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# Relevance of Oxygen Free Radicals and Antioxidants in Sperm Production and Function

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**Abstract:** All the cells living under aerobic conditions constantly face the oxygen paradox. Oxygen is required to support life, but its metabolites such as Reactive Oxygen Species (ROS) can modify cell functions, endanger cell survival, or both. Sperm cell membranes are high in polyunsaturated fatty acids and are sensitive to oxygen free radical induced damage mediated by lipid peroxidation. Oxygen free radicals are naturally produced by mitochondrial and sperm plasma membrane and have beneficial and detrimental effects that cannot be overlooked. Limited endogenous mechanisms exist to reverse these damages. The excessive generation of oxygen free radicals in the form of ROS by abnormal spermatozoa and by contaminating leukocytes has been identified as one of the few defined etiologies for male infertility. However, the over production of oxygen free radicals in sperm membrane decreases the antioxidant defense activity thereby leading to low sperm motility and viability, DNA fragmentation and protein denaturation. To counteract the negative effects of excess ROS, spermatozoa and seminal plasma have several mechanisms to neutralize free radicals. They include enzymatic and non-enzymatic antioxidant systems which work synergistically to prevent harmful effects of the by-products from aerobic metabolism. Since, ROS is essential for the normal sperm physiology, rationale use of antioxidants is advocated. Therefore, this review will attempt to discuss the relevance of free radicals and antioxidants in spermatozoa production and function.

**Key words:** Free radicals, antioxidant, spermatozoa

## INTRODUCTION

Numerous evidence have accumulated to implicate cellular damage arising from oxygen free radicals, at least in part, in the etiology and pathophysiology of diseases such as neurodegenerative disorders, inflammation, infertility, viral infections, autoimmune pathologies and digestive system disorders such as gastrointestinal inflammation and ulcer (Repetto and Llesuy, 2002; Aruoma, 2003; Surh and Fergusson, 2003). In addition, chemical mobilization of fat stores under various conditions such as lactation, exercise, fever, infection and even fasting, can result in increased radical activity and damage, in particular, to the immune and nervous systems, while the stress hormones secreted by the adrenal glands under conditions of continuing and excessive emotional stress, are metabolized into simpler, albeit, free radical molecules.

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Free radical now appears to be the fundamental mechanism underlying a number of neurologic and other disorders. For instance in diabetes, increased oxidative stress which co-exist with reduction in the antioxidant status has been postulated: Oxygen free-radical can initiate peroxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes and play a role in the long term complication of diabetes (Collier *et al*., 1990; Baynes, 1991; Sabu and Kuttan, 2002).

The excessive generation of oxygen free radicals in the form of reactive oxygen species by abnormal spermatozoa and by contaminating leukocytes has been identified as one of the few defined etiologies for male infertility. Mammalian sperm cells present highly specific lipidic composition, high content of polyunsaturated fatty acids, plasmalogenes and sphingomyelins. This unusual structure of sperm membrane is responsible for its flexibility and the functional ability of sperm cells. However, spermatozoa's lipids are the main substrates for peroxidation, which may provoke severe functional disorder of sperm. On the other hand, low levels of lipid peroxidation reflect the influence of reactive oxygen species on sperm metabolism thus enhancing the ability of spermatozoa to interact with zona pellucida (Aitken *et al*., 1989a).

In normal physiological functions, there is a balanced generation of reactive oxygen species and antioxidant enzymes (Kovalski *et al*., 1992; Plante *et al*., 1994; Aitken *et al*., 1995a, b). However, overproduction of ROS and decreased antioxidant defense activity cause low sperm motility and viability, DNA fragmentation and protein denaturation (Agarwal and Said, 2005; Kankofer *et al*., 2005). To counteract oxidative damage, spermatozoa and seminal plasma have several mechanisms to neutralize free radicals. Enzymatic and non-enzymatic antioxidant systems work synergistically to prevent harmful effects of by-products from aerobic metabolism (De Laraminde *et al*., 1993; De Lamirande *et al*., 1998). For example, mammalian semen (mainly seminal plasma) has many compounds with non-enzymatic antioxidant activity (e.g., ascorbic acid, α-tocopherol, taurine and albumin (Alvarez and Storey, 1983). However, the presence of specific antioxidant enzymes suggests that they also play a major role in the protection of spermatozoa against reactive oxygen species. This study is an attempt to discuss the relevance of oxygen free radicals and antioxidants on spermatozoa production and functions.

Over the past two decades, an expanding body of evidence from epidemiological and laboratory studies have demonstrated that some antioxidant have substantial protective effects on carcinogenesis (Tsao *et al*., 2004; Kinghorn *et al*., 2004).

**Spermatozoa**

Semen is a mixture of spermatozoa produced by testicles and seminal plasma secreted at different sites by accessories glands and by the epididymis, which are combined at the time of ejaculation (Castellini, 2008). Like the blood, semen consists of two compartments, the cellular compartment and non cellular compartment. Seminal plasma also contains other particles of different size which affect the spermatozoa behaviour during the transit along the female reproductive tract (Castellini, 2008). It is found that semen are highly fragile medium and must be handled carefully. Semen is a whitish, milky fluid, very viscous, containing water, enzymes, vitamins, minerals and small amounts of salt, protein and fructose.

Recent scientific evidence has shown that the particulate fraction of seminal plasma plays an important role in reproductive physiology of several mammal species (Castellini, 2008). These particles are secreted by different accessory glands of the male reproductive

apparatus and thus their biochemical composition and function vary from species to species (Castellini, 2008). Seminal particles have different dimensions: large granules about the sperm head dimension, abundant in rabbit semen (Zaniboni *et al*., 2004) and small particles described in several other mammalian species (Fornes *et al*., 1991; El-Hajj-Ghaoui *et al*., 2004).

The lipid composition of plasma membrane of mammalian spermatozoa is markedly different from those of mammalian somatic cells. They have very high levels of phospholipids, sterols, saturated and polyunsaturated fatty acids therefore sperm cells are particularly susceptible to the peroxidation damage induced by excessive free radicals released.

**Spermatozoa Membrane Lipids**

A characteristic feature of most, if not all, biological membranes is an asymmetrical arrangement of lipids within the bilayer. The lipid composition of plasma membrane of mammalian spermatozoa is markedly different from those of mammalian somatic cells. They have very high levels of phospholipids, sterols, saturated and polyunsaturated fatty acids (Alvarez and Storey, 1995) (Table 1, 2). Therefore sperm cells are particularly susceptible to the peroxidation damage induced by excessive ROS release (Aitken and Clarkson, 1987; Mack *et al*., 1986; Alvarez and Storey, 1995).

Lipids are the major substances responsible for the fluidity of membrane lipid bilayers and changes in composition of the plasma membranes of sperm cells from their epididymal maturation to their capacitation in the female reproductive tract. Sperm cells undergo changes in lipid content during their passage through the epididymis. As a consequence of these changes the plasmalogenes (ether-linked lipids) become a major phospholipid component

Table 1: Lipid components in human spermatozoa

| Components | nmol $10^{-8}$ cells |
|---|---|
| **Phospholipid** | |
| Choline diacylglycerophospholipid | 37.0 |
| Ethanolamine diacylglycerophospholipid | 31.5 |
| Choline plasmalogen | 12.5 |
| Ethanolamine plasmalogen | 20.0 |
| Phosphatidilserine | 8.5 |
| Phosphatidylinositol | 6.1 |
| Phosphatidyloglycerol | 0.6 |
| Sphingomyelin | 20.0 |
| Cardiolipin | 2.1 |
| Total phospholipids | 138.3 |
| **Sterols** | |
| Cholesterol | 133.0 |
| Desmosterol | 78.5 |
| Total sterol | 211.5 |
| Glycolipids | 6.4 |

Table 2: Fatty acids components in human spermatozoa

| Components | nmol $10^{-8}$ cells |
|---|---|
| **Saturated fatty acids** | |
| Hexadecanoic (palmitic) | 105.5 |
| Octadecanoic (stearic) | 35.9 |
| **Unsaturated fatty acids** | |
| Octadenoic (oleic) | 32.6 |
| Octadecadienoic (linoleic) | 23.2 |
| Icosatrienoic | 14.9 |
| Docosahexaenoic | 20.1 |
| Icosatetraenoic (arachidonic) | 108.0 |

in the caudal epididymis and 2-fold increase of cholesterol/phospholipid molar ratio is observed during sperm migration from the seminiferous tubules (Aveldano *et al*., 1992). Very high amounts of polyunsaturated fatty acids-especially docosahexaenoic (DHA) are found in the plasma membrane of sperm. The DHA is thought to play a major role in regulating membrane fluidity in sperm and in the regulation of spermatogenesis (Hall *et al*., 1991; Haidl and Opper, 1997; Ollero *et al*., 2000). The DHA content is significantly higher in immature germ cells and immature spermatozoa as compared to mature sperm and indicates that there is a net decrease in DHA content during the process of sperm maturation (Ollero *et al*., 2000).

Another sperm lipid, phosphatidylserine, is known to translocate during capacitation. Before capacitation this phospholipid was found mainly in the midpiece but after capacitation, the localisation of phosphatidylserine was changed and it was identified also in the acrosomal region but never in the equatorial area (Kotwicka *et al*., 2002). Sperm also contains desmosterol, which is lost during capacitation (Cross, 2003). Other phospholipid, sphingomyelin in the sperm influences the rate of capacitation by slowing down the loss of sterols and the exogenous sphingomyelinase accelerates capacitation promoting the loss of sterols and generating ceramide (Cross, 2000). Cholesterol is known to regulate the fluidity and the permeability of cell membrane. Cholesterol efflux during capacitation enables the massive influx of extracellular $Ca^{2+}$. Increased intracellular $Ca^{2+}$ concentration plays an important role in the acrosome reaction (Schroeder *et al*., 1991; Zaneveld *et al*., 1996).

The high level of in polyunsaturated fatty acids (PUFA) in mammalian spermatozoa membranes makes it susceptible to oxygen free radical induced damage mediated by lipid peroxidation (LPO). Therefore spermatozoa are unique in structure, function and susceptibility to damage by LPO caused by free radicals (Alvarez *et al*., 1987).

**Free Radicals**

Free radicals are short-lived reactive chemical intermediates, which contain one or more electrons with unpaired spin. By definition a free radical is any atom (e.g., oxygen, nitrogen) with at least one unpaired electron in the outermost shell and is capable of independent existence. These include single oxygen, hydrogen peroxide, superoxide anion radicals, peroxyl radicals and hydroxyl radicals. The sources of these free radicals in the living body are respiratory chain, phagocytes, arachidonic acid metabolism, cytophosphokinase, non-enzymatic reaction of oxygen and ionizing radiations. These compounds sometimes working together with some common metals like copper, iron and cobalt attack an important group of tissue constituents-notably lipids-creating a number of deleterious effects. Stress, anesthesia, anti-cancer drugs, pain killers and even normal energy metabolism also will release free radicals in varying amounts.

The free radicals are highly reactive and oxidize lipids, amino acids and carbohydrates as well as causing DNA mutations. Any free radical involving oxygen can be referred to as Reactive Oxygen Species (ROS). Reactive Oxygen Species (ROS) therefore may have been implicated in the pathogenesis of a growing number of diseases (Chin *et al*., 1992; Rosen *et al*., 1993; White *et al*., 1994). Enhanced, pathological ROS generation in living organisms may be caused by several mechanisms like: ionizing radiation (Sadani and Nadkarni, 1997; Zhang *et al*., 1998), bio-activation of xenobiotics (Akiyama, 1999), inflammatory cells (Villegas *et al*., 2003), increased cellular metabolism (Hollan, 1996), decompartmentalisation of transition metal ions (Huang *et al*., 2001), activation of oxidases and oxygenases (Davydov, 2001) and loss of antioxidant capacity (Aitken and Sawyer, 2003).

**Free Radical Formation**

Atoms are most stable in the ground state. An atom is considered to be in a ground state when every electron in the outermost shell has a complimentary electron that spins in the opposite direction. A free radical is easily formed when a covalent bond between entities is broken and one electron remains with each newly formed atom.

Free radicals are very unstable and react quickly with other compounds, trying to capture the needed electron to gain stability. Free radicals are highly reactive due to the presence of unpaired electron(s). Generally, free radicals attack the nearest stable molecule, stealing its electron. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction. Once the process is started, it can cascade, finally resulting in the disruption of a living cell.

Any free radical involving oxygen can be referred to as reactive oxygen species. Oxygen centered free radicals contain two unpaired electrons in the outer shell. When free radicals steal an electron from a surrounding compound or molecule a new free radical is formed in its place. In turn the newly formed radical then looks to return to its ground state by stealing electrons with anti-parallel spins from cellular structures or molecules. Thus the chain reaction continues and can be thousand of events long (Goldfarb, 1999). The electron transport chain, which is found in the inner mitochondrial membrane, utilizes oxygen to generate energy in the form of adenosine triphosphate. Oxygen acts as the terminal electron acceptor within the electron transport chain.

**Types of Free Radicals**

There are numerous types of free radicals that can be formed within the body. The most common free radicals are: the superoxide anion ($O_2^-$), the hydroxyl radical ($OH^-$), singlet oxygen ($^1O_2$) and hydrogen peroxide ($H_2O_2$) (Table 3). Superoxide anions are formed when oxygen ($O_2$) acquires an additional electron, leaving the molecule with only one unpaired electron. Within the mitochondria $O_2^-$ is continuously being formed. The rate of formation depends on the amount of oxygen flowing through the mitochondria at any given time. Hydroxyl radicals are short-lived, but the most damaging radicals within the body. This type of free radical can be formed from $O_2^-$ and $H_2O_2$ via the Harber-Weiss reaction. The interaction of copper or iron and $H_2O_2$ also produce $OH^-$ as first observed by Fenton. These reactions are significant as the substrates are found within the body and could easily interact (Halliwell and Gutteridge, 1984). Hydrogen peroxide is unique in that it can be converted to the highly damaging hydroxyl radical or be catalyzed and excreted harmlessly as water. Glutathione peroxidase is essential for the conversion of glutathione to oxidized glutathione, during which $H_2O_2$ is converted to water (Alessio and Blasi, 1997). If $H_2O_2$ is not converted into water singlet oxygen ($^1O_2$) is formed.

Table 3: Examples of free radicals

| Free radical | Structures |
|---|---|
| Nitric oxide | $NO^-$ |
| Nitric dioxide | $NO_2^-$ |
| Hypochlorus acid | ClOH |
| Hypobromus acid | BrOH |
| Hypoiodus acid | JOH |
| Peroxy radical | $ROO^-$ |
| Peroxide | ROOH |
| Superoxide anion | $O_2^-$ |
| Hydrogen peroxide | $H_2O_2$ |
| Hydroxyl radical | $OH^-$ |

Source: Dorota and Maciej (2004)

Singlet oxygen is not a free radical, but can be formed during radical reactions and also cause further reactions. Singlet oxygen violates Hund's rule of electron filling in that it has eight outer electrons existing in pairs leaving one orbital of the same energy level empty. When oxygen is energetically excited one of the electrons can jump to empty orbital creating unpaired electrons. Singlet oxygen can then transfer the energy to a new molecule and act as a catalyst for free radical formation. The molecule can also interact with other molecules leading to the formation of a new free radical.

**Oxygen Free Radical [Reactive Oxygen Species (ROS)]**

Reactive oxygen species are highly reactive oxidizing agents belonging to the class of free-radicals. Reactive oxygen species are produced in the cells by cellular metabolism and other exogenous environmental agents. They are generated by a process known as redox cycling and are catalysed by transition metals, such as $Fe^{2+}$ and $Cu^{2+}$ (Halliwell and Gutteridge, 1999). Overproduction of ROS can damage cellular biomolecules like nucleic acids, lipids, carbohydrates, proteins and enzymes, resulting in several diseases.

Living systems have specific pathways to overcome the adverse affects of various damages. However, sometimes these repair mechanisms fail to keep pace with such deleterious effects (Halliwell, 1995; Nilsson *et al*., 2004). The most common ROS that have potential implications in reproductive biology include superoxide anions ($O_2^-$), hydrogen peroxide ($H_2O_2$), peroxyl ($ROO^-$) radicals and hydroxyl ($OH^-$) radicals. The nitrogen derived free radical Nitric Oxide (NO) and peroxynitrite anion ($ONOO^-$) also appear to play a significant role in the reproduction and fertilization. The ultimate effects of Nitric Oxide ($NO^-$) depend upon its concentration and interactions with hydrogen peroxide. Peroxynitrite (oxoperoxonitrate) anion may be formed *in vivo* from superoxide and nitric oxide and actively reacts with glutathione, cysteine, deoxyribose and other thiols/thioethers (Koppenol *et al*., 1992). This can form a strongly nitrating species in the presence of metal ions or complexes. Reactive oxygen species are known to damage cellular membranes by inducing lipid peroxidation (LPO) in spermatozoa (Rama Devi and Prasad, 1998).

**Oxygen Free Radical and Peroxidation of Membrane Lipids**

A free radical prefers to steal electrons from the lipid membrane of a cell, initiating a free radical attack on the cell known as lipid peroxidation. Polyunsaturated fatty acids (PUFAs) are abundant in cellular membranes and in Low-Density Lipoproteins (LDL) (Dekkers *et al*., 1996). The PUFAs allow for fluidity of cellular membranes. Reactive oxygen species target the carbon-carbon double bond of polyunsaturated fatty acids. Peroxidation of polyunsaturated fatty acids (PUFAs) in sperm cell membranes is an autocatalytic, self-propagating reaction, which can give a rise to cell dysfunction associated with loss of membrane function and integrity. The first step in the peroxidation process, called-initiation is the abstraction of a hydrogen atom from an unsaturated fatty acid. The second step propagation-is the formation of a lipid alkyl radical followed then by its rapid reaction with oxygen to form a lipid peroxyl radical. The peroxyl radical is capable of abstracting a hydrogen atom from an unsaturated fatty acid with the concomitant formation of a lipid radical and lipid hydroperoxide. Since, the peroxyl and alkyl radicals are regenerated, the cycle of propagation could continue indefinitely or until one of the substrates is consumed or terminated in the radical-radical reaction.

The products of peroxidation of polyunsaturated fatty acids have been implicated in a wide variety of pathological conditions including infertility, cardiac and cerebral ischaemic-reperfusion injury and inflammatory joint diseases amongst others.

**Lipid Peroxidation Products**

The most popular product of lipid peroxidation is malondialdehyde (MDA). There are a lot of other products of lipid peroxidation such as: conjugated dienes and secondary peroxidation products, which include saturated and unsaturated aldehydes, ketones, oxo and hydroxyl acids and saturated and unsaturated hydrocarbons (e.g., ethane, pentane). Lipid peroxidation in biological membranes causes impairment of membrane functioning, decreased fluidity, inactivates of membrane-bound receptors and enzymes and increased non-specific permeability to ions. Moreover, lipid hydroperoxides decompose upon exposure to copper while iron chelates the other factors including metals as haem, haemoglobin or myoglobin. Cytotoxic aldehydes are formed as a consequence of lipid hydroperoxide degradation. Malondialdehyde (MDA) and 4-hydroxynonenal are hydrophilic and are released from Low-Density Lipoproteins (LDL) into aqueous surroundings. Hydroxynonenal is biologically active and can cause severe cell dysfunction both on protein and DNA levels. Hydroxynonenal is chemotactic for polymorphonuclear leukocytes (PMNs) at picomolar concentrations, inhibits cell proliferation and is mutagenic (Esterbauer *et al*., 1990; Aitken *et al*., 1993).

**Biological Implications of Lipid Peroxidation (LPO) Products on Spermatozoa**

Spermatozoa, unlike other cells, are unique in structure, function and susceptibility to damage by LPO (Alvarez *et al*., 1987). In general, the most significant effect of 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. Minor alterations in sperm membranes in selected cases of dyspermia can be reversed by GSH therapy (Lenzi *et al*., 1994). Studies on how these cellular changes caused by LPO affect seminal parameters and sperm function and reversal of these effects are open to further investigations.

Besides membrane effects, LPO can damage DNA and proteins, either through oxidation of DNA bases or through covalent binding to MDA resulting in strand breaks and cross-linking (Ernster, 1993). The ROS can also induce oxidation of critical-SH groups in proteins and DNA, which will alter structure and function of spermatozoa with an increased susceptibility to attack by macrophages (Aitken *et al*., 1994). The oxidative damage to mitochondrial DNA is well known to occur in all aerobic cells which are rich in mitochondria and this may include spermatozoa. In addition, the redox status of spermatozoa is likely to affect phosphorylation and ATP generation with a profound influence on its fertilizing potential (Cummins *et al*., 1994). Aitken *et al*. (1995a) recently showed that stimulation of endogenous NADPH-dependent ROS generation in sperm appears to regulate acrosome reaction via tyrosine phosphorylation. In general, the oxidizing conditions increase tyrosine phosphorylation with enhanced sperm function while reducing conditions have the opposite effect.

**Oxygen Free Radical and Spermatozoa**

Presence of leukocytes in semen has been linked with severe male factor infertility cases (Wolff and Anderson, 1988; Aitken *et al*., 1992). There has been much speculation as to whether the origin of ROS in semen is from spermatozoa or from infiltrating leukocytes (Kessopoulou *et al*., 1992; Krausz *et al*., 1994). Iwasaki and Gagnon (1992) reported that the leukocyte free percoll fractions of semen samples obtained from nonazoospermic infertile men generate detectable levels of ROS when compared to the semen of normal and azoospermic men suggesting that damaged spermatozoa are likely to be the source of ROS. Also, higher

levels of ROS were correlated with a decreased number of motile sperm; conversely, greater sperm motility was observed in samples with lesser amounts of detectable ROS (Iwasaki and Gagnon, 1992). It is important for the clinician to recognize that assisted reproductive techniques (percoll gradients/sperm washing/centrifugation) may induce damage to spermatozoa by increasing ROS generation by spermatozoa (Aitken and Clarkson, 1987).

**Free Radical and Sperm Function**

The balance of ROS can be termed as the balance of creation and destruction. Under normal circumstances, there is an appropriate balance between pro-oxidants and anti-oxidants. Theoretically, cellular damage in the semen is the result of an improper balance between ROS generation and scavenging activities. A shift in the levels of ROS towards pro-oxidants in semen and vaginal secretions can induce an oxidative stress on spermatozoa. The scavenging potential in gonads and seminal fluid is normally maintained by adequate levels of antioxidants superoxide dismutase (SOD), catalase and probably glutathione (GSH) peroxidase and reductase (Sikka *et al*., 1995). This balance can be referred to as Oxidative Stress Status (OSS) and its assessment may play a critical role in monitoring sperm damage and infertility.

A situation in which there is a shift in this ROS balance towards pro-oxidants, because of either excess ROS or diminished anti-oxidants, can be classified in terms of positive Oxidative Stress Status (OSS). At present, there is no true ROS detection method available which will evaluate this balance. However, assessment of OSS, or a similar paradigm when monitored more objectively, would be a good indicator of sperm damage caused by oxidative stress (Sikka *et al*., 1995). Chronic asymptomatic genitourinary inflammation can be regarded as a condition with positive OSS, which may be the real cause of idiopathic infertility in such patients. Superoxide dismutase (SOD) may directly act as antioxidant enzymes involved in the inhibition of sperm LPO (Alvarez *et al*., 1987). A high GSH/GSSG ratio will help spermatozoa to combat oxidative insult (Irvine, 1996).

Nitric Oxide ($NO^-$) radical and Reactive Nitrogen Species (RNS) have recently been found to have biological roles in inflammation and in mediating many cytotoxic and pathological events (Darley-Usmar *et al*., 1995). Synthesis of $NO^-$ in response to infection and inflammation could contribute to poor sperm motility and function and may lead to infertility (Rosselli *et al*., 1995). The RNS (e.g., $NO^-$) like ROS may normally be useful for maintaining sperm motility but can be toxic in excess. Other RNS such as nitrogen dioxide ($NO_2$) radical and peroxynitrite ($ONOO^-$) anion are considered to be damaging. The primary mechanism of nitric oxide-induced sperm damage is likely to be inhibition of mitochondrial respiration and DNA synthesis (Hibbs *et al*., 1987). Nitric oxide induced toxicity is also mediated indirectly through its interaction with superoxide anions and formation of peroxynitrite anion, which when protonated, decomposes to form $OH^-$ and $NO_2$, both of which are cytotoxic agents (Beckman *et al*., 1990).

**Interaction Between Free Radicals, Leukocytes and Sperm Functions**

Reactive Oxygen Species (ROS) have been implicated in over a hundred of disease states which range from arthritis and connective tissue disorders to carcinogenesis, ageing, toxin exposure, physical injury, infection, acquired immunodeficiency syndrome and leukocytospermia (Joyce, 1987). The exact site of origin of these leukocytes in semen, their mode of action and the role that bacteria, viruses and subsequent genitourinary-inflammation might have on sperm function are not clear. Experimentally, ROS production by human spermatozoa and contaminating leukocytes can be stimulated by phorbol esters and certain

formyl peptides with deleterious effects on sperm motility and fertilization (Krausz *et al*., 1994). Although, the presence of leukocytes in semen did not diminish the *in vitro* fertilizing capacity of spermatozoa however, the introduction of leukocytes into washed sperm preparations did reduce sperm function by the production of ROS (Aitken *et al*., 1994). This finding seems paradoxic but does indicate that seminal plasma has significant antioxidant or ROS scavenging capacity which may prevent sperm damage by leukocytes. An association between leukocytospermia and ROS has been recently found to correlate with increased chemokine (IL-8) and decreased SOD activity of the semen (Rajasekaran *et al*., 1995). This demonstrates that increased oxidative stress during leukocytospermia is caused by a defective ROS scavenging system which, in turn, can be modulated by certain pro-inflammatory cytokines. A significant shift towards increased production of pro-inflammatory chemokine (GRO-a) compared to anti-inflammatory cytokine (IL-10) during leukocytospermia suggests an active chemotactic pro-inflammatory response (Rajasekaran *et al*., 1996). This shift may be responsible for a significant oxidative stress to spermatozoa due to leukocytes in the semen of the infertile patient (Sikka *et al*., 1995).

**Bio-negative Effects of Oxygen Free Radicals**

Infertility has been a major medical problem since the dawn of humanity. Despite the enormous progress in research and reasoning, most of the blame for infertility, until recently, was placed on the female. Only during the last 15-20 years, advances in understanding of gonadal/sperm function and dysfunction led to a dramatic increase in our knowledge of male infertility. Defective sperm function is the most prevalent cause of male infertility and a difficult condition to treat (Hull *et al*., 1985).

Many environmental, physiological and genetic factors have been implicated in the poor sperm function and infertility. Although, techniques like intracytoplasmic sperm injection (ICSI) offer considerable promise to such male infertility factor, the indiscriminate use of such assisted fertility treatments, especially when the etiology of sperm dysfunction is poorly understood is not warranted. Thus, it is very important to identify the factors which affect normal sperm function. Free radical-induced oxidative damage to spermatozoa is one such condition which is recently gaining a considerable attention for its role in inducing poor sperm function and infertility. Understanding of how such conditions affect sperm function will help designing new and effective treatment strategies.

Mammalian spermatozoa are rich in polyunsaturated fatty acids and thus are very susceptible to ROS attack which results in a decreased sperm motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability and increased midpiece morphology defects with deleterious effects on sperm capacitation and acrosome reaction (Lamirande and Gagnon, 1992). Lipid peroxidation of sperm membrane is considered to be the key mechanism of this ROS-induced sperm damage leading to infertility (Alvarez *et al*., 1987). Limited amounts of ROS can also interfere physiologically in the regulation of sperm functions thus may lead to infertility.

Some forms of infertility are caused by age related degenerative disorders of the testis. As apparent from the declining sperm population over the last two generations, this problem is increasing in industrialized societies (Johnson, 1989). Although, the decline in sperm numbers is considered to be associated with male fetal and/or neonatal exposure to increased level of the environmental estrogen, the idiopathic male infertility may also be explained as a form of premature or differential ageing of the testis induced by ischemia and oxidative stress associated with defective mitochondrial genome that controls the oxidative phosphorylation (Cummins *et al*., 1994). Presence of retained cytoplasmic droplets on spermatozoa due to imperfect spermiation in ageing testis may be a sign of reduced fertility.

Decreased vascularity, increased spermatogenic failure and reduced sperm output occur with senescence in a variety of animal species and in humans (Kerr, 1992). Degenerated germ cells are presumably phagocytosed by Sertoli cells, resulting in lipid accumulations that increase progressively with age. Increased levels of lipofuscin and lipid are seen intracellularly, suggesting presence of mitochondrial dysfunction possibly compounded by oxidative stress in the older population (Reichel, 1968). These degenerative changes in gonads associated with accumulation of lipofuscin pigment and multiple nuclei are considered to be due to ROS-induced lipid peroxidation with age (Reichel, 1968). Most of these changes are strikingly similar to that seen in men with idiopathic testicular failure, probably due to induced gonadotoxicity. However, functional abnormalities in mature spermatozoa leading to infertility have not been demonstrated in normal ageing men.

It is known that lipid peroxidation (LPO) and mid piece anomalies are linked (Rao *et al*., 1989) and that the increased rate of LPO and Creatine Kinase (CK) activity in immature sperm is due to incomplete cytoplasmic extrusion during terminal spermatogenesis (Huszar and Vigue, 1994). In addition, sertoli cells abnormality in infertile men may well be central to the development of spermatogenic failure due to faulty spermiation and maybe related to genetic defects, oxidative stress, or even ageing of the gonads.

**Bio-Positive Effect of Free Radicals**

**Immune System**

Although, the previous section has focused only on the negative effects of oxygen free radical production. However, oxygen free radicals are naturally produced by some systems within the body and have beneficial effects that cannot be overlooked. The immune system is the main body system that utilizes free radicals. Oxygen free radicals play an important role in regulation of vasotonus and in antimicrobial defense. Foreign invaders or damaged tissue is marked with free radicals by the immune system. This allows for determination of which tissue need to be removed from the body. Because of this, there are divided opinions on the need for antioxidant supplementation, as they believe supplementation can actually decrease the effectiveness of the immune system.

**Fertility**

The assumption that free radicals can influence male fertility has received substantial scientific support (Gagnon *et al*., 1991). The proposed mechanism for loss of sperm function upon oxidative stress has been shown to involve excessive generation of ROS (Aitken and Clarkson, 1987). The hydrogen peroxide has both beneficial and damaging effects on sperm and thus can influence the fertilization process. Hence, oxygen free radicals are associated with oxidative stress and are likely to play a number of significant and diverse roles in reproduction.

The generation of ROS occurs physiologically during normal cell metabolism. Mitochondrial respiration is the main biological source of superoxide anion radicals under physiological conditions. During the tetravalent reduction of oxygen to water by the mitochondrial cytochrome c oxidase, these radicals can leak to the cell. At low concentrations reactive oxidants have a bio-positive effect and act selectively (De Lamirande *et al*., 1997a). They act on the metabolism of prostanoids, in gene regulation or in the regulation of cellular growth, intracellular signaling and the other types of signal transduction. It has been observed that low amounts of free radicals in human semen enhance spermatozoa ability to bind zona pellucida. In addition, the incubation of sperm cells with low concentrations of hydrogen peroxide was found to stimulate sperm capacitation, hyperactivation, acrosome reaction and oocyte fusion (De Lamirande and Gagnon, 1993; Griveau and Le Lannou, 1997; De Lamirande *et al*., 1998).

**Assessment of Oxygen Free Radicals**

In many complex biological systems including semen, the true ROS status leading to oxidative stress reflects a relative balance between the ROS-generated and ROS-scavenged. The measurement of the rate of ROS generation by luminol induced chemiluminescence has been the most common method for quantitating ROS. Assessment of the rate of ROS production using luminol as a probe can be a dynamic measure of oxidative stress (Aitken and Clarkson, 1987). However, clinically the evaluation of this ROS generation is limited by a very short half life of these free radicals (Kessopoulou *et al*., 1992). Hence, since using luminol as a probe in ROS 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 (Sikka *et al*., 1995). The methods commonly used for measuring ROS can be categorized into:

- Reactions involving nitroblue tetrazolium (NBT) or cytochrome c-$Fe^{3+}$ complexes which measure ROS on the cell membrane surface
- Reactions that measure ROS (generated inside or outside the cell) utilizing luminol-dependent chemiluminescence
- The electron spin resonance methods which are more sensitive and can identify the type of ROS generated inside the cell but require skillful operation, accurate interpretations and expensive instrumentation

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

Electrolysis of physiological buffer under defined conditions also generates ROS which can damage sperm motion (Rajasekaran *et al*., 1994). Selective modifications of these defined conditions can identify:

- The free radicals involved
- Their mode of action on spermatozoa
- The evaluation of selective protective mechanisms. Hence, the basic and clinical research on the involvement of spermatogenesis, reactive oxygen species and antioxidants in maintaining normal sperm function is very much warranted

**Antioxidant**

The word antioxidant comes from the Greek anti meaning against, plus oxys referring to oxidation. So, antioxidants, in general, are compounds and reactions which dispose, scavenge and suppress the formation of ROS, or oppose their actions. Certain oxygen molecules, called free radicals, are normally produced during body metabolism. But over production of oxygen free radicals can cause problems. Many factors can cause your body to produce more free radicals than are needed. These may include smoking, alcohol, too much fat in your diet, exposure to sun, even too much exercise and too many pollutants in the air you breathe. When your body produces too many free radicals, the extra free radicals prey on healthy molecules. But high concentration of these free radicals can directly damage sperm cells (Madding *et al*., 1986). Antioxidant functions chiefly to protect cells and tissues from the ravages of oxygen.

**Mode of Action of Antioxidant**

Antioxidants work to protect lipids from peroxidation by free radicals. Antioxidants are effective because they are willing to give up their own electrons to free radicals. When a free radical gains the electron from an antioxidant it no longer needs to attack the cell and the chain reaction of oxidation is broken (Dekkers *et al*., 1996). After donating an electron, an antioxidant becomes a free radical by definition. Antioxidants in this state are not harmful because they have the ability to accommodate the change in electrons without becoming reactive. The human body has an elaborate antioxidant defense system. Antioxidants are manufactured within the body and can also be extracted from the food eat such as fruits, vegetables, seeds, nuts, meats and oil. There are two lines of antioxidant defense within the cell. The first line, found in the fat-soluble cellular membrane consists of vitamin E, beta-carotene and coenzyme Q (Kaczmarski *et al*., 1999). Of these, vitamin E is considered the most potent chain breaking antioxidant within the membrane of the cell. Inside the cell water soluble antioxidant scavengers are present. These include vitamin C, glutathione peroxidase, superoxide dismutase (SOD) and catalase (Dekkers *et al*., 1996).

**Antioxidant Enzyme and Spermatozoa**

Antioxidants can scavenge free radicals before they cause damage, or prevent oxidative damage from spreading out. The antioxidant defense systems in the human body are extensive and consist of multiple layers that protect at different sites and against different types of free radicals. An important part of the intracellular antioxidant defense systems are antioxidant enzymes such as superoxide dismutase (SOD), catalase and peroxidases.

On the other hand, every ejaculate has intra and extracellular antioxidants of enzymatic and non-enzymatic systems. Enzymic and low-molecular weight antioxidants exist in semen to scavenge free radicals as self-protection mechanisms (Fraga *et al*., 1991; Molianen *et al*., 1993; Lewis *et al*., 1995). The most important antioxidants in semen are the enzymic antioxidants: Superoxide dismutase, catalase glutathione peroxidase.

**Enzymatic Antioxidants**

**Superoxide Dismutase**

Superoxide dismutase (SOD) scavenges both extracellular and intracellular superoxide anion and prevents lipid peroxidation of the plasma membrane. In order to act against hydrogen peroxide ($H_2O_2$), it must be conjugated with catalase or glutathione peroxidase (Jeulin *et al*., 1989). The SOD also prevents premature hyper activation and capacitation induced by superoxide radicals before ejaculation (De Lamirande and Gagnon, 1995a). The biological antioxidants, SOD and its two isozymes and catalase have a significant role. The SOD spontaneously dismutates ($O_2^-$) anion to form oxygen ($O_2$) and $H_2O_2$ while catalase converts $H_2O_2$ to $O_2$ and water ($H_2O$). SOD protects spermatozoa against spontaneous $O_2$ toxicity and LPO (Alvarez *et al*., 1987). The SOD and catalase also remove ($O_2^-$) generated by NADPH-oxidase in neutrophils and may play an important role in decreasing LPO and protecting spermatozoa during genito-urinary inflammation (Aitken *et al*., 1995b).

Glutathione peroxidase/reductase system: Glutathione peroxidase, a selenium-containing antioxidant enzyme with glutathione as the electron donor removes peroxyl ($ROO^-$) radicals from various peroxides including $H_2O_2$ (Calvin *et al*., 1981). The most important metabolic role of selenium is its function in the active site of selenoenzymes glutathione peroxidase (GSH-Px), which may vary from monomeric to tetrameric form. This enzyme together with SOD and catalase protect cells against damage caused by free radicals and hydroperoxides or lipoperoxides (Carlsen *et al*., 1995). This enzyme not only allows the removal of $H_2O_2$ and

toxic lipid peroxides moiety but also permits the regeneration of membrane lipid molecule through reacylation. GSH-Px constitutes a family of enzymes among which selenoperoxidase contain selenocysteine at their active site and this amino acid is involved in the catalytic cycle.

Glutathione peroxidase has been demonstrated in rat sperm mitochondria which play a significant role in this peroxyl scavenging mechanism and in maintaining sperm motility (Calvin *et al*., 1981). It would be interesting to explore the mechanism of action of this antioxidant in spermatozoa. In addition, GSH peroxidase and GSH-reductase may directly act as antioxidant enzymes involved in the inhibition of sperm LPO (Lenzi *et al*., 1994). GSH has a likely role in sperm nucleus decondensation and may alter spindle microtubule formation in the ovum, thus affecting the outcome of pregnancy. In this context, the g-glutamyl transpeptidase (gGGT) considered being present in the mid piece and acrosomal regions of spermatozoa of certain mammalian species may further affect GSH content of oocyte at the time of sperm penetration (Lenzi *et al*., 1994; Irvine, 1996). Thus, in view of the great number of mitochondria in spermatozoa, these antioxidant mechanisms are important in the maintenance of sperm motility, the rate of hyperactivation and the ability of sperm to undergo acrosome reaction during sperm preparation techniques especially in the absence of seminal plasma.

**Non-Enzymatic Antioxidants**

In addition to these antioxidant enzymes, there are several small molecule antioxidants that also play an important role in antioxidant defense systems, particularly in the extracellular space, where antioxidant enzymes are absent or present in small quantities only. The small molecule antioxidants can be separated into Coenzyme Q-10, Glutathione, beta-carotene, lipid-soluble and water-soluble antioxidants. The lipid-soluble antioxidants are localized to membranes and lipoproteins, whereas the water-soluble ones are present in extracellular and intracellular fluids.

**Ascorbic Acid**

Antioxidants have been found to slow, block or reverse oxidative changes in body cells. For example, ascorbic acid prevents the conversion of nitrates (from tobacco smoke, smog, bacon, lunch meats and some vegetables) into cancer-causing substances. Vitamin C consists of a lactone ring that has two hydroxyl groups on it, two chiral carbons and has uniqueness because it does not contain a carboxyl group but is an acidic molecule. Vitamin C is a water soluble antioxidant which means that it will be located in the extracellular areas of the body. Ascorbic acid has much polarity because of the numerous hydroxyl groups making it dissolve in water easily. This is advantageous because ascorbic acid will be converted from the body more readily. Thus, vitamin C is able to react with aqueous free radicals and reactive oxygen.

Dawson *et al*. (1987) reported that low level of vitamin C lead to infertility and increased damage to sperm's genetic material. Dawson *et al*. (1987) showed the effect of vitamin C on male infertility when 30 infertile but otherwise healthy men were given a placebo, 200 and 1000 mg of vitamin C daily. After one week, the group receiving 1000 mg $day^{-1}$ had a 140% and 200 mg $day^{-1}$ group had 112% increases in sperm count and there was no change in placebo group. Most important is that by the end of 60 days study, every participant in vitamin C group had impregnated their partner and no pregnancy in placebo group (Dawson *et al*., 1987). Although, vitamins E and C may protect spermatozoa against endogenous oxidative DNA and membrane damage they have minimal effects in improving the post thaw sperm parameters.

**Vitamin E**

It is a well documented antioxidant and have been shown to inhibit free radical induced damage to sensitive cell membrane (Aitken *et al*., 1989b). Lipid peroxidation in seminal plasma and spermatozoa was estimated by malondialdehyde (MDA) concentration. Vitamin E retards cellular ageing due to oxidation. It also helps to block oxidation that converts Low-Density Lipoprotein (LDL) cholesterol from a form that stays in the blood to a form that can stick to and clog arteries (atherosclerotic plaque build up). Looking at the structure of vitamin E, one can see that the molecule is very non polar, the only remnant of polarity is the hydroxyl group and oxygen contained in the six-carbon ring, but the benzene ring and the long non polar hydrocarbon chain makes it a non polar molecule. Because of the hydrophobic nature of vitamin E, it is located in biological membranes and lipoproteins. It is a chain-breaking antioxidant which means it will disrupt the radical chain reactions of lipid peroxidation. The effect of vitamin E supplementation in combination with IVF techniques is a worthy notion. Further controlled clinical studies will determine if many of these putative antioxidants can improve infertility. Hence, the application of ROS scavengers (e.g., SOD, catalase, vitamin E, GSH-enzymes) is likely to improve sperm motility and function. Pentoxifylline, a sperm motility stimulator, can also act as a suppressor or scavenger of ROS (Sikka *et al*., 1993).

**Beta-Carotene**

Carotenoids (beta-carotene) and ubiquinols may also play a role in quenching singlet oxygen and reducing lipid derived free radicals with detrimental effects on sperm LPO (Ernster, 1993). Beta-carotene is like tocopherol because it is also a lipid-soluble antioxidant. Compared to tocopherol, beta-carotene acts as a weak antioxidant and is only present in a 1/20 concentration of alpha-tocopherol and will only be used up after all other antioxidant defenses have been used. Beta-carotene consists of a long non polar chain and will therefore be located in cell membranes and lipoproteins. It is capable of radical trapping and is a radical scavenger. Beta carotene has been shown to reverse pre-cancerous changes in cells that line the mouth and cervix.

**Coenzyme Q-10**

In sperm cells coenzyme Q-10 (coQ10) is concentrated in mitochondrial mid piece for energy production. It also functions as an antioxidant, preventing lipid peroxidation of sperm membrane. CoQ 10 (60 mg) was given to 17 infertile patients for a mean 103 days showed a significant increase in sperm count and motility with resulting improvement in fertilization rate (Sandler and Faragher, 1948; Lewin and Lavon, 1997).

**Glutathione**

Glutathione is vital to sperm antioxidative defenses. Glutathione and selenium are essential to formation of phospholipids hydroperoxide glutathione peroxidase, an enzyme present in spermatid. This is a structural protein of mitochondria in mid place of mature spermatozoa. Deficiency of both lead to instability of mid piece resulting in defective motility (Lenzi *et al*., 1993). Glutathione and selenium was used in 2 months, placebo controlled, double blind, cross over trial over 20 infertile men. It showed a statistically significant effect on sperms motility rather than placebo group (Hansen and Deguchi, 1996).

**Physiologic Roles of Antioxidants**

**Antioxidants and Spermatogenesis**

Although, oxidative stress was suggested as an important contributor to disruption of sperm function over the past five decades, the importance of oxidative stress has gained a

wider understanding in the last decade (Sharma and Agarwal, 1996). In normal physiological functions, there is a balanced generation of Reactive Oxygen Species (ROS) and antioxidant enzymes (Kovalski *et al*., 1992; Plante *et al*., 1994; Aitken *et al*., 1995a, b). The ROS have a physiological role in signaling events controlling sperm capacitation and induction of the acrosome reaction in many species including equine (De Laraminde and Gagnon, 1993; De Lamirande *et al*., 1997b; Baumber *et al*., 2003). However, overproduction of ROS and decreased antioxidant defense activity cause low sperm motility and viability, DNA fragmentation and protein denaturation (Agarwal and Said, 2005; Kankofer *et al*., 2005).

The cell structure of spermatozoa makes them potentially susceptible to damage from free radicals (De Lamirande and Gagnon, 1995b; Sikka, 2004). Sperm membranes are rich in polyunsaturated fatty acids and can easily undergo lipid peroxidation in the presence of ROS, leading to changes in membrane fluidity (Alvarez and Storey, 1982), which finally results in decreased fertilizing capacity. In addition, low cytoplasm content remaining after spermatogenesis contributes to sperm cell fragility, limiting the potential for DNA and protein repair (Baumber *et al*., 2005; Bustamante Filho *et al*., 2005).

To counteract reactive oxygen species, spermatozoa and seminal plasma have several mechanisms to neutralize free radicals. Enzymatic and non-enzymatic antioxidant systems work synergistically to prevent harmful effects of by-products from aerobic metabolism (De Laraminde and Gagnon, 1993; De Laraminde *et al*., 1993). For example, mammalian semen (mainly seminal plasma) has many compounds with non-enzymatic antioxidant activity (e.g., ascorbic acid, α-tocopherol, taurine and albumin; Alvarez and Storey, 1983). However, the presence of specific antioxidant enzymes suggests that they also play a major role in the protection of spermatozoa against ROS. Three enzyme systems (catalase, glutathione peroxidase and superoxide dismutase) have superoxide radicals and hydrogen peroxides as substrates (Alvarez and Storey, 1989; Zini *et al*., 1993; Ball *et al*., 2000). The observed increase in semen quality parameters of rabbit bucks administered soymilk could be attributed in part by the antioxidant activity of locally prepared semen extender made from the bark of *Saccoglotis gabonensis* (Umesiobi *et al*., 1998, 2002; Umesiobi, 2004) Antioxidants act by reducing the production of deleterious residues from oxidative physiological metabolism. In bovine semen, a decrease in antioxidant activity following cryopreservation has been reported by Bilodeau *et al*. (2000). Furthermore, freeze-thawing of equine and bovine spermatozoa has been associated with an increase in ROS generation (Bilodeau *et al*., 2000).

Numerous studies have evaluated effects of antioxidants on male fertility in several species (Hsu *et al*., 1998; Bruemmert *et al*., 2002; Foote *et al*., 2002). Although, many clinical trials demonstrated a beneficial effect of antioxidants in selected cases of male infertility, other studies failed to verify similar benefits. Investigators have used different antioxidants in different combinations, making it difficult to reach a definitive conclusion. Deichsel *et al*. (2008), working with oral antioxidant supplementation (tocopherol 300 mg $day^{-1}$; ascorbic acid 300 mg $day^{-1}$; L-carnitine 4000 mg $day^{-1}$; folic acid 12 mg $day^{-1}$), have not found a pronounced effect on semen quality of stallions. Conversely, Arlas *et al*. (2008) found a higher total radical trapping potential in stallions supplemented with rice oil containing gamaoryzanol. The poor semen quality observed in laboratory animals fed the leaf meals of some ethnoveterinary plants could be explained in part to the indirect inhibitory effects of their active compounds on the antioxidant activity of these plants on seminal endogenous antioxidant enzyme activity (Ogbuewu *et al*., 2009b; Umesiobi *et al*., 2004).

**Antioxidants and Inflammatory Diseases**

Inflammation is known by a respiratory burst of activated neutrophils and macrophages, leading to destruction of invading micro-organisms. The mechanism is a useful function

protecting against attack, however, the inflammatory response can also be detrimental as it is non specific and may lead to the development of inflammatory diseases such as rheumatoid arthritis, systemic Lupus Erythrometosis, inflammatory bowel disease and psoriasis.

There is also strong relationship between reactive oxygen species and inflammatory diseases. Free radicals are thought to act indirectly as cellular messengers and elicit any inflammatory response. Over-production of these species may cause oxidative modification of biological molecules e.g., trypsin, collagen, LDL, DNA and lipids.

Reactive oxygen species and other free radicals also activate a series of enzyme systems including protein kinase, protein phosphatases, transcription factors and heat shock proteins. The ROS are also critical for gene expressions which encode inflammatory proteins e.g., proteinases involved in tissue destruction such as collagenases and gelatinases. Nuclear factor-κβ (NF-κβ) has been implicated in AIDS, as HIV is NF-κβ dependent. In the case of rheumatoid arthritis, rheumatoid factor binds IgG when it is exposed to free radicals. This binding stimulates the production of more free radicals which then attack the cartilage matrix.

There is evidence that antioxidant supplementation can alleviate inflammation. Vitamin E in the synovial fluid of patients suffering from rheumatoid arthritis was significantly lower than those in healthy individual (Blake, 1981). Studies have implicated low level of vitamin E, β-carotene and selenium to be associated with increased risk of rheumatoid arthritis (Heliovaara *et al*., 1994).

**Antioxidants and Ageing**

Ageing is a universal phenomenon. It is the accumulation of changes responsible for the sequential alteration that accompany advancing age and associated progressive increases in the chances of death. The free radical theory of aging (Harman, 1996) states that aging is the result of cumulative damage incurred by free radical reactions as well as progressive defects in protection against free radical reactions with the passage of time. Free radical mediated lipid peroxidation in lysosome membrane leak out lysosomal hydrolases which cause dystrophic changes in muscle fibers. As a result, muscles become weak with growing age (Singh *et al*., 2005a). It has been undoubtedly accepted and successfully explained that antioxidant enzymes can slow the ageing process by scavenging free radicals (Orr and Sohal, 1994). GSHPx, a selenium containing antioxidant enzyme which scavenges $H_2O_2$ and prevents the initiation of free radical chain reaction, has been theorized to extend life span and prevent age related functional disorders.

**Antioxidants and Cancer**

Over the past three decades, extensive efforts were made to treat cancer. However, as recent statistics show, the incidence and mortality from cancer have in general not reduced but are on the increase rather (Jemal *et al*., 2002). Reactive oxygen species are essential for various cell defense mechanisms; they can also cause oxidative damage to DNA, proteins and lipids, resulting in potentially enhanced risk of cancer. Several antioxidants like vitamin C, vitamin E and beta carotene can help maintain appropriate balance between the desirable and undesirable cellular effects of reactive oxygen species. Vitamin C is considered to be one of the most prevalent antioxidant components of fruits and vegetables and it could exert chemopreventive effects without apparent toxicity at higher than the current RDA of 60 mg $dL^{-1}$. Bowie and O'Neill (2000) demonstrated that vitamin C inhibits expression of

transcription factor NF-kβ through p38 mediated protein kinase. It is found that that vitamin C can prevent hydrogen peroxide induced mutation in human cells which are inadvertently linked to tumor initiation and tumorogenesis. Vitamin E's role to prevent carcinogenesis has been a topic of much interest due to its strong antioxidant properties.

The role of antioxidant minerals in the etiogenesis of cancer has also been reviewed (Rahul and Geeta, 2007). Among various antioxidant minerals, selenium is emerging as a dietary factor that may prove to be of major significance as a prophylactic agent against cancer. Low blood selenium concentration and incidence of carcinogenesis have been well observed in both animals (Ip, 2003) as well as in human studies (Shamberger, 1970). In addition, it has been demonstrated in a double blind randomized cancer prevention trial in humans that increased selenium intake has a significant role in the treatment of cancer (Clark *et al*., 1996). Various investigators have reported the role of antioxidants as an inhibitor of carcinogenesis in various organs including liver, skin, stomach, mammary gland and oral cavity etc. (Ip and Medina, 1987; Ip and Daniel, 1985). *In vitro* and *in vivo* studies on dietary antioxidant supplementation also suggested that antioxidant inhibits cell growth and DNA synthesis in a variety of cell lines leading to the normalization of regulatory pathways that are affected in early stages of carcinogenesis (Watrach *et al*., 1984; Fico *et al*., 1986). There is a large body of epidemiological evidence that shows correlation between low dietary intake of antioxidant and increased risk of carcinogenesis (Diplock, 1990; Salonen *et al*., 1984). Klein (2003) on the basis of antioxidants (selenium and vitamin E) cancer prevention trial in humans concluded that treatment with a high dose of selenium in combination with vitamin E can prevent the incidence of prostrate cancer. This reflects the importance of antioxidant in the etiology of cancer (Klein, 2003).

Over the past two decades, an expanding body of evidence from epidemiological and laboratory studies have demonstrated that some edible plants (spices and herbs) as a whole, or their identified ingredients with antioxidant properties have substantial protective effects on human carcinogenesis (Surh and Fergusson, 2003; Tsao *et al*., 2004; Kinghorn *et al*., 2004). Spices and herbs are recognized as sources of natural antioxidants that can protect from oxidative stress and thus play an important role in the chemoprevention of diseases that has their etiology and pathophysiology in reactive oxygen species.

**Antioxidant and Cardiovascular Diseases**

Coronary artery disease is the major cause of death in developed countries (Murray and Lopez, 2005). It may present as any of the three manifestations encompassed in term acute coronary syndrome. Despite the availability of good coronary care units and reduced in-hospital mortality rate, we see that about 75% of the Myocardial Infarction (MI) patients are not even able to make it to the hospital. 20% of the cases of sudden death is the first and only manifestation (Ruston *et al*., 1998; Kannel *et al*., 1984; Kendall *et al*., 1995).

Oxidative stress is involved in the pathogenesis of atherosclerosis and antioxidants have been used in the clinical studies in the past few years for the prevention and treatment of atherosclerosis. Soy genistein has been reported to suppress the oxidative stress (Ogbuewu *et al*., 2009a). In studies with macrophages, soy genistein inhibited the activation of nuclear factor-κβ (NK-κβ), which is inducible by oxidative stress and regulates the expression of genes involved in immune and inflammatory responses (Ogbuewu *et al*., 2009a). A great deal of evidence exists that supports the idea that lipid peroxidation products are damaging to coronary arteries and that antioxidants help prevent such damage. It now appears that Low-Density Lipoprotein (LDL) must first undergo oxidation before plague

accumulations can occur. These modified LDL particles do not bind readily to the endogenous LDL receptor and are therefore not cleared from the circulation by this mechanism. They penetrate the arterial intima more easily, are more readily oxidized, possibly because they contain less antioxidant protection and are taken up by the macrophage scavenger receptors, accelerating foam cell formation. Because LDL is the primary carrier of fat soluble vitamin it seems logical that antioxidant vitamin supplementation can fortify LDL with antioxidant power and increase resistance of LDL to oxidation.

In an epidemiological study, Salonen *et al.* (1982) observed an excess of incidence of mortality from ischemic heart disease in Eastern Finnish men and women with low serum selenium concentrations suggesting that low selenium levels have a causal effect in development and deterioration of ischemic heart disease. In addition, it has been documented that *in vitro* and *in vivo* studies on Oxygen Free Radicals (OFRs) suggest that free radicals are toxic to the myocardium and can cause tissue damage that leads to extensive necrosis, myocytolysis and cellular edema (Kloner *et al*., 1989). The biological function of antioxidant against OFRs mediated injury in mammals appears to be expressed through different biological active compounds in serum and tissue. The remarkable contribution of antioxidant in protecting against oxidant result has been also observed in on atherosclerotic patients (Singh *et al*., 2005b). Antioxidants scavenge free radicals and are associated with reduced risk of cancer and cardiovascular diseases.

**Antioxidant and Alzheimer's Diseases**

The Alzheimer's disease is known by regional degeneration, synaptic loss and the presence of nuerofibrillary tangles and senile plagues (Pfeifer *et al*., 2002). It has the potential to become the most overwhelming public health concern of this century owing to increasing life expectancy and growth in the ageing population (Souder *et al*., 2002).

A decade of studies has suggested that ROS may contribute to the neuronal damage in Alzheimer's disease. *In vitro* studies have reported a strong relationship between A-β protein and generation of free radicals (Markesbery, 2001; Behl, 1997). The increased production and deposition of A-β protein are early events in the pathogenesis of Alzheimer's disease, thus precede other changes such as the formation of tau, amyloid production and deposition and may be associated with increased oxidative stress. Hence, the presence of antioxidants may provide protection to neurons and preserve cognitive function (Engelhart *et al*., 2002). Charlton *et al*. (2004) and Helmer *et al*. (2003) have shown the decrease plasma levels of vitamin c and E are correlated with the risk of Alzheimer's disease.

**Future Directions**

Future research efforts should be directed towards understanding the sensitive balance between free radicals, antioxidants and spermatozoa functions. Free radicals can be considered to be advantageous in sperm function under special physiologic conditions and in such situation the administration of exogenous antioxidants would be inappropriate. On the other hand, over production of free radicals inhibits the physiological processes of spermatozoa functions, the therapeutic intervention in form of antioxidant administration would be beneficial. However, the administration of exogenous antioxidant should be done with care since there are divided opinions on the need for exogenous antioxidant administration, as they believed that antioxidant supplementation can actually decrease the effectiveness of the body immune system.

## CONCLUSIONS

Oxygen toxicity is an inherent challenge to aerobic life forms, including the spermatozoa. Increased oxidative damage to sperm membranes (indicated by increased LPO), proteins and DNA is associated with alterations in signal transduction mechanisms that affect fertility. Spermatozoa possess an inherent but limited capacity to generate ROS which may help the fertilization process. A variety of defense mechanisms encompassing antioxidant enzymes and antioxidant enzymes are involved in biological systems. A balance between the benefits and risks from ROS and antioxidants appears to be necessary for the survival and normal functioning of spermatozoa. In conclusion research efforts should be geared towards better understanding of the etiology of free radicals on spermatozoa, their benefits and potential risks and the appropriate quantities of exogenous antioxidants to be administered.

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