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Supraphysiological Free Radical Levels and their Pathogenesis in Male Infertility

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Abstract

Oxidative stress is an important aetiological factor which leads to sperm DNA damage and infertility. It damages all biomolecules and both mitochondrial and nuclear DNA and adversely affects sperm membrane fluidity and motility. This acts like a biological safeguard however use of such sperm for ART/ICSI can lead to pre and post implantation losses, major and minor congenital malformations and even childhood cancer. Thus it is important to know the causes of oxidative stress and how the levels of free radicals be maintained at physiological levels when they are beneficial for normal function. It is also important to develop techniques to identity cases with high free radical levels and also adopt certain life style measures which can minimise oxidative stress and improve male reproductive health.

Keywords: Oxidative stress; Free radicals; Semen; Infertility; Oligozoospermia; Azoopsermia

Introduction

Infertility is one of the major health problems. Nearly 30% of the couples in reproductive age group are not being able to conceive within one year of unprotected sexual intercourse. Of these the male factor is solely responsible in about 20% of the cases and is contributory in other 30-40% cases [1]. It is estimated that globally, 60-80 million couples suffer from infertility every year and of which probably 15-20 million are in India alone. Various pre-testicular, testicular and post-testicular causes like varicocele, Y chromosome micro-deletions, injuries, infections, hormonal disorders and obstruction are known to cause infertility. Despite extensive investigations in about 40-50% no aetiology is identified. In recent years it has been shown that infertile men with normal and abnormal sperm parameters may have significantly high free radical levels and sperm DNA damage.

Oxidative stress is one of the major causes of infertility at the molecular level. Oxidative stress is a condition in which free radical levels are very high and overwhelms the antioxidant defence mechanisms. In such cases high free radical levels damage all bio molecules like lipids, carbohydrates, proteins and both mitochondrial and nuclear DNA. Supraphysiological Reactive Oxygen Species (ROS) mediated damage to sperm is found in 30-80% of infertile men. Oxidative Stress has also been implicated in the pathogenesis of many other diseases like atherosclerosis, cancer, diabetes, liver damage, rheumatoid arthritis, cataract, AIDS, Inflammatory Bowel Disease (IBD), Central Nervous System (CNS) disorders, Parkinson's disease, motor neuron disease and premature birth. ROS can be generated from exogenous and endogenous sources and they cause damage to different molecules and parts of spermatozoa, a highly polarized cell. In this review we are going to analyse the different mechanisms by which ROS can damage the spermatozoa and those who are at risk for oxidative stress induced damage. The adequate knowledge of these helps us to delineate those who will benefit from antioxidant therapy. These cannot be predicted by routine semen analysis. Thus standard semen parameters are poor predictors of fertility potential.

Free Radical Biochemistry

ROS are product of normal cellular metabolism. Most of body's energy is produced by oxidative phosphorylation within the mitochondria. During this very abrupt reduction to produce energy, free radicals are formed [2]. A free radical is defined as an oxygen molecule containing one or more unpaired electrons in atomic or molecular orbit. The addition of one electron to dioxygen forms the superoxide anion radical, the primary form of ROS. This superoxide ion can then be directly or indirectly converted to secondary ROS such as hydroxyl radical, peroxyl radical or hydrogen peroxide. ROS represent a broad category of molecules that indicate the collection of radicals (hydroxyl ion, superoxide, nitric oxide, peroxyl, etc.) and non-radicals (ozone, single oxygen, lipid peroxides, hydrogen peroxide) and oxygen derivatives [3]. Among these Nitric oxide (NO) has been shown to have detrimental effects on normal sperm functions inhibiting both motility and sperm competence for zone binding [3].

Free radicals participate in chemical reactions that relieve them of their unpaired electrons resulting in oxidation of lipids in membranes, amino acids in proteins and carbohydrates within nucleic acids [4].

ROS in small amounts (physiological levels) are necessary for spermatozoa to acquire fertilizing capabilities and essential for fertilization, acrosome reaction, hyper activation, motility and oocyte fusion. Lipid peroxidation caused by low levels of ROS leads modification of the plasma membrane, facilitating sperm-oocyte adhesion. In other tissues of the body, ROS participates in various functions like signalling molecules, gene transcription factors.

Sources of ROS in the Semen

Supraphysiological ROS levels are detected in the semen of infertile men with normal and abnormal semen parameters (agglutination, viscosity, motility etc.). Within semen, there are two principal sources of free radicals; leukocytes and sperm midpiece. Activation of leukocytes play an important role in determining the ROS output. This is established by the positive correlation between the seminal ROS production and the pro-inflammatory cytokines such as IL-6 [5], IL-8 [6] and TNF-α [6]. The contribution of leukocyte to ROS can be studied using the specific leukocyte activator, N-Formyl methionine. – Leucine-Phenylalanine (FLMP). Activated leukocyte produces 100 fold

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higher levels of free radicals and thus infections (TB, malaria, fever) and chronic inflammatory disorders are associated with increased free radical levels.

Sperm isolation techniques like density centrifugation gradient (DCG) show that spermatozoa themselves are also capable of producing ROS [7]. It has been further proved when the leukocytes are further depleted using magnetic beads coated with leukocyte specific CD-45 antibodies, the ROS are still present in the semen [8] and morphologically abnormal sperm and sperm with impaired motility produce high free radical levels [9].

The ability of sperm to produce ROS inversely correlates with their maturational states. During spermiogenesis there is a loss of cytoplasm to allow the sperm to form its condensed, elongated form. Immature teratozoospermic sperm are often characterized by the presence of excess cytoplasmic residue in the mid-piece. These residues are responsible for the generation of ROS via the NADPH-HMP pathway. [10-12]. This shows that morphologically abnormal and immature sperm can generate more ROS than morphologically normal sperm. Thus there is a need to segregate sperm subpopulations of morphologically normal and abnormal sperm so that high free radical produced by morphologically abnormal sperm may not induce oxidative damage to sperm with normal parameters.

The rate of production of ROS by leukocytes is reported to be 1000 times higher than that of spermatozoa at capacitation [13], making leukocytes the likely dominant producer of seminal ROS. When seminal ROS production is divided into that produced by the sperm themselves (intrinsic ROS) and that made by the leukocytes (extrinsic), an interesting observation was found [14]. While both intrinsic and extrinsic ROS production is negatively correlated with sperm DNA integrity, the relationship is significantly stronger for intrinsic ROS production. This suggests that while leukocytes produce more ROS than sperm on a per cell basis, the close proximity between intrinsic ROS production and sperm DNA makes intrinsic ROS production a more important variable in terms of fertility potential.

Oxidative Stress –What Does it Mean?

Oxidative stress (OS) is a condition that occurs when the production of ROS overwhelms the antioxidant defence mechanisms. In male reproductive pathologies, OS significantly impairs spermatogenesis and sperm function, which may lead to male infertility. Unlike somatic cells, spermatozoa are highly vulnerable to free radical attack and the induction of a lipid peroxidation process that disrupts the integrity of plasma membrane and impairs sperm motility [15]. Sperm is a highly polarized, terminally differentiated cell, lacks cytosolic antioxidants (as majority of the cytoplasm is shed during spermiogenesis) and very rich in Polyunsaturated Fatty Acids (PUFA).

Presence of polyunsaturated fatty acids (PUFA) in the sperm is necessary for membrane fluidity required for membrane fusion events associated with fertilization particularly acrosomal exocytosis and fusion with oolemma. Thus as much as 50% of the fatty acid in a human spermatozoon is docosahexaenoic acid with six double bonds per molecule [15]. Unfortunately, such highly unsaturated fatty acids are particularly prone to oxidative attack because the conjugated nature of the double bonds facilitates such processes as hydrogen abstraction, which initiates the lipid peroxidation cascade. The latter can be promoted by the presence of transition metals such as iron and copper that can vary their valency state by gaining or losing electrons. Significantly, there is sufficient free iron and copper in human seminal plasma to promote lipid peroxidation once this process has been initiated [16]. Such transition metals can also promote the ability of ROS to attack another important substrate in mammalian spermatozoa-the DNA present in the sperm nucleus and mitochondria.

Oxidative stress has been reported to cause abnormal denaturation of DNA into single stranded DNA and double-stranded DNA breaks, DNA base-pair oxidation, chromatin cross linking, and chromosome micro-deletion. It can damage both nuclear and mitochondrial DNA [17,18]. Out of these, mitochondrial DNA is more vulnerable to oxidative attack because it is a naked nucleus not bound by histones and also it is the first site of production of ROS and ROS induced oxidative damage [19]. Sperm exists in a state of oxygen paradox. It requires oxygen for their metabolism and experience oxidative damage. On the other hand, Sperm nuclear DNA is less vulnerable to oxidative damage as it is tightly compacted with protamines, and are further stabilized by the creation of inter- and intra-molecular disulphide bonds to form a crystalline toroid [20,21]. Nevertheless, free radicals can still damage nuclear DNA, engaging in H-abstraction reactions with the ribose unit and inducing the formation of DNA base adducts. Sperm nuclear DNA is organized into two compartments, a central protamine bound condensed fraction (85%) and a fraction lying in the peripheral portion of nucleus bound to histones (15%). It is the histone bound nucleosomal DNA that is prone to oxidative injury. Interestingly it is this fraction of DNA (nucleosomal) which transmits genes of developmental importance (like HOX, WNT) and it is most vulnerable to environmental insults and oxidative damage.

Both of these processes greatly destabilize the DNA structure and may ultimately result in the formation of DNA strand breaks [22].

One of major oxidised base adduct formed when the DNA is subjected to attack by ROS, is 8-hydroxy 2' deoxyguanosine (8-OHdG). This has been used as a marker of oxidative DNA damage and has been highly correlated with DNA strand breaks, as assessed by TUNEL assay. To measure the efficiency of chromatin remodelling during spermiogenesis, the DNA-sensitive fluorochrome, chromomycin A3, CMA3, was employed. The latter competes with nucleoproteins for binding sites in the minor groove of GC-rich DNA and serves as marker for the efficiency of DNA protamination during spermiogenesis. Accordingly, staining with this probe has been shown to be positively correlated with the presence of nuclear histones [23] and ultra structural evidence of poor chromatin compaction (Iranpour et al. [24]). This oxidized product is premutagenic and can lead to transversions, single and double strand breaks. As telomeric DNA (at chromosomal ends), is rich in guanine repeats, guanine having a lower oxidative potential preferentially accumulates oxidative damage and form 8-oxo- guanine. This accelerates telomere shortening. As telomeres serve as biological molecular clock, telomere attrition may lead to accelerated testicular aging of germ cells with reduced replicative span and thus leading to oligozoospermia, azoospermia and infertility. Telomere shortening is also associated with genomic and chromosomal instability leading to chromosomal re-arrangements and aberrant recombinations. This may lead to segregation anomalies during meiosis and thus to meiotic arrest.

Mitochondria and ROS

The location of mitochondria, in sperm midpiece is unique in as it is positioned at the site of maximum energy requirement. It has been well established that mitochondria make ATP by the coupling of respiration generated proton gradient with the proton-driven phosphorylation of ATP. It is associated with the inner mitochondrial membrane where highly mutagenic oxygen radicals are generated as by-product of OXPHOS in the respiratory chain73 and leakage of these free radicals from the respiratory chain makes the mitochondria

as a major intracellular source of ROS [25]. These unique features are probably the cause of 10-15 times faster accumulation of the mutations and single nucleotide polymorphisms in mt DNA than nuclear DNA. Mitochondrial dysfunction in such cases may be measured as mitochondrial membrane potential (MMP), which has been reported to decrease in the spermatozoa of infertile men with raised ROS levels [26]. Several studies have reported that human cells harbouring mutated mt DNA have lower respiratory function and show increased production of superoxide anions, hydroxyl radicals and H_2O_2 [27,28]. It has been reported that morphologically abnormal sperm and sperm with impaired motility have increased mt DNA copy number. Shamsi et al. [18] reported that axonemal defects (partially formed, disorganized microtubules) in cases harbouring a high number of non-synonymous pathogenic mt DNA mutations [29]. These variations adversely affect ATP production but result in increased production of free radicals. Thus low ATP levels leads to disruption of OXPHOS results in impaired differentiation of germ cells with resultant motility defects and increased mt DNA damage.

A correlation was found between ROS and mitochondria in apoptosis, as high levels of ROS disrupt the inner and outer mitochondrial membrane and result in release of cytochrome C from the mitochondria [30]. Cytochrome C protein activates the caspases and induces apoptosis, which has been reported to be significantly higher in oxidative stress induced infertile men [26]. The number of mt DNA in sperm (1.2 mt DNA per mt) is far fewer than in somatic cell, (1000 to 1 lakh). Mutations and sequence variations in mt DNA result in early phenotypic variations [29] leading to morphological defects in spermatozoa. It has been reported that during sperm remodelling not only sperm nuclear genome undergo extensive reorganization but also mitochondrial copy number is reduced to minimize chances of paternal transmission of mt DNA.

Jc-1, is a micro-tracker dye that can report the functional state of the mitochondria and depending on the redox potential it can be driven inside the mitochondrial membrane [30].

Nuclear DNA damage and ROS: nuclear DNA experiences changes mainly due to three mechanisms- environmental pollutants, persistence of which following meiosis causes defective chromatin packaging. Several other factors can also induce DNA damage, these include altered protamine1: protamine2 ratio, higher temperature, varicocele, electromagnetic radiation, xenobiotics and supraphysiological ROS levels.

The mechanism of DNA damage is predominantly seen in oxidative stress induced by xenobiotic exposure. One of the first hypotheses to be advanced concerning the origins of DNA damage in the male germ line, focused on the physiological strand breaks created by topoisomerase during spermiogenesis as a means of relieving the torsional stresses created as DNA is condensed and packaged into the differentiating sperm head [31,32]. Normally these strand breaks are marked by a histone phosphorylation event (gamma-H2AX; H2A histone family, member X) and fully resolved by topoisomerase before spermatozoa are released from the germinal epithelium during spermiogenesis [33]. If these repair mechanisms are impaired, which occurs especially on exposure to xenobiotics and irradiation, high levels of DNA damage will be noticed, with double strand breaks with persistent expression of gamma-H2AX and DNA repair/maintenance proteins like RAD50 (radiation sensitive) and 53BPI (Binary Protein Interaction) [34].

Sperm with DNA damage that fertilize oocyte are repaired by oocyte repair mechanisms before first round of replication, to prevent replication of damaged DNA. But if the damage is too extensive and especially accumulation of oxidative by-products like etheno nucleosides can inhibit oocyte nucleotide excision repair mechanisms and thereby result in propagation of damaged DNA. This maybe the underlying mechanisms of pre and post implantation failure following IVF/ ICSI and increased incidence of major and minor congenital malformations in children conceived through these techniques.

A two-step hypothesis has been proposed regarding the DNA damage in the germ line [35]. According to this hypothesis the first step in the DNA damage cascade has its origins in spermiogenesis when the DNA is being remodelled prior to condensation. Defects in the chromatin remodelling process result in the production of spermatozoa that are characterized by an overall reduction in the efficiency of protamination, an abnormal protamine1 to protamine2 ratio and relatively high nucleohistone content [30,36,37]. These defects in the chromatin remodelling process create a state of vulnerability, whereby the spermatozoa become susceptible to oxidative damage. In the second step of this DNA damage cascade, the chromatin is attacked by high free radical levels.

Sources of ROS are 1) generation of reactive oxygen species (ROS) by leukocytes as a consequence of male genital tract infections; 2) electromagnetic radiation, including heat or radio frequency radiation in the mobile phone range; 3) redox cycling metabolites or xenobiotics, such as catechol estrogens or quinones; 4) ROS generated as a consequence of electron leakage from the sperm mitochondria; and 5) deficiency in the antioxidant protection afforded to these vulnerable cells during their transit through the male reproductive tract [35,38].

Oxidative Stress can activate endonucleases [39] which can trigger DNA breaks It is also reported that sperm chromatin possess two different topoisomerases [40]. It is still being determined if topoisomerase- inhibitor can be used to ameliorate oxidative stress induced DNA damage [22].

Apoptosis in oxidative stress

It has been in consensus for a long time that the spermatozoa undergo regulated cell death via activation of the intrinsic apoptotic cascade like other cells. But how it differs from the usual apoptotic pathway in somatic cells? Sperm are transcriptionally and translationally silent. Secondly the chromatin has reduced nucleosome content due to extensive protamination and so cannot exhibit the characteristic DNA laddering seen in somatic cells and the last, the physical architecture of these cells prevents endonucleases activated in the cytoplasm or released from the mitochondria from physically accessing the DNA [22]. As it is well established that the mitochondria play the pivotal role in apoptosis, there can be a correlation between the oxidative stress induced by mitochondria and apoptosis.

Oxidative stress and Y chromosome

The Y chromosome is particularly vulnerable to DNA damage; partly because of its genetic structure, aberrant recombination events between areas of homologous or similar sequence repeats (for example, Alu repeats or gene families) between the X and Y chromosomes or within the Y chromosome itself by unbalanced sister chromatid exchange [41]. The instability of the Y chromosome may also be related to the high frequency of repetitive elements clustered along the deletion interval 6 on the long arm of Y chromosome and partly because it cannot correct double-stranded DNA deletions by homologous recombination. The fact that such damage to the Y chromosome frequently results in infertility might be regarded as another safety mechanism that serves to limit the extent to which mutations are propagated in the germ line. If the DNA damage does not induce infertility through an effect on the Y

chromosome but involves an oncogene, the result will be an increased risk of cancer in the offspring. Such associations are illustrated by the increased risk of childhood cancer seen in the children of men who possess high DNA fragmentation in their spermatozoa as a consequence of heavy smoking. Moreover, because the mutation is fixed in the germ line, it has the potential to impact upon the health and well-being of all the future descendants of a given individual [42]. The correlation between OS and Yq micro-deletions has to be validated further. But now it is believed that male infertility may be an early marker of testicular cancer and is associated with 4 to 5 fold increased risk of extragonadal tumours.

Possible Origins of Oxidative Stress in Our Body

Though both exogenous and endogenous factors induce oxidative stress, some clinically important causes are being mentioned below.

Infections and varicocele are important causes of oxidative stress. Infections can be systemic or localized genitourinary infections.

Male Accessory Gland Infections and Oxidative Stress

Male accessory gland infection (MAGI) has been identified among those diagnostic categories which have a negative impact on the male infertility [43]. MAGI is a hypernym which groups the following different clinical categories: prostatitis, prostate-vesiculitis and prostate-vesiculo-urethritis. Some of the characteristics they share are: common pathogenic organisms, with a chronic course, may cause obstruction of the seminal pathways, can have an unpredictable spread to one or more sexual accessory glands of the reproductive tract, as well as to one or both sides [44].

The association between MAGI and oxidative stress is evident from the fact that these groups of infections are associated with altered secretory function of the prostate, seminal vesicles and vesico-urethral glands and presence of leukocytes. This causes reduction in the antioxidant properties in the seminal plasma and also increases the oxidative damage to the spermatozoa due to the increased amount of free radicals and cytokines produced by these infections. The damage produced can range from functional and structural damage to spermatozoa to sub-clinical obstruction of the tract [44].

The presence of MAGI in the patients with Chonic Bacterial Prostatitis (CBP) plus Inflammatory Bowel Syndrome (IBS) was associated with a significantly lower sperm concentration, total number, and forward motility, and with a higher seminal leucocyte concentration compared with the patients with CBP alone and MAGI [45]. It is also shown that those with MAGI have increased seminal viscosity. Semen viscosity of patients with male accessory gland infection (28.6 ± 2.2 cps) was significantly ($P < 0.05$) higher than that in the controls (10.7 ± 0.6 cps). Significantly increasing values were observed in patients with involvement of multiple gland inflammation (prostatitis < prostatovesiculitis < prostatovesiculo-epididymitis) [46].

The presence of 2 million or more peroxidase-positive white blood cells per ml of semen, or the diagnosis of male accessory gland infection, is associated with important biochemical and biological changes in semen plasma and in the spermatozoa, reducing their fertilizing potential *in vitro* and *in vivo* [47]. Even though WBCs are beneficial in smaller amount they are liable to produce damage in larger amount due to the production of excess ROS from them. In subfertile patients with or without leukocytospermia, increase in the number of WBC was associated with lower α-glucosidase levels and β-glutamyltransferase activity [48]. These were correlated with the overproduction of ROS, interleukin-1 (IL-1), and IL-receptor antagonist, suggesting that in cases with male accessory gland infection, the deleterious effects on sperm quality may be exerted through the production of ROS and/or of particular cytokines produced locally and by WBC.

The measurement of these cytokines in semen may provide clinically useful information for the diagnosis of male accessory gland infection and in the absence of WBC where it can provide information about certain mechanisms of male reproductive function and dysfunction. IL-6 concentration in seminal plasma is the most specific marker for a sensitivity of 95% in discriminating between cases with or without MAGI, and that ROS, IL-la and IL-6 have a comparable sensitivity for a specificity of 95% in discriminating between cases with or without MAGI [49].

Combinations of lipopolysaccharides and interferon- γ are detrimental to human spermatozoa and may contribute to male infertility in patients with chronic genitourinary inflammation [50]. In the present study, we have strongly indicated that the activity of the antioxidant system is dependent on particular interleukins. The probable molecular mechanism behind oxidative stress in MAGI is transcription factor (nuclear factor- κB (NF κB)). NF κB-dependent transcription is inhibited by antioxidants and its activation is induced or potentiated by ROS [51-53]. It has been known that TNF a may increase IL-6 gene expression through the activation of NF κB, and that the antioxidants can suppress TNF a -dependent IL-6 expression, thereby inhibiting the activation of the transcriptionally active NF κB [54].

From the above discussion it is clear that the male accessory gland infections are prone to produce oxidative stress and cause a double pronged attack on male fertility status both by causing the alteration in the antioxidant levels and also by producing oxidative damage to the sperm. To combat this, a course of systemic antioxidants must be added to those with genitourinary infections along with antibiotics.

Genitourinary infections

It is recorded that 50% of men experience prostatitis and it may be chronic in 10% of cases [55]. Bacteria responsible for prostate infection may originate from the urinary tract or can be sexually transmitted [56,57]. Typical non-sexually transmitted pathogens include Streptococci (*S. viridans* and *S. pyogenes*), coagulase-negative Staphylococci (*S.epidermidis*, *S. haemolyticus*), gram-negative bacteria (*E. coli*, *Proteus mirabilis*) and atypical mycoplasma strains (*Ureaplasma urealyticum*, *Mycoplasma hominis*) and Chlamydia infections. These may cause influx of polymorphonuclear leucocytes which kills these organisms either by NADPH-halide pathway or other pathways involving free radicals.

Among the different viral groups analyzed HSV appears to have a possible role in the initiation of oxidative damage to sperm. Herpes simplex DNA is found in 4–50% of infertile men's semen [58,59], with IgM antibodies towards HSV being associated with a 10-fold increase in the rate of leukospermia (Krause et al. [60]). It is also found that the sperm motility also decreases in men positive for seminal HSV DNA [58].

The leukocytes which infiltrate entering the seminal fluids in an activated, free radical-generating state, they are potentially capable of inducing oxidative damage in the spermatozoa. Whether this is the case depends on a number of factors such as: (i) the number and sub-type of leukocytes involved, (ii) when, where and how they were activated and (iii) how efficient the male reproductive tract fluids were in protecting the spermatozoa from oxidative stress. In as much as infection is the

major cause of leukocytic infiltration into the male tract, the leukocytes can be encountered by the antioxidants in the seminal fluid. It has been reported that in acute oxidative stress, the antioxidant levels increase however severe and chronic oxidative stress are associated with low antioxidant levels [61].

Does this mean that leukocytes are always detrimental and have no positive effect in infertility? Leucocytes may also be instrumental in creating iatrogenic sperm DNA damage in assisted conception cycles, when the protective action of seminal plasma is removed and the spermatozoa are inadvertently co-cultured with contaminating leukocytes in media that may contain catalytic amounts of transition metals [62]. Under these circumstances, there is every possibility that leukocyte derived ROS will impede oocyte fertilization and development. Indeed a good prediction of in vitro fertilization success has been secured using sperm morphology and leukocyte contamination (measured with FLMP provocation Test) as the only independent variables in a multiple regression equation [63].

Systemic infections and other inflammatory disorders

It has been shown from various studies that in systemic infections like leprosy, typhoid and tuberculosis, hepatitis B and C there would be generalised oxidative stress which adversely affects the testis [64,65]. The pathophysiology between the chronic inflammatory disorders has been well studied in patients with chronic nonbacterial prostatitis. One report has linked a polymorphism of the TH-2 cytokine IL-10 with chronic non-bacteria prostatitis [66]. A lack of this TH-2 cytokine may tip the immune balance towards the TH-1 direction leading to the generation of T lymphocytes reactive against prostate antigens. These T cells will liberate cytokines such as IFN-γ, TNF-α and IL-1β that stimulate chemotaxis and activation of leukocytes, leading to increased seminal oxidative stress [67,68]. There are also evidences of oxidative stress in patients with diabetes; uraemia even after hemodialysis, hyperhomocysteinemia but the exact mechanisms behind the OS is still to be explored. In hyperhomocysteinemia the mechanism can be linked to the toxic accumulation of homocysteine which is further supported by the presence of SNPs (C677T and others) in the MTHFR gene in some infertile men [69,70] and DAZL in some infertile men [71].

OS in varicocele

Varicocele is defined as enlargement of veins within the scrotum and it is one of the highly correlated causes of oxidative stress associated with low sperm production, sperm quality and infertility [72]. Clinical or subclinical varicocele [73] has been shown to cause male infertility in about 15 per cent of infertile couples [74]. These patients have increased ROS in serum, testes, and semen samples. Increased nitric oxide also has been demonstrated in the spermatic veins of patients with varicocele [75], which may be responsible for the spermatozoal dysfunction [76]. ROS in patients with varicocele 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 decreases the ROS level in the semen [72] and increases the concentrations of antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, and vitamin C, in seminal plasma as well as improves sperm quality [77]. Dada et al. [72] also reported a very rapid and significant decline in ROS levels within 1 month post surgery and oxidative injury to DNA showed significant decline after 3 months post surgery. A significant correlation between ROS levels and varicocele grade also exists. The researchers demonstrated that ROS levels were significantly higher in men with grade 2 and 3 varicocele than in those with grade 1 [78] and the level of 8-OHdG was high in those with varicocele [79]. The conclusion from a meta-analysis was that oxidative stress parameters (such as ROS and lipid peroxidation) are significantly increased in infertile patients with varicocele as compared with normal sperm donors, and antioxidant concentrations were significantly lower in infertile varicocele patients compared with controls [80].

Lifestyle and OS

Among the lifestyle factors inducing OS, smoking stands as the first and foremost contributor. Smoking results in a 48% increase in seminal leukocyte concentrations and a 107% increase in seminal ROS levels [81]. 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 [82], as well as axonemal damage [83] and decreased sperm count [84]. Smokers have decreased levels of seminal plasma antioxidants such as Vitamin E [85] and Vitamin C [86].

Sperm from smokers have been found to contain higher levels of DNA strand breaks [87]. 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%) [88]. Thus, smoking does, in fact, affect the quality and quantity of sperm present within a male.

The next major lifestyle factor influencing OS is dietary influence. With the advancing lifestyle factors, the intake of junk foods and chemicals in the diet, obviously there is increase in the systemic oxidative insult. Adding to this, there is also decrease in the intake of antioxidants adding to OS. The Age and Genetic Effects in Sperm (AGES) study examined the self-reported dietary intake of various antioxidants and nutrients (vitamins C and E, b-carotene, folate and zinc) in a group of 97 healthy non-smokers and correlated this with sperm quality [89]. This study did observe a significant correlation between vitamin C intake and sperm concentration and between vitamin E intake and total progressively motile sperm. Fertile men with low levels of oxidative attack may not be as dependant on seminal antioxidants for protection of their sperm DNA integrity. Therefore, a dietary deficiency in antioxidants may not lead to sperm oxidative DNA damage in this fertile cohort [90].

It is also to be noted that alcohol induces oxidative stress. A study of 46 alcoholic men of reproductive age has suggested the presence of oxidative stress within the testicle by reporting a significant reduction in plasma testosterone, increase in serum lipid peroxidation by-products and significantly lower levels of antioxidants [91]. However, no study to date has directly examined the link between alcohol intake and sperm oxidative damage.

Obesity produces oxidative stress as adipose tissue releases pro-inflammatory cytokines that increase leukocyte production of ROS [92]. Furthermore, accumulation of adipose tissue within the groin region results in heating of the testicle which has been linked with oxidative stress and reduced sperm quality [38]. On the other hand strenuous exercise also induces oxidative stress high impact exercise is linked with oxidative stress since muscle aerobic metabolism creates a large amount of ROS [93]. Thus exercise in moderation and yoga aid in reducing free radical production. Also it is well established from studies conducted worldwide that oxidative stress increases with aging. Animal studies using the Brown Norway rat, an established model of male reproductive aging, confirm that sperm from older animals produce more free radicals than from young animals and have a reduced enzymatic antioxidant activity, resulting in an increase in ROS-mediated sperm DNA damage [93,94].

Xenobiotics and OS

Oxidative stress and DNA damage could also be induced in the male germ line by xenobiotics that either redox cycle or activate free radical production by the spermatozoa. Human beings now live in a sea of estrogens and polychlorobiphenyls. Such compounds undergo enterohepatic recirculation and thereby lead to accumulation in the body. Recent analyses of the impact of quinones and catechol estrogens on free radical production by human spermatozoa indicated that these cells have the one electron reduction/oxidation machinery needed to activate such compounds and initiate ROS generation [95-97]. It is also well known that we are at present living in an environment of plastics and they contain phthalate esters which are difficult to be degrade. Oral administration of phthalate esters to rats is reported to increase the generation of ROS within the testis and a concomitant decrease in antioxidant levels, culminating in impaired spermatogenesis [98]. Several other pollutants like pesticides [99], preservatives and diesel [100] have been related to oxidative stress. Paternal exposure to heavy metals such as lead, arsenic and mercury is associated with decreased fertility and pregnancy delay according to recent studies [101]. Oxidative stress is hypothesized to play an important role in the development and progression of adverse health effects due to such environmental exposure due to heavy metals [102]. Many drugs like cyclophosphamide [103] and acetaminophen [3] are also found to increase the seminal ROS levels. Also electromagnetic radiations from cell phones especially when kept in trousers create oxidative stress in testis [104] which has yet to be confirmed in large population based studies which can determine the exact duration and use of cell phones which can adversely affect sperm function.

Apart from these causes it is seen from various studies that oxidative stress can be seen in idiopathic cases also. Based on the discussion above, we know that ROS can be generated even from normal spermatozoa and more so from dysmature and teratozoospermic cells. As approximately one-third of infertile men exhibit teratozoospermia [105], it is not surprising that sperm oxidative stress is commonly identified in the idiopathic infertile male population. Thus there is a need to evaluate free radical levels in men with both normal and abnormal sperm parameters.

OS and Erectile Dysfunction

Erectile dysfunction may not be classified under causes of infertility but considering male reproductive health under a holistic approach and erectile dysfunction is a predictor for many other lethal diseases, a brief note is added in this review. Also it is understood from various reviews that NO, which is a free radical is the main mediator involved in erection. It is noteworthy to find out how other free radicals interact with NO to impair erectile dysfunction.

NO interacts with superoxide to form peroxynitrite, which has been reported to play a central role in atherogenesis [106]. Peroxynitrite reacts with the tyrosyl residue of proteins, which inactivates superoxide dismutase and leads to decreased removal of superoxide [107]. This further increases the formation of peroxynitrite and reduces the available NO concentration. Peroxynitrite causes smooth-muscle relaxation and is less potent than NO. Khan et al. [108] studied the effect of NO and peroxynitrite on stripped cavernosal tissue from rabbits. They reported that relaxation induced by NO is short lived and immediate in onset, compared with that due to peroxynitrite, which is prolonged and slow in onset. Moreover, the tissues returned to original tension immediately with NO, whereas with peroxynitrite, the tissues were unable to recover their original tension. These mechanisms ultimately produce an ineffective relaxation in cavernosal tissue, which produces ED. Peroxynitrite and superoxide have been reported to increase the incidence of apoptosis in the endothelium. This leads to denudation of endothelium and further reduction of available NO [106,108]. Recently, low concentrations of oxidative stress were reported to have a more prominent proliferative effect on cavernosal smooth muscle than high concentrations, which inhibit cell growth [109]. Increased production of ROS (superoxide and peroxynitrite) reduces the effective NO concentration available for cavernosal muscle relaxation. The reduced availability of NO in acute disease and long-term endothelial damage are the 2 most important causes of ED. This also holds true in age related erectile dysfunction.

Laboratory Methods to Detect Oxidative Stress

As oxidative stress is one of the chief underlying cause of many diseases and disorders it has provoked the emergence of many tests for its diagnosis. They are either based on detecting the signs of oxidative damage, chromatin remodelling, lipid peroxidation or the measurement of ROS itself. Furnishing the detailed protocol of all the tests is beyond the scope of this basic review and so the mechanism involved in each test has been discussed here. For descriptive purpose these tests can be classified under following groups:

On chronological point of view, based on history and some red-flag signs in routine semen analysis we can strongly suspect OS in some cases when there is:

1. Reduced motility especially asthenozoospermia (<32% of progressively motility) (WHO guidelines, 2010) is one of the most important indicators of OS [110,111];
2. Hyper-viscosity of semen is also linked to elevated seminal plasma level of MDA [112] and reduced seminal plasma antioxidant status [113];
3. Infection with Ureaplasma urealyticum in the past can also increase the seminal viscosity [114];
4. Presence of more round cells in the semen which may be either immature spermatozoa or leucocytes both of which are prone to generate ROS at increased levels [115];
5. More than 50% of dead sperm i.e. teratozoospermia (WHO guidelines, 2010);
6. Poor sperm membrane integrity as shown by hypo osmotic swelling test [116];
7. Presence of morphologically abnormal sperm with impaired motility (Thilagavathi et al. [9])

Direct methods

These assays measure damage created by excess free radicals against the sperm lipid membrane or DNA [90]. As oxidative stress is the result of an in balance between ROS production and total antioxidant capacity (TAC), direct tests reflect the net biological effect between these two opposing forces and the net damage caused either in the lipid membrane or the sperm DNA. The tests which come under this category are:

Measurement of MDA by LPO-thiobarbiturate assay: Malondialdehyde (MDA) is an end product of lipid peroxidation (LPO) which is measured through thiobarbituric acid (TBA) assay [117]. TBA reactive substances (TBARS) are mainly formed during the determination of LPO in vitro (Gotz et al. [118]).

Normally MDA levels in sperm are quite low and therefore

require the use of sensitive high-pressure liquid chromatography (HPLC) equipment [119,120] or the use of iron-based promoters and spectrofluorimetry measurement [117]. Seminal plasma levels of MDA are 5–10-fold higher than sperm, making measurement on standard spectrophotometers possible [121].

Measurement of MDA appears to be of some clinical relevance since its concentration within both seminal plasma and sperm is elevated in infertile men with excess ROS production, compared with fertile controls or normozoospermic individuals [121]. Other direct tests of sperm membrane lipid peroxidation such as measurement of the isoprostane 8-Iso-PGF/PGF2α [122] and the c11-BODIPY assay [123] (Kao et al. [124]) have shown promise but are not yet in common usage. Due to the development of other advanced tests the measurement of MDA has gained little importance. As compared to MDA, measurement of 8-Isoprostane (8-IP) is more reliable as 8-IP is more reliable as 8-IP is more stable and its levels do not fluctuate with dietary intake of lipids.

Measurement of 8-OHdG: We have already discussed the importance of 8-OHdG in oxidative stress. This can be measured in sperm or seminal plasma by HPLC [125], enzyme-linked immunosorbent assay [126] or directly within sperm using immunofluorescence (Kao et al. [124]). De Iuliis et al. correlated 8OHdG levels with the degree of sperm DNA damage. The formation of 8OHdG also was correlated with the degree of DNA damage ($P < 0.01$, $R=0.253$, $n=94$), and this association was particularly marked in the high-density Percoll fraction ($P < 0.001$, $R=0.756$). The relationship between 8OHdG formation and superoxide anion production by the spermatozoa from donors and found a significant correlation ($P<0.05$, $R=0.303$, $n=50$) across high- and low-density Percoll fractions that was particularly marked within the high-density Percoll fraction ($P < 0.05$, $R=0.443$, $n=25$) [127]. The 8OHdG assay employed in a study gave a linear response when populations of human spermatozoa were subjected to progressively increasing levels of oxidative stress generated by a combination of $H_2O_2$and Fe^{2+} [127]. Assessment of 8OHdG levels are important as this base is highly mutagenic and can result in transversions and single strand breaks, thus its estimation is of clinical significance.

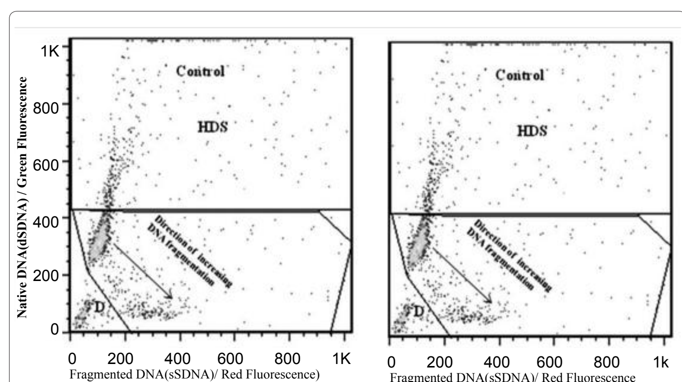

Figure 1: Pseudo Color dot plot cytograms of control semen samples by SCSA. X-axis represents fragmented DNA and Y- axis represents native DNA.

Tests for sperm DNA damage: There are a panel of tests for assessing the sperm DNA damage which occurs as a consequence of oxidative stress. They are:

Sperm chromatin structure assay (SCSA): This assay is based on the premise that DNA in sperm with abnormal chromatin structure is more prone to acid or heat denaturation [128]. Using the metachromatic properties of acridine orange (AO), SCSA measures susceptibility of sperm DNA to acid-induced denaturation in situ. By quantifying this metachromatic shift of AO from green to red after acid treatment using flow cytometry, the extent of DNA denaturation is determined [129]. The parameter obtained by SCSA most commonly referred to in the literature is DNA fragmentation index (DFI), a measure of DNA denaturation. A cut-off value has been established (DFI infertile men=42.32 ± 7.93; Control=13.38 ± 3.21) in a study conducted in our laboratory [130]. Figure 1 depicts the control whereas figure 2 depicts cases with mild to moderate DNA fragmentation. This test requires flow cytometry and is developed as a modification of acridine orange test which is less sensitive.

Toluidine blue (TB): TB is a basic dye used to evaluate sperm chromatin integrity. Toluidine Blue (TB) test [131,132]. The test measures the availability of the sperm chromatin DNA phosphate residues for staining with TB, which is dependent on both the protein state and DNA integrity. Four cell groups with different optical densities can be distinguished by the TB test [132]. These correspond to the following visual TB colours: dark violet cells (TBDCs; abnormal chromatin structure), light blue cells (TBLCs; normal chromatin structure) and two intermediate forms: light violet and dark blue (probably with less damaged chromatin structure). A threshold of TBDCs at 45% is a predictor of male in vivo infertility, providing additional prognostic information to that obtained by sperm concentration and motility assessment. This finding is quite understandable because a high proportion of sperm with impaired chromatin structure hinders fertilization in vivo. The disadvantage of the TB test is that only a limited number of sperm can be assessed when compared with the 5–10,000 sperm assessed in the SCSA. Similarly aniline is an acidic dye which binds to residual histones,

TUNEL assay: The terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labelling

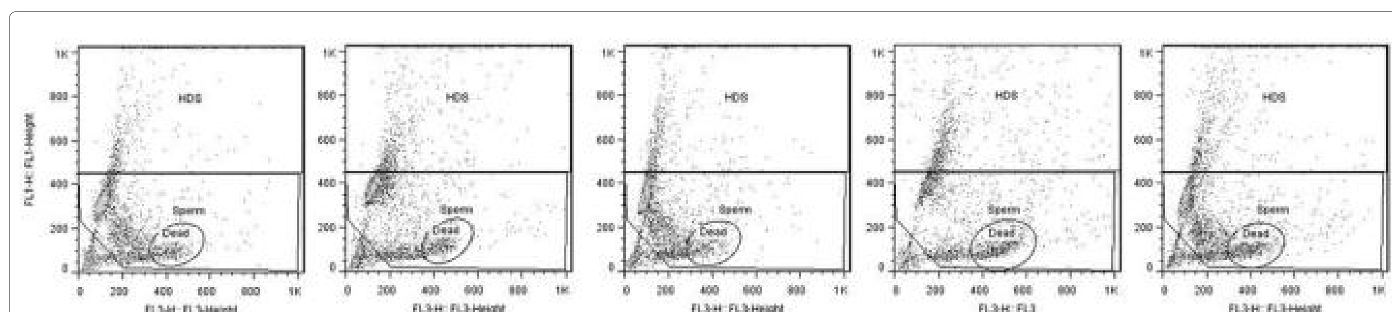

Figure 2: Representative cytograms of 25 infertile semen samples with mild, moderate to higher DNA fragmentation by SCSA. X-axis represents fragmented DNA and Y-axis represents native DNA.

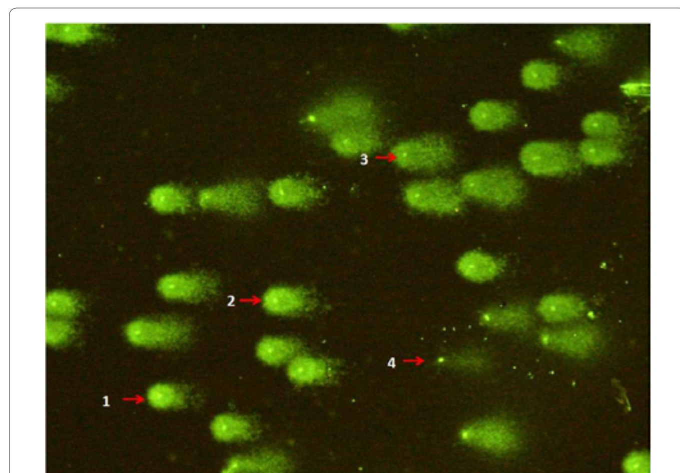

Figure 3: Sperm Comet image (200X) showing DNA fragmentation.
1. Sperm with least DNA damage, only a circular halo is visible
2 and 3. Sperm with moderate DNA damage, smaller DNA fragments have migrated to tail, while non fragmented DNA is present in comet head
4. Sperm with damaged DNA, most of the DNA has migrated to tail

assay (TUNEL) is a direct quantification of sperm DNA breaks [133]. dUTP is incorporated at single-stranded and double stranded DNA breaks in a reaction catalyzed by the enzyme TdT. The DNA breaks based on the incorporated dUTP are then labelled and can be measured using bright field or fluorescent microscopy as well as flow cytometry [133]. Sperm are then classified as TUNEL positive or negative and expressed as a percentage of the total sperm in the population. The in situ Nick Translation assay works in similar mechanism but it only identifies single-stranded DNA breaks in a reaction catalyzed by the template dependent enzyme, DNA polymerase I [134].

Comet assay: The single-cell gel electrophoresis (Comet) assay is another test for direct assessment of sperm DNA breaks [135]. Decondensed sperm are suspended in an agarose gel, subjected to an electrophoretic gradient, stained with fluorescent DNA-binding dye, and then imaged. Low-molecular weight DNA, short fragments of both single-stranded and double-stranded DNA, will migrate during electrophoresis giving the characteristic comet tail [136]. High-molecular weight intact segments of DNA will not migrate and remain in the head of the "comet." Imaging software is then use to measure comet tail length and tail fluorescent intensity, which are increased in sperm with high levels of DNA strand breaks [137]. A cut-off value has been established [DFI Infertile men=49.7 $\pm$ 12.8 control=14.37 $\pm$ 4.39] in our laboratory [61,138] (Figure 3).

Comet assay has the unique ability to measure DNA damage within an individual cell as opposed to an aggregate measure of damage versus undamaged cells in other tests as SCSA or TUNEL. The other advantage of comet assay is that it requires fewer sperm (100 cells) for analysis so it is particularly useful for men with low sperm count and for DNA damage analysis on testicular sperm. However for technical and biological reasons, the comet assay underestimates the true frequency of DNA breaks. This may be due to several possible causes: (i) masking, overlapping and entangling of migrating fragments (ii) incomplete chromatin decondensation may not allow all breaks to be revealed, (iii) due to loss of small pieces of DNA from agarose during various steps involved in the comet assay there may be fragments which are too small to be visualized. Thus the DNA damage observed is less than the actual DNA damage providing an approximate assessment for level of DNA damage [61]. The major limitation of this assay is that it is labor intensive, has observer subjectivity and requires experience to evaluate the comets. Expensive softwares are commercially available to analyze the comets [138].

Sperm chromatin dispersion test: The sperm chromatin dispersion (SCD) test is based on induced condensation which is directly linked with sperm DNA fragmentation [139]. Intact sperm are immersed in an agarose matrix on a slide, treated with an acid solution to denature, and then treated with a lysis buffer to remove sperm membranes and proteins giving rise to nucleotides with a central core and a peripheral halo of dispersed DNA loops. Sperm can be stained with Wright's stain for visualization under bright field microscopy or an appropriate fluorescent dye for visualization under fluorescent microscopy [139].

Sperm Chromatin Dispersion (SCD) like comet assay requires the sperm to be embedded in the agarose but without electrophoresis, thus it is comparatively fast and easy. Neither does it requires colour or flouresence determination which makes its interpretation simple and without the use of any complex instrument. During the SCD, processing of agarose embedded sperm remove the protamine molecules. This removal leads to breakage of disulfide bonds in the otherwise tightly looped and compact sperm genome. As the disulfide bonds break, the loops of DNA relax, forming haloes around the residual nuclear central structure. Spermatozoa with fragmented DNA showed evidence of restricted DNA loop dispersions, showing very limited haloes or absence of them, unlike the sperm with non fragmented DNA [140]. A cut-off value has been established (DFI infertile men=47.32 ± 12.7 control=12.71 ± 3.78) in our laboratory (Figure 4).

Indirect methods

The indirect tests depend on the measurement of ROS levels by chemiluminescence assay or Total Antioxidant Capacity score.

Estimation of ROS: The chemiluminescence assay quantifies both intracellular and extracellular ROS and thus measures global ROS levels. It uses sensitive probe such as luminol (5-amino-2,3, dihydro 1,4, phthalazinedione) and lucigenin for quantification of redox activities

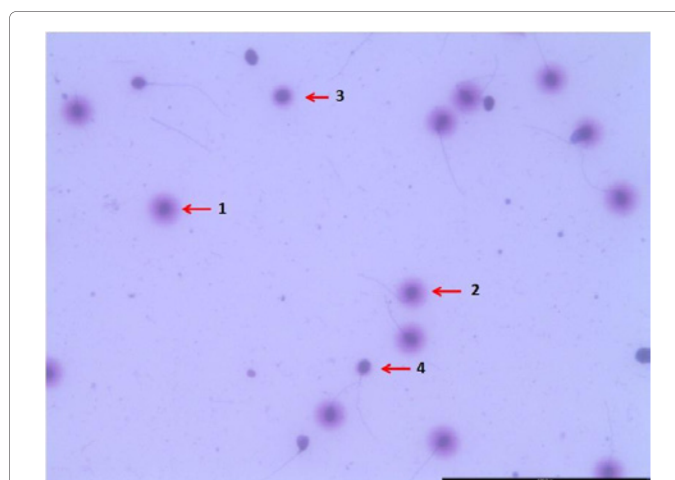

Figure 4: Sperm chromatin Dispersion assay image showing DNA fragmentation.
1. Sperm with maximum halo representing intact sperm genome
2 and 3. Moderate level of sperm DNA damage represented by intermediate size of halo
4. Sperm with highest sperm DNA fragmentation, represented by no halo around the nuclear head
As discussed above, Jc-1 can be used to measure mitochondrial membrane potential and CAM-3 to measure chromatin remodeling defects

of spermatozoa [17]. 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 lucigenin can measure only the superoxide radical released extracellularly and lucigenin can undergo auto-oxidation to produce superoxide ions and false positive results. Hence, by using both the probes on the same sample, it is possible to accurately identify intracellular and extracellular ROS generation [17,141]. 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 $\times 10^6$ counted photons per minute (cpm) per 20×10^6 sperm. Normal ROS levels in washed sperm suspensions range from 0.10 to 1.0×10^6 cpm/20×10^6 sperm. In a recent study, ROS levels of 0.145×10^6 cpm per 20×10^6 sperm were defined as the optimum cut-off value in unprocessed ejaculated samples [142,143]. But these values are variable depending on the standardization of the equipments, depending on the probe used and other factors.

As the luminometer used is very expensive and difficult to maintain, the primitive model of this assay can be used which involves microscopic quantification of Nitro Blue Tetrazolium (NBT) activity. NBT is a yellow water soluble compound that reacts with superoxide anions within cells to produce a blue pigment diformazan. The amount of diformazan crystals seen within a leukocyte or sperm reflects its superoxide anion production. The NBT assay has been shown to correlate well with traditional chemiluminescence techniques [144] but has two distinct advantages. First, the NBT assay is inexpensive to set up as it only requires a light microscope. Secondly, the NBT assay can discriminate between production of ROS by sperm and leukocytes without the need for addition of activating peptides (FMLP) used in chemiluminescence assays [145].

TAC measurement: Measurement of TAC within semen can be conducted in a variety of ways. The ability of seminal plasma to inhibit chemiluminescence elicited by a constant source of ROS (horse-radish peroxidase) is a commonly used technique. The TAC is usually quantified against a Vitamin E analogue (Trolox) and expressed as a ROS-TAC score [146]. However, colorimetry techniques based on the colour change of ABTS (2,20-azinobis3-ethylbenzo-thiazoline-6-sulphate) are now becoming more popular as they are cheaper and easier to perform [147,148]. The reduced ABTS molecule is oxidized to ABTS+ using hydrogen peroxide and a peroxidase to form a relatively stable blue-green colour measured at 600 nm with a standard spectrophotometer. Antioxidants present within seminal plasma suppress this colour change to a degree that is proportional to their concentrations. Again the antioxidant activity is quantified using Trolox. The average ROS-TAC score for fertile healthy men was 50 ± 10, which was significantly higher ($p \leq 0.0002$) compared to infertile patient (35.8 ± 15). The probability of successful pregnancy is estimated at <10% for values of ROS-TAC <30, but increased as the ROS-TAC score increased [149]. These findings suggest that ROS measurement should be used as a diagnostic tool in infertile men especially in cases of idiopathic infertility and that the reference values of ROS in neat semen can be used to define the pathologic levels of ROS in infertile men and may guide for therapeutic interventions.

Management of Oxidative Stress

The answer to this depends upon the trigger inducing the oxidative stress. By the above mentioned tests we can label whether a patient has oxidative stress. Then from the history and examination we can find out the reason behind it. The treatment must be targeted against the cause.

Lifestyle modifications

As various lifestyle factors like smoking, excessive use of cell phone, exposure to insecticides and pesticides are some of the main causes of oxidative stress, lifestyle modifications like quitting smoking and alcohol, consuming diet rich in antioxidants, fruits, vegetables, maintaining optimal weight and doing exercise in moderation can substantially reduce excess free radical production. Those persons subjected to occupational exposure and to xenobiotics / pollutants must be provided with adequate ventilation, protective equipment, clothes and duty on rotation.

Treating infections

We have already discussed above the effect of infections especially genitourinary tract in oxidative stress. So the infections especially Chlamydia and Ureaplasma must be adequately treated with prolonged course of antibiotics. One relatively large and well conducted study randomized men with Chlamydia or Ureaplasma infection to either 3 months of antibiotics or no treatment [150]. Compared with the controls, the antibiotic treated group exhibited a significant fall in seminal leukocytes and ROS production at 3 months, an improvement in sperm motility and a significant improvement in natural conception (28.2 vs 5.4%,P=0.009).

In addition to antibiotic treatment, Non-Steroidal Anti-Inflammatory (NSAID) drugs may also reduce seminal leukocytes production of free radicals. In one study men with antibiotic treated Chlamydia or Ureaplasma infection were randomized to either a NSAID or carnitine antioxidant and monitored for improvements in sperm quality over the next 4 months [151]. In addition, a one month course of a COX-2 anti-inflammatory along with 2 months of carnitine has been shown to significantly reduce sperm leukocyte count, while improving sperm motility, morphology and viability [152].

Treating the surgical causes

Several investigators have reported that surgical treatment of varicocele can reduce seminal ROS levels and improve sperm DNA integrity [72,153]. At present, selective ligation of grade II/III varicocele is the treatment of choice in men with poor reproductive outcome despite antioxidant therapy.

If there is obstruction in the pathway of sperm, ROS levels can be increased. Most ROS-mediated damage occurs during storage in the epididymis [154]. Two studies have compared sperm DNA quality in the same individual using either ejaculate [154] or surgically aspirated epididymal sperm [155] with sperm surgically extracted from the testicle. Both of these studies report significant improvements in sperm DNA quality in the testicular aspirated samples. This can be used as a rescue measure if obstruction of the testicular pathway is the cause of oxidative stress and if all antioxidant measures fail.

Antioxidants *vs.* OS

This is one of the main weapons to counteract the oxidative stress in the body. Spermatozoa are protected by various enzymatic and non enzymatic antioxidants in the seminal plasma or in spermatozoa itself to prevent oxidative damage [156]. An antioxidant that reduces oxidative stress and improves sperm motility could be useful in the management of male infertility [157]. Antioxidants are the agents, which break the oxidative chain reaction, thereby, reduce the oxidative stress [158].

Endogenous antioxidants

These are glutathione peroxidise, superoxide dismutase and catalase. Antioxidant protection is particularly critical for spermatozoa because these cells are relatively deficient in ROS-scavenging enzymes as a consequence of the limited volume, and restricted distribution, of cytosolic space [22]. As a result, these cells are particularly dependent on the antioxidant protection offered by the male reproductive tract. This is of major importance in the epididymis where spermatozoa are stored and complete the first stage of their post-testicular maturation. In order to protect the spermatozoa during their sojourn in the epididymis this organ secretes a complex array of antioxidant factors into the lumen of the epididymal tubules including small molecular mass free radical scavengers (vitamin C, uric acid, taurine, thioredoxin) and highly specialized extracellular antioxidant enzymes, including unique isoforms of superoxide dismutase and glutathione peroxidase, particularly glutathione peroxidase 5 (GPx5) [159].

GPx5 is an unusual glutathione peroxidase in that it is solely expressed in the caput epididymis under androgenic control. It is also unusual in that it lacks a selenocysteine residue while still retaining its antioxidant properties [159,160]. This protein associates with the sperm surface during epididymal transit and protects the spermatozoa from peroxide mediated attack as they are undergoing maturation [159,161]. The functional significance of this molecule has recently been demonstrated with publication of the phenotype of the GPx5 knockout mouse [162]. This mouse exhibits an age dependent increase in oxidative damage to sperm DNA which is, in turn, associated with high rates of miscarriage in mated females as well as birth defects in the offspring. Male factor infertility has been linked with a reduction in seminal plasma [163] and spermatozoa [164] GPX activity, further supporting an important role for this enzyme in male fertility. In addition, men exhibiting leukospermia-associated oxidative stress have been reported to have significantly reduced GPX activity within their spermatozoa [165]. Finally, the continued activity of GPX depends on the regeneration of reduced glutathione by glutathione reductase (GTR). Selective inhibition of GTR reduces the availability of reduced glutathione for maintaining GPX activity, thereby exposing sperm to oxidative stress [166]. The coordinated activity of GPX, GTR and glutathione clearly play a pivotal role in protecting sperm from oxidative attack.

Superoxide dismutase (SOD) and catalase are enzymatic antioxidants which inactivate the superoxide anion (O2•--) and peroxide (H_2O_2) radicals by converting them into water and oxygen. SOD is present within both sperm and seminal plasma [167,168]. The addition of SOD to sperm in culture has been confirmed to protect them from oxidative attack [169].

Antioxidants in the seminal plasma are the basis for the TAC score carried out in the seminal fluid as it evaluates the oxidative stress in the Andrology laboratory. Unlike the epididymis, sperm spend very little time in seminal plasma. Nevertheless, the animal data tell us that the secondary sexual glands are essential for reproductive success. If these glands are surgically removed then the animals exhibit high levels of oxidative sperm DNA damage and the development of the embryos is impaired, leading to physical and behavioural defects in the offspring [170,171]. In non-smoking males there is also some data to suggest that DNA damage in spermatozoa is associated with a reduction in the antioxidant capacity of human semen as reflected in the levels of, for example, vitamin C [172], carnitine [173] and co-enzyme Q10 [174]. Similarly, the total antioxidant capacity of human semen has been measured and been shown to be negatively associated with oxidative stress and fertility status [175,176]. Sperm are therefore vulnerable to oxidative damage during epididymal transit, especially when there is epididymal inflammation such as male genital tract infection. In addition, testicular biopsies from men with varicocele-associated oxidative stress have shown an increase in oxidative DNA damage within spermatogonia and spermatocytes [177]. Therefore, while seminal plasma antioxidants may help minimize ejaculated sperm oxidative stress, they have no capacity to prevent oxidative damage initiated 'upstream' at the level of the testis and epididymis [90]. ROS production in the ejaculate consumes antioxidant equivalents from seminal plasma lowering the level of protection that can be afforded to the viable cells in the ejaculate [22].

Exogenous antioxidants

Vitamin E is a major chain-breaking antioxidant in the sperm membranes and appears to have a dose-dependent effect. It scavenges all three types of free radicals, namely, superoxide, H_2O_2, and hydroxyl radicals. 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 [178]. Also, pregnancy rates consequently increased significantly (21% in treatment group as compared with placebo group).

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 [179]. It neutralizes hydroxyl, superoxide, and hydrogen peroxide radicals and prevents sperm agglutination. It prevents lipid peroxidation, recycles vitamin E and protects against DNA damage induced by the H_2O_2 radical. Administration of 200 mg of vitamin C orally along with vitamin E and glutathione for two months significantly reduced 8-OH-dG levels [180].

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 [181]. It is present in the sperm midpiece and recycles vitamin E and prevents its pro-oxidant activity [182]. 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 [181]. 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 [181]. Also many other antioxidants like N-acetyl cysteine, carnitine, trehalose, hyaluranon, bovine serum albumin, inositol and carotenoids have been used in animal models.

The major antioxidant in green tea (epigallocatechin gallate) can covalently cross-link sperm DNA to the point where fertilization would be impossible [183]. But high doses of the some antioxidants have also been shown to inhibit IVF in a porcine model [184]. This is because ROS play an important role in regulating the signal transduction cascades that drive sperm capacitation, it should be ensured that any antioxidants employed in vitro do not compromise the fertilizing potential of these cells [185] (Aitken and Baker) [111].

Oxidative Stress and Assisted Reproduction

ROS are produced during ART mainly by oocytes, embryos, cumulus cells and immature spermatozoa [3]. Sperm preparation techniques can be used to decrease ROS production to enhance and maintain sperm quality after ejaculation. The most common sperm preparation techniques used to preserve and optimize sperm quality after ejaculation is density gradient centrifugation, migration-

sedimentation, glass wool filtration, and conventional swim-up [186]. The first three preparation techniques are more effective in reducing levels of free radicals than the conventional swim-up technique [186]. However, repeated centrifugation causes mechanical injury to spermatozoa and increases ROS production [3]. Currently use of antioxidants and other substances to prevent ROS generation during sperm preparation processes are under use but these levels must be adjusted as not to impair the 'induced' fertilization during IVF or impair normal physiological functions. There is a significant correlation between ROS levels in spermatozoa and the fertilization rate after IVF (estimated overall correlation 0.374, 95% CI 0.520 to 0.205) [3].

What role ROS has in fertilization? Oocyte provides a glutathione-mediated reducing intracellular environment within which sperm chromatin decondensation occurs. During ART, an oocyte in the Petri dish becomes very susceptible to oxidative damage due to depletion of the intracellular glutathione pool. It also becomes incapable of decondensing the sperm nucleus, resulting in ART failure. Because oxygen is toxic to the embryo, an increase in oxidative stress will have a significant impact on the developmental potential of the mammalian embryo [187]. In an interesting study, the arrest in embryonic development in mice at the 2-cell stage probably because of the activation of an apoptotic pathway was shown to be associated with the sudden production of hydrogen peroxide by the embryo [188]. It is also shown that the culture media in which viable human embryos were maintained retained the antioxidant activity, but the media recovered from incubations involving fragmenting defective human embryos showed a significant loss of antioxidant activity with time (Paszkowski and Clark [189]). There are various protocols used in ART for stimulation of oocyte-sperm interaction using Platelet activating factor, pentoxifylline and carnitine (Zhang et al. [190]). The currently popular response of resorting to mechanical techniques such as IVF-ICSI in all cases of male factor infertility is unlikely to be 'best practice' since ROS damaged paternal DNA will result in poor quality blastocysts, less than optimal pregnancy rates and an increase in miscarriage (Zorn et al. [191]). Thus it can be concluded that attenuating ROS levels along with appropriate antioxidants and sperm stimulants can be used to increase the success rate of ART.

Conclusion

Limited amount of free radicals have an important role in modulating many physiological functions in reproduction. ROS are being constantly produced in small controlled amounts by spermatozoa and leukocytes in the semen. But if the amount exceeds the antioxidant defence against it, then OS develops.

This basic review about OS and its role in male infertility just provides a bird's eye view regarding the effects of OS, patho-physiological mechanism behind it, the different laboratory tests used to identify it, aetiologies and the treatment for those triggers. Lot of advances have been made in this field in the past 20 years. But the cost involved in it, lack of standardization in the procedures used and lack of awareness among people make them restricted only to the laboratories involved in research and in some ART centres. Also, it is important to establish reference values for ROS above which antioxidants could be used for male infertility treatment. The dose and duration of these antioxidants should also be determined and standardized.

Now if OS is persistent, DNA damage may occur which worsens the condition. Although ART is able to compensate for the impairment of sperm chromatin integrity, transmission of abnormal genetic material through ART may result in birth of offspring with congenital malformations and childhood cancer.

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