Clinical Relevance of Oxidative Stress in Male Factor Infertility: An Update

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Male factor has been considered a major contributory factor to infertility. Along with the conventional causes for male infertility such as varicocele, cryptorchidism, infections, obstructive lesions, cystic fibrosis, trauma, and tumors, a new, yet important cause has been identified: oxidative stress. Oxidative stress (OS) is a result of the imbalance between reactive oxygen species (ROS) and antioxidants in the body, which can lead to sperm damage, deformity and eventually male infertility. This involves peroxidative damage to sperm membrane and DNA fragmentation at both nuclear and mitochondrial levels. OS has been implicated as the major etiological factor leading to sperm DNA damage. OS-induced DNA damage can lead to abnormalities in the offspring including childhood cancer and achondroplasia. In this article, we discuss the need of ROS in normal sperm physiology, the mechanism of production of ROS and its pathophysiology in relation to male reproductive system. The benefits of incorporating antioxidants in clinical and experimental settings have been enumerated. We also highlight the emerging concept of utilizing OS as a method of contraception and the potential problems associated with it.

Introduction

Infertility is a major clinical problem, affecting people medically and psychosocially. Fifteen percent of all couples in the US are infertile and the male factor is responsible for 25% of these cases.[1] Of the many causes of male infertility, oxidative stress (OS) has been attributed to affect the fertility status and thus, it has been studied extensively in recent years. Spermatozoa, like any other aerobic cell is 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, its breakdown products such as ROS can prove to be detrimental to cell function and survival.[3] OS has also been implicated in the pathogenesis of many other human diseases such as atherosclerosis, cancer, diabetes, liver damage, rheumatoid arthritis, cataracts, AIDS, inflammatory bowel disease, Parkinson disease, motor neuron disease, and conditions associated with premature birth.[4]

Physiological role of ROS in male reproductive system

Until recently, ROS was considered toxic exclusively to human spermatozoa. Substantial evidence exists to suggest 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] Co-incubation of spermatozoa with low concentrations of hydrogen peroxide has been shown to stimulate sperm capacitation, hyperactivation, acrosome reaction, and oocyte fusion.[10–13] ROS such as nitric oxide (NO) and the superoxide anion have also shown to

promote capacitation and the acrosome reaction.[14] Furthermore, ROS have also been implicated in sperm oocyte interaction.[15]

Sources of ROS

Reactive oxygen species represent a broad category of molecules including radical (hydroxyl ion, super-oxide, NO, peroxyl etc.) and non-radical (ozone, sin-glet oxygen, lipid peroxide, hydrogen peroxide) and oxygen derivatives.[4] Reactive nitrogen species (nitrous oxide, peroxynitrite, nitroxyl ion etc) are free nitrogen radicals and considered a subclass of ROS.[16,17] NO has been shown to have detrimental effects on normal sperm function inhibiting both motility and sperm competence for zona binding.[18]

Virtually every human ejaculate is considered to be contaminated with potential sources of ROS.[12] 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 (excess residual cytoplasm), explains the missing link between poor sperm qual-ity and increased ROS generation. These structures, which are a result of defective spermiogenesis are a major source of ROS.[20] Studies indicate that reten-tion of residual cytoplasm by spermatozoa is posi-tively 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 through two ways: (i) nicotin-amide adenine dinucleotide phosphate (NADPH) oxi-dase system at the level of the sperm plasma membrane[21] and (ii) NADPH-dependent oxidoreduc-tase (diphorase) at the mitochondrial level.[22]

World Health Organization (WHO) defines leuko-cytospermia (increased leukocyte infiltration in semen) as the presence of peroxidase-positive leuko-cytes in concentrations of $>1 \times 10^6$ per milliliter of semen. Controversy exists over the clinical signifi-cance of leukocytospermia.[23] Sperm parameters such as poor quality, decreased hyperactivation, and defective sperm function[24] have been attributed to leukocytospermia. On the other hand, no correlation was reported between seminal leukocyte concentra-tions and impaired sperm quality[25] or defective sperm function.[26]

Our laboratory has shown that ROS levels were lower in fertile men than in subfertile patients in non-leukocytospermic samples in unprocessed (neat) and washed semen. Similarly, we demonstrated that in samples with leukocytes, ROS levels were lower in fertile men in neat and washed semen.[27] Further-more, we have shown that OS correlates with the ris-ing leukocyte count.[28] Thus, it can be concluded that OS occurs even in patients with a very low seminal leukocyte count (between 0 and $1 \times 10^6/mL$) and increases with an increase in leukocyte count.

Peroxidase-positive leukocytes include polymor-phonuclear leukocytes (PMNL), which represent 50–60% of all seminal leukocytes, and macrophages, which represent another 20–30%.[23] These PMNL are mainly contributed to the human ejaculate by the prostate gland and the seminal vesicles.[24] Infection and inflammation may activate the leukocytes,[29] and these activated leukocytes can produce up to 100-fold higher amounts of ROS compared with non-activated leukocytes.[30,31]

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 on the nature and amount of ROS involved and also on the duration of ROS exposure and extra-cellular factors such as temperature, oxygen tension and the com-position of the surrounding environment (e.g. ions, proteins, and ROS scavengers).

Lipid peroxidation

Lipids are considered to be the most susceptible mac-romolecules and are present in sperm plasma mem-brane in the form of polyunsaturated fatty acids (PUFA); fatty acids that contain more than two car-bon–carbon double bonds. ROS attacks PUFA in the cell membrane leading to a cascade of chemical reac-tions called lipid peroxidation. One of the by-prod-ucts of lipid peroxidation is malondialdehyde (MDA), which has been used in various biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa.[26,32] Results of such assays exhibit an excellent correlation when examining the relationship between impaired sperm function, dis-cussed in terms of motility, and the capacity for sperm-oocyte fusion.[33] Recently peroxidative dam-age to the sperm has been measured using a probe,

BODIPY (581/591), which gets incorporated into the cells and when attacked by ROS undergo a spectral emission.[34]

Effect on motility

Increased ROS levels have been correlated with decreased sperm motility.[35–37] However, the exact mechanism through which this occurs is not understood. One hypothesis suggests that H_2O_2 diffuses across the membranes into the cells and inhibits the activity of some vital enzymes such as glucose-6-phosphate dehydrogenase (G6PD) via the hexose monophosphate shunt controls the intracellular availability of NADPH, which is then used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase.[38] 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.[39]

Loss of motility observed when spermatozoa are incubated overnight is highly correlated with lipid peroxidation status of the spermatozoa.[40] Furthermore, the ability of antioxidants (α-tocopherol) to revive sperm motility both *in vivo* and *in vitro* is evidence that lipid peroxidation is a major cause for motility loss in spermatozoa.[41]

DNA damage by OS

DNA damage in the male germ line cells is associated with poor fertilization rates following IVF, defective pre-implantation embryonic development and high rates of miscarriage and morbidity in the offspring. Many possible causes of DNA damage include abortive apoptosis, infection, defective spermatogenesis, and OS.[34] DNA damage is often induced by OS, rather than being the result of other processes such as defective apoptosis.[42–44]

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.[44] OS is also associated with high frequencies of single and double strand DNA breaks (Fig. 1).[44,45] ROS can also cause gene mutations such as point mutation and polymor-
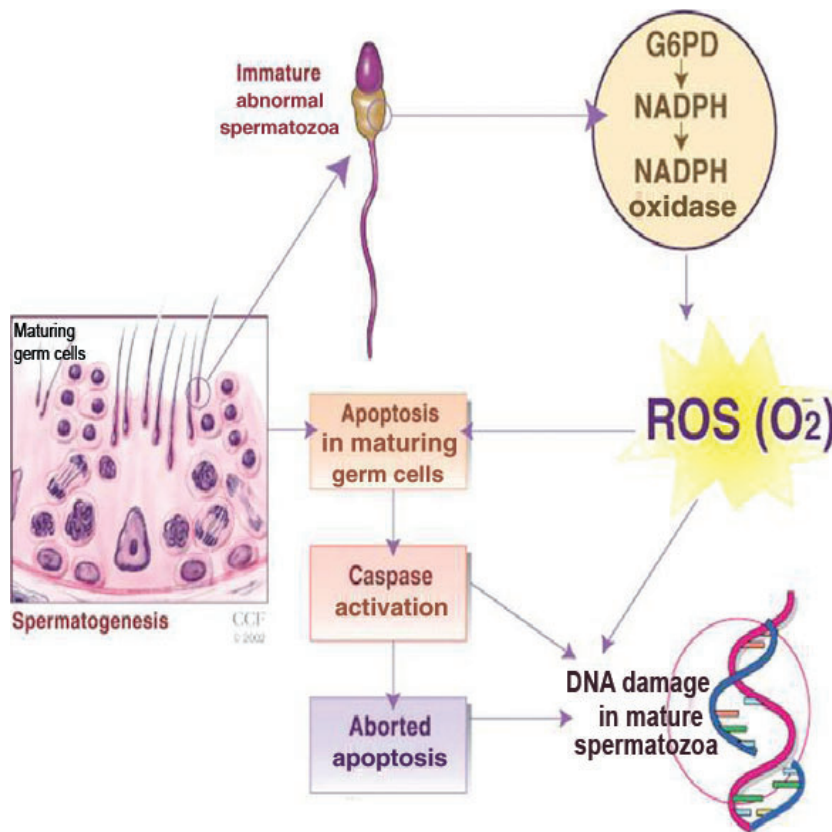

Fig. 1 Mechanism of DNA damage caused by ROS.

phism, resulting in decreased semen quality.[46,47] Other mechanisms such as denaturation and DNA base-pair oxidation may also be involved.[42] When DNA damage is small, spermatozoa can undergo self-repair.[15] The oocyte is also capable of repairing damaged DNA of spermatozoa.[15] However, if the damage is extensive, apoptosis and embryo fragmentation can occur. DNA damage in the Y chromosome can also cause gene deletion in the Y chromosome of the offspring leading to infertility.[45]

Oxidative stress and apoptosis

Apoptosis is a non-inflammatory response to tissue damage characterized by a series of morphological and biochemical changes. It helps in elimination of abnormal spermatozoa.[48] 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 may also be initiated by ROS-independent pathways involving the cell surface protein Fas.[49] Samples with low sperm concentrations are more likely to have a high proportion of Fas-positive spermatozoa.[48] In patients with male factor infertility, increased sperm damage by ROS was shown to correlate with higher levels of cytochrome c and caspase 9 and 3 indicative of apoptosis.[50] Annexin-V staining assay used as a marker of early apoptosis was significantly increased in mature spermatozoa from infertile patients compared with the mature spermatozoa from a control group of normal sperm donors.[51]

Varicocele and OS

Clinical or subclinical varicocele[52] has been shown to cause male infertility in about 15% of infertile couples.[53] These patients have increased ROS in serum, testes, and semen samples.[54] Increased NO has also been demonstrated in the spermatic veins of patients with varicocele,[55,56] which could lead to spermatozoal dysfunction.[57] Varicocelectomy has been shown to increase the concentrations of antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, and vitamin C in seminal plasma and improve the sperm quality.[58] ROS levels were significantly higher in men with grade 2 and 3 varicocele compared to grade 1 but showed no correlation with testicular volumes.[59] In a meta-analysis reported recently, infertile patients with varicocele

showed significantly increased OS and reduced antioxidant concentrations compared with controls.[60]

Smoking, oxidative stress, and infertility

Animal studies have indicated deleterious effects of cigarette smoke on sperm maturation and ability of sperm to establish a viable pregnancy.[61] Significant positive association has been reported between active smoking and sperm DNA fragmentation[62] as well as axonemal damage[63] and decreased sperm count.[64] Sperm from subjects who smoked are significantly more sensitive to acid-induced DNA denaturation than non-smokers.[65] In a study carried out on 655 smokers and 1131 non-smokers, it was shown that cigarette smoking was associated with significant decrease in sperm density, total sperm count, and total number of motile sperm.[66]

Assessment of ROS by chemiluminescence

The most common method of measuring ROS is a chemiluminescence assay. Luminol (5-amino-2, 3, dihydro 1, 4, phthalazinedione) and lucigen probes can be used for quantification of redox activities of spermatozoa.[67] Luminol can measure both intracellular and extracellular ROS whereas lucigen can only measure the superoxide radical released extracellularly. ROS levels can be determined by a luminometer. The results are expressed as $\times 10^6$ counted photons per min (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 and 0.145×10^6 cpm per 20×10^6 sperm in unprocessed ejaculated samples.[27] Direct methods such as pulse radiolysis and electron-spin resonance spectroscopy are limited by problems of relatively low volume of seminal plasma, short life span of ROS, and the need to evaluate in fresh samples.[15]

Antioxidants

Studies have shown that antioxidants protect spermatozoa from ROS producing abnormal spermatozoa, scavenge ROS produced by leukocytes, prevent DNA fragmentation, improve semen quality in smokers, reduce cryodamage to spermatozoa, block premature sperm maturation and stimulate spermatozoa and improve ART outcome. Seminal plasma contains superoxide dismutase, catalase, and glutathione peroxidase/glutathione reductase in addition

to non-enzymatic antioxidants such as ascorbate, urate, vitamin E, pyruvate, glutathione, albumin, vitamin A, ubiquitol, taurine, and hypotaurine.

Major antioxidants

Vitamin E

Vitamin E is a major chain-breaking antioxidant in the sperm membranes and appears to have a dose-dependent effect.[68] It scavenges all the three types of free radicals, namely superoxide, H_2O_2, and hydroxyl radicals.[8] Administration of 100 mg of Vitamin E three times a day for 6 months in a group of asthenozoospermic patients with normal female partners showed a significant decrease in lipid peroxidation and increased motility and pregnancy rates.[41]

Vitamin C

Vitamin C is important chain-breaking antioxidant. 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 H_2O_2 radical. Administration of 200 mg of vitamin C orally along with vitamin E and glutathione for 2 months significantly reduced hydroxyguanine(8-OH-dG) levels in spermatozoa and also led to an increase in sperm count ($P < 0.05$).[42]

Coenzyme Q-10

Coenzyme Q-10 is a non-enzymatic antioxidant that is related to low density lipoproteins and protects against peroxidative damage.[69] It is an energy-promoting agent and enhances sperm motility.[70] It is present in the sperm mid-piece and recycles vitamin E and prevents its pro-oxidant activity.[71] Oral supplementation of 60 mg/day of coenzyme Q-10 was shown to improve fertilization rate using intracytoplasmic sperm injection (ICSI) in normospermic infertile males.[70]

Role of antioxidants in motility

Antioxidants such as vitamin E and C, glutathione, *N*-acetyl cysteine, SOD, catalase, albumin, taurine and hypotaurine prevent reduction in sperm motility and *N*-acetyl cysteine and coenzyme Q-10 increase sperm motility. Incubation of sperm sample from asthenozoospermic infertile male for 24 hr in Ham's F-10 medium with 50 μM coenzyme Q-10 improved sperm motility.[70] Oral supplementation of 2–3 g/day of carnitines for >2 months improved sperm concentration and motility.[72]

Role of antioxidants in preventing cryodamage

Sperm freezing and thawing procedures cause a significant and irreversible decrease in motility and metabolic activity of sperm along with disruption of plasma membrane.[73] Vitamin E (10 mmol/L) and Rebamipide (300 mmol/L) have been shown to decrease the cryodamage during freeze-thaw procedure and improves post thaw motility.[74] *In vitro* supplementation of 300 μmol/L of Rebamipide in semen sample during incubation (37°C) and cryopreservation (−196°C, 3 days) showed significant decrease in ROS level.[75]

Role of antioxidants in preventing DNA damage

Antioxidants have been demonstrated to decrease DNA fragmentation induced by OS. Daily oral supplementation of 1 g vitamins C & E for 2 months reduced the number of TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) positive spermatozoa from 22.1% to 9.1% while the amount of spermatozoa with DNA fragmentation remained the same in the placebo group (22.4–22.9%).[76] Moreover, a marked improvement of clinical pregnancy (48.2% *versus* 6.9%) and implantation (19.6% *versus* 2.2%) rates after antioxidant treatment as compared with the pre-treatment outcomes of ICSI was also reported.[77] Addition of Vitamin E or C to the sperm preparation media during density gradient separation protected sperm from DNA damage.[78] Albumin also helps neutralize lipid peroxide-mediated damage to the sperm plasma membrane and DNA.[79]

ROS in ART

Oxidative stress-induced DNA damage may have important clinical implications in the context of assisted reproductive techniques (ART). Studies have indicated that significantly increased levels of ROS production occur in response to repeated cycles of centrifugation involved in conventional sperm preparation techniques used for ART.[36]

Spermatozoa selected for ART usually originate from the environment experiencing OS, and a high percentage of these sperm may have damaged DNA.[42] When intrauterine insemination 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.[6,80] In ART procedures, ROS can be produced by oocyte and embryo metabolism, cumulus cells, leukocyte contamination during sperm preparation and culture media. A meta-analysis study by our group in 2004 concluded that ROS has 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 the IVF outcome. We have also 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.[81]

Assisted reproduction techniques may show significant improvement in *in-vitro* supplementation of antioxidants.[82] A dose-dependent decrease in % blastocyst development rate (BDR) was shown with increasing concentrations of H_2O_2 indicating that H_2O_2 (>60 μM) is embryotoxic.[83] In the clinical ART setting, various antioxidants such as vitamin E,[84] vitamin C,[85] cysteine,[86] and taurine and hypotaurine[87] when added to the culture medium have been shown to improve the developmental ability of the embryos by reducing the effects of ROS.

Oxidative stress and contraception

Lipid peroxidation induced by H_2O_2 not only disrupts sperm motility but also impairs all the sperm functions, which are dependent on the integrity of plasma membrane, including sperm-oocyte fusion and ability to undergo acrosomal exocytosis.[88] Such findings have raised the possibility that hydrogen peroxide or reagents producing them on contact with spermatozoa might be an effective way of contraception.[89]

Conclusion

In the last decade, there has been a phenomenal growth in our knowledge of male reproduction, sperm function and development of diagnostic tools and treatment modalities. In addition, our understanding of OS has given rise to several new treatment modalities, which are now being investigated for improving male infertility. Many new antioxidants are now available that can decrease OS and improve sperm quality but a major concern in their usage is lack of scientific evidence of their effectiveness, which has led to their non-approval by FDA. Several sperm preparation techniques such as density gradient centrifugation, glass wool filtration and migration-sedimentation significantly reduce the level of ROS by removing leukocytes.[71,90] Another technique that has been recently used is magnetic activated cell separation (MACS). MACS has been shown to yield motile, viable, morphologically normal spermatozoa that displays significant cryopreservation tolerance and higher fertilization potential.[91–97] It allows separation of apoptotic spermatozoa, which may be one of the causes of ART failure even in patients with otherwise normal sperm parameters.

However, OS being only one of the many causes of male infertility, it is recommended that antioxidant therapy should be tried only in cases of increased OS or established DNA damage.

Future strategies

Evaluation of OS status and use of antioxidants is not routine in clinical practice. The immediate need is to simplify and validate the evaluation of ROS and OS status so that it can be performed routinely without the use of sophisticated equipment. 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. With the increase in the use of ART procedures there should be an effort to develop optimum combinations of antioxidants to supplement sperm preparation media. MACS could also be used to isolate spermatozoa with compromised genetic integrity but its value in sperm preparation prior to ICSI requires further investigation. The proposal to use hydrogen peroxide-producing agents as a possible means of contraception warrants further investigation.

References

1 Sharlip ID, Jarow JP, Belker AM, Lipshultz LI, Sigman M, Thomas AJ, Schlegel PN, Howards SS, Nehra A, Damewood MD, Overstreet JW, Sadovsky R: Best practice policies for male infertility. *Fertil Steril* 2002; 77:873–882.

2 Sies H: Strategies of antioxidant defense. *Eur J Biochem* 1993; 215:213–219.

3 de Lamirande E, Gagnon C: Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. *Hum Reprod* 1995; 10(Suppl. 1):15–21.

4 Agarwal A, Prabakaran SA: Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. *Indian J Exp Biol* 2005; 43:963–974.

5 Aitken RJ: Molecular mechanisms regulating human sperm function. *Mol Hum Reprod* 1997; 3:169–173.

6 Aitken RJ: The Amoroso Lecture. The human spermatozoon–a cell in crisis?. *J Reprod Fertil* 1999; 115:1–7.

7 Gagnon C, Iwasaki A, De Lamirande E, Kovalski N: Reactive oxygen species and human spermatozoa. *Ann N Y Acad Sci* 1991; 637:436–444.

8 Agarwal A, Nallella KP, Allamaneni SS, Said TM: Role of antioxidants in treatment of male infertility: an overview of the literature. *Reprod Biomed Online* 2004; 8:616–627.

9 Griveau JF, Le Lannou D: Reactive oxygen species and human spermatozoa: physiology and pathology. *Int J Androl* 1997; 20:61–69.

10 de Lamirande E, Eiley D, Gagnon C: Inverse relationship between the induction of human sperm capacitation and spontaneous acrosome reaction by various biological fluids and the superoxide scavenging capacity of these fluids. *Int J Androl* 1993; 16:258–266.

11 Griveau JF, Renard P, Le Lannou D: An in vitro promoting role for hydrogen peroxide in human sperm capacitation. *Int J Androl* 1994; 17:300–307.

12 Aitken RJ: Free radicals, lipid peroxidation and sperm function. *Reprod Fertil Dev* 1995; 7:659–668.

13 Kodama H, Kuribayashi Y, Gagnon C: Effect of sperm lipid peroxidation on fertilization. *J Androl* 1996; 17:151–157.

14 Griveau JF, Dumont E, Renard P, Callegari JP, Le Lannou D: Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *J Reprod Fertil* 1995; 103:17–26.

15 Agarwal A, Prabhakaran SA, Sikka SC: Clinical relevance of oxidative stress in patients with male factor infertility: evidence-based analysis. *AUA Update Series* 2007; 26:1–12.

16 Sikka SC: Relative impact of oxidative stress on male reproductive function. *Curr Med Chem* 2001; 8:851–862.

17 Darley-Usmar V, Wiseman H, Halliwell B: Nitric oxide and oxygen radicals: a question of balance. *FEBS Lett* 1995; 369:131–135.

18 Wu TP, Huang BM, Tsai HC, Lui MC, Liu MY: Effects of nitric oxide on human spermatozoa activity, fertilization and mouse embryonic development. *Arch Androl* 2004; 50:173–179.

19 Garrido N, Meseguer M, Simon C, Pellicer A, Remohi J: Pro-oxidative and anti-oxidative imbalance in human semen and its relation with male fertility. *Asian J Androl* 2004; 6:59–65.

20 Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ: Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl* 1996; 17:276–287.

21 Aitken RJ, Buckingham DW, West KM: Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. *J Cell Physiol* 1992; 151:466–477.

22 Gavella M, Lipovac V: NADH-dependent oxidoreductase (diaphorase) activity and isozyme pattern of sperm in infertile men. *Arch Androl* 1992; 28:135–141.

23 Thomas J, Fishel SB, Hall JA, Green S, Newton TA, Thornton SJ: Increased polymorphonuclear granulocytes in seminal plasma in relation to sperm morphology. *Hum Reprod* 1997; 12:2418–2421.

24 Wolff H: The biologic significance of white blood cells in semen. *Fertil Steril* 1995; 63:1143–1157.

25 Tomlinson MJ, Barratt CL, Cooke ID: Prospective study of leukocytes and leukocyte subpopulations in semen suggests they are not a cause of male infertility. *Fertil Steril* 1993; 60:1069–1075.

26 Aitken J, Krausz C, Buckingham D: Relationships between biochemical markers for residual sperm cytoplasm, reactive oxygen species generation, and the presence of leukocytes and precursor germ cells in human sperm suspensions. *Mol Reprod Dev* 1994; 39:268–279.

27 Athayde KS, Cocuzza M, Agarwal A, Krajcir N, Lucon AM, Srougi M, Hallak J: Development of normal reference values for seminal reactive oxygen species and their correlation with leukocytes and semen parameters in a fertile population. *J Androl* 2007; 28:613–620.

28 Sharma RK, Pasqualotto AE, Nelson DR, Thomas AJ Jr, Agarwal A: Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. *J Androl* 2001; 22:575–583.

29 Pasqualotto FF, Sharma RK, Potts JM, Nelson DR, Thomas AJ, Agarwal A: Seminal oxidative stress in patients with chronic prostatitis. *Urology* 2000; 55:881–885.

30 Plante M, de Lamirande E, Gagnon C: Reactive oxygen species released by activated neutrophils, but not by deficient spermatozoa, are sufficient to affect normal sperm motility. *Fertil Steril* 1994; 62:387–393.

31 Shekarriz M, Sharma RK, Thomas AJ Jr, Agarwal A: Positive myeloperoxidase staining (Endtz test) as an indicator of excessive reactive oxygen species formation in semen. *J Assist Reprod Genet* 1995; 12:70–74.

32 Aitken RJ, Clarkson JS, Fishel S: Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* 1989; 41:183–197.

33 Aitken RJ, Harkiss D, Buckingham DW: Analysis of lipid peroxidation mechanisms in human spermatozoa. *Mol Reprod Dev* 1993; 35:302–315.

34 Aitken RJ, Wingate JK, De Iuliis GN, McLaughlin EA: Analysis of lipid peroxidation in human spermatozoa using BODIPY C11. *Mol Hum Reprod* 2007; 13:203–211.

35 Lenzi A, Lombardo F, Gandini L, Alfano P, Dondero F: Computer assisted sperm motility analysis at the moment of induced pregnancy during gonadotropin treatment for hypogonadotropic hypogonadism. *J Endocrinol Invest* 1993; 16:683–686.

36 Agarwal A, Ikemoto I, Loughlin KR: Relationship of sperm parameters with levels of reactive oxygen species in semen specimens. *J Urol* 1994; 152:107–110.

37 Armstrong JS, Rajasekaran M, Chamulitrat W, Gatti P, Hellstrom WJ, Sikka SC: Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic Biol Med* 1999; 26:869–880.

38 Aitken RJ, Fisher HM, Fulton N, Gomez E, Knox W, Lewis B, Irvine S: Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol Reprod Dev* 1997; 47:468–482.

39 de Lamirande E, Gagnon C: Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J Androl* 1992; 13:379–386.

40 Gomez E, Irvine DS, Aitken RJ: Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. *Int J Androl* 1998; 21:81–94.

41 Suleiman SA, Ali ME, Zaki ZM, el-Malik EM, Nasr MA: Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl* 1996; 17:530–537.

42 Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T: Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril* 1997; 68:519–524.

43 Barroso G, Morshedi M, Oehninger S: Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod* 2000; 15:1338–1344.

44 Kemal Duru N, Morshedi M, Oehninger S: Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. *Fertil Steril* 2000; 74:1200–1207.

45 Aitken RJ, Krausz C: Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 2001; 122:497–506.

46 Spiropoulos J, Turnbull DM, Chinnery PF: Can mitochondrial DNA mutations cause sperm dysfunction? *Mol Hum Reprod* 2002; 8:719–721.

47 Sharma RK, Said T, Agarwal A: Sperm DNA damage and its clinical relevance in assessing reproductive outcome. *Asian J Androl* 2004; 6:139–148.

48 Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U: Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod* 1999; 4:31–37.

49 Lee J, Richburg JH, Younkin SC, Boekelheide K: The Fas system is a key regulator of germ cell apoptosis in the testis. *Endocrinology* 1997; 138:2081–2088.

50 Wang X, Sharma RK, Sikka SC, Thomas AJ Jr, Falcone T, Agarwal A: Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertil Steril* 2003; 80:531–535.

51 Agarwal A, Saleh RA, Bedaiwy MA: Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 2003; 79:829–843.

52 Gonda RL Jr, Karo JJ, Forte RA, O'Donnell KT: Diagnosis of subclinical varicocele in infertility. *AJR Am J Roentgenol* 1987; 148:71–75.

53 Schoor RA, Elhanbly SM, Niederberger C: The pathophysiology of varicocele-associated male infertility. *Curr Urol Rep* 2001; 2:432–436.

54 Koksal IT, Usta M, Orhan I, Abbasoglu S, Kadioglu A: Potential role of reactive oxygen species on testicular pathology associated with infertility. *Asian J Androl* 2003; 5:95–99.

55 Mitropoulos D, Deliconstantinos G, Zervas A, Villiotou V, Dimopoulos C, Stavrides J: Nitric oxide synthase and xanthine oxidase activities in the spermatic vein of patients with varicocele: a potential role for nitric oxide and peroxynitrite in sperm dysfunction. *J Urol* 1996; 156:1952–1958.

56 Romeo C, Ientile R, Santoro G, Impellizzeri P, Turiaco N, Impala P, Cifala S, Cutroneo G, Trimarchi F, Gentile C: Nitric oxide production is increased in the spermatic veins of adolescents with left idiophatic varicocele. *J Pediatr Surg* 2001; 36:389–393.

57 Ozbek E, Turkoz Y, Gokdeniz R, Davarci M, Ozugurlu F: Increased nitric oxide production in the spermatic vein of patients with varicocele. *Eur Urol* 2000; 37:172–175.

58 Mostafa T, Anis TH, El-Nashar A, Imam H, Othman IA: Varicocelectomy reduces reactive oxygen species levels and increases antioxidant activity of seminal plasma from infertile men with varicocele. *Int J Androl* 2001; 24:261–265.

59 Allamaneni SS, Naughton CK, Sharma RK, Thomas AJ Jr, Agarwal A: Increased seminal reactive oxygen species levels in patients with varicoceles correlate with varicocele grade but not with testis size. *Fertil Steril* 2004; 82:1684–1686.

60 Agarwal A, Prabakaran S, Allamaneni SS: Relationship between oxidative stress, varicocele and infertility: a meta-analysis. *Reprod Biomed Online* 2006; 12:630–633.

61 Aitken RJ, Koopman P, Lewis SE: Seeds of concern. *Nature* 2004; 432:48–52.

62 Sun JG, Jurisicova A, Casper RF: Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. *Biol Reprod* 1997; 56:602–607.

63 Zavos PM, Correa JR, Karagounis CS, Ahparaki A, Phoroglou C, Hicks CL, Zarmakoupis-Zavos PN: An electron microscope study of the axonemal ultrastructure in human spermatozoa from male smokers and nonsmokers. *Fertil Steril* 1998; 69:430–434.

64 Vine MF, Tse CK, Hu P, Truong KY: Cigarette smoking and semen quality. *Fertil Steril* 1996; 65:835–842.

65 Potts RJ, Newbury CJ, Smith G, Notarianni LJ, Jefferies TM: Sperm chromatin damage associated with male smoking. *Mutat Res* 1999; 423:103–111.

66 Kunzle R, Mueller MD, Hanggi W, Birkhauser MH, Drescher H, Bersinger NA: Semen quality of male smokers and nonsmokers in infertile couples. *Fertil Steril* 2003; 79:287–291.

67 Aitken RJ, Baker MA, O'Bryan M: Shedding light on chemiluminescence: the application of chemiluminescence in diagnostic andrology. *J Androl* 2004; 25:455–465.

68 Hull MG, North K, Taylor H, Farrow A, Ford WC: Delayed conception and active and passive smoking. The Avon Longitudinal Study of Pregnancy and Childhood Study Team. *Fertil Steril* 2000; 74:725–733.

69 Frei B, Kim MC, Ames BN: Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations. *Proc Natl Acad Sci U S A* 1990; 87:4879–4883.

70 Lewin A, Lavon H: The effect of coenzyme Q10 on sperm motility and function. *Mol Aspects Med* 1997; 18(Suppl.):S213–219.

71 Karbownik M, Gitto E, Lewinski A, Reiter RJ: Induction of lipid peroxidation in hamster organs by the carcinogen cadmium: melioration by melatonin. *Cell Biol Toxicol* 2001; 17:33–40.

72 Lenzi A, Sgro P, Salacone P, Paoli D, Gilio B, Lombardo F, Santulli M, Agarwal A, Gandini L: A placebo-controlled double-blind randomized trial of the use of combined l-carnitine and l-acetyl-carnitine treatment in men with asthenozoospermia. *Fertil Steril* 2004; 81:1578–1584.

73 Meseguer M, Garrido N, Martinez-Conejero JA, Simon C, Pellicer A, Remohi J: Role of cholesterol, calcium, and mitochondrial activity in the susceptibility for cryodamage after a cycle of freezing and thawing. *Fertil Steril* 2004; 81:588–594.

74 Park NC, Park HJ, Lee KM, Shin DG: Free radical scavenger effect of rebamipide in sperm processing and cryopreservation. *Asian J Androl* 2003; 5:195–201.

75 Askari HA, Check JH, Peymer N, Bollendorf A: Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. *Arch Androl* 1994; 33:11–15.

76 Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J: Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl* 2005; 26:349–353.

77 Greco E, Romano S, Iacobelli M, Ferrero S, Baroni E, Minasi MG, Ubaldi F, Rienzi L, Tesarik J: ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment. *Hum Reprod* 2005; 20:2590–2594.

78 Hughes CM, Lewis SE, McKelvey-Martin VJ, Thompson W: The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. *Hum Reprod* 1998; 13:1240–1247.

79 Twigg J, Irvine DS, Houston P, Fulton N, Michael L, Aitken RJ: Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective

significance of seminal plasma. *Mol Hum Reprod* 1998; 4:439–445.

80 Twigg JP, Irvine DS, Aitken RJ: Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Hum Reprod* 1998; 13:1864–1871.

81 Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE, Thornton J, Agarwal A: Differential growth of human embryos in vitro: role of reactive oxygen species. *Fertil Steril* 2004; 82:593–600.

82 Sikka SC: Role of oxidative stress and antioxidants in andrology and assisted reproductive technology. *J Androl* 2004; 25:5–18.

83 Zhang X, Sharma RK, Agarwal A, Falcone T: Effect of pentoxifylline in reducing oxidative stress-induced embryotoxicity. *J Assist Reprod Genet* 2005; 22:415–417.

84 Esfandiari N, Falcone T, Agarwal A, Attaran M, Nelson DR, Sharma RK: Protein supplementation and the incidence of apoptosis and oxidative stress in mouse embryos. *Obstet Gynecol* 2005; 105:653–660.

85 Tatemoto H, Ootaki K, Shigeta K, Muto N: Enhancement of developmental competence after in vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-O-alpha-glucoside during in vitro maturation. *Biol Reprod* 2001; 65:1800–1806.

86 Ali AA, Bilodeau JF, Sirard MA: Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. *Theriogenology* 2003; 59:939–949.

87 Guerin P, Guillaud J, Menezo Y: Hypotaurine in spermatozoa and genital secretions and its production by oviduct epithelial cells in vitro. *Hum Reprod* 1995; 10:866–872.

88 Aitken RJ, Harkiss D, Buckingham D: Relationship between iron-catalysed lipid peroxidation potential and human sperm function. *J Reprod Fertil* 1993; 98:257–265.

89 Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS: Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998; 59:1037–1046.

90 Wroblewski N, Schill WB, Henkel R: Metal chelators change the human sperm motility pattern. *Fertil Steril* 2003; 79(Suppl. 3):1584–1589.

91 Aziz N, Said T, Paasch U, Agarwal A: The relationship between human sperm apoptosis, morphology and the sperm deformity index. *Hum Reprod* 2007; 22:1413–1419.

92 Grunewald S, Paasch U, Glander HJ: Enrichment of non-apoptotic human spermatozoa after cryopreservation by immunomagnetic cell sorting. *Cell Tissue Bank* 2001; 2:127–133.

93 Grunewald S, Paasch U, Said TM, Rasch M, Agarwal A, Glander HJ: Magnetic-activated cell sorting before cryopreservation preserves mitochondrial integrity in human spermatozoa. *Cell Tissue Bank* 2006; 7:99–104.

94 Said TM, Grunewald S, Paasch U, Rasch M, Agarwal A, Glander HJ: Effects of magnetic-activated cell sorting on sperm motility and cryosurvival rates. *Fertil Steril* 2005; 83:1442–1446.

95 Said TM, Grunewald S, Paasch U, Glander HJ, Baumann T, Kriegel C, Li L, Agarwal A: Advantage of combining magnetic cell separation with sperm preparation techniques. *Reprod Biomed Online* 2005; 10:740–746.

96 Said TM, Agarwal A, Grunewald S, Rasch M, Glander HJ, Paasch U: Evaluation of sperm recovery following annexin V magnetic-activated cell sorting separation. *Reprod Biomed Online* 2006; 13:336–339.

97 Said T, Agarwal A, Grunewald S, Rasch M, Baumann T, Kriegel C, Li L, Glander HJ, Thomas AJ Jr, Paasch U: Selection of nonapoptotic spermatozoa as a new tool for enhancing assisted reproduction outcomes: an in vitro model. *Biol Reprod* 2006; 74:530–537.