

Oxidative Damage to Sperm DNA: Clinical Implications

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Abstract

Background: Sperm DNA is susceptible to oxidative damage due to intrinsic and extrinsic factors which cause oxidative stress and due to limited DNA damage detection and repair mechanism. Reactive oxygen species (ROS) are the chief cause of sperm DNA damage. So, this study was planned to assess oxidative stress levels and correlate with sperm DNA damage.

Material and Method: The study included 35 men who had fathered a child in the last one year and 54 male partners of couple experiencing primary infertility. Semen analysis was done according to World Health Organization (1999) criteria. ROS measurement was done by direct chemiluminescence method using luminol as a probe. DNA damage was assessed by sperm chromatin structure assay (SCSA) and expressed as percentage DFI. 8-Hydroxy-2'-deoxyguanosine (8-OHdG) estimation was carried out by competitive ELISA.

Results: The seminal ROS level (RLU/sec/million sperm) was significantly higher (40.52 ± 18.32) in infertile men as compared to fertile controls (14.04 ± 6.67) ($p < 0.0001$). The mean values of 8-OHdG levels (pg/ml) were also significantly higher in patients (30.92 ± 3.27) as compared to fertile controls (14.29 ± 2.24) ($p < 0.0001$) and mean DFI (%) of infertile men was found to be 35.48 ± 12.95 , which was higher as compared to controls (24.18 ± 8.76). There was a strong positive correlation between these parameters.

Conclusion: Majority of the sperm DNA damage in infertile men is caused by oxidative damage to the genomic DNA. In presence of limited DNA damage detection and repair mechanism in sperm, prevention of oxidative stress by simple lifestyle interventions may actually be therapeutic.

Keywords: Infertility, Sperm, DNA damage, DFI, 8-OHdG, ROS

Introduction

Men in the reproductive age group may experience qualitative and quantitative defect in sperm production, but there are men with normal sperm parameters who are infertile, such cases are classified with idiopathic infertility. Men with idiopathic infertility generally present with significantly higher seminal ROS levels and lower antioxidant potential than fertile controls [1].

Male factor accounts for 20% cases of infertility, female factor for 38 % cases and in 27% cases of infertility both partners are involved. Traditionally, the diagnosis of male infertility is based upon macroscopic and microscopic assessment and analysis of sperm concentration, motility and morphology as routine indicators of semen quality. These indicators provide fundamental information about sperm production upon which clinicians base their initial diagnosis [2]. However, even with appropriate quality assurance, traditional semen parameters provide a limited degree of prognostic and diagnostic information. The semen parameters assessed by traditional methods provide modest information regarding fertilizing capacity of sperm. Sperm chromatin integrity is essential in the

process of fertilization, implantation and proper embryonic development and birth of healthy off spring [3]. Sperm and ova carry all the necessary information required for fertilization and embryonic development. Any form of damage to the paternal (sperm DNA) or maternal (ova) can have serious consequences in the form of pre and post implantation losses, impaired embryonic development, childhood morbidity and even cancer [4]. Thus DNA damage assessment may be a diagnostic measure in cases with idiopathic infertility especially in men with normal sperm parameters [5]. The major forms of DNA damage in the male gamete include chromosomal aberrations (mostly deletions and aneuploidies), epigenetic modifications, mutations, base oxidation and sperm DNA fragmentation (SDF). As compared to ova (which develops in a relatively hypoxic environment in the ovarian cortex), sperm exists in a state of oxygen paradox. By virtue of it being transcriptionally inert, lacking cytosolic antioxidants, being endorsed with high polyunsaturated fatty acid content and deficient in DNA damage detection and repair mechanisms sperm is most vulnerable to oxidative damage by both external and internal insult [6]. Several factors like morphologically abnormal sperm, infection, varicocele, and sedentary life style, exposure to organic pollutants, psychological stress, high temperature, smoking, and obesity can lead to oxidative stress [7]. As sperm has a limited DNA damage repair mechanism and a highly truncated basic repair mechanisms, preventing or minimizing

exposure to free radicals may be the only option to prevent DNA damage.

SDF can originate in the testis, after spermiation and during storage and transit in the male genital tract, when they undergo final maturation in the epididymis. During the process of IVF after ejaculation, when the sperm cells are separated from the seminal plasma and incubated in vitro sperm are more vulnerable to oxidative damage as protective antioxidants are removed due to sperm washing [8]. These insults to the sperm DNA may pose problems in the process and outcome of ART. Fragmentation of the sperm DNA also occurs during spermatogenesis in the testis as part of the apoptotic process (which is known as the abortive apoptosis) or during chromatin compaction, and also during replacement of histones by protamines [9]. Presence of sperm DNA damage is an indicator of cell lethality and cellular death. Such sperm would be rejected in vivo and would not be able to successfully bypass various barriers of fertilization, however use of such sperm with compromised DNA in vitro in assisted conception may affect foetal well-being. Unlike oocyte which is vulnerable to segregation anomalies, DNA damage in the form of mutations accumulates in sperm as sperm cells undergo several rounds of replication and each replication event is a source of mutations.

Unlike somatic cell, sperm is enriched with arginine and cysteine-rich protamines that helps in formation of strong disulphide bonds to maintain sperm DNA integrity. In order to reduce the volume of the sperm chromatin and the sperm as well to make the sperm head more hydrodynamic for its smooth movement and function there occurs vigorous compaction the sperm chromatin in the form of replacement of histones by transition proteins followed by protamines [10]. Apart from these anatomical and physiological factors there are several physical (environmental exposures, radiations, food habits, life style factors) and chemical agents (pesticides, chemotherapy, persistent organic pollutants) that alter the sperm DNA and introduce damage in the form of mutations, deletions and base modifications. Sperm genome is not merely a vector of paternal DNA, but transfers at time of fertilization not only DNA, but also transcripts and miRNA that are critical for early embryonic development. Therefore, understanding the nature of sperm DNA damage and its impact on the normal functions of the sperm cells and hence the fertilization capacity and outcomes is pivotal in the management of infertility [11-13].

Increased reactive oxygen species (ROS) levels are the chief cause of DNA damage and previous studies have shown that high seminal ROS levels are implicated in infertile men with normal and abnormal semen parameters. One of the leading causes of increased free radical production by mitochondria is disruption of OXPHOS electron transport pathway by electrophilic aldehydes (accumulate as a result of peroxidation of lipids). This arises due to dietary deficiency, age, varicocele, life style factors and low antioxidant levels. Hence it is essential to assess sperm DNA damage in the infertility management which is not included in the normal, traditional methods of semen assessment. Oxidative stress is thought to be the main mechanism responsible for the occurrence of DNA fragmentation and DNA base oxidation. Oxidative stress oxidizes the DNA bases preferentially guanine. Sperm has a highly truncated BER mechanism and thus OGG1 removes oxidized Guanine and create basic sites. These are repaired only by oocyte at fertilization. However ageing oocyte and presence of extensive sperm DNA damage may overwhelm the oocyte repair mechanism with persistence of these mutagenic bases. This can have deleterious impact on foetal health and may manifest as infertility, pre and post implantation losses, children with congenital

malformation and even cancers. In an ongoing study in our laboratory we have found very high levels of sperm 8-hydroxy 2-deoxyguanosine levels and DNA damage in fathers of children who developed Retinoblastoma by 2 years of age.

Men with abnormal semen parameters (sperm motility and morphology) have high levels of DNA fragmentation [14,15]. Sperm DNA quality is important in the process of fertilization and fertility outcomes both in vivo and in vitro. However, sperm DNA quality assessment is still not included in the routine assessment of infertility. It is important in clinical practice to take a threshold value with discriminatory power to distinguish DNA damage in fertile and infertile population; routine semen analysis is not able to do so. As DNA fragmentation approaches 20, the chances to sustain conception decreases and beyond DFI of 30 it is nearly impossible to achieve conception. Recent studies from our lab have shown that a DFI threshold of 26 results in pre and post implantation losses [16]. Concomitant with increased oxidative DNA damage in sperm there is accumulation of highly mutagenic oxidized bases with 8-OHdG. This preferentially occurs in Telomeres causing telomere shortening (due to OGG1 action) and thus accumulates Single strand and double strand breaks. This rapid telomeric attrition leads to chromosomal instability, rearrangements, aneuploidies during segregation and also genomic instability. This may be the common underlying factor by which sperm DNA damage leads to idiopathic infertility, idiopathic RSA, congenital malformation and even childhood cancer.

As mentioned earlier oxidative stress is the major cause of DNA damage. Oxidative stress is the condition when the free radicals and reactive oxygen species production exceeds the antioxidant capacity of the cell. These free radicals and reactive oxygen species are produced in various physiological processes, disease conditions and various pathologies. These oxidants when cross the physiological levels cause oxidative damage to DNA and cell membranes along with many other biomolecules like proteins and lipids. Oxidative damage to DNA can contribute to mutations, histone and base modifications and hamper the sperm capacity in fertilization [17-19]. Although many different oxidative DNA damage products have been identified, the guanine-derived lesion 8OHGua (8-hydroxyguanine) and its corresponding deoxynucleoside 8-OHdG (8-hydroxy 2-deoxyguanosine) have been the subject of intensive study. The 8-hydroxy-2'-deoxyguanosine (8-OHdG) is one of the best-characterized oxidized bases [20]. 8-OHdG in DNA could lead to mis-incorporation of adenines opposite the 8-OHdG lesion thus inducing G:C to T:A transversions in genomic DNA.

Ongoing studies from our laboratory have documented that oxidative DNA damage may be the cause of accelerated testicular ageing which manifests as oligo and azoospermia due to shorter sperm telomere. Telomere shortening may also impair cleavage and lead to pre and post implantation losses [16]. Thus evaluation of DNA damage, oxidized DNA adducts and detecting the cause of DNA damage are not only important diagnostic marker but are invaluable for appropriate management of such couples.

Materials and Methods

The study was initiated after institutional ethical clearance and informed consent from patient and controls. The female partners of all these cases were normal after complete clinical, gynaecological, hormonal and radiological examination. Human ejaculates were obtained from 35 fertile age matched controls, and 54 male partners of

couple experiencing primary infertility within age group of 18-45 years were enrolled. Semen analysis was assessed by World Health Organization (1999) criteria. These patients after through clinical examination were referred from the Department of Gynaecology and Obstetrics and Department of Urology, AIIMS, New Delhi. Statistical analysis was done using Student t test and Pearson correlation.

Measurement of ROS

ROS detection by chemiluminescence assay in neat semen: The ROS production in 400 µl of liquefied neat semen was measured after addition of 10 µl of 5 mM solution of luminol in DMSO (dimethylsulphoxide, Sigma Chemical Co.). A tube containing 10 µl of 5 mM luminol (5-amino-2,3-dihydro- 1,4-phthalazinedione, Sigma Chemical Co., St. Louis, MO, USA) solution in DMSO was used as a blank. Chemiluminescence was measured for 10 min using the Berthold detection luminometer (USA). Results were expressed in relative light units (RLU) per second and per 20 × 10⁶ spermatozoa. The RLU/sec was then recalculated according to the original spermatozoa concentration in semen sample and expressed as RLU/sec per 20 × 10⁶ spermatozoa.

Sperm Chromatin Structure Assay (SCSA)

Preparation of samples: The SCSA was performed according to the procedure described by Evenson et al. [17]. The aliquot from each ejaculate was thawed in a water bath at 37°C for 30 seconds and diluted to a concentration of 2×10⁶ sperm/mL in TNE buffer to a total of 200 mL in a falcon tube. Immediately, 0.4 mL of acid detergent solution (0.08 mol/L HCl, 0.15 mol/L NaCl, 0.1% v/v Triton X-100, pH 1.2) was added to the Falcon tube. After exactly 30 seconds, 1.2ml of AO-staining solution (6 mg AO[chromatographically purified:Polysciences, Inc. USA] per mL citrate buffer [0.037 mol/L citric acid, 0.126 mol/L Na₂HPO₄, 1.1 mmol/L EDTA disodium, 0.15 mol/L NaCl, pH 6.0]) was added. For every 6 test samples, 1 standard reference sample was analyzed to ensure instrument stability.

Flow Cytometric Measurements

The samples were analyzed using a FAC Scan flow cytometer (BD Biosciences), with an air cooled argon laser operated at 488 nm and a power of 15 mW. The green fluorescence (FL1) was collected through a 515-545 nm band pass filter, and the red fluorescence (FL3) was collected through a 650 nm long pass filter. The sheath/ sample was set on “low,” adjusted to a flow rate of 200 events/s when analyzing a sample containing 2×10⁶ sperm/mL. Immediately after the addition of the AO staining solution, the sample was placed in the flow cytometer and run through the flow system. All the samples were assessed in duplicate at 1-month interval and the average was taken. After complete analysis of the sample, the X-mean (red fluorescence) and Y-mean (green fluorescence) values were recorded manually after selecting gate for sperm cells using Flowjo software (Oregon). Strict quality control was maintained throughout the experiment.

DFI calculation: Post-acquisition DFI calculation was performed offline in the Flowjo software. The sperm cells are gated after excluding debris and high DNA stainability (HDS) cells and mean values of red and green fluorescence were recorded manually. The DFI was then calculated by the formula, DFI = mean red fluorescence/ (mean red fluorescence + mean green fluorescence).

8-Hydroxy-2'-deoxyguanosine (8-OHdG) Estimation

Total 8-OHdG was estimated in sperm DNA. Sperm cells were first isolated and separated using 80 & 40% density gradient media - (Pureception,USA). From pure sperm cells DNA was isolated by standard method. Then DNA was digested with DNase 1 and Alkaline phosphatase enzyme. The quantification of 8-OHdG was achieved by using Cayman's EIA kit [18]. Protocol was followed essentially as described by the manufacturer for the quantification of 8-OHdG (Promega).

Results

Sperm parameters like sperm count (p=0.003) and forward motility (p=0.0002) were significantly lower in infertile men compared to controls and no significant difference in the age, seminal volume, and pH was observed between infertile men and controls. Out of 54 cases 22 men had normal semen parameters as per WHO 1999 guidelines.

Category	S C*(p=0.003)	FM* (p=0.0002)	pH	Volume	Age
Infertile N=54	41.43 ± 36.13	44.16 ± 19.94	7.52 ± 0.64	3.03 ± 1.39	31.83 ± 4.3
Controls N=35	70.66 ± 57.98	60.14 ± 18.33	7.61 ± 0.38	2.96 ± 1.07	32.23 ± 4.0

Table1: Comparison of Semen Parameters and Age of Infertile Men and Controls

SC: Sperm Count; FM: Forward Motility (A+B grade in %)

ROS levels

The seminal ROS level (RLU/sec/million sperm) was significantly higher (40.52 ± 18.32) in infertile men than fertile controls (14.04 ± 6.67) (p<0.0001). Out of 54 cases 32 cases had ROS > 22 RLU/sec/million sperm and 22 cases had <22 RLU/sec/million sperm (Figure 1).

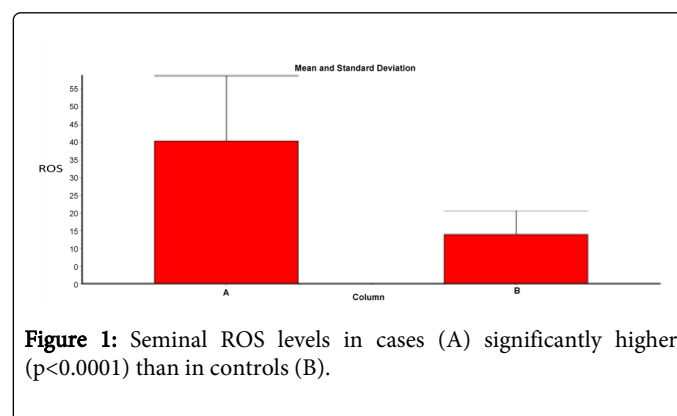


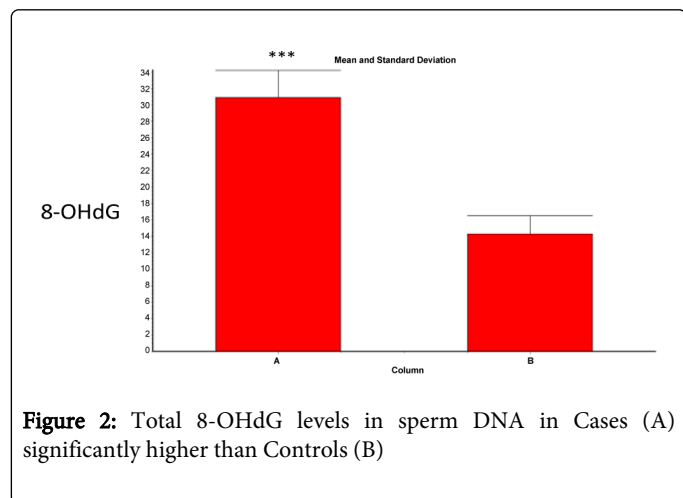
Figure 1: Seminal ROS levels in cases (A) significantly higher (p<0.0001) than in controls (B).

***= p < 0.0001

8-OHdG Level

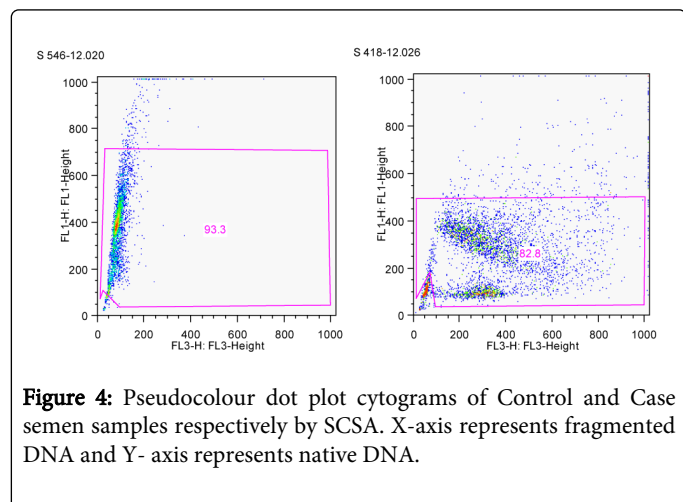
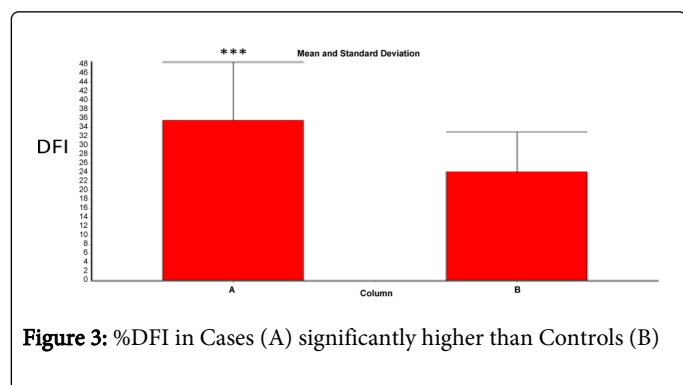
The total 8-OHdG level was measured in DNA isolated from the spermatozoa after removal of somatic cells. For the whole study population (infertile and fertile men), the mean values of 8-OHdG

levels (pg/ml) were respectively 30.92 ± 3.27 and 14.29 ± 2.24 . This difference was highly statistically significant ($p < 0.0001$) (Figure 2).



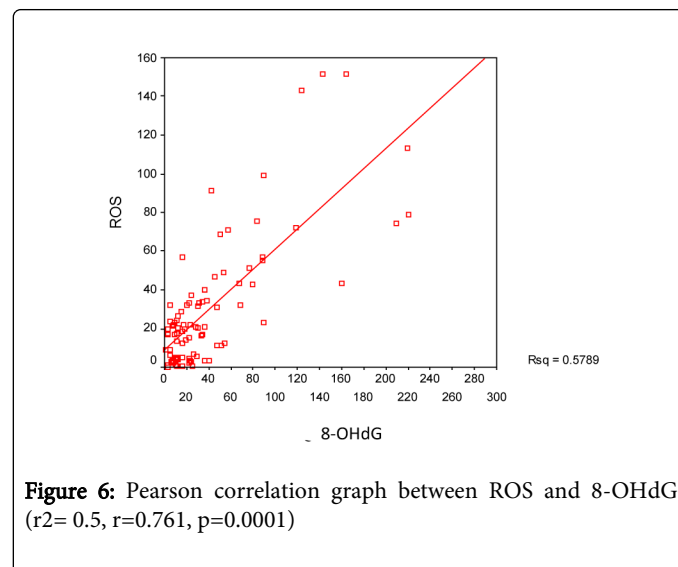
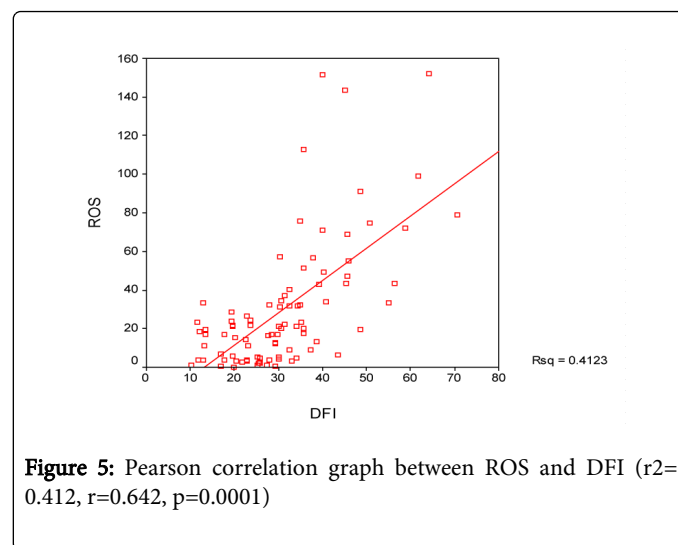
DNA Fragmentation Index (%DFI)

The mean DFI (%) of infertile men was found to be 35.48 ± 12.95 , which was higher as compared to controls (24.18 ± 8.76). The median DFI of infertile men was 33.5 (Minimum-11.9, Maximum- 70.6) significantly higher when compared to controls 22.8 (Minimum-10.1, Maximum- 45.3). Out of 54 cases 36 cases had $>30\%$ DFI and 18 cases had $<30\%$ DFI (Figure 3,4).



From total 54 cases 28 cases had increased ROS (>22 RLU/sec/million sperm) and increased DFI ($>30\%$).

In our study, we observed a strong positive correlation (Pearson correlation) between ROS and DFI (r value= 0.642 , p value= 0.0001). Strong positive correlation of ROS with DFI explains that the chief cause of DNA damage is oxidative stress, which is also indicated by raised total 8OHdG levels. The strong positive correlation between ROS level and 8-OHdG indicates that oxidative stress is chief cause of accumulation of mutagenic base. There was found a positive correlation of DFI with 8-OHdG ($r=0.628$, $p=0.0001$) and also between ROS and 8-OHdG ($r=0.761$, $p=0.0001$) (Figure 5,6).



Discussion and Conclusion

Sperm DNA integrity is important for accurate transmission of genetic information to the offspring. There are different school of thoughts that explain the origin and impact of sperm DNA damage. Oxidative stress has been established as a major cause of sperm DNA damage along with other causes like abortive apoptosis, sperm chromatin re-modelling and defective packaging processes [23]. The sperm genome acts as a database of information required for

fertilization, implantation and embryonic development. Any insult to the sperm genome may disturb these crucial processes and result in implantation losses and impaired embryonic development, congenital malformations and recurrent pregnancy loss. Sperm genome is partitioned into 2 compartments. The central protamine bound compact crystalline toroid is relatively stable and immune to damage, however the peripheral histone bound compartment is loosely packaged and is vulnerable to environmental insults and oxidative stress. This histone bound compartment has promoter of genes of critical developmental importance also has telomeric DNA and several regulatory RNA. These are thus the sites of oxidative DNA damage.

As sperm has a very basic repair mechanism and we have previously documented low levels of PARP1, we must identify cause of DNA damage and minimize or prevent exposure to factors which cause DNA damage [24].

Oxidative stress is the focus of major research. Our study is based on the hypothesis that infertility is associated with accelerated testicular ageing. One of the hall marks of ageing is Oxidative stress. As shown in these study 32 infertile men (both normal and abnormal sperm parameter) had raised ROS levels. This is also evident by the accumulation of highly mutagenic base 8-OH-2-deoxyguanosine. As sperm has a highly truncated DNA detection and repair mechanism, thus majority of DNA damage is corrected by the oocyte after fertilization. But extensive DNA damage may overwhelm the oocyte repair capacity. The presence of this mutagenic base in the embryo may also be the cause of pre and post implantation losses, congenital malformation and even cancer. These oxidative stress markers need to be extensively studied for the impact they have on the sperm DNA health. Our study found a positive correlation between ROS, 8-OHdG and DFI indicating that the major DNA damage is oxidative in nature.

In a study from our laboratory, we have documented high seminal free radical levels and sperm DNA damage in father of children with retinoblastoma (presented at International Symposium of Genetics of male Infertility, 2013, Florence). Majority of fathers were smokers and had advanced age (35 years) and usually the child affected was the last born child. Thus oxidative sperm DNA damage may not lead to infertility but may also cause recurrent spontaneous abortions, congenital malformation and even cancer [25]. Sperm exists in a state of oxygen paradox. Previous studies from our laboratory has documented that sperm from infertile men had significant higher number of non-synonymous mitochondrial variation and had lower antioxidant capacity and higher free radical levels leading to oxidative stress [26]. Humans now live in a sea of free radicals with ever increasing exposure to both exogenous and endogenous source of free radicals (electromagnetic radiation, persistence organic pollutants, insecticides, pesticides, high temperature, psychological stress, smoking, alcoholic, sedentary life style, varicocele, infection and inflammation). Majority of these causes of oxidative stress are preventable and exposure to them can be minimized. As sperm has a very basic DNA repair mechanism and low levels of DNA damage detection mechanism prevention of oxidative stress may be better than curative measures as the effect of antioxidants is highly variable. In a study from our laboratory we found only slight significant improvement in sperm motility with no effect on DNA integrity and total antioxidant capacity after adyzoa administration [27]. We have also analyzed that lifestyle interventions like yoga and meditation are actually therapeutic and showed a significant decline in blood free radical levels, levels of inflammatory markers and cortisol and significant increase in levels of β endorphins and upregulation in

activity of telomerase enzyme. This study is now being extended analyse effect of yoga and meditation on sperm DNA. Thus oxidative stress to sperm is chief cause of sperm DNA damage and may be the common underlying cause of infertility, cancer, RSA and congenital malformations and adoption of healthy stress free lifestyle may actually improve DNA health.

References

1. Tremellen K (2008) Oxidative stress and male infertility--a clinical perspective. *Hum Reprod Update* 14: 243-258.
2. Zini A, Libman J (2006) Sperm DNA damage: clinical significance in the era of assisted reproduction. *CMAJ* 175: 495-500.
3. Smith R, Kaune H, Parodi D, Madariaga M, Rios R, et al. (2006) Increased sperm DNA damage in patients with varicocele: relationship with seminal oxidative stress. *Hum Reprod* 21: 986-993.
4. Kumar K, Thilagavathi J, Deka D, Dada R (2012) Unexplained early pregnancy loss: role of paternal DNA. *Indian J Med Res* 136: 296-298.
5. Venkatesh S, Shamsi MB, Deka D, Saxena V, Kumar R, et al. (2011) Clinical implications of oxidative stress & sperm DNA damage in normozoospermic infertile men. *Indian J Med Res* 134: 396-398.
6. Saleh RA, Agarwal A, Nelson DR, Nada EA, El-Tonsy MH, et al. (2002) Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study. *Fertil Steril* 78: 313-318.
7. Aitken RJ, Baker MA, De Iulii GN, Nixon B (2010) New insights into sperm physiology and pathology. *Handb Exp Pharmacol* : 99-115.
8. Esteves SC1, Agarwal A (2011) Novel concepts in male infertility. *Int Braz J Urol* 37: 5-15.
9. Aitken RJ, De Iulii GN, Finnie JM, Hedges A, McLachlan RI (2010) Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum Reprod* 25: 2415-2426.
10. Venkatesh S, Riyaz AM, Shamsi MB, Kumar R, Gupta NP, et al. (2009) Clinical significance of reactive oxygen species in semen of infertile Indian men. *Andrologia* 41: 251-256.
11. Aitken RJ, Henkel RR (2011) Sperm cell biology: current perspectives and future prospects. *Asian J Androl* 13: 3-5.
12. Zini A, Bielecki R, Phang D, Zenzes MT (2001) Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril* 75: 674-677.
13. Carrell DT1, Liu L, Peterson CM, Jones KP, Hatasaka HH, et al. (2003) Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch Androl* 49: 49-55.
14. Zini A, Bielecki R, Phang D, Zenzes MT (2001) Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril* 75: 674-677.
15. Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA, et al. (2000) DNA integrity in human spermatozoa: relationships with semen quality. *J Androl* 21: 33-44.
16. Kumar K, Deka D, Singh A, Mitra DK, Vanitha BR, et al. (2012) Predictive value of DNA integrity analysis in idiopathic recurrent pregnancy loss following spontaneous conception. *J Assist Reprod Genet* 29: 861-867.
17. Barratt CL, Aitken RJ, Björndahl L, Carrell DT, de Boer P, et al. (2010) Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications--a position report. *Hum Reprod* 25: 824-838.
18. Tamburrino L, Marchiani S, Montoya M, Elia Marino F, Natali I, et al. (2012) Mechanisms and clinical correlates of sperm DNA damage. *Asian J Androl* 14: 24-31.
19. Lefièvre L, Bedu-Addo K, Conner SJ, Machado-Oliveira GS, Chen Y, et al. (2007) Counting sperm does not add up any more: time for a new equation? *Reproduction* 133: 675-684.

20. Guz J, Gackowski D, Foksinski M, Rozalski R, Zarakowska E, et al. (2013) Comparison of oxidative stress/DNA damage in semen and blood of fertile and infertile men. *PLoS One* 8: e68490.
21. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, et al. (1999) Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod* 14: 1039-1049.
22. Weimann A, Broedbaek K, Henriksen T, Stovgaard ES, Poulsen HE (2012) Assays for urinary biomarkers of oxidatively damaged nucleic acids. *Free Radic Res* 46: 531-540.
23. Venkatesh S, Singh A, Shamsi MB, Thilagavathi J, Kumar R, et al. (2011) Clinical significance of sperm DNA damage threshold value in the assessment of male infertility. *Reprod Sci* 18: 1005-1013.
24. Mishra S, Kumar R, Malhotra N, Dada R (2013) Expression of PARP1 in primary infertility patients and correlation with DNA fragmentation index a pilot study. *Journal of the Anatomical Society of India* 62: 98-104
25. Kumar M, Kumar K, Jain S, Hassan T, Dada R (2013) Novel insights into the genetic and epigenetic paternal contribution to the human embryo. *Clinics (Sao Paulo)* 68 Suppl 1: 5-14.
26. Thilagavathi J, Venkatesh S, Dada R (2013) Telomere length in reproduction *Andrologia* 45: 289-304.
27. Kumar R, Saxena V, Shamsi MB, Venkatesh S, Dada R (2011) Herbo-mineral supplementation in men with idiopathic oligoasthenoteratospermia : A double blind randomized placebo-controlled trial. *Indian J Urol* 27: 357-362.