Role of Oxidative Stress and Antioxidants in Male Infertility

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ABSTRACT

Infertility is defined as the inability to conceive after one year of unprotected intercourse by couples of reproductive age. Out of many causes of male infertility, oxidative stress (OS) has been attributed to affect the fertility status. The excessive production of reactive oxygen species (ROS) by abnormal spermatozoa, leukocytes can cause damage to sperm. Mammalian spermatozoa membrane is very sensitive to ROS attack as they are rich in polyunsaturated fatty acids (PUFA). ROS attacks the fluidity of sperm membrane, damages mitochondria & DNA integrity. This review highlights the need of ROS in normal sperm physiology, the mechanisms of ROS production and pathophysiological roles of ROS in relation to the male reproductive system. We also highlight the depletion of antioxidants due to overproduction of ROS & need to evaluate oxidative stress and antioxidant in seminal plasma.

Key words: Male infertility, Lipid peroxidation, Oxidative stress, Antioxidants

INTRODUCTION

Infertility is defined as inability to achieve conception in a period of one year in a couple, despite regular and adequate unprotected sexual intercourse. [¹] The condition affects 15% of couples, and the male is responsible in 50% of cases. [²] Oxidative stress has been attributed to affect the fertility status of males and thus, it has been studied extensively in recent years. Spermatozoa, like any other aerobic cell is constantly facing the oxygen paradox. [³] Oxygen is essential to sustain life as physiological levels of reactive oxygen species (ROS) are necessary to maintain normal cell function. Reactive oxygen species can have beneficial or detrimental effects on sperm functions depending on the nature and the concentration of the reactive oxygen species as well as the location and length of exposure to reactive oxygen species. [⁴] Oxidative stress has become the focus of interest as a potential cause of male infertility. High concentration of reactive oxygen species was detected in the semen of 30-80% of infertile men. Normally equilibrium exists between ROS production and antioxidant scavenging activities in the male reproductive tract. Under physiological condition, spermatozoa produces small amount of reactive oxygen species, which are needed for capacitation, acrosome
reaction and fertilization. However excessive amount of reactive oxygen species produced by leukocytes and immature spermatozoa cause damage to the normal spermatozoa by inducing lipid peroxidation and deoxyribonucleic acid (DNA) damage. [5]

Human sperm cells in contrast with other cells are particularly susceptible to oxidation of their plasma membranes due to existence of a high concentration of polyunsaturated fatty acids (PUFA) in the membrane. [6] Polyunsaturated fatty acids play an important role in ion transport and sperm membrane fluidity, therefore oxidation of sperm membrane PUFA by oxidants (ROS) can cause deficiency of membrane function and sperm death. [7] The intracellular antioxidant enzymes cannot protect the plasma membrane that surrounds the acrosome and the tail, forcing the spermatozoa to supplement their limited intrinsic antioxidant defences by depending on the protection afforded by the seminal plasma. Hence, any excess ROS must be continuously inactivated in order to maintain normal cell function. This function is done by antioxidants present in seminal plasma. [4]

Physiological Role of ROS in Male Reproductive Tract:

Pioneering work in the field of reactive oxygen species was conducted by Aitken and his group. According to his research, ROS was exclusively considered toxic to human spermatozoa. Based on these studies, small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities. [8] Under physiological conditions, spermatozoa produce small amounts of reactive oxygen species, which are needed for capacitation, acrosomal reaction, hyperactivation, motility and fertilization. [9,10] Capacitation is a maturation process of spermatozoa that take place in the female genital tract. During this process, the levels of intracellular calcium, reactive oxygen species, and tyrosine kinase all increase, leading to an increase in cyclic adenosine monophosphate (cAMP). This increase in cAMP facilitates hyperactivation of spermatozoa, a condition in which they are highly motile. [11] However, only capacitated spermatozoa exhibit hyperactivated motility and undergo a physiological acrosome reaction. Studies have shown that co-incubation of spermatozoa with low concentrations of hydrogen peroxide stimulate sperm capacitation, hyperactivation, acrosome reaction, and oocyte fusion. [12] Free radicals are also involved in the fusion of spermatozoa with the oocyte. [13] Nitric oxide plays a role in the sperm’s ability to fuse with oocyte, but it has no action in zonapellucida binding. Low concentration of hydrogen peroxide cause tyrosine phosphorylation, which in turn results in the binding of the spermatozoal membrane proteins with ZP-3 proteins on the zonapellucida and ultimately, spermatozoa oocyte fusion. [14,15]

Sources of Reactive Oxygen Species in Male Reproductive System: [16]

1. Leukocytes, particularly neutrophils and macrophages, have been associated with excessive reactive oxygen species production, and they ultimately cause sperm dysfunction.

2. Another important source of reactive oxygen species is immature and morphologically abnormal spermatozoa.

3. The production of reactive oxygen species is also increased by lifestyle factors such as smoking and pollutions. Smoking increases ROS production, causing sperm DNA damage, and suppresses antioxidants in both semen and serum.

Reactive Oxygen Species Production by Spermatozoa:
Cytoplasmic droplets, or excess residual cytoplasm, explain the missing link between poor sperm quality and increased ROS generation. Cytoplasmic droplets, a result of defective spermiogenesis, are a major source of ROS. During spermatogenesis, a defect of the cytoplasmic extrusion mechanism results in release of spermatozoa from germinal epithelium carrying surplus residual cytoplasm. The resulting spermatozoa are thought to be immature and functionally defective. Retention of residual cytoplasm by spermatozoa is, in fact, positively correlated with ROS generation via mechanisms that may be mediated by the cytosolic enzyme glucose-6-phosphate dehydrogenase.

Spermatozoa may generate ROS in two ways:
1. As a result of the NADPH-oxidase system at the level of the sperm plasma membrane.
2. As a result of the NADH-dependent oxidoreductase (diphorase) at the level of mitochondria.

The mitochondrial system is the major source of reactive oxygen species in spermatozoa in infertile men. Spermatozoa are rich in mitochondria because they need a constant supply of energy for their motility. Production of reactive oxygen species is significantly increased in dysfunctional mitochondria which in turn affect mitochondrial function in spermatozoa. The primary reactive oxygen species generated in human spermatozoa is the superoxide anion (O$_2^-$). This one-electron reduction product of O$_2$ secondarily reacts with itself in a dismutation reaction, which is greatly accelerated by superoxide dismutase, to generate hydrogen peroxide (H$_2$O$_2$). In the presence of transition metals such as iron and copper, H$_2$O$_2$ and O$_2^-$can interact to generate the extremely pernicious hydroxyl radical (OH') (Haber-Weiss reaction) as shown in the following equation:

$$O_2^- + H_2O_2 \rightarrow OH^+ + OH + O_2'$$

Alternatively, the hydroxyl radical can be produced from hydrogen peroxide (Fenton reaction), which requires a reducing agent such as ascorbate or ferrous ions, as shown in the equation:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^+ + OH'$$

The hydroxyl radical is thought to be an extremely powerful initiator of the lipid peroxidation cascade and can precipitate loss of sperm functions.

Reactive Oxygen Species Production by Leukocytes:

Leukocytes particularly neutrophils and macrophages, have been associated with excessive reactive oxygen species production, and they ultimately cause sperm dysfunction. Leukocytes act either directly by reactive oxygen species synthesis or indirectly by other neighboring white cells via soluble factors as cytokines. Peroxidase-positive leukocytes are the major source of reactive oxygen species in semen. Peroxidase-positive leukocytes include polymorphonuclear leukocytes (PMNL)
which represents 50–60% of all seminal leukocytes, and macrophages, which represent 20–30% of all seminal leukocytes. [22-24] These PMNL are mainly contributed to the human ejaculate by the prostate gland and seminal vesicle. [25] Leukocytes may be activated in response to various stimuli such as infection and inflammation, [11] and these activated leukocytes can produce up to 100-fold higher amounts of reactive oxygen species compared with non-activated leukocytes. [26] This is mediated by an increase in NADPH production via the hexose monophosphate shunt. The myeloperoxidase system of both polymorphonuclear leukocytes and macrophages is also activated, leading to a respiratory burst and production of high levels of reactive oxygen species. Sperm damage from leukocyte derived reactive oxygen species may occur when seminal leukocyte concentrations are abnormally high, such as in leukocytospermia. [18]

**Reactive Oxygen Species and lipid Peroxidation:**

Lipids are considered to be the most susceptible macromolecules and are present in sperm plasma membrane in the form of polyunsaturated fatty acids (PUFA), fatty acids that contain more than two carbon-carbon double bonds. [27] Most membrane PUFA contain unconjugated double bonds that are separated by methylene groups. The presence of a double bond adjacent to a methylene group makes the methylene carbon-hydrogen bond weaker, and as a result, the hydrogen is more susceptible to abstraction. When this abstraction has occurred, the radical produced is stabilized by the rearrangement of double bonds. The PUFA rearranges to form a conjugated diene radical that subsequently can be oxidized. [11,28] The PUFA are necessary for the plasma membrane fluidity and normal physiological function of sperm. Also ion pumps located in the membrane help to maintain concentrations of nutrients and ions (calcium and sodium) in the cell. The normal function of the pump is related to membrane fluidity. Changes in membrane fluidity impair activity of this pump thereby cause accumulation of ions inside the cell and leads to disruption of the normal functioning of the cell. [29] Reactive oxygen species attacks PUFA in the cell membrane leading to a cascade of chemical reactions called lipid peroxidation. One of the byproduct of lipid peroxidation is malondialdehyde, which has been used as an end product in biochemical arrays to monitor degree of peroxidative damage to spermatozoa lipid peroxidation results in loss of membrane fluidity, which is essential for sperm motility and sperm oocyte fusion. [4,27]

**Reactive Oxygen Species and Sperm Motility:**

Increased ROS levels have been correlated with decreased sperm motility. [4,30] However, the exact mechanism through which ROS causes decreased motility is not understood. Thus, many hypothesis have been proposed to explain the link between ROS and decreased motility. [11,18,31]

One hypothesis suggests that H2O2 can diffuse across the membranes into the cells and inhibit the activity of some vital enzymes such as glucose-6-phosphate dehydrogenase (G6PD). G6PD is an enzyme that controls the rate of glucose flux via the hexose monophosphate shunt and in turn, controlling the intracellular availability of NADPH. This is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase. [18] Decreased G6PDH leads to a decrease in the availability of NADPH and a concomitant accumulation of oxidized glutathione. These changes can cause a decrease in the antioxidant defenses of the spermatozoa,
which ultimately leads to the peroxidation of membrane phospholipids. Another hypothesis involves a series of interrelated events resulting in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion.

**DNA Damage and Apoptosis Induced by Reactive Oxygen Species:**

Two factors protect spermatozoa DNA from oxidative stress, one the characteristic tight packaging of sperm DNA and other the antioxidants defence in seminal plasma. Increased reactive oxygen species formation and lipid peroxidation results in oxidative stress which can damage mitochondrial DNA. 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. ROS induces DNA damage in the form of modification of all bases (primarily guanine via lipid peroxyl or alkoxyl radicals), production of base-free sites, deletions, frame shifts, DNA cross-links through covalent binding to MDA, and chromosomal rearrangements. Reactive oxygen species 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. Oxidative stress is also associated with high frequencies of single and double strand breaks. Reactive oxygen species can also cause gene mutations such as point mutation and polymorphism, resulting in decreased sperm quality. Other mechanism such as denaturation and DNA base pair oxidation may also be involved. 8-hydroxy-2-deoxyguanosine (8-OH-2-deoxyguanosine), a common byproduct of DNA oxidation, has been considered a key biomarker of this oxidative DNA damage. When DNA damage is small, spermatozoa can undergo self-repair. The oocyte is also capable of repairing damaged DNA of spermatozoa. However, if damage is extensive, apoptosis and embryo fragmentation can occur.

![Mechanistic pathway showing sperm DNA damage due to oxidative stress.](image-url)

**Apoptosis (programmed cell death)** is a physiological phenomenon characterized by cellular morphological and biochemical alteration that cause a cell to die. It helps in elimination of abnormal spermatozoa. Apoptosis is strictly regulated by extrinsic and intrinsic factors and can be triggered by a variety of stimuli. Examples of extrinsic stimuli are irradiation, chemotherapy, and toxin exposure. Reactive oxygen species generated from abnormal spermatozoa may stimulate the process of apoptosis, resulting in death of spermatozoa. Mitochondria play a key role in the mechanism of apoptosis. The integrity of mitochondria is established by the presence of cytochrome C in the inner membrane space. High levels of reactive oxygen species disrupt the inner and outer mitochondrial membranes resulting in release of cytochrome-C protein from the mitochondria that activates the caspases and induces apoptosis. Apoptosis in sperm may also be initiated by ROS independent...
pathways involving the cell surface protein Fas. \[42\] Fas is a member of the tumour necrosis factor receptor family. When ROS levels are raised pathologically, the process of apoptosis may also be initiated in mature spermatozoa. The process of apoptosis may be accelerated by ROS induced DNA damage, which ultimately leads to a decline in the sperm count. As a result, patients may present with azoospermia. \[4\]

Antioxidants:
Seminal plasma contains enzymatic as well as non-enzymatic antioxidants.

**Enzymatic Antioxidants:**
Enzymatic antioxidants are also known as natural antioxidants; they neutralize excess ROS and prevent it from damaging the cellular structure.

1) **Superoxide dismutase (SOD) and catalase:**
SOD protects sperm from superoxide anions by catalyzing the conversion of superoxide into oxygen and H$_2$O$_2$, thereby preventing lipid peroxidation and improving motility. On the other hand, catalase aids in the decomposition of H$_2$O$_2$ into water and oxygen. \[43\]

\[
\text{SOD} \
\begin{array}{c}
2O_2^- + 2H^- \\
\rightarrow H_2O + O_2
\end{array}
\]

\[
\text{Catalase} \
\begin{array}{c}
H_2O_2 \\
\rightarrow H_2O + \frac{1}{2}O_2
\end{array}
\]

Superoxide dismutase scavenges both extracellular and intracellular superoxide anion and prevents lipid peroxidation of the plasma membrane. SOD also prevents premature hyper activation and capacitation induced by superoxide radicals before ejaculating. Catalase detoxifies both intracellular and extracellular H$_2$O$_2$ to water and O$_2$. In addition, catalase activates NO$^-$ induced sperm capacitation, which is a complex mechanism involving H$_2$O$_2$. Thus, both SOD and catalase assist in removing ROS that has the potential to damage sperm. \[4\]

2) **Glutathione peroxidase/reductase:**
Glutathione peroxidase/reductase system forms an excellent protection against lipid peroxidation of plasma membrane of spermatozoa. Glutathione peroxidase (Se-GSH-Px) with glutathione (GSH) as the electron donor removes peroxyl (ROO) radicals from various peroxides including H$_2$O$_2$. Glutathione reductase (GSH-Red), then regenerates reduced GSH from GSSG as shown in following equation:

\[
\begin{align*}
\text{Se-GSH-Px} & \quad 2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \\
\text{GSSG} + \text{NADPH} + \text{H}^+ & \rightarrow 2\text{GSH} + \text{NADP}^+
\end{align*}
\]

It scavenges lipid peroxides thereby arresting the progressive chain reaction of lipid peroxidation. It also scavenges hydrogen peroxide (H$_2$O$_2$) which is responsible for the initiation of lipid peroxidation; Glutathione reductase (GRD) stimulates the reduction of glutathione disulfide to reduced glutathione. This ensures a steady supply of the reductive substrate (NADPH) to glutathione peroxidase. G6PD is required for the conversion of nicotinamide adenine dinucleotide phosphate (NADP$^+$) to its reduced form (NADPH). \[4\]

**Non-Enzymatic Antioxidants:**
The important non-enzymatic antioxidants are:

1. **Vitamin E:**
   It is the major lipid-soluble antioxidant, and plays a vital role in protecting membranes from oxidative damage. Its primary activity is to trap peroxyl radicals in cellular membranes. \[16\] It scavenges all three types of free radicals, namely, superoxide, H$_2$O$_2$, and hydroxyl radicals. \[9\] Administration of 100mg of vitamin E three times a day for 6 months in
a group of asthenozoospermic patients with normal female partners showed a significant decrease in Lipid peroxidation and increased motility and pregnancy rates. [44]

2. Vitamin C (Ascorbic acid):
Vitamin C is another important chain-breaking antioxidant, contributing up to 65 per cent of the total antioxidant capacity of seminal plasma found intracellularly and extracellularly. It neutralizes hydroxyl, superoxide, and hydrogen peroxide radicals and prevents sperm agglutination. [9] It also appears to participate in recycling vitamin E radicals. Interestingly, vitamin C also functions as a pro-oxidant under certain circumstances. [16] Administration of 200 mg of vitamin C orally along with vitamin E and glutathione for 2 months significantly reduced hydroxyl glutathione levels in spermatozoa and also led to an increase in sperm count. [45]

3. Coenzyme Q10:
Coenzyme Q10 is a non-enzymatic antioxidants that is related to low density lipoproteins and protects against peroxidative damage. [46] It is an energy promoting agent and enhances sperm motility. [47] It is present in sperm mid piece and recycles vitamin E and prevents its pro-oxidant activity. [48] Oral supplementation of 60 mg/day of coenzyme Q10 was shown to improve fertilization rate using intra cytoplasmic sperm injection (ICSI) in normospermic infertile males. [47]

4. Glutathione:
This is the most important intracellular defense against damage by reactive oxygen species. It is a tripeptide (glutamylcysteinyl- glycine). The cysteine provides an exposed free sulphhydryl group (SH) that is very reactive, providing an abundant target for radical attack. Reaction with radicals oxidizes glutathione, but the reduced form is regenerated in a redox cycle involving glutathione reductase and the electron acceptor NADPH. [16] In addition to these, there are few other minor antioxidants that contribute to relieving OS, such as carnitine, carotenoids, cysteines, pentoxifylline, albumin, taurine/hypotaurine, inositol and some metals.

CONCLUSION
Production of low and controlled concentrations of reactive oxygen species play an important role in normal sperm physiological processes such as capacitation, hyperactivation, acrosome reactions, and signaling processes to ensure appropriate fertilization. Oxidative stress caused by excessive production of ROS significantly impairs sperm function. These impairments have resulted in male infertility via mechanisms involving the induction of peroxidative damage to the sperm plasma membrane, DNA damage, and apoptosis. ROS must be maintained at appropriate levels to ensure appropriate physiological function, while preventing pathological damage to the spermatozoa. Incorporation of standard protocol for assessment of seminal oxidative stress and antioxidant in routine andrology workup can help in the diagnosis and management of male infertility.

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