane creates the fluidity necessary by the spermatozoa to perform normal physiological functions (Bell et al, 1993). Also, mem-

brane-bound ATPases that function as ion pumps help maintain the correct intracellular concentrations of nutrients and ions (such as sodium or calcium), and the functioning of these pumps is critically dependent on membrane fluidity (Aitken and Clarkson, 1987; Ernster, 1993). If the latter is lost, then the ATPases cease to function and the cells suddenly start to accumulate these ions at a rate that ultimate leads to their destruction.

In general, the most significant effect of membrane lipid peroxidation (LPO) in all cells is the perturbation of membrane structure and function. 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 (Alvarez and Storey, 1989). Such altered intracellular calcium balance is likely to affect sperm motility (Aaberg et al, 1989). Minor alterations in sperm membranes in selected cases of dyspermia can be reversed by glutathione (GSH) therapy (Irvine, 1996). Studies on how these cellular changes caused by LPO affect seminal parameters and sperm function, and whether these could be reversed by antioxidants are open to further investigations.

Oxidative Stress and Protein Damage

Besides affecting membrane components and fluidity, ROS-induced peroxidation of critical thiol groups in proteins will alter structure and function of spermatozoa and ova with an increased susceptibility to attack by macrophages (Alvarez and Storey, 1989; Thomas et al, 1995). In addition, the redox status of human spermatozoa is likely to affect phosphorylation and adenosine triphosphate (ATP) generation with a profound influence on its fertilizing potential. Stimulation of endogenous NADPH-dependent ROS generation in human sperm appears to regulate acrosome reaction via tyrosine phosphorylation (Leclerc et al, 1997). In general, the oxidizing conditions increase tyrosine phosphorylation with enhanced sperm function, whereas reducing conditions have the opposite effect (Aitken et al, 1995). Although this has been debated for a long time, it is still not clear whether sperm have an NADPH-dependent oxygenase system (Armstrong et al, 2002). Nonetheless, how membrane changes regulate specific sperm function because of altered tyrosine phosphorylation is an interesting area for further investigation in research laboratories. Similarly, ROS-induced protein glycosylation may also contribute to such damage (Jain and Palmer, 1997). It is not clear whether certain antioxidants (eg, vitamin E) will protect against such ROS-induced peroxidation, glycosylation, and phosphorylation of many important proteins. In association with new information and concepts obtained through proteomics, such mechanisms may open a new series of diagnostic modalities to assess sperm function and prevent sperm damage to help manage clinical infertility.

Oxidative Stress and Apoptosis as Related to Sperm DNA Damage and Role of ART

Current focus on male factor infertility during oxidative stress suggests damage to integrity of DNA in the sperm nucleus resulting in base modification, DNA fragmentation, and chromatin cross-linking (Evenson et al, 2002; Agarwal and Said, 2003). Such DNA damage may accelerate the process of germ cell apoptosis, also known as programmed cell death. In the testis (Sinha-Hikim and Swerdloff, 1999), that can lead to a decline in sperm counts and result in infertility (Sun et al, 1997). Recent studies have indicated a significant increase in the levels of apoptotic spermatozoa in the semen of infertile men (Sakkas et al, 1999, 2002). Patients who were inseminated with samples containing higher degrees of DNA damage (>12%) had poor embryo quality and/or experienced miscarriages (Duran et al, 2002). Sperm, surgically extracted from the epididymis or testicular tissue in patients with obstructive azoospermia undergoing ICSI, revealed a significantly high percentage of DNA fragmentation (Sakkas et al, 1999). The oxidative damage to mitochondrial DNA (mtDNA) is also known to occur in all aerobic cells that are rich in mitochondria including spermatozoa. Multiple mtDNA deletions in spermatozoa could arise through a free radical–driven event occurring at the spermatogonial cell stage and can account for reproductive failure in some men (Kao et al, 1998). Although standard sperm parameters are not predictive of high levels of apoptosis, it may be an independent phenomenon that plays an important role in the pathophysiology of male infertility.

It is not clear how ROS-induced DNA-damaged spermatozoa impair the process of fertilization and embryo development. An ART facility with routine sperm DNA integrity assessment by sperm chromatin structure assay (SCSA) or Comet assay can play a significant role in managing such issues in infertility (Evenson et al, 2002; Agarwal and Said, 2003). At present, the key questions in this direction that remain unanswered are the following:

1. Is active apoptosis present in the spermatozoa in semen, and if yes, what is the molecular mechanism of such an apoptotic pathway?
2. Is apoptosis a significant contributor to DNA damage in the seminal spermatozoa?
3. Is this sperm DNA damage via transmembrane receptors and adapter molecules or via mitochondrial membrane damage or both?
4. What is the molecular mechanism(s) of ROS-induced sperm DNA damage? Is it via activation of apoptosis or via some independent action on the DNA integrity?

Considering that oxidative stress plays a significant role in sperm DNA damage and infertility, it becomes even

more important to look into the role of antioxidants in vitro and in vivo that can protect against such oxidative damage to spermatozoa and improve their fertilization potential. Further research is required to elucidate the exact mechanism(s) of association of apoptosis with male factor infertility and the role of antioxidants.

What Are Antioxidants and Their Role in ART and Infertility?

Antioxidants, in general, are free radical scavengers that suppress the formation of ROS and/or oppose their actions. Superoxide dismutase (SOD), catalase, and glutathione peroxidase are well-known biological antioxidants that convert superoxide (O_2^-) and peroxide (H_2O_2) radicals to form O_2 and H_2O via the following equations:

$$2(O_2^-) + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$H_2O_2 \xrightarrow{Catalase} H_2O + 1/2\,O_2$$

SOD protects against spontaneous O_2 toxicity and LPO (Fridovich, 1985). SOD and catalase also remove (O_2^-) generated by NADPH-oxidase in neutrophils and can play an important role in protecting spermatozoa during genitourinary inflammation (Baker et al, 1996). Glutathione peroxidase, a selenium-containing antioxidant enzyme with glutathione as the electron donor, removes peroxyl radicals from various peroxides including H_2O_2 to improve sperm motility. Glutathione reductase regenerates reduced GSH from its oxidized form (GSSG).

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

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

Because GSH has a likely role in sperm nucleus decondensation and may alter spindle microtubule formation in the ovum, it may help protect ova and embryo during ART, thus affecting the outcome of pregnancy. Another enzyme, α-glutamyl transpeptidase that is present in the midpiece and acrosomal regions of spermatozoa may further regulate GSH content of oocyte at the time of sperm penetration (Irvine, 1996). Thus, in view of increased generation of ROS because of the great number of mitochondria present in spermatozoa, these antioxidant mechanisms are important in the maintenance of sperm motility, capacitation, and the ability of sperm to undergo acrosome reaction during sperm preparation techniques, especially in the absence of seminal plasma. Human serum albumin (HSA) present in culture media serves as a powerful antioxidant by providing such thiol groups for chain breaking antioxidant activity (Ernster, 1993; Armstrong et al, 1998). Also, vitamin E supplementation has been shown to restore glutathione levels of red blood cells and inhibit membrane LPO (Jain et al, 2000). In light of this, considering that a high GSH/GSSG ratio should help spermatozoa to combat oxidative insult, evaluation of these GSH enzymes and how antioxidant supplements affect certain mechanisms related to infertility is an important area for further investigation in ART facilities.

Within the category of chemical antioxidants that include both natural and synthetic products, their usefulness in reproduction and management of infertility has not yet been demonstrated. It is possible that pentoxifylline, a known sperm motility stimulator, can also act as a suppressor or scavenger of ROS to improve sperm motion in leukocytospermia (Sikka and Hellstrom, 1991; Sikka et al, 1993). Also, the role of vitamins E and C, the chain-breaking antioxidants that scavenge intermediate peroxyl and alkoxyl radicals in protecting spermatozoa against endogenous oxidative DNA and membrane damage, should be evaluated for their effects in improving the postthaw sperm parameters. Carotenoids (beta-carotene) and ubiquinols are also known to quench singlet oxygen and may play a role in reducing detrimental effects on sperm. Hence, the application of ROS scavengers (eg, SOD, catalase, vitamin E, GSH enzymes) is likely to improve sperm motility, function, and outcome of ART (Zini et al, 1993). Further controlled clinical trials will determine whether any of these putative antioxidants will improve infertility in selected groups of patients (Table 1).

Seminal plasma is well endowed with an array of antioxidants that act as free radical scavengers to protect spermatozoa against oxidative stress (Aitken and Fisher 1994; Alkan et al 1997, Armstrong et al, 1998; Agarwal and Saleh 2002). This defense mechanism compensates for the loss of sperm cytoplasmic enzymes that occur when the cytoplasm is extruded during maturation. This, in turn, diminishes endogenous repair mechanisms and enzymatic defenses (Aitken, 1994; Donnelly et al, 1999). Seminal plasma contains a number of enzymatic antioxidants such as SOD, catalase (Zini et al, 1993), and glutathione peroxidase (Alvarez and Storey, 1989). In addition, it contains a variety of nonenzymatic antioxidants such as ascorbate, urate, α-tocopherol, pyruvate, glutathione, taurine, and hypotaurine (Saleh and Agarwal, 2002). Antioxidants present in the seminal plasma are the most important form of protection available to spermatozoa against ROS (Aitken et al, 1994; Sikka et al, 1995, 2001; Sharma and Agarwal, 1996). They provide defense mechanisms through three levels of protection: 1) prevention, 2) interception, and 3) repair. Because ROS generation is a major source of sperm DNA damage, antioxidants may play a role in decreasing apoptosis during spermatogenesis, sperm storage, and transit in the genital tract. These may also protect against infection and in turn improve the sperm quality and reduce sperm DNA damage. There is great interest in vitamin E supplementation, and its role in ART and infertility needs further exploration.

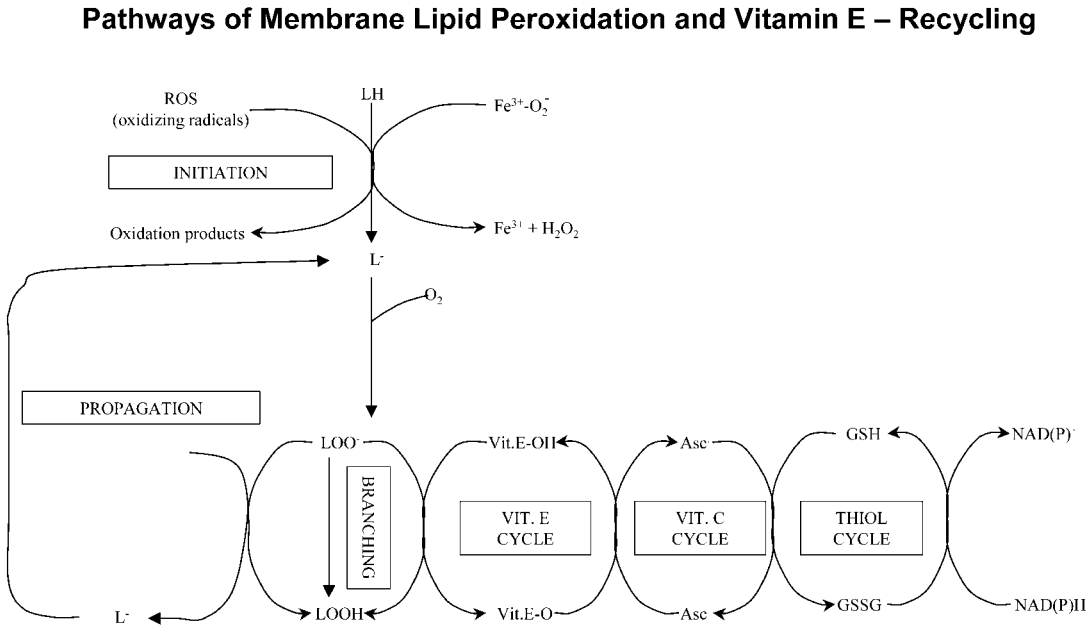

Figure 2. Pathways of membrane lipid peroxidation and vitamin E recycling.

In Vitro Protection by Antioxidants

When added in vitro during IVF preparation or as sperm wash media, ascorbic acid (600 μmol/L), alpha-tocopherol (30 and 60 μmol/L), and urate (400 μmol/L) have each been reported to provide significant protection from subsequent sperm DNA damage (Hughes et al, 1996; Geva et al, 1998). Many isoflavones (eg, genistein and equol) are plant products with antioxidant activity and may prevent sperm damage. Compared with ascorbic acid and alpha-tocopherol, genistein was shown to be more potent antioxidant when added to culture media. In addition, human serum albumin in ART media acts as a powerful antioxidant that prevents oxidative stress–induced damage (Armstrong et al, 1998; Aitken et al, 1994). Thus, any substitution of IVF media with other potential antioxidants may not have additional benefits to improve the success of ART procedures. However, all such restraints are removed when ICSI is performed. No overt increase in early pregnancy loss with ICSI, compared with IVF, has been reported, and live birth rates are no worse, possibly better. This means either that the DNA repair mechanisms in the embryo are extremely efficient or that the consequences of the DNA damage will appear only in late life. Well-controlled dose-response studies will help in establishing any benefits of such supplementation in vitro.

In vivo dietary supplementation

It is believed that for males with idiopathic infertility, dietary supplementation with a combination of well-tolerated, clinically efficacious, and noninvasive vitamins and vitamin-like agents, such as L-carnitine, acetylcartinine, vitamins E and C, fructose, citric acid, selenium, and zinc provide an alternate pharmacological therapy to improve sperm quality and greater likelihood of success of ART procedures (Bayer, 1960; Dawson et al, 1992; Kessopoulou, 1995; Wong et al, 2000). It is not clear whether some of these antioxidants will have the same beneficial effects when combined together. Genistein and equol, when added in combination, were more protective against sperm damage than when added singly (Sierens et al, 2002). Similarly, antioxidant activity of vitamin E is synergistically enhanced in the presence of vitamin C (Rolf et al, 1999) and possibly by ubiquinones (Figure 2). It is argued that because daily intake of these vitamins and other supplements by very large populations have not shown any adverse events, they are presumably safe. However, very high doses of vitamin E can increase hemorrhagic incidence in susceptible individuals. It is possible that excessive intake of some of these antioxidants, especially in combination, may cause other deleterious side effects, and their use needs to be carefully monitored. Table 1 lists dietary requirements and recommended daily doses of some of the antioxidants.

Myths and Facts About Vitamin E as Antioxidant

Vitamin E, discovered in 1922, is the name given to a group of naturally occurring lipid-soluble antioxidants, the tocopherols and the tocotrienols, that are found in certain plant oils. Vitamin E is the major, if not the only, chain-breaking antioxidant in membranes. However, its membrane concentration is very low, usually equal to or

Table 1. *The cellular antioxidants of human biology*

Intracellular	Mechanism of Action
Superoxide dismutase (SOD)	
Catalase	Enzymatic
Glutathione peroxidase/glutathione	
Extracellular	
Ascorbic acid (vitamin C)	
Urate; ubiquinone	
Sulphydryl groups (proteins, glutathione)	Chain-breaking antioxidants
Bilirubin	
Vitamin E (tocopherols)	
Membranes	
Tocopherols (vitamin E)	
Carotenoids (vitamin A)	Preventative antioxidants
Transferrin, ceruloplasmin	
Haptoglobin, hemopexin	

less than 0.05–0.1 nmol/mg of protein (less than 1 per 1000–2000 membrane phospholipids). On the other hand, the rate of lipid radical generation in membranes can be very high, about 1–5 nmol/mg protein per minute under certain circumstances. Nevertheless, under normal conditions oxidation of membrane lipids and proteins does not occur and vitamin E deficiency is seldom found in adult humans. This is mainly due to the vitamin E cycle (Figure 2).

Vitamin E acts catalytically, being efficiently reduced from its free radical quenching form back to its native state. The presence of a reductant, either hydrophilic (as vitamin C or thiols) or lipophilic (as ubiquinol), causes the vitamin E radical to be regenerated. During nonenzymatic recycling pathway for vitamin E regeneration that occurs in membranes, vitamin C reduces chromanoxyl radicals to recycle vitamin E and eventually being itself consumed by the process (Packer, 1993). On the other hand, enzymatic recycling of vitamin E by NADPH/NADH electron transfer occurs in mitochondria and microsomes. The efficiency of such transfer depends on the concentration of ubiquinol. Thus, ubiquinol is also a partner with vitamin E in protecting membranes against oxidation, by enhancing the antioxidant activity of vitamin E through recycling. Vitamin C or ubiquinone alone (in vitamin E deficiency) does not enhance such protection against membrane peroxidation. Under conditions in which these auxiliary systems act synergistically to keep the steady-state concentration of vitamin E radicals low, the loss or consumption of vitamin E is prevented. Thus, vitamin E will be lost only when these backup systems, either in the aqueous or membrane domains, become rate limiting. At this point, increased rates of lipid and protein oxidation, destruction of membrane function, and inactivation of membrane enzymes and receptors will accompany the loss of vitamin E. Thus, vitamin E not only has an antioxidant action but also acts as a biological response modifier. This vitamin E cycle provides a clear example of a synergistic effect and nature's amazing way of recycling.

Vitamin E Requirement

Health status, lifestyle, diet, and environment markedly influence the requirements for vitamin E. Although demonstrable vitamin E inadequacy in apparently healthy adults is rare, vitamin E requirements may vary fivefold in individuals, depending on the dietary intake of polyunsaturated fat, tissue composition, the steady state concentrations of other interactive antioxidants in tissues, and genetic factors. D-alpha tocopherol and d-alpha tocotrienol, two forms of vitamin E, are natural membrane antioxidants. D-alpha tocotrienol may have higher physiological activity than d-alpha tocopherol under conditions of oxidative stress. The concept of vitamin E recycling may prove to be crucial in the development of new strategies for the treatment of acute and chronic conditions involving oxidative stress, especially sperm damage in case of infertility. Because vitamin E and other antioxidants prevent or minimize oxidative damage in biological systems, the key question is how adequate the antioxidant defense should be to protect the body from excessive free radical concentrations.

During leukocytospermia, the superoxide anion produced by leukocytes is an oxygen radical that along with high levels of inflammatory chemokines may be important for bacterial killing (Rajasekaran et al, 1995). Because vitamin E is an antioxidant, the bacterial-killing mechanism of leukocytes may well be inhibited by large doses of vitamin E that will raise its levels in leukocytes. Because during ART procedures spermatozoa are washed and leukocyte contaminants are removed, it is debated whether use of such antioxidant supplementation may

Table 2. *Target dosages for optimal dietary supplementation with potential antioxidant properties**

Nutrient	USRDA†	ORD‡
Mixed carotenoids (alpha and beta carotene, etc)	N/A	25 000 IU
Vitamin C (ascorbic acid)	60 mg	
Vitamin D (ergocalciferol)	400 IU	[200–600] IU
Vitamin E (d-alpha tochopherol; mixed-tocopheroles preferred)	30 IU	[200–800] IU
Zinc (nicotinate)	15 mg	[25–50] mg
Chromium (picolinate)	N/A	[100–800] mg
Selenium	55 mg	
N-acetyl-L carnitine	N/A	[500–2000] mg
Alpha-lipoic acid (ALA)	N/A	[100–600] mg
Co-enzyme Q-10 (ubiquinone)	N/A	[30–100] mg
Pycnogenol (pine bark extract or grape seed extract)	N/A	[60–100] mg
Phosphatidyl choline (lecithin)	N/A	[250–1200] mg
Garlic extract (odor free)	N/A	[100–1200] mg
Ginseng (standardized)	N/A	[250–500] mg
Ginkgo biloba	N/A	[150–350] mg
Flaxseed and fish oils	N/A	[1000–3000] mg
Lycopene	N/A	300 mg

* Modified from Coles LS, 2003.

† USRDA = US recommended daily dietary allowance; ORD = optimal range of recommended daily intake.

‡ Their role in infertility practice is not yet established.

have additional benefits, especially in the presence of HSA in various culture media. HSA itself is a strong antioxidant (Aitken et al, 1994; Armstrong et al, 1998). Besides, unexpectedly elevated intracellular concentrations of vitamin E may have unknown adverse effect on various physiological processes as well as ART outcome. Thus, indiscriminate use of megadoses of vitamin E and other antioxidants both in vitro and as dietary supplements should be carefully monitored. Table 2 describes some potent antioxidants and their recommended daily intake; beyond that, caution is desired (Coles, 2003). Clinical trials using antioxidants in human reproduction have resulted in a major debate (Martin-Du Pan and Sakkas, 1998, Wong et al, 2000). Further in vivo and in vitro research is required before one can be optimistic about a role for antioxidants in the treatment of infertile men.

Sperm Preparation Techniques and ART

The recovery of desired sperm population can be achieved, depending on the type of andrology/ART procedure. However, the results vary in different ART facilities. Discontinuous Percoll density gradient (95.0% and 47.5%) has been used in the past with a great degree of success, resulting in improved DNA integrity (Larson et al, 2000). Use of Percoll as density gradient for andrology/ART procedures raised certain concerns a few years back, and since then it has been replaced by other commercially available density gradient agents for such purposes. Sperm sample prepared by PureSperm density gradients resulted in a significant decrease in the percentage of sperm DNA fragmentation as shown by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling (TUNEL) assay (Sakkas et al., 2002). However, swim-up technique has been shown to be better than density gradient in protecting sperm DNA integrity (Zini et al, 2000). Semen samples after swim-up or density gradient centrifugation can be enriched with morphologically normal spermatozoa with improved nuclear integrity (Colleu et al, 1996). This beneficial effect coincides with the relatively high post-IVF fertilization rate (76% ± 5.3%) after use of simple swim-up technique (Younglai et al, 2001).

Ejaculation directly in prewarmed sperm wash medium has been proposed to increase the proportion of antibody-free spermatozoa in semen samples that have been evaluated to contain antisperm antibodies by direct assay, thus enhancing the fertilization rate in vitro (Elder et al, 1990). Also, the addition of medium before liquefaction could inhibit the binding of bacteria to the sperm surface and subsequently diminish DNA damage caused by ROS. This method has also proven beneficial in semen samples with an increased percentage of DNA-damaged spermatozoa, allowing improved fertilization (Zollner et al, 2001).

On the other hand, many factors play a role in generating ROS during ART that induce spermatozoa damage when processed in vitro. These include leukocytes, abnormal spermatozoa, transition metals present in the culture medium, and centrifugation steps. Considering this fact, it is surprising to find that in vitro culture of testicular spermatozoa does not increase their susceptibility to DNA damage. In fact in vitro culture for 48–72 h at 37°C has been reported to improve the motility and postthaw recovery rate of testicular spermatozoa (Emiliani et al, 2000). Also, immature germ cell culture for 48 h facilitated the selection of TUNEL-negative spermatids (Tesarik et al, 1999). For patients with obstructive azoospermia, the proportion of spermatozoa containing single-stranded DNA damage decreases significantly after 3 days of culture (Emiliani et al, 2001). It is not clear whether it is due to the disintegration of single-strand DNA-damaged spermatozoa and the parallel development of immature double-strand DNA spermatids.

Sperm chromatin is a very complex structure, and its capability to decondense is one of the essential criteria for considering a spermatozoon to be fertile. DNA integrity in sperm is essential for the accurate transmission of genetic information and, in turn, the maintenance of good health in future generations. It is an independent measure of sperm quality that provides better diagnostic and prognostic capabilities than standard sperm parameters for male fertility potential. Numerous studies have reported

a negative correlation of in vitro fecundity with the percentage of DNA-damaged spermatozoa in semen samples. Several methods are currently used to assess DNA damage. However, the establishment of a cut-off point between normal levels in the average fertile population and the minimal levels of sperm DNA integrity required to achieve pregnancy using these different assays is still lacking.

Assessment of Oxidative Stress Status, Sperm Damage, and ART

In many complex biological systems including seminal fluid, the true OSS 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 (Krausz et al, 1994; Sikka et al, 1995). The methods commonly used for measuring ROS can be categorized into: 1) reactions involving nitroblue tetrazolium or cytochrome c-Fe^{3+} complexes that measure ROS on the cell membrane surface; 2) reactions that measure ROS (generated inside or outside the cell) utilizing luminol-dependent chemiluminescence; and 3) the electron spin resonance methods, which are more sensitive and can identify the type of ROS generated inside the cell, require skillful operation, accurate interpretations, and expensive instrumentation.

Assessment of Lipid Peroxidation of Spermatozoa

LPO is the most extensively studied manifestation of oxygen activation in biology. The most common types of LPO are nonenzymatic membrane LPO and enzymatic (NADPH and adenosine diphosphate (ADP) dependent) LPO. The enzymatic reaction involves NADPH-cytochrome P-450 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 (Ernster, 1993; Bell et al, 1993). Formation of MDA can be assayed by the thiobarbituric acid reaction, which is a simple and useful diagnostic tool for the measurement of LPO for in vitro and in vivo systems (Taourel et al, 1992). Currently, evaluation of isoprostanes (eg, 8-Iso-PGF2a) is commonly employed to indicate lipid peroxidation and oxidative stress response in vivo (Lawson et al, 1999).

Measurement of ROS and Nuclear DNA Damage

Levels of ROS ($O_{2}^{-\cdot}$, OH^{-}, and H_2O_2) are determined by either flow cytometry or more commonly by chemiluminescence assay using luminol (5-amino-2, 3 dihydro-1, 4 pthalazinedione) and lucigenin as the probes (Armstrong et al, 1999). Luminol measures both extracellular and intracellular ROS, whereas lucigenin detects extracellular O_{2}^{-}. The SCSA measured by flow cytometry detects susceptibility of sperm nuclear DNA to acid-induced denaturation or DNA breaks in situ and secondarily provides information on the extent of chromatin condensation (Sun et al, 1997; Evenson et al, 2002; Saleh and Agarwal, 2002). Normal, native chromatin remains structurally intact and produces a narrow α_t distribution. DNA in spermatozoa with abnormal chromatin structure shows increased red fluorescence (Evenson et al, 2002). Because sperm membrane–oocyte interactions are no longer relevant during ICSI, an evaluation of sperm DNA integrity is even more important in such cases. This lower-than-expected fertilization rate and/or embryo development could be due to chromatin structure defects in sperm DNA that can be monitored by SCSA or the comet assay (Evenson et al, 2002). In a recent study by Saleh and Agarwal (2002), semen samples were obtained from a randomly selected group of infertile men with a history of infertility of more than 1 year. Levels of apoptosis were detected using annexin-V staining assay. Sperm nuclear DNA damage was assessed by SCSA, and results were expressed as DNA fragmentation index. Apoptotic spermatozoa were identified by the expression of phosphatidylserine on the outer surface of the plasma membrane. Their results supported the recent hypothesis of a significant role of apoptosis in the pathogenesis of sperm nuclear DNA damage in men who failed to initiate a pregnancy after assisted reproductive techniques. Such an increase may be related to high levels of seminal oxidative stress presumably caused by prostatitis leading to leukocytospermia that can be easily detected microscopically (Muller et al, 2001). Recently the degree of DNA fragmentation as a marker of apoptosis was assessed using flow cytometric TUNEL assay. The data were used as a predictor of IUI success (Molina et al, 1995). It is possible that this TUNEL assay can be a potentially useful prognostic test in couples about to embark on IVF treatment.

What Is TAC and How Is It Measured?

A growing body of evidence suggests that low seminal total antioxidant capacity (TAC) is related to male infertility (Smith et al, 1996; Lewis et al, 1997; Sharma et al, 1999; Sikka, 2001). Thus, it is important to ensure that any measurement of seminal TAC is accurate and reliable and yet easy to use as a diagnostic tool in the evaluation and follow-up of male infertility. Several methods have been developed to measure TAC in biological fluids, such as the oxygen radical absorbance capacity (Cao and Prior, 1998), the ferric-reducing ability (Benzie and Strain, 1996), and the phycoerythrin fluorescence-based assay (Glazer, 1990). The enhanced chemiluminescence assay,

however, is the most commonly used method for measuring TAC in seminal fluid (Lewis et al, 1995; Sharma et al, 1999; Kobayashi et al, 2001; Pasqualotto et al, 2000). To perform the enhanced chemiluminescence assay, a signal reagent (luminol + para-iodophenol), which is a source of chemiluminescence, is mixed with horseradish peroxidase (HRP)-linked immunoglobulin (HRP-linked Ig) to produce ROS, which in turn is mixed with a substrate, hydrogen peroxide (H_2O_2). The power of the antioxidants in the seminal plasma to reduce the chemiluminescence of the signal reagent is compared with that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble alpha-tocopherol analogue, and is measured as molar Trolox equivalents (Sharma et al, 1999). However, this assay is cumbersome, requires a luminometer, and is often not readily available in a physician's office.

Another technique based on colorimetric assay has been recently described for estimating the TAC in human seminal plasma (Said et al, 2003). This assay is based on the formation of a stable ethylbenzothiazoline-6-sulphonic acid derivative with blue-green color that is measured at 600 nm. Antioxidants in the sample suppress this color production to a degree that is proportional to their concentrations. Another assay for measuring TAC is by monitoring the oxygen radical absorbance capacity. However, that requires a fluorescence detector takes a longer time than the colorimetric assay. There is a lack of consensus in the literature regarding what are normal TAC values in seminal plasma despite the strong correlation between low levels of seminal TAC and male infertility. Therefore, the standardization of any simple and inexpensive assay should be considered the first step to reach such a consensus.

Monitoring Oxidative Stress and Antioxidant Capacity of Media to Determine Health of Oocyte and the Embryo Quality in an ART Laboratory

Oocyte provides a glutathione-mediated reducing intracellular environment within which sperm chromatin decondensation occurs. Once an oocyte is retrieved into the Petri dish during processing for ART, it becomes very susceptible to oxidative damage. Depletion of the intracellular glutathione pool may further render the oocyte vulnerable to oxidative stress and incapable of decondensing the sperm nucleus, resulting in ART failure. Because oxygen is toxic to the embryo, an increase in oxidative stress will have a significant impact on the developmental potential of the mammalian embryo. In an interesting study, the arrest in embryonic development in mice at the 2-cell stage probably because of the activation of an apoptotic pathway was shown to be associated with the sudden production of hydrogen peroxide by the embryo (Nasr-Isfahani et al, 1990). This suggests that the excessive generation of ROS by the defective human embryo can similarly induce apoptosis with demonstrable nuclear changes typical of apoptotic cells. The culture media frequently used by such facilities are usually supplemented with antioxidants (eg, HSA), and as such the antioxidant capacity in the media can be easily monitored in the laboratory. Paszkowski and Clarke (1996) showed that the culture media in which viable human embryos were maintained retained the antioxidant activity, but the media recovered from incubations involving fragmenting defective human embryos showed a significant loss of antioxidant activity with time. This presumably confirms the hypothesis that the intrinsic generation of excess ROS will result in increased oxidative stress leading to failure in the normal development of embryo. It is not clear whether additional supplementation of specific antioxidants during these ART procedures will combat this oxidative stress and help improve fertilization process, embryo quality, and better success rate. Once confirmed, frequent sampling of a small amount of culture media to monitor antioxidant capacity can provide the basis of a simple noninvasive technique for assessing progress in embryo quality in ART programs especially prior to implantation. Thus, understanding of the role of these antioxidants is at present a very challenging area to focus in an ART laboratory and clinical practice.

Sperm Stimulation and ART

Poor motility of sperm samples retrieved after cryopreservation, electroejaculation, and vibratory stimulation and in idiopathic infertility patients with genitourinary inflammation has been associated with low success rate after IUI. Sperm cryopreservation continues to gain importance in assisted reproduction, especially for donor programs and for patients having AIDS, cancer, irradiation, vasectomy, and other aging issues who later desire to have children. Men with spinal cord injury who desire children have invariably low sperm motility in their samples collected from vibratory stimulation or electroejaculation. Several reports suggest improvement in sperm motility and progression by use of many biological or pharmacological stimulants in vitro. Methyl xanthines like pentoxifylline (PTX) and caffeine, which are cyclic adenosine 5′-monophosphate (cAMP) phosphodiesterase inhibitors, improve sperm motility via cAMP pathway (Sikka and Hellstrom, 1991; Bell et al, 1993). For andrology/ART purposes, it is recommended that PTX should be used at doses less than 3 mM for a maximum of 1-hour incubation. Adenosine analog (2-deoxyadenosine) increases cAMP by stimulating adenyl cyclase activity and should be used at doses less than 2.5 mM (Sharma and Agarwal, 1996). PAF, a naturally occurring phosphatidyl derivative in membranes, is known to stabilize membranes and may protect against oxidative stress–in-

duced cryodamage to improve recovery of motile spermatozoa and improve IUI outcome (Wang et al, 1993; Minhas, 1993; Wild and Roudebush, 2001). These studies also demonstrated that both PTX and PAF can act synergistically in improving ART results because of different modes of actions (Wang et al, 1994). L-carnitine is the biologically active stereoisomer of 3-hydroxyaminobutyrate and at a dose of more than 3 g/day when administered orally for 6 months is known to improve sperm motility because of increased mitochondrial energy metabolism (Costa et al, 1994). Some of these sperm stimulants (eg, pentoxifylline, L-carnitine) are also ROS scavengers and may demonstrate antioxidant properties besides affecting cellular metabolism. However, onset of rapid capacitation and early acrosome reaction by using such stimulants can affect proper timing for sperm-ovum interaction. Thus, with such stimulated sperm samples, IUI should be performed within 1–2 hours of stimulation and washing procedures. Besides timing, female fertility status and number of progressively motile spermatozoa used for insemination for IUI (more than 1 million sperm) or IVF (2000-20 000 sperm under mineral oil) are other major critical factors for ART success in presence of such sperm stimulants. With renewed interest in these stimulants for andrology/ART purposes, it will be interesting to see their role in the outcome of pregnancy for such infertile patients.

Concluding Remarks

One in 7 couples suffers from infertility issues during their reproductive lives, and male factor is implicated as the cause in up to 50% of these cases. Although an ART laboratory may demonstrate normal parameters on standard semen analysis, defective sperm function is the most prevalent cause of idiopathic male infertility and a difficult condition to treat. At present, there is no single reliable test, which can evaluate normal sperm function, in spite of many efforts in this direction (Bar-Chama and Lamb, 1994; Muller, 2000). Andrology/ART procedures like IUI and IVF still have great potential even in this ICSI era. Many sperm stimulation protocols have been described to improve the success rate for these procedures. However, timing of insemination, number of progressively motile sperm (eg, more than 1 million for IUI and about 10 000 to 20 000 for IVF), and female fertility status are important factors to be considered for such success.

Spermatozoa and oocytes possess an inherent but limited capacity to generate ROS, which may help the fertilization process. However, excessive ROS generation as a result of oxygen toxicity or inflammatory process will induce a state of oxidative stress that is damaging to spermatozoa, oocytes, and the embryo and may affect the results of various andrology/ART procedures. How this oxidative stress affects interaction of sperm with the ovum and causes embryo toxicity is still unknown. Increased oxidative damage to sperm membranes, proteins, and DNA is associated with alterations in signal transduction mechanisms that affect fertility. On the other hand, ROS-induced apoptosis may be particularly important to ensure that genetically damaged cells do not contribute to the future fetus. 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 both in vivo and in vitro appears to be necessary for the survival and normal reproductive functioning. An assay system for the evaluation of 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 nonresponders to any putative antioxidant therapy. Most dietary antioxidants appear to have only modest physiological effects. It is believed that for males with idiopathic infertility, recommended daily dietary supplementation with a combination of well-tolerated, clinically efficacious and noninvasive vitamins and vitamin-like agents, such as L-carnitine, acetylcartinine, and vitamins E and C, may provide an alternate pharmacological approach to improve sperm quality and greater likelihood of success of ART. Although the therapeutic use of such antioxidants appears attractive with minimal side effects, the proper multicenter clinical trials are required to determine efficacy and any reproductive toxicity before clinicians could recommend such therapeutic alternatives. The key unanswered question pertaining to their usage is to find out how much is success and beyond that it becomes excess.

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