Review Article

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Oxidative stress & male infertility

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The male factor is considered a major contributory factor to infertility. Apart from the conventional causes for male infertility such as varicocoele, cryptorchidism, infections, obstructive lesions, cystic fibrosis, trauma, and tumours, a new and important cause has been identified: oxidative stress. Oxidative stress is a result of the imbalance between reactive oxygen species (ROS) and antioxidants in the body. It is a powerful mechanism that can lead to sperm damage, deformity and eventually, male infertility. This review discusses the physiological need for ROS and their role in normal sperm function. It also highlights the mechanism of production and the pathophysiology of ROS in relation to the male reproductive system and enumerate the benefits of incorporating antioxidants in clinical and experimental settings.

Key words Antioxidants - apoptosis - male infertility - oxidative stress - reactive oxygen species - sperm

Introduction

Infertility is a major clinical problem, affecting people medically and psychosocially. Statistics indicate that 15 per cent of all couples in the United States are infertile, and the male factor is responsible for 25 per cent of these cases[1]. Of the many causes of male infertility, oxidative stress (OS) has been identified as one factor that affects fertility status and thus, has been extensively studied in recent years. Spermatozoa, like any other aerobic cell, are constantly facing the "oxygen-paradox"[2]. Oxygen is essential to sustain life as physiological levels of reactive oxygen species (ROS) are necessary to maintain normal cell function. Conversely, breakdown products of oxygen such as ROS can be detrimental to cell function and survival[3]. Reactive oxygen species are present as free radicals. Examples of ROS include the hydroxyl ion, superoxide, hydrogen peroxide, peroxyl radical, and hypochlorite ion. These are the common forms of ROS that have been considered injurious to sperm survival and function when present in abundance**.**

OS is a consequence of an imbalance between the production of ROS and the body's antioxidant defense mechanisms. OS also has been implicated in the pathogenesis of many other human diseases such as atherosclerosis, cancer, diabetes, liver damage, rheumatoid arthritis, cataracts, AIDS, inflammatory bowel disease, central nervous system disorders, Parkinson's disease, motor neuron disease, and conditions associated with premature birth[4]. This article briefly enumerates the pathophysiology of ROS generation, its physiological and pathological effects on the male reproductive system, its importance in the field of assisted reproductive technology, and finally,

the possible ways of preventing and minimizing oxidative stress with the goal of achieving positive results in infertile couples with male factor infertility.

Physiological role of ROS in male reproductive system

Pioneering work in the field of reactive oxygen species was conducted by Aitken and his group in the mid eighties. Until recently, ROS was exclusively considered toxic to human spermatozoa. However, substantial evidence suggests that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities[5-7]. Low levels of ROS have been shown to be essential for fertilization, acrosome reaction, hyperactivation, motility, and capacitation[8,9]. Capacitation has been shown to occur in the female genital tract, a process carried out to prepare the spermatozoa for interaction with the oocyte. During this process, the levels of intracellular calcium, ROS, 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[10,11]. However, only capacitated spermatozoa exhibit hyperactivated motility and undergo a physiological acrosome reaction, thereby acquiring the ability to fertilize[12]. Co-incubation of spermatozoa with low concentrations of hydrogen peroxide has been shown to stimulate sperm capacitation, hyperactivation, acrosome reaction, and oocyte fusion[5,10,13,14]. Other ROS such as nitric oxide and the superoxide anion also are shown to promote capacitation and the acrosome reaction[15]. ROS also have been implicated in sperm oocyte interaction[16]. Lipid peroxidation caused by low levels of ROS leads to modification of the plasma membrane, thus facilitating sperm-oocyte adhesion[14].

Sources of ROS

ROS represent a broad category of molecules, including a collection of radical (hydroxyl ion, superoxide, nitric oxide, peroxyl, *etc.*) and non-radical (ozone, singlet oxygen, lipid peroxide, hydrogen peroxide) oxygen derivatives[4]. These derivatives participate in a cascade of reactions that give rise to free radicals that ultimately can damage organic substrates. Reactive nitrogen species (nitrous oxide, peroxynitrite, nitroxyl ion, *etc*.) are also a class of free radicals derived from nitrogen and considered a subclass of ROS[17,18]. Virtually every human ejaculate is considered to be contaminated with potential sources of ROS[10] as human semen is known to contain different types of cells, such as mature and immature spermatozoa, round cells from different stages of spermatogenesis, leukocytes, and epithelial cells. Of these different cell types, leukocytes and spermatozoa have been shown to be the two main sources of ROS[19].

Cytoplasmic droplets, or excess residual cytoplasm, explain the missing link between poor sperm quality and increased ROS generation. Gomez *et al* [20] showed that 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. Studies have suggested that 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[6]. The generation of ROS by spermatozoa has been proposed to occur in two ways: (*i*) nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the level of the sperm plasma membrane[21], and (*ii*) NADPH-dependent oxidoreductase (diphorase) at the mitochondrial level[22].

Immature, morphologically abnormal spermatozoa and seminal leukocytes are the main sources of ROS in human ejaculates[23] Spermatozoa are rich in mitochondria because they need a constant supply of energy for their motility. Unfortunately, when spermatozoa contain dysfunctional mitochondria, increased production of ROS occurs, affecting mitochondrial function[24]. Such a relationship could be due to two mutually interconnected phenomena: ROS causing damage to the mitochondrial membrane and the damaged mitochondrial membrane causing an increase in ROS production.

World Health Organization (WHO) defines leukocytospermia (increased leukocyte infiltration in semen) as the presence of peroxidase-positive leukocytes in concentrations of $>1 \times 10^6$ per milliliter of semen[25]. However, controversy exists over the clinical significance of leucocytospermia[26]. On one side, sperm parameters such as poor quality, decreased hyperactivation, and defective sperm function[27] have been attributed to leucocytospermia**.** On the other side, no correlation was established between seminal leukocyte concentrations and impaired sperm quality[28] or defective sperm function[29].

Studies conducted in our laboratory have shown that in non leukocytospermic samples, ROS levels were lower in fertile men than in subfertile patients in unprocessed (neat) samples (0.29 vs. 0.94, *P*=0.001) and washed semen (5.73 vs. 23.4, *P*=0.001)[30]. Similarly, samples with leukocytes were found to have lower ROS levels in fertile men in neat (0.75 vs. 2.0, *P*=0.001) and washed semen (15.85 vs. 239.83, *P*<0.001)[31]. Furthermore, in an earlier study, oxidative stress correlated with the rising leukocyte count[32]. Thus, after reviewing this new information, it can be concluded that oxidative stress occurs even in patients with a very low seminal leukocyte count (between 0 and 1x 10^6 /ml), and a rise in ROS occurs with an increase in leukocyte count. Also, it has been concluded that the presence of any leukocytes is associated with oxidative stress and may, therefore, impair infertility.

Peroxidase-positive leukocytes include polymorphonuclear leukocytes, which represent 50 to 60 per cent of all seminal leukocytes, and macrophages, which represent another 20 to 30 per cent[26]. The prostate gland and the seminal vesicles are the main sources of these peroxidase-positive leukocytes in human ejaculate[27]. Leukocytes may be activated in response to various stimuli such as infection and inflammation[32], and these activated leukocytes can produce up to 100-fold higher amounts of ROS compared with non-activated leukocytes[33]. 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 ROS. Sperm damage from ROS that is produced by leukocytes, occurs if seminal leukocyte concentrations are abnormally high, such as in leukocytospermia[34] or if seminal plasma is removed during sperm preparation for assisted reproduction[35].

Effects of OS

All cellular components, including lipids, proteins, nucleic acids, and sugars are potential targets of OS. The extent of OS-induced damage depends not only on the nature and amount of ROS involved, but also on the duration of ROS exposure and on extracellular factors such as temperature, oxygen tension, and the composition of the surrounding environment (*e.g.,* ions, proteins, and ROS scavengers)[3,4,6,9,17,36].

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. 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. Once 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[10,14,15,35-39].

ROS attacks PUFA in the cell membrane, leading to a cascade of chemical reactions called lipid peroxidation. ROS have a tendency toward chain reactions; that is, a compound carrying an unpaired electron will react with another compound to generate an unpaired electron, in such a manner that "radical begets radical". The reactions proceed through three main steps- initiation, propagation, and termination[10,14,15,36-39].

During initiation, the free radicals react with fatty acid chains and release lipid free radicals. This lipid free radical may further react with molecular oxygen to form the lipid peroxyl radical. Peroxyl radicals can react with fatty acids to produce lipid free radicals, thus propagating the reaction[10,14,15,36-39]. One of the byproducts of lipid peroxidation is malondialdehyde. This byproduct has been used in various biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa[36,37]. Results of such an assay exhibit an excellent correlation when examining the relationship between impaired sperm function, discussed in terms of motility, and the capacity for sperm-oocyte fusion[38].

Effect on motility

Increased ROS levels also have been correlated with decreased sperm motility[40-42]. However, the exact mechanism through which ROS causes decreased motility is not understood. Thus, many hypotheses have been proposed to explain the link between ROS and decreased motility. One hypothesis shows that H_2O_2 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[43]. 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[44].

DNA damage by OS

Two factors protect spermatozoa DNA from oxidative stress: the characteristic tight packaging of sperm DNA and the antioxidants in seminal plasma[45]. Exposing the sperm to artificially produced ROS causes DNA damage in the form of modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross- links, and chromosomal rearrangements[46]. Oxidative stress also is associated with high frequencies of single- and double-strand DNA breaks[46,47]. ROS also can cause various types of gene mutations such as point mutations and polymorphism, resulting in decreased semen quality[31,48]. Other mechanisms such as denaturation and DNA base - pair oxidation also may be involved[49]. A common byproduct of DNA oxidation, 8-hydroxy-2-deoxyguanosine (8-OH-2-deoxyguianosine), has been considered a key biomarker of this oxidative DNA damage[50].

When the extent of DNA damage is small, spermatozoa can undergo self-repair, and moreover, the oocyte also is capable of repairing damaged DNA of spermatozoa[16]. However, if the damage is extensive, apoptosis and embryo fragmentation can occur. Decreased fertilization rates and poor embryo cleavage and quality have been reported in infertility cases where sperm samples contain a high frequency of damaged DNA[51]. DNA damage in the Y chromosome also can cause gene deletion in the Y chromosome of the offspring, leading to infertility[47].

Oxidative stress and apoptosis

Apoptosis is a non-inflammatory response to tissue damage characterized by a series of morphological and biochemical changes[52-59]. In the context of male reproductive tissue, it helps in elimination of abnormal spermatozoa, thus maintaining the nursing capacity of the Sertoli cells[54]. High levels of ROS disrupt the inner and outer mitochondrial membranes, inducing the release of the cytochrome-C protein and activating the caspases and apoptosis. Apoptosis in sperm also may be initiated by ROS-independent pathways involving the cell surface protein Fas[60]. Fas is a type I membrane protein that belongs to the tumour necrosis factor-nerve growth factor receptor family and mediates apoptosis[61]. When Fas ligand or agonistic anti-Fas antibody binds to Fas, apoptosis occurs[62]. On the other hand, *bcl-2*, the inhibitor gene of apoptosis, protects the cell, most likely by mechanisms that reduce ROS production[63].

Although the Fas protein often leads to apoptosis, some of the Fas-labelled cells may escape apoptosis through abortive apoptosis. This result in a failure to clear all of the spermatozoa destined for elimination and thus, leads to a large population of abnormal spermatozoa in the semen. This failure to clear Fas- positive spermatozoa may be due to a dysfunction at one or more levels. First, the production of spermatozoa may not be enough to trigger apoptosis in men with hypospermatogenesis. In this case, Fas-positive spermatogonia may escape the signal to undergo apoptosis. Second, Fas-positive spermatozoa also may exist because of problems in activating Fas-mediated apoptosis. In this scenario, apoptosis is aborted and fails to clear spermatozoa that are earmarked for elimination by apoptosis[52]. In men with abnormal sperm parameters (oligozoospermia, azoospermia), the percentage of Fas-positive spermatozoa can be as high as 50 per cent. Samples with low sperm concentrations are more likely to have a high proportion of Fas-positive spermatozoa[52].

Mitochondrial exposure to ROS results in the release of apoptosis inducing factor (AIF), which directly interacts with the DNA and leads to DNA fragmentation[64,65]. In another study by our group, a positive correlation was demonstrated between increased sperm damage by ROS and higher levels of cytochrome C and caspase 9 and 3, which indicate positive apoptosis in patients with male factor infertility[66]. Activation of caspases 8, 9, 1, and 3 in human ejaculated spermatozoa have been studied to examine the main pathways of apoptosis. Potential functional impact of this phenomenon and possible activation mechanisms were examined by subjecting cells to freezing and thawing, and testing the dependence of caspase activity on membrane integrity[67].

In an earlier study carried out by our group, annexin V staining assay was used to study the externalization of phosphatidylserine, a marker of early apoptosis. It was shown that mature spermatozoa from infertility patients had significantly higher levels of apoptosis compared with the mature spermatozoa from a control group of normal sperm donors[68].

Varicocele and OS

Clinical or subclinical varicocoele[69] has been shown to cause male infertility in about 15 per cent of infertile couples[70]. These patients have increased ROS in serum, testes, and semen samples[71]. Increased nitric oxide also has been demonstrated in the spermatic veins of patients with varicocoele[72,73], which could be responsible for the spermatozoal dysfunction[74]. ROS in patients with varicocoele are formed due to the excessive presence of xanthine oxidase, a source of superoxide anion from the substrate xanthine and nitric oxide in dilated spermatic veins. On the other hand, it has also been recorded that varicocelectomy increases the concentrations of antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, and vitamin C, in seminal plasma as well as improves sperm quality[75]. One study showed a significant correlation between ROS levels and varicocele grade[76]. The researchers demonstrated that ROS levels were significantly higher in men with grade 2 and 3 varicocoele than in those with grade 1, and that no correlation existed between ROS levels and testicular volumes. Patients with varicocoele had increased 8-hydroxy-2:deoxyguanosine (8-OHdG), indicating oxidative DNA damage[77,78]. The conclusion from a meta-analysis was that oxidative stress parameters (such as ROS and lipid peroxidation) are significantly increased in infertile patients with varicocoele as compared with normal sperm donors, and antioxidant concentrations were significantly lower in infertile varicocoele patients compared with controls[79].

Smoking, oxidative stress and infertility

Tobacco smoke consists of approximately 4,000 compounds such as alkaloids, nitrosamines and inorganic molecules, and many of these substances are reactive oxygen or nitrogen species. Significant positive association has been reported between active smoking and sperm DNA fragmentation[80], as well as axonemal damage[81] and decreased sperm count[82].

Sperm from smokers have been found to be significantly more sensitive to acid- induced DNA denaturation than those from non smokers because the smokers' sperm have been shown to contain higher levels of DNA strand breaks[83]. In a study carried out on 655 smokers and 1131 non smokers, cigarette smoking was associated with a significant decrease in sperm density (-15.3%), total sperm count (-17.5%), and total number of motile sperm (-16.6%)[84]. Thus, smoking does, in fact, affect the quality and quantity of sperm present within a male.

Assessment of ROS by chemiluminescence

To accurately quantify oxidative stress, levels of ROS and antioxidants should be measured in fresh samples. Direct methods such as pulse radiolysis and electron-spin resonance spectroscopy have been useful for many systems of the body but have limitations in their use in the male reproductive system. These methods are faced with the problems of a relatively low volume of seminal plasma, short life span of ROS, and the need to perform the evaluation in fresh samples[16]. Thus, another method is needed that avoids the problems encountered by the direct methods. Recently, one of the most widespread methods of measuring ROS is chemiluminescence assay. This method seems to quantify both intracellular and extracellular ROS. It uses sensitive probes such as luminol (5-amino-2, 3, dihydro 1, 4, phthalazinedione) and lucigen for quantification of redox activities of spermatozoa[85]. Luminol is an extremely sensitive, oxidizable substrate that has the capacity to react with a variety of ROS at neutral pH. Furthermore, it can measure both intracellular and extracellular ROS, whereas lucigen can measure only the superoxide radical released extracellularly. Hence, by using both the probes on the same sample, it is possible to accurately identify intracellular and extracellular ROS generation[57,85,86]. The reaction of luminol with ROS results in production of a light signal that is converted to an electrical signal (photon) by a luminometer. Levels of ROS are assessed by measuring the luminal-dependent chemiluminescence with the luminometer. The results are expressed as $x10^6$ counted photons per minute (cpm) per 20 x 10^6 sperm. Normal ROS levels in washed sperm suspensions range from 0.10 to 1.0 x 10^6 cpm/20 x 10^6 sperm. In a recent study, ROS levels of 0.145 x 10^6 cpm per 20 x 10^6 sperm were defined as the optimum cut-off value in unprocessed ejaculated samples[87].

Antioxidants

ROS have physiological and pathological roles. Spermatozoa, due to the paucity of cytoplasmic enzymes, are unable to repair oxidative damage. Studies have shown that antioxidants have a widespread effect in andrology. These protect spermatozoa from ROS producing abnormal spermatozoa, scavenge ROS produced by leucocytes, prevent DNA fragmentation, improve semen quality in smokers, reduce cryodamage to spermatozoa, block premature sperm maturation, and stimulate spermatozoa and improve assisted

reproductive techniques (ART) outcome. Three different antioxidant protection systems play important and interdependent roles in reducing OS in males: dietary antioxidants, endogenous antioxidants, and metal-binding proteins[88-96].

Endogenous antioxidants comprise antioxidants present in seminal plasma and spermatozoa. Seminal plasma contains three main enzymatic antioxidants: superoxide dismutase (SOD), catalase, and glutathione peroxidase/glutathione reductase (GPX/GRD), in addition to a wide range of non enzymatic antioxidants like ascorbate, urate, vitamin E, pyruvate, glutathione, albumin, vitamin A, ubiquitol, taurine, and hypotaurine. Spermatozoa possess primarily enzymatic antioxidants, with SOD being the most predominant. Dietary antioxidants are usually present in the form of vitamin C, vitamin E, beta-carotenes, carotenoids, and flavonoids. Metal-binding proteins such as albumin, ceruloplasmin, metallothionein, transferrin, ferritin, and myoglobin function by inactivating transition metal ions that otherwise would have catalyzed the production of free radicals[2,92,93,95,96]. Metal chelators such as transferrin, lactoferrin, and ceruloplasmin that are present in human semen also control lipid peroxidation of the sperm plasma membrane, protecting its integrity[91,97].

In vivo antioxidants

(i) Vitamin E: Vitamin E is a major chain-breaking antioxidant in the sperm membranes and appears to have a dose-dependent effect[98]. It scavenges all three types of free radicals, namely, superoxide, H_2O_2, and hydroxyl radicals[8]. Suleiman *et al*[98] showed that administration of 100 mg of vitamin E three times a day for six months in a group of asthenozoospermic patients with normal female partners led to a significant decrease in lipid peroxidation and increase in motility. Also, pregnancy rates consequently increased significantly (21% in treatment group as compared with placebo group).

(ii) Vitamin C: 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[8]. It prevents lipid peroxidation, recycles vitamin E and protects against DNA damage induced by the H_2O_2 radical. Kodama *et al*[49] showed that administration of 200 mg of vitamin C orally along with vitamin E and glutathione for two months significantly reduced 8-OH-dG levels in spermatozoa and also led to an increase in sperm count.

(iii) Coenzyme Q10: Coenzyme Q-10 is a non enzymatic antioxidant that is related to low-density lipoproteins and protects against peroxidative damage. Since it is an energy-promoting agent, it also enhances sperm motility[89]. It is present in the sperm midpiece[99] and recycles vitamin E and prevents its pro-oxidant activity[39]. It has been shown that oral supplementation of 60 mg/day of coenzyme Q10 improves fertilization rate using intracytoplasmic sperm injection (ICSI) in normospermic infertile males[89].

Role of antioxidants in motility

Not only do antioxidants prevent reduction in sperm motility (mainly vitamin E and C, glutathione, N-acetyl cysteine, SOD, catalase, albumin, taurine, and hypotaurine), these also increase sperm motility (N-acetyl cysteine and coenzyme Q10). A randomized double-blind controlled trial has shown that vitamin E administered orally (300 mg/day) results in a decrease in malondialdehyde (a marker for lipid peroxidation) concentration in spermatozoa and improved sperm motility[98]. Another study has shown that incubation of sperm samples from asthenozoospermic infertile males for 24 h in Ham's F-10 medium with 50 μM coenzyme Q10 improves sperm motility[89]. Lenzi *et al*[90] reported that oral supplementation of 2-3 g/day of carnitines for >2 months improved sperm concentration and motility. In another study incubating sperm with D-penicillamine significantly increased sperm motility[91].

The results of *in vitro* trials using antioxidants are not better than the results of *in vivo* trials[92] and the potential advantages of antioxidants in assisted reproduction are still under debate[93]. One study showed that supplementing the sperm preparation media with a combination of vitamins C and E was associated with decreased ROS production by the sperm[94]. In another study, superoxide supplementation was associated with improved rates of acrosome reaction and preservation of sperm motility[9]. In the clinical ART setting, various antioxidants such as vitamin E[100], vitamin C[101], cysteine[102], and taurine and hypotaurine[103] added to the culture medium can improve the developmental ability of the embryos by reducing the effects of ROS.

Role of antioxidants in preventing cryodamage

Sperm freezing and thawing procedures cause a significant and irreversible depression of motility and

metabolic activity of sperm along with disruption of plasma membrane[104]. Park *et al* [105] have shown that vitamin E (10 mmol/l) and rebamipide (300 mmol/l) decreased the cryodamage during the freeze-thaw procedure and improve post- thaw motility. *In vitro* supplementation of 300 micromol/l of rebamipide in semen samples during incubation (37°C) and cryopreservation (-196°C, 3 days) has been shown to significantly decrease the ROS level[106].

Role of antioxidants in preventing DNA damage

Antioxidants have been shown to decrease the DNA fragmentation induced by oxidative stress. Daily oral supplementation of 1 *g* vitamins C and E for two months is reported to reduce the number of TUNEL-(terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) positive spermatozoa from 22.1 to 9.1 per cent, while the amount of spermatozoa with DNA defragmentation remained the same in the placebo group[95]. Moreover, the same group also showed a marked improvement of clinical pregnancy and implantation rates after antioxidant treatment compared with the pre-treatment outcomes of ICSI[107]. Vitamin E or C was added to the sperm preparation media during density gradient separation using Percoll, and thus, spermatozoa were protected from DNA damage[96]. On the contrary, using a combination of the above vitamins demonstrated an increase in DNA damage. Twigg *et al* [45] found that albumin can be an important means of neutralizing lipid peroxide-mediated damage to the sperm plasma membrane and DNA.

ROS in assisted reproductive techniques

OS-induced DNA damage may have important clinical implications in the context of ART. Studies have indicated that human spermatozoa significantly increased levels of ROS production in response to repeated cycles of centrifugation involved in conventional sperm preparation techniques used for ART [41]. Spermatozoa selected for ART usually originate from an environment experiencing oxidative stress, and a high percentage of these sperm may have damaged DNA[49]. When intrauterine insemination (IUI) or *in vitro* fertilization (IVF) is used; such damage may not be a cause of concern because the collateral peroxidative damage to the sperm plasma membrane ensures that fertilization cannot occur with a DNA-damaged sperm.

When (ICSI) is used, this natural selection barrier is bypassed and a spermatozoon with damaged DNA is directly injected into the oocyte[6, 44]. However, ROS can be produced in a number of ways in ART procedures. Oocytes and embryo metabolism, cumulus cells, leukocyte contamination during sperm preparation, and culture media are the major sources. Oral *et al*[108] demonstrated that higher MDA levels in follicular fluid of females was an indicator of lower pregnancy rates, and thus, MDA can be used as a potential marker for predicting ART outcomes. A meta-analysis by our group[109] concluded that ROS have a statistically significant effect on the fertilization rate after IVF, and that the measurement of ROS levels in semen specimens before IVF may be useful in predicting IVF outcomes. We also have reported that high day 1 ROS levels in culture media were associated with low blastocyst rate, low fertilization rate, low cleavage rate, and high embryonic fragmentation with ICSI but not with conventional IVF; however, high day 1 ROS levels in culture media were associated with lower pregnancy rates in both IVF and ICSI cycles[110].

Assisted reproduction techniques may show significant improvement in *in vitro* supplementation of antioxidants and metal chelators to achieve a better success[111]. Excellent results were obtained with the use of many compounds like rebamipide, pentoxyfylline, vitamins E and C, SOD, catalase, *etc.* In a study on 740 embryos, Zhang *et al* [112] showed a dose-dependent decrease in % BDR (blastocyst development rate) with increasing concentrations of H_2O_2, indicating that H_2O_2 (>60 mM) is embryotoxic, and the administration of pentoxyfylline at 500 μM could reduce the embryotoxic effect of hydrogen peroxide.

Conclusion

In the last decade, a phenomenal growth has occurred in our knowledge of male reproduction, sperm function, and development of diagnostic tools and treatment modalities for male infertility. In addition, knowledge regarding oxidative stress has given rise to several new treatment modalities that are now being tried to improve male infertility. Many new antioxidants are now available that can decrease oxidative stress and improve sperm quality, but a major concern in their usage is lack of scientific evidence of their effectiveness, which has led to denial of their approval by the US Food and Drug Administration. Evidence exists that supports the use of systemic antioxidants as well as antioxidants in sperm preparation techniques. Moreover, several newer sperm preparation techniques such as density gradient

centrifugation, glass wool filtration and migration-sedimentation have significantly reduced the level of ROS by removing leucocytes. However, OS being only one of the causes of male infertility, antioxidant therapy should be tried only in cases of increased oxidative stress or established DNA damage.

Evaluation of OS status and the use of antioxidants is not a routine in clinical practice. Immediate attention should be directed at simplifying and validating the evaluation of reactive oxygen species and OS status so that it can be performed routinely without the use of sophisticated equipment. Also, a threshold ROS level above which antioxidants could be used for male infertility, should be determined. The dose and duration of these antioxidants should also be determined and standardized. With the increased use of ART procedures, efforts should be directed at developing optimum combinations of antioxidants to supplement sperm preparation media. Adding testicular sperm extraction and percutaneous epididymal sperm aspiration to our ART armamentarium and improving cryopreservation techniques may help patients, especially in cases of cancer and azoospermia.

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