

Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach

ASHOK AGARWAL and TAMER M. SAID

Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Glickman Urological Institute and Department of Obstetrics and Gynecology, The Cleveland Clinic Foundation, Cleveland, Ohio, USA

Accepted for publication 27 July 2004

KEYWORDS

male infertility, reactive oxygen species, antioxidants, sperm preservation

INTRODUCTION

All living aerobic cells are normally exposed to some reactive oxygen species (ROS) but if ROS levels rise, oxidative stress (OS) occurs, which results in oxygen and oxygen-derived oxidants, and in turn increases the rates of cellular damage. OS has been shown to be a major cause of male infertility; a large proportion of infertile men have elevated levels of seminal ROS. Several forms of sperm DNA damage are caused by ROS, e.g. chromatin cross-linking, chromosome deletion, DNA strand breaks and base oxidation. Moreover, ROS are important in mediating apoptosis by inducing cytochrome c and caspases 9 and 3, which in turn result in a high frequency of single- and double-stranded DNA strand breaks [1]. Therefore, in the context of male infertility, seminal OS, sperm DNA damage and apoptosis are interlinked, and constitute a unified pathogenic molecular mechanism. The impact of these factors on male infertility, their clinical significance and management options has always been a subject of controversy.

In this review we provide an overview of patients who are at risk of seminal OS, sperm DNA damage and extensive apoptosis, as well as the methods available for their diagnosis. We also describe how these phenomena can affect potential male fertility, and discuss the different strategies used in their prevention and treatment.

CLINICAL AND LABORATORY PRESENTATIONS

Infertility is the main complication arising in patients with high levels of OS, apoptosis or sperm DNA damage. These variables are

clearly independent measures of sperm quality. Therefore, pathogenic ROS levels or poor-quality sperm chromatin structure may be considered indicative of male subfertility [1,2].

IDIOPATHIC INFERTILITY

Men with idiopathic infertility generally present with significantly higher seminal ROS levels and lower antioxidant properties than healthy controls [3]. Therefore, it appears that the presence of OS in infertile normozoospermic men may be the cause behind previously unexplained cases of infertility. Similarly, sperm DNA damage analysis may reveal hidden sperm DNA abnormalities in infertile men with normal standard sperm values who were diagnosed with idiopathic infertility. The increase in sperm DNA damage in these patients may be partly related to high levels of seminal OS.

Finally and importantly, some conditions may pass unnoticed but still affect the sperm genomic integrity. In one case report [4], a fertile patient who had influenza and a 1-day fever of 39.9 °C presented with a relatively high percentage of sperm with damaged DNA (36%) 18 days after the onset of his fever.

GENITAL TRACT INFECTIONS

Genital tract infections are usually associated with leukocytospermia and elevated ROS levels, as leukocytes represent the major source of ROS production in ejaculates. Although leukocytes are a constant component of human ejaculates and virtually no semen sample is free of them, if the prevalence of leukocytes exceeds normal values ($1 \times 10^6/\text{mL}$), spermatozoa can be compromised. However, interestingly, leukocytes may cause OS even at concentrations below the WHO threshold.

Most importantly, leukocytospermia has been associated with occult sperm DNA damage; this may occur directly in the form of

leukocytospermia, a manifestation of inflammation that is associated with cytokines, which can potentially alter spermatogenesis and cause DNA aberrations, or indirectly as a result of pathological ROS levels, which are frequent in leukocytospermic patients. In one study, levels of the oxidative DNA damage marker 8-hydroxy-2'-deoxyguanosine (8-OH-dG) were significantly elevated in infertile male patients [5].

VARICOCELE

The exact pathways by which a varicocele damages spermatogenesis and sperm quality remain poorly understood. ROS may be an important factor, as elevated levels have been detected in infertile patients with varicocele, along with reduced levels of both seminal and blood plasma antioxidants. Levels of ROS positively correlate with the degree of varicocele and are expected to decrease after varicocelectomy [6].

In a recent study from our group, infertile patients with varicocele had a significantly higher sperm DNA fragmentation index (DFI) than healthy controls. In addition, infertile patients with varicocele had significantly higher levels of OS than the infertile patients with normal genital examination and the controls. Therefore, it appears that infertile men with varicocele have significantly greater spermatozoal DNA damage, which can be related to high levels of OS in semen [7]. Another potential cause of sperm DNA damage in patients with varicocele is apoptosis. Levels of apoptosis are higher in ejaculated spermatozoa from such patients than in spermatozoa from healthy men.

TERATOZOOSPERMIA

Teratozoospermia occurs as a result of defective spermatogenesis and is characterized by an abundance of spermatozoa carrying surplus residual cytoplasm. The retention of residual

cytoplasm promotes spermatozoa to generate endogenous ROS via mechanisms that may be mediated by the cytosolic enzyme glucose-6-phosphate dehydrogenase [8]. Therefore, patients presenting with teratozoospermia are at greater risk of developing pathogenic levels of ROS, apoptosis and sperm DNA damage (Fig. 1).

In general, ROS production is highest in immature spermatozoa from males with abnormal semen values. However, immature spermatozoa with cytoplasmic retention are not the only abnormal male germ cells that are associated with high levels of DNA damage and ROS production. Spermatozoa with abnormal head morphology, midpiece defects and tail defects also have the same characteristics. Production of ROS positively correlates with the sperm deformity index, calculated by dividing the total number of deformities observed by the number of sperm evaluated [9].

ASTHENOZOOSPERMIA

ROS can directly damage spermatozoa by inducing peroxidation of the lipid-containing sperm plasma membrane, which decreases its integrity, and may also affect sperm motility by damaging the axonemal structure [10]. Therefore, high levels of OS are important in the impairment of sperm motility and the occurrence of asthenozoospermia. Unbalanced apoptosis may be another contributing factor. Apoptotic mediators such as the caspases have been detected in high levels in spermatozoa characterized by low motility [11].

AZOOSPERMIA

Apoptosis and DNA damage may prevent sperm from maturing; as a result, patients may present with azoospermia as a result of an imbalance in these pathways [1]. Under physiological conditions, apoptosis maintains the number of germ cells within the supportive capacity of Sertoli cells. However, disturbances in this pathway can interrupt the spermatogenic cascade. High levels of apoptosis were detected at spermatogenic stages where major developmental blocks occur, and frequencies of DNA damage were higher in less mature germ cells (round spermatids compared with elongated spermatids) [12].

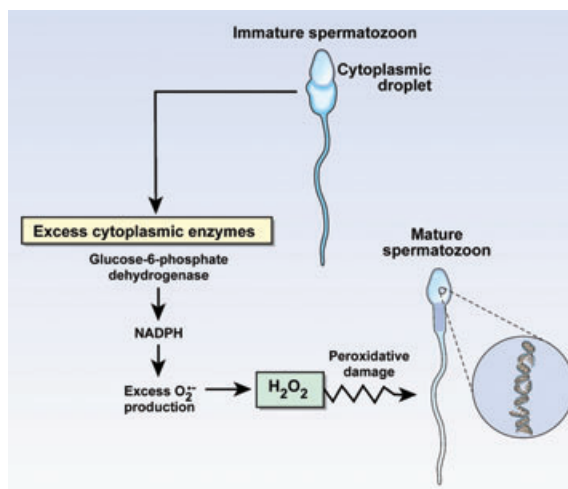


FIG. 1. The mechanism for the link between OS and sperm DNA damage. Immature spermatozoa characterized with cytoplasmic droplets have deranged redox metabolic activity and greater ability to produce ROS. In turn, OS leads to damage of the sperm DNA.

PREDISPOSING CONDITIONS

Cancer treatments are well known to adversely affect male fertility. Radiation and alkylating agents used in treating malignant conditions can induce DNA damage in the male genome, which can cause various complications ranging from miscarriage to persistent mutations and carcinogenesis in offspring. Although sperm DNA damage may be assessed using several techniques, these techniques cannot provide information on the mutational risk of the offspring.

Infertility associated with malignant disease was always considered to be a side-effect of drugs and irradiation used during the course of treatment. However, it is now widely thought that there is decreased fertility before exposure to treatment. Patients with cancer may present with DNA that is intact or severely damaged. However, the prevalence of DNA damage is significantly higher in patients with untreated Hodgkin's and non-Hodgkin's diseases than in healthy controls [13].

Infertile men who smoke have higher levels of seminal OS than infertile non-smokers. The link between cigarette smoking and high seminal ROS can be attributed in part to the associated increase in seminal leukocytes. Indeed, smoking can increase leukocyte concentrations by as much as 48% [14]. In addition, cigarette smoke contains a variety of ROS. Smoking may also damage the chromatin structure and produce endogenous DNA strand breaks in human sperm. Levels of DNA damage tend to be higher in smokers [15].

PROGNOSIS

Apoptosis and OS are involved in mediating DNA damage in the germ line. The Y chromosome is particularly susceptible to damage leading to gene deletions because the haploid genome is unable to retrieve lost genetic information. In turn, Y chromosome deletions and microdeletions can lead to infertility in the offspring [16].

Whereas the impact of sperm DNA fragmentation on fertilization rates remains controversial, there is wider agreement about its negative effects on embryo development and pregnancy rates [17]. Although low levels of sperm DNA damage can be repaired by the oocyte, apoptosis and embryo fragmentation can occur if the damage is extensive. Decreased embryo cleavage rates and embryo quality have been reported in sperm samples containing a high frequency of damaged DNA [2].

Sperm DNA damage may also serve as a prognostic tool of human fertility. When couples were followed for 12 menstrual cycles [18], all male partners of couples who achieved a pregnancy during months 1–3 had <30% sperm with fragmented DNA. On the other hand, 10% of the couples who achieved pregnancy in months 4–12 and ≈20% of those who never achieved a pregnancy had >30% sperm with fragmented DNA. In the same study, 84% of the men who initiated pregnancy during the first 3 months had sperm DNA damage levels of <15%.

MANAGEMENT

LABORATORY INVESTIGATIONS

Because the assays that are used to identify OS, DNA damage and apoptosis are complex, testing should be limited to cases where an abnormality is suspected. Routine semen analysis remains the backbone of evaluating male infertility. Most infertile men do not require advanced and sometimes expensive tests that diagnose defects at the molecular level.

Because OS is the result of an imbalance between ROS levels and the total antioxidant capacity (TAC) of seminal plasma, it is important to assess both variables when evaluating the OS status of a given sample. The chemiluminescence assay is one of the most commonly used methods to detect free radicals. The assay is accurate and reliable when the sperm concentration is $>1 \times 10^6/\text{mL}$ and the samples are analysed within the first hour after specimen collection [19]. Values of $>1 \times 10^6$ counted photons per minute are considered high.

Although accurate for measuring TAC in seminal plasma, the enhanced chemiluminescence assay is cumbersome and time-consuming, and requires expensive instrumentation (e.g. a luminometer). However, colorimetry is currently gaining considerable acceptance [20]. Currently, the characterization of average TAC values is lacking. With no clear demarcation between fertile and infertile patients, assessing TAC does not currently appear to be of high value.

Although several methods are currently used to assess apoptosis and sperm DNA damage, establishing a threshold between normal levels in the average fertile population and the minimal levels of sperm DNA integrity required for achieving pregnancy remains extremely challenging. All methods currently lack a threshold, except for the sperm chromatin structure assay; this assesses the ability of the DNA to resist denaturation by acid or heat, using flow cytometry, and the damage is expressed as the DFI [21]. In clinical applications, the DFI not only distinguished fertile men from those who were infertile, but also identified samples that were compatible with *in vivo* and *in vitro* pregnancy ($<28\text{--}30\%$) [2].

At present, the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling and Comet assays for apoptosis are commonly used in research applications. Both assays correlate well with fertility and *in vitro* fertilization [17] but the subjectivity and variability of their results does not currently allow their use as distinctive identifiers of samples with impaired fertility.

TREATMENT

Oral: Levels of ROS can be reduced by augmenting the scavenging capacity of the seminal plasma with antioxidants [22]. Combined therapy is much more beneficial in managing infertile men, because antioxidants act by different mechanisms on different free radicals. Patients diagnosed with male accessory gland infections may benefit from carnitines (L-carnitine 1 g and acetyl-carnitine 0.5 g) twice daily for 3 months, as it results in a significant reduction in ROS levels in semen samples. Nevertheless, treatment with antioxidants in these cases is mainly an adjunctive therapy and does not replace proper antibiotic regimens. Other combinations of vitamins A and E with N-acetyl-cysteine may also be used.

A few clinical trials report the positive effects of antioxidant administration on sperm DNA integrity. When given for 2 months, vitamin C (200 mg), combined with vitamin E (200 mg) and glutathione (400 mg), significantly decreased 8-OH-dG levels, considered as a marker of OS-induced sperm DNA damage. Similarly, N-acetyl-cysteine and/or a mixture of essential fatty acids and natural vitamins A and E reduced levels of 8-OH-dG [22].

When the molecular framework of apoptosis is identified, specific apoptotic inhibitors may have a role in promoting germ-cell survival. Current research is underway to identify agents that may rationally manipulate the apoptotic machinery for therapeutic benefits. Sphingosine-1-phosphate is an example of an apoptotic inhibitor; at 1 and 10 $\mu\text{mol/L}$ it suppressed apoptosis in germ cells extracted from testicular tissue by 30% [23]. Similarly, N-acetyl-cysteine is a potential regulator of germ-cell death, mainly because it is a well established inhibitor of physiological cell death in several systems, and because it is a compound known to act on human semen as a survival factor. When given in concentrations of 125, 100, 50 and 25 mmol/

L, N-acetyl-cysteine suppressed germ cell death in a dose-dependent manner [24]. These agents have been evaluated mainly *in vitro*; their efficacy still remains to be validated *in vivo*.

Sperm preparation methods are used during assisted reproduction techniques (ART) to recover a selected healthy population of cells. The 'swim-up' method, glass-wool filtration and density-gradient centrifugation all help to ensure that semen samples contain DNA-intact spermatozoa [2].

During sperm preparation techniques, media may be supplemented with a variety of antioxidant(s) to guard against OS. Adding different concentrations of vitamin C (300 and 600 $\mu\text{mol/L}$) and vitamin E (40 and 60 $\mu\text{mol/L}$) to sperm preparation medium significantly reduced hydrogen peroxide. The superoxide anion can be also reduced by 29–72% by adding 10 mmol/L pentoxifylline [22].

The effects of antioxidants *in vitro* are more marked in samples that were originally characterized by high levels of ROS. N-acetyl-cysteine (0.1, 1 and 5 mg/mL) has a dose-dependent effect in reducing ROS levels; the reduction was greater in patients with high levels of ROS than in those with low levels [25].

ARTs, which include intrauterine insemination, *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), currently have a major role in treating infertility. Success rates depend on a variety of factors, but most important is the structural and functional integrity of the gametes used. Therefore, the exact nature of the infertility, and its cause and pathogenesis, should be considered before the treatment is started. Unidentified factors may adversely affect the end result, and add to the financial, social and emotional problems of the patients.

Semen samples that contain high levels of DNA damage are often associated with decreased fertilization rates and/or embryo cleavage after IVF and ICSI, and may be linked to early embryo death. Although the most normal-appearing and motile spermatozoa are selected during ART, there is always a chance that sperm containing varying degrees of DNA damage may be used. The miscarriage rate is highest after ICSI, which possibly reflects the fact that genomically compromised spermatozoa are sometimes

used and lead to irreparable DNA damage in the embryo [26].

In patients with obstructive azoospermia, spermatozoa are often surgically retrieved from the epididymis or testicular tissue. In general, surgically extracted spermatozoa tend to have higher percentages of DNA strand breaks. However, the use of testicular spermatozoa is preferred over epididymal spermatozoa, because the former have less DNA damage and a better developmental potential [27].

Sperm cryopreservation is extensively used in ART programmes. Despite various advances in cryopreservation methods, the recovery rate of functional spermatozoa after thawing remains unsatisfactory. ROS are produced during the freezing and thawing of spermatozoa, which may be the cause for the decrease in sperm function after cryopreservation [28]. Cryopreservation induces many changes in sperm cells, including membrane disorders and cell death; it also acts as an inducer of apoptosis in sperm cells and in turn, it clearly facilitates DNA damage.

Whether the cryopreservation of raw semen samples or prepared (after washing) samples yield the best results is a matter of controversy. Sperm that is frozen unprepared in seminal fluid may be more resistant to freezing damage because of the presence of protective seminal plasma. In our opinion, separating the mature healthy spermatozoa from leukocytes and immature spermatozoa is more important, as using media supplements *in vitro* can always compensate for the antioxidant properties of the seminal plasma.

PREVENTIVE MEASURES

It is extremely important for natural and assisted conception to reduce the levels of seminal OS. Minimizing the interaction between ROS-producing cells in semen (sperm with cytoplasmic droplets/leukocytes) and the mature spermatozoa may help to protect spermatozoa that have the potential to fertilize from the detrimental effects of ROS. Any underlying causal factor such as inflammation, infection or cigarette smoking should be managed properly.

The sperm preparation techniques used for ART (e.g. density gradient and swim-up) can

be used to separate the mature spermatozoa with the best ability to fertilize and to decrease their interaction with ROS-producing cells. However, during these techniques, the semen samples are repeatedly centrifuged and the antioxidant-rich seminal plasma is removed, which may result in increased ROS levels. Shortening the duration of the centrifugation may reduce the risk of OS-induced injury to the sperm.

Currently, the presence of spermatozoa in media appears to promote sperm DNA integrity. *In vitro* culture of testicular spermatozoa for 72 h reduces the proportion of spermatozoa containing single-stranded DNA, thereby increasing the availability of double-stranded DNA spermatozoa for ICSI use [29]. Moreover, the immediate addition of sperm-wash media after collecting semen samples or ejaculating in media, may also decrease the incidence of sperm DNA damage [30].

CONCLUSION

OS, sperm DNA damage and apoptosis are clearly implicated in the pathogenesis of male infertility. These interlinked molecular events are associated with various clinical and laboratory manifestations that may be present in infertile males. Although standardized assays for diagnosing these conditions need to be improved, assessment is advised in selected cases where the exact diagnosis is suspected. Identifying the exact nature of the defect will help in selecting proper management, which in turn will improve natural and assisted reproduction success rates, and help to ensure healthy offspring.

CONFLICT OF INTEREST

None declared.

REFERENCES

- 1 Said T, Paasch U, Glander H, Agarwal A. Role of caspases in male infertility. *Hum Reprod Update* 2004; **10**: 39–51
- 2 Agarwal A, Said T. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update* 2003; **9**: 331–45
- 3 Pasqualotto F, Sharma R, Kobayashi H,

- Nelson D, Thomas A Jr, Agarwal A. Oxidative stress in normospermic men undergoing infertility evaluation. *J Androl* 2001; **73**: 459–64
- 4 Evenson D, Jost L, Corzett M, Balhorn R. Characteristics of human sperm chromatin structure following an episode of influenza and high fever: a case study. *J Androl* 2000; **21**: 739–46
- 5 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**: 519–24
- 6 Barbieri E, Hidalgo M, Venegas A, Smith R, Lissi E. Varicocele-associated decrease in antioxidant defenses. *J Androl* 1999; **20**: 713–7
- 7 Saleh R, Agarwal A, Sharma R, Said T, Sikka S, Thomas A Jr. Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. *Fertil Steril* 2003; **80**: 1431–6
- 8 Aitken R. The Amoroso lecture. The human spermatozoon – a cell in crisis? *J Reprod Fertil* 1999; **115**: 1–7
- 9 Aziz N, Saleh R, Sharma R *et al.* Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. *Fertil Steril* 2004; **81**: 349–54
- 10 Saleh A, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. *J Androl* 2002; **23**: 737–52
- 11 Weng S, Taylor S, Morshedi M *et al.* Caspase activity and apoptotic markers in ejaculated human sperm. *Mol Hum Reprod* 2002; **8**: 984–91
- 12 Tesarik J, Greco E, Cohen-Bacrie P, Mendoza C. Germ cell apoptosis in men with complete and incomplete spermiogenesis failure. *Mol Hum Reprod* 1998; **4**: 757–62
- 13 Kobayashi H, Larson K, Sharma R *et al.* DNA damage in cancer patients before treatment as measured by the sperm chromatin structure assay. *Fertil Steril* 2001; **75**: 469–75
- 14 Saleh R, Agarwal A, Sharma R, Nelson D, Thomas AJ. Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. *Fertil Steril* 2002; **78**: 491–9
- 15 Potts R, Newbury C, Smith G, Notarianni L, Jefferies T. Sperm chromatin damage associated with

- male smoking. *Mutation Res* 1999; **423**: 103–11
- 16 Aitken R, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 2001; **122**: 497–506
- 17 Sharma R, Said T, Agarwal A. Sperm DNA damage and its clinical relevance in assessing reproductive outcome. *Asian J Androl* 2004; **6**: 139–48
- 18 Evenson DP, Jost LK, Marshall D *et al.* Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod* 1999; **14**: 1039–49
- 19 Kobayashi H, Gil-Guzman E, Mahran A *et al.* Quality control of reactive oxygen species measurement by luminol-dependent chemiluminescence assay. *J Androl* 2001; **22**: 568–74
- 20 Said T, Kattal N, Sharma R *et al.* Enhanced chemiluminescence assay vs colorimetric assay for measurement of the total antioxidant capacity of human seminal plasma. *J Androl* 2003; **24**: 676–80
- 21 Evenson D, Larson K, Jost L. Sperm chromatin structure assay. its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J Androl* 2002; **23**: 25–43
- 22 Agarwal A, Nallella K, Allamaneni S, Said T. Role of antioxidants in treatment of male infertility: an overview of the literature. *Reprod Biomedicine Online* 2004; **8**: 616–27
- 23 Suomalainen L, Hakala J, Pentikainen V *et al.* Sphingosine-1-phosphate in inhibition of male germ cell apoptosis in the human testis. *J Clin Endocrinol Metab* 2003; **88**: 5572–9
- 24 Erkkila K, Hirvonen V, Wuokko E, Parvinen M, Dunkel L. N-acetyl-L-cysteine inhibits apoptosis in human male germ cells in vitro. *J Clin Endocrinol Metab* 1998; **83**: 2523–31
- 25 Oeda T, Henkel R, Ohmori H, Schill WB. Scavenging effect of N-acetyl-L-cysteine against reactive oxygen species in human semen: a possible therapeutic modality for male factor infertility? *Andrologia* 1997; **29**: 125–31
- 26 Carrell D, Wilcox A, Lowy L *et al.* Elevated sperm chromosome aneuploidy and apoptosis in patients with unexplained recurrent pregnancy loss. *Obstet Gynecol* 2003; **101**: 1229–35
- 27 Steele E, McClure N, Maxwell R, Lewis S. A comparison of DNA damage in testicular and proximal epididymal spermatozoa in obstructive azoospermia. *Mol Hum Reprod* 1999; **5**: 831–5
- 28 Wang A, Zhang H, Ikemoto I, Anderson D, Loughlin K. Reactive oxygen species generation by seminal cells during cryopreservation. *Urology* 1997; **49**: 921–5
- 29 Emiliani S, Van den Bergh M, Vannin A, Biramane J, Verdoodt M, Englert Y. Evidence of reduced single-stranded testicular sperm DNA from obstructive azoospermic men after three days of *in vitro* culture. *Hum Reprod* 2001; **16**: 1200–3
- 30 Zollner U, Zollner K, Dietl J, Steck T. Semen sample collection enhances the implantation rate following ICSI in patients with severe oligoastheno-teratozoospermia. *Hum Reprod* 2001; **16**: 1110–4

Correspondence: Ashok Agarwal, Professor of Surgery, Director, Center for Advanced Research in Human Reproduction, Infertility and Sexual Function, Glickman Urological Institute and Department of Obstetrics and Gynecology, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Desk A19.1, Cleveland, OH 44195, USA.
e-mail: agarwaa@ccf.org

Abbreviations: ROS, reactive oxygen species; OS, oxidative stress; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; DFI, DNA fragmentation index; TAC, total antioxidant capacity; ART, assisted reproduction technique; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.