REVIEW ARTICLE

OXIDATIVE STRESS AND ROLE OF ANTIOXIDANTS IN MALE INFERTILITY

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INTRODUCTION

With regular cohabitation without any protection if women do not conceive for at least a period of one year is labelled as infertility. It is based on the observation that most of the normal couples achieve conception within a year of initiation of regular sex.[1]

Infertility affects approximately 15% of all couples trying to conceive. It's a major clinical problem, affecting people medically and psychologically. Male factor infertility is the major cause in roughly half of the cases, and no identifiable cause can be found in over 25% of infertile males.[2]

Out of many causes of male infertility Oxidative stress (OS) has been attributed to affect the fertility status and thus, it has been studied extensively in recent years.[3] Excessive production of free radicals or reactive oxygen species (ROS) can damage sperm and ROS has been extensively studied as one of the major mechanism of infertility.[4]

Spermatozoa, like any other cell is constantly facing the oxygen paradox.[5] Oxygen is essential to sustain life as physiological levels of reactive oxygen species (ROS) are necessary to maintain normal cell function. Conversely it's metabolites such as ROS can modify cell functions, endanger cell survival.[6,7] Reports indicate that high levels of ROS are detected in the semen of 25% to 40% of infertile men.[7,8]

Spermatozoa are particularly susceptible to the damage induced by excessive ROS because their plasma membrane contain large quantities of polyunsaturated fatty acids[9] (PUFA) and their cytoplasm contains low concentrations of scavenging enzymes.[7] In addition 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.[10,11] 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.

Physiological role of ROS in male reproductive system

ROS can have beneficial or detrimental effects on sperm functions depending on the nature and the concentration of the ROS as well as the location and length of exposure to ROS.[12] During epididymal transit, sperm acquire the ability to move progressively. However, they acquire the ability to fertilize, in the female tract through a series of physiological changes called 'Capacitation'.[13] Under physiological conditions, spermatozoa produce small amounts of ROS, which are needed for capacitation and acrosomal reaction, hyper activation, motility and fertilization.[14,15] Co-incubation of spermatozoa with low concentration of H_2O_2 has been shown to stimulate sperm capacitation, hyperactivation, acrosome reaction and oocyte fusion.[16,17] According to one study superoxide and nitric oxide also take part in these processes.[18] Free radicals are also involved in the fusion of spermatozoa with the Oocyte.[19] Nitric oxide plays a role in the sperm's ability to fuse with oocyte, but it has no action in zona pellucida 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 zona pellucida and ultimately, spermatozoa Oocyte fusion.[20,21]

Mechanism of oxidant generation in human sperm ROS production by spermatozoa

Studies suggest than human sperm can generate ROS.[22] Levels of ROS produced by spermatozoa were negatively correlated with the quality of sperm in the original semen.[23] Spermatozoa under goes a remarkable transformation during the final stage of sperm differentiation and loose their cytoplasm to become mature spermatids. When spermatogenesis is impaired, the cytoplasmic extrusion mechanism is defective. In this situation following spermiation, any residual cytoplasm that is associated with spermatozoa is retained in the mid-piece region as an irregular cytoplasmic mass.[24] Under these circumstances the spermatozoa that are released after spermiation are thought to be immature and functionally defective. They are capable of producing increased amounts of ROS generation via mechanisms that may be mediated by the cytosolic enzyme glucose-6-phosphate dehydrogenase.[25]

Mitochondria—Source and target of ROS

Spermatozoa may generate ROS in two ways that is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the level of sperm plasma membrane and (NADH) dependent oxido-reductase (diphorase) at the level of mitocondria.[26]

Spermatozoa are rich in mitochondria because they need a constant supply of energy for their

motility. Production of ROS is significantly increased in dysfunctional mitochondria, which in turn affect mitochondrial function in spermatozoa.[27] The primary ROS generated in human spermatozoa is the superoxide anion (O^*_2). This one electron reduction product of O_2 secondarily react with itself in a dismutation reaction, which is greatly accelerated by superoxide dismutase to generate hydrogen peroxide (H_2O_2). In the presence of transition metals such as iron and copper, H_2O_2 and O^*_2 can interact to generate the extremely pernicious hydroxyl radical (OH^-) (Haber-Weiss reaction). Alternatively, the hydroxyl radical can be produced from hydrogen peroxide (Fenton reaction), which requires a reducing agent such as ascorbate or ferrous ions. The hydroxyl radical is thought to be an extremely powerful initiator of the lipid peroxidation cascade and can precipitates loss of sperm function.

Reactive oxygen species produced by leukocytes

Peroxidase-positive leukocytes are the major source of ROS in semen and one largely contributed by the prostate and seminal vesicles.[28] Peroxidase-positive leukocytes include polymorphonuclear leukocytes which represents 50–60% of all seminal leukocytes, and macrophages, which represent 20–30% of all seminal leukocytes.[29] The capacity of leukocytes to generate ROS is related to their activation in response to inflammation and infection. During activation NADPH production is increased, and the myloperoxidase system of leukocytes is activated, leading to a respiratory burst with subsequent release of high levels of ROS.[30] These activated leukocytes can produce up to 100 fold higher amounts of ROS compared with non-activated leukocytes.[31]

Sperm damage from leukocyte derived ROS may occur when seminal leukocyte concentrations are abnormally high, such as in leukocytospermia.[32] WHO defines leukocytospermia (increased leukocyte infiltration in semen) as the presence of peroxidase-positive leukocytes in concentration of $>1\times10^6$ per millilitre of semen.[33] Sperm damage may happens even at leukocyte concentration below the WHO cut-off value for leukocytospermia.[34]

Effects of ROS

All the free radicals are highly toxic to all types of biological molecules including DNA, lipids, protein and carbohydrates. The extent of free radical damage depends on the nature and amounts of ROS involved and also on the duration of ROS exposure and extra cellular factors such as temperature, oxygen tension and the composition of the surrounding environment. (e.g., ions, proteins and ROS scavengers).

Lipid peroxidation of sperm's plasma membrane

Lipids are the most susceptible macromolecules present in sperm's plasma membrane in the form of polyunsaturated fatty acids (PUFA); fatty acid that contain more than two carbon-carbon double bonds. ROS attacks PUFA in the cell membrane leading to a cascade of chemical reactions called lipid peroxidation. One of the by product of lipid peroxidation is malondialdehyde, which has been used as an end product in biochemical arrays to monitor degree of peroxidative damage to spermatozoa,[35] lipid peroxidation results in loss of membrane fluidity, which is essential for sperm motility and sperm oocyte fusion.

Effects on sperm motility

Increased ROS formation has been associated with decreased sperm motility.[36] However; the exact mechanism through which it occurs is not understood. One hypothesis suggests that H_2O_2 diffuses across the cell membrane in to the cells and inhibit the activity of enzymes such as G_6PDH which via the hexose-monophosphate shunt controls the intracellular availability of NADPH, which is then used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH Oxidase.[37] Decreased G_6PDH 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.[38]

Another hypothesis involve 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.[39]

DNA damage and apoptosis induced by ROS

The oxidation damage to mitochondrial DNA is well known to occur in all aerobic cells, which are rich in mitochondria and this, may include spermatozoa. Two factors protect the sperm DNA from oxidative insult; the characteristic tight packing of the DNA and the anti-oxidants present in the seminal plasma.[40] Oxidative damage can cause base degradation, DNA fragmentation and cross linking of proteins.[41] Spermatozoa with damaged DNA lose their ability to fertilize the ooctye. ROS can also cause gene mutations such as point mutation and polymorphism resulting in decreased semen quality.[41] When DNA damage is small spermatozoa can undergo self repair. The oocyte is also capable of repairing damaged DNA of spermatozoa.[42] However if the damage is extensive, apoptosis and embryo fragmentation can occur.

Apoptosis, described as 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.[43] 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. ROS generated from abnormal spermatozoa may stimulate the process of apoptosis, resulting in death of spermatozoa. High levels of ROS disrupt the inner and outer mitochondrial membranes resulting in release of cytochrome-C protein from the mitochondria that activates the caspases and induces apoptosis.[44]

Apoptosis in sperm may also be initiated by ROS independent pathways involving the cell surface protein Fas [45] (Fas) is a member of the tumour necrosis factor (TNF) 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.

Role of antioxidants (Potential scavengers of ROS)

Since the ROS has both physiological and pathological roles, an array of antioxidants maintains a steady state of ROS in the seminal plasma. Antioxidants act as free radical scavengers to protect spermatozoa against ROS. These antioxidants are superoxide dismutase (SOD). Catalase and glutathione peroxidase (GPx). In addition, semen contains a variety of non-enzymatic antioxidant molecules such as vitamin C, vitamin E, Pyruvate, glutathione and carnitine[12](Table-1). These antioxidants compensate for the loss of sperm cytoplasmic enzymes as the cytoplasm is extruded during spermiogenesis, which in turn, diminishes endogenous repair mechanisms and enzymatic defenses.[17]

Among the well known biological antioxidants, SOD and its two isozymes and catalase have a significant role. SOD spontaneously dismutase (O^-_2) anion to form O_2 and H_2O_2, while catalase converts H_2O_2 to O_2 and H_2O.

$$2(O^-_2) + 2H \xrightarrow{SOD} H_2O_2 + O_2$$

$$H_2O_2 \xrightarrow{Catalase} H_2O + ½\ O_2$$

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.[46]

Catalase detoxifies both intracellular and extracellular H_2O_2 to water and O_2. In addition, catalase activates NO^- induced sperm capacitation, which is a complex mechanism involving H_2O_2.[47]

Table-1: Antioxidants and their mechanism of action

Antioxidant	Mechanism of action
Vitamin E	It is major chain breaking anti-oxidant in plasma membrane. Directly neutralizes superoxide anion, hydrogen peroxide and hydroxyl radical. It suppresses lipid peroxidation. Enhance fusion of spermatozoa with oocyte and to improve zona pellucida binding.
Vitamin C	Chain breaking antioxidant. Competitively protects the lipoprotein from peroxyl radicals. Recycles vitamin E.
Albumin	Reacts against peroxyl radical and prevents per oxidative damage of sperms. Neutralizes lipid peroxide mediated damage to sperm plasma membrane and DNA.
Glutathione	Neutralizes super-oxide anion. Reduced glutathione metabolizes H_2O_2 and OH radical.
Superoxide-dismutase	Neutralizes super-oxide anion by both intra and extra cellular.
Catalase	Neutralizes hydrogen peroxide. It can reduce the loss of motility caused by leukocyte generated ROS.
Super oxide dismutase	Neutralizes super-oxide anions (intra and extra cellular). Improves rate of acrosome reaction and preservation of sperm motility.
Coenzyme Q_{10}	It is an energy promoting agent, and reduces generation of super oxide anion.
N-Acetyl-L-Cysteine	It acts as a precursor of glutathione.

Glutathione peroxidase/reductase system forms an excellent protection against LPO 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_2O_2. Glutathione reductase (GSH-Red), then regenerates reduced GSH from GSSG as shown in following equation:

$$2\ GSH + H_2O_2 \xrightarrow{Se-GSH-Px} GSSG + 2H_2O$$

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

It scavenges lipid peroxides thereby arresting the progressive chain reaction of lipid peroxidation. It also scavenges hydrogen peroxide (H_2O_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 adeninedinucleotidephosphate ($NADP^+$) to its reduced form (NADPH).

Vit. E is a major chain breaking antioxidant in the sperm membrane and appears to have a dose dependent effect.[48] It scavengers superoxide, H_2O_2 and hydroxyl radicals. Administration of 100mg of vitamin E three times a day for 6months in a group of asthenozoospermic patients with normal female

partners showed a significant decrease in Lipid peroxidation and increased motility and pregnancy rates.[49]

Vitamin C is another important chain breaking and hydrogen peroxide radicals and prevents sperm agglutination. It prevents lipid peroxidation, recycles vitamin E and protects against DNA damage induced by H_2O_2 radical. 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.[50]

Coenzyme Q_{10} is a non-enzymatic antioxidants that is related to low density lipoproteins and protects against peroxidative damage.[51] It is an energy promoting agent and enhances sperm motility.[52] It is present in sperm mid piece and recycles vitamin E and prevents its pro-oxidant activity.[53] Oral supplementation of 60 mg/day of coenzyme Q_{10} was shown to improve fertilization rate using intra cytoplasmic sperm injection (ICSI) in normospermic infertile males.[52]

Antioxidants such as vitamin E and C, glutathione, N-Acetyl Cysteine, SOD, Catalase, Albumin, Taurine and Hypotaurine prevents reduction in sperm mortility and N-acetyl cysteine and coenzyme Q_{10} increases sperm motility.

Assessment of oxidative stress

However many men who demonstrate normal parameters on standard semen analysis remain infertile suggesting the routine semen analysis (measurement of seminal volume, spermetozoal motility, density, viability and morphology), does not necessarily provide complete diagnostic information.[54] As a result of active research in the area of evaluation of human semen, a series of sperm function assays have been developed (Table-2). However, no single test is capable of evaluating all the steps involved in fertilization.

Table-2: Laboratory Assays for Evaluation of Human Semen

Routine Evaluation	Seminal fluid volume, sperm count, motility, Morphology, Viability, Leukocytes in semen, sperm antibodies.
Specialised Sperm Function	Membrane integrity, Sperm-cervical mucus interaction, CASA, Capacitation, Acrosome reaction, Zona pellucida binding, zona pellucida penetration, Oocyte-sperm fusion.
Sperm Function Assays	HOST, Postcoital test, Tru-Trax, Penetrak, SPA, Acrosome reaction tests, Mannose receptor level. HZA, IVF

IVF: *in vitro* fertilization; CASA: computer –aided sperm analysis; SPA: sperm penetration Assay; HZA: hemizona assay; HOST: hypo-osmotic swelling test

At present only combination of assays can provide a comprehensive evaluation of sperm function.[55] The most common method for quantitating ROS is the measurement of rate of ROS generation by luminol induced chemiluminescense. This rate may not accurately reflect the status of sperm damaging ROS. The methods commonly used for measuring ROS are:

a) Reactions involving nitroblue tetrazolium (NBT) or cytochrome C-Fe^{3+} complexes which measure ROS on the cell membrane surface.
b) Reactions that measure ROS generated inside or outside the cell, utilising luminal dependent chemiluminescence.
c) The Electron spin resonance methods which are more sensitive and can identify the ROS generated inside the cell but require skilful operation and expensive instruments.

Evaluation of oxidative stress status (OSS)

The Balance of ROS is called as balance of creation and destruction. Under normal condition there is an appropriate balance between Oxidants and antioxidants. A shift in the levels of ROS towards pro-oxidants and oxidants in semen and vaginal secretions can induce an oxidative stress on spermatozoa. Similarly a decrease in antioxidant activities (e.g., SOD, Catalase, Se-GSH-Px, GSH-Red, GSH) in semen correlates with idiopathic infertility.[56] It is possible that an increased rate of ROS production (suggesting high oxidative stress) may inhibit the action of these antioxidant enzymes or alternatively the inherent decreased expression of these antioxidant enzymes may cause increased oxidative stress.[25] This will result in increased LPO, decreased sperm motility, viability and function, and ultimately leads to infertility.

Direct detection of free radicals is only possible by electron spin resonance (ESR or EPR for electron paramagnetic resonance). Unfortunately this method is restricted to expensive laboratory equipment and even more limiting to cell free systems, tissue culture and small organisms.

Peroxidases are the most important ROS generated by free radical action. There are several different methods for their detection. The most important ones are luminometric and colorimetric methods, which are based on the peroxide-peroxidase reaction, which leads to a light emission or colour production. Assessment of the rate of ROS production/generation using luminol as a probe can be a dynamic measure of oxidative stress.[57] However clinically the evaluation of this ROS generation is limited by a very short half life of these free radicals.[58]

Many indirect methods are available for the detection of ROS induced damage to lipid membrane. The thiobarbituric acid assay is commonly used. Thiobarbituric acid (TBA) reactive substances such as malondialdehyde (MDA) are measured by spectrophotometry analysis.[59]

Measuring TBA-MDA activity as in indicator of LPO remains one of the most efficacious method for assessing the oxidative damage to sperm.[60] This TBA-MDA measurement will needed to be combined with other assays which would be able to measure the rate of ROS production and antioxidant production for the overall assessment of OSS in infertility.

CONCLUSION

It is crucial for andrologist to understand free radicals their sources, mechanism of generation, and the damage they can cause to the male reproductive system. In addition, it is also essential to be aware of the various diseases that increase ROS generation in the blood, plasma and seminal fluids. A multifaceted approach is required for the treatment of male infertility induced by free radicals. Methods can be used to decrease ROS production. (e.g. the addition of antioxidants during sperm preparation techniques). With the abundance of many synthetic and natural antioxidants, it is very important to use them judiciously. Clinical trials using antioxidants in vivo and in vitro have resulted in a major debate, and further research is required before one can be optimistic about a role for antioxidants in the treatment of infertile man.

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