

RESEARCH ARTICLE

Oxidative Stress Induced Damage to Paternal Genome and Impact of Meditation and Yoga - Can it Reduce Incidence of Childhood Cancer?

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Abstract

Background: Sperm DNA damage is underlying aetiology of poor implantation and pregnancy rates but also affects health of offspring and may also result in de novo mutations in germ line and post fertilization. This may result in complex diseases, polygenic disorders and childhood cancers. Childhood cancer like retinoblastoma (RB) is more prevalent in developing countries and the incidence of RB has increased more than three fold in India in the last decade. Recent studies have documented increased incidence of cancers in children born to fathers who consume alcohol in excess and tobacco or who were conceived by assisted conception. The aetiology of childhood cancer and increased disease burden in these children is linked to oxidative stress (OS) and oxidative DNA damage (ODD) in sperm of their fathers. Though several antioxidants are in use to combat oxidative stress, the effect of majority of these formulations on DNA is not known. Yoga and meditation cause significant decline in OS and ODD and aid in regulating OS levels such that reactive oxygen species mediated signal transduction, gene expression and several other physiological functions are not disrupted. Thus, this study aimed to analyze sperm ODD as a possible etiological factor in childhood cancer and role of simple life style interventions like yoga and meditation in significantly decreasing seminal oxidative stress and oxidative DNA damage and thereby decreasing incidence of childhood cancers. **Materials and Methods:** A total of 131 fathers of children with RB (non-familial sporadic heritable) and 50 controls (fathers of healthy children) were recruited at a tertiary center in India. Sperm parameters as per WHO 2010 guidelines and reactive oxygen species (ROS), DNA fragmentation index (DFI), 8-hydroxy-2'-deoxy guanosine (8-OHdG) and telomere length were estimated at day 0, and after 3 and 6 months of intervention. We also examined the compliance with yoga and meditation practice and smoking status at each follow-up. **Results:** The seminal mean ROS levels ($p < 0.05$), sperm DFI ($p < 0.001$), 8-OHdG ($p < 0.01$) levels were significantly higher in fathers of children with RB, as compared to controls and the relative mean telomere length in the sperm was shorter. Levels of ROS were significantly reduced in tobacco users ($p < 0.05$) as well as in alcoholics ($p < 0.05$) after intervention. DFI reduced significantly ($p < 0.05$) after 6 months of yoga and meditation practice in all groups. The levels of oxidative DNA damage marker 8-OHdG were reduced significantly after 3 months ($p < 0.05$) and 6 months ($p < 0.05$) of practice. **Conclusions:** Our results suggest that OS and ODD DNA may contribute to the development of childhood cancer. This may be due to accumulation of oxidized mutagenic base 8OHdG, and elevated MDA levels which results in MDA dimers which are also mutagenic, aberrant methylation pattern, altered gene expression which affect cell proliferation and survival through activation of transcription factors. Increased mt DNA mutations and aberrant repair of mt and nuclear DNA due to highly truncated DNA repair mechanisms all contribute to sperm genome hypermutability and persistent oxidative DNA damage. Oxidative stress is also associated with genome wide hypomethylation, telomere shortening and mitochondrial dysfunction leading to genome hypermutability and instability. To the best of our knowledge, this is the first study to report decline in OS and ODD and improvement in sperm DNA integrity following adoption of meditation and yoga based life style modification. This may reduce disease burden in next generation and reduce incidence of childhood cancers.

Keywords: Childhood cancer - sperm DNA damage - telomeres - DNA fragmentation index

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Introduction

Retinoblastoma (RB) is the most common intraocular malignancy in children and is reported to affect 1 in 15,000

to 1 in 18,000 live births (Purkayastha et al., 2016). It represents almost 4% of all pediatric malignancies. RB is caused by mutations in tumor suppressor gene on 13q14 RB1 (MIM 180200). Studies from India show

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a two- to three-fold higher incidence of tumors of the eye (majority of which are RB) in children. Sixty-nine percent of the cases are unilateral and 30.5% bilateral; familial cases are usually bilateral, while sporadic cases are usually unilateral. RB arises retinal cells that have lost the function of both alleles of RB1 (Purkayastha et al., 2016). Children born inheriting a mutation in the RB1 gene have a 95% chance of developing RB and other tumors like sarcoma, osteosarcoma and leukemia (Ward et al., 2014). In cases with unaffected parents a de novo germ line mutation, may be the cause of non-familial sporadic heritable RB. Recent studies have documented increased incidence of childhood cancers in children born to older fathers and men who smoke (Kumar et al., 2015). An increase in incidence of cancer has also been reported in children conceived by assisted technology (ART) (Aitken et al., 2014). This prompted us to undertake this study to evaluate seminal oxidative stress and sperm DNA damage in fathers of such children. Previous studies from our lab have shown that seminal oxidative stress is associated with damage to both nuclear and mt genome (Shamsi et al., 2010), shorter telomeres (Thilagavathy 2013a,b; Shamsi, 2012;) aberrant expression of various genes (Kumar et al., 2012), low total antioxidant capacity and reduced DNA damage and detection mechanisms in men with high DNA fragmentation index. (Mishra et al., 2016). Single gene mutations increase with paternal age and about 75% new mutations occur in the male germ line; it is also reported that an additional two mutations are found in male germ line with each advancing year of paternal age (Forster et al., 2015). Hence majority of mutations can be attributed to the male germ line and tend to accumulate at a much higher rate, if DNA is damaged. (Kumar et al., 2015) showed that sporadic RB usually affected the last born child when the fathers age was above 35 years. This may be due to accumulation of mutations in both mt and nuclear DNA due to oxidative stress and replication errors. Sperm with its limited DNA damage repair and detection system accumulates mutations. This is also increased due to presence of accumulation of oxidized base (8-OHdG) and elevated MDA levels which result in MDA MDA dimers which are mutagenic. This results in denovo germ line mutations or if this not repaired by oocyte due to inefficient or aberrant repair it results in denovo mutations post fertilization. Thus, sperm DNA damage whether due to endogenous or exogenous causes may lead to childhood diseases and is believed to be cause of several autosomal dominant disorders, complex neuropsychiatric disorders, increase disease burden in children and even childhood cancers (Wiener-Megnazi et al., 2012; Aitken et al., 2014). Sperm DNA damage arises due to persistence of nicks following meiosis, abortive apoptosis due to dysfunctional mitochondria, defective protamination, due to altered P1/P2 ratio and mutations in genes coding for protamines8. Oxidative stress is the major cause of DNA damage. Previous studies from our laboratory have documented that acute stress is associated with elevated levels of enzymatic and nonenzymatic antioxidants however chronic severe oxidative stress is associated with low total antioxidant capacity and disturbs the equilibrium between free radical production and scavenging (Shamsi

MB et al., 2010). This may be perturbed by a number of adverse internal and external environmental factors namely presence of genitourinary or systemic infection, inflammation, varicocele, immature and morphologically abnormal sperm in ejaculate, exposure to xenobiotics, endocrine disrupting chemicals, insecticides, pesticides, electromagnetic radiation, high temperature, heavy metals, iatrogenic, psychological stress, poor diet deficient in fruits and vegetables, smoking, excess alcohol consumption, obesity, sedentary lifestyle, depression and advanced paternal age (Tremellen, 2008; Kumar et al., 2015).

Oxidative stress targets the guardian of our genome, the telomeres which consist of repetitive guanine rich sequences and associated proteins that cap the ends of linear chromosomes to maintain chromosomal stability and genomic integrity. Notably, telomeres erode rapidly due to oxidative stress, suggesting that they are highly sensitive to oxidative damage (Thilagavathy et al., 2013a,b; Mishra et al., 2016). Telomere dysfunction is associated with chromosome instability and apoptosis, leading to carcinogenesis or aging and their shortening leads to chromosomal instability and loss of genomic integrity.

It is important to apply an appropriate biomarker of oxidative stress, the most reliable methodology to evaluate oxidative stress includes measuring of oxidative DNA damage, which can be assessed by determination of 8-hydroxy-2'-deoxyguanosine level (mutagenic by product of oxidative damage) in cellular DNA. Therefore, various parameters, such as 8-hydroxy-2'-deoxyguanosine, ROS, sperm chromatin structure assay (SCSA) and telomere length were analyzed in fathers of children with non-familial sporadic RB and compared with age-matched controls in order to find out a possible association between loss of sperm DNA integrity (by quantifying oxidative DNA damage) and non-familial sporadic heritable RB.

Cigarette smoking and tobacco use causes up to 30% of all cancer deaths (World Health Organization, 1997). The carcinogenic vapor phase compounds in puff of cigarette include nitrogen oxides, benzene, lead, and cadmium. (Hoffmann et al., 2001). The particulate phase contains many carcinogens including polycyclic aromatic hydrocarbons, N-nitrosamines, aromatic amines, and metals (Hoffmann et al., 2011).

Also, cigarette smoking has been reported to be associated with reduced sperm density and motility and increased percentage of abnormal forms of sperm, although these findings have not been consistent (Liu et al., 2010). Some study also highlighted that the smoking is another source of oxidative stress that causes tissues to mimic a state of inflammation and also leads to reduced antioxidant levels (Pham-Huy et al., 2008). Use of alcohol increases the urge to smoke (Businelle, 2013). In addition smoking results in higher cadmium levels which results in lower level of OGG1 the only BER enzyme which remains active in sperm. Thus smoking not only results in accumulation of various cyto and genotoxic chemicals in the cell but also downregulates the BER machinery in the sperm and thus accumulation of the oxidized DNA adduct 8OH2DG. Sperm is dependant on oocyte for completely

the repair of abasic sit created by OGGI as it lacks APE and XRCC1 thus it has to work with oocyte to repair its DNA lesions however maternal aging and suboptimal quality of oocytes with inefficient and aberrant DNA repair mechanism results in persistence of these mutagenic bases and increases susceptibility to denovo mutations post fertilization and increase disease burden in children and even childhood cancer. Oxidative stress can result in carcinogenesis by not only increasing mt and nuclear genome hypermutability, but also by causing aberrant DNA methylation, and modifying expression of genes involved in cellular proliferation, survival and apoptosis and enhanced expression of cyclins and cyclin dependant kinases and resulting in accelerated attrition of telomeres (Thilagavathy et al., 2013a,b). Free radicals also function as second messengers involved in activation of NFkB following binding of TNF to its receptor mediated by hydrogen peroxide. Supraphysiological ROS levels also cause lipid peroxidation and build up of electrophilic aldehydes like 4HNE, acrolein and MDA (Aitken et al., 2014). MDA form dimers which are mutagenic and induce mutations in tumour suppressors and oncogenes and also lead to loss of cekk cycle control and altered gene expression and in addition products of lipid peroxidation bind to electrophilic proteins of ETC like SDH and cause leakage of electrons and mt dysfunction. Such mt produce more free radicals and results in a vicious cycle resulting in more mt DNA damage and fragmentation and such mt DNA fragments can insert into nuclear DNA and cause activation of oncogenes. Oxidative stress also disrupt assembly of gap junction proteins and decrease cell to cell communication. And thus a host of factors cause oxidative stress which can disrupt/modify cellular homeostasis, mt and nuclear DNA, signal transduction, gene transcription and sperm epigenome and thus affect lifelong health of offspring. Thus it is pertinent to improve our lifestyle by quitting smoking and avoiding drinking and adopt a lifestyle which results in lower systemic and testicular free radical levels. Though there are several antioxidants available and may cause significant improvement in standard semen parameters but their impact of DNA damage is still controversial and whether at therapeutic doasage they impact nuvlear DNA integrity. On the other hand indiscriminate usage of antioxidants can disrupt several redox sensitive physiological processes .

Therefore, there is an urgent need to address the issues like smoking and drinking which is life style habit of over 1 million people all over the world. Some study demonstrated that meditation and yoga may improve cognitive deliberation which is needed to make effective choices and avoid smoking in tempting situations. (Bock, 2014) and improve quality of life in stressed and depressed individuals (Dada et al., 2016; Mohanty et al., 2016). A brief, 12week yoga based lifestyle intervention for infertile men with MDD showed significant improvements in basic semen parameters based on WHO 2010 criteria by reduction in both oxidative and psychological stress. Our results showed significant increase in sperm count and progressive sperm motility (Type A+B) in 80% patients after intervention as a primary outcome. Secondary outcomes included improvement in

quality of life (WHOQOL-BREF scale) and reduction in depression severity (Beck Depression Inventory-II (BDI-II)) (Tolahunase et al., 2016). Owing to the ability of Yoga and meditation to address both mind and body, it works through a well-defined psychoneuro-endocrine pathway which then affects a wide range of processes from basic metabolism, epigenetics, DNA repair, oxidative bioprocesses to aging, blood pressure, organ system maintenance, subjective well-being, and reproductive health. Therefore, the main aim of present study was to evaluate impact of lifestyle on sperm molecular factors and examine the impact of yoga and meditation on seminal oxidative stress and oxidative damage to sperm DNA.

Materials and Methods

The study was performed on a cohort of fathers of RB affected children who presented to the RB Clinic of AIIMS, NDelhi at Dr. R.P. Centre for Ophthalmic Sciences between October, 2012 and March, 2014. Of 131 children that were recruited, 126 (96%) children were diagnosed with unilateral and 5 (4%) children with bilateral RB. Out of 131 fathers, 53 declined to take part in the study 9 were unable to ejaculate and 3 were excluded due to somatic mutation in RB gene in their peripheral blood; 10 men were excluded due to previous history of vasectomy, varicocele and diabetes. Thus, a total of 56 men (43% of eligible subjects) were included for the study. Both parents had normal karyotype. Control group comprised of 50 age-matched men with a healthy child born in the last one year. Written informed consent was obtained from both study groups.

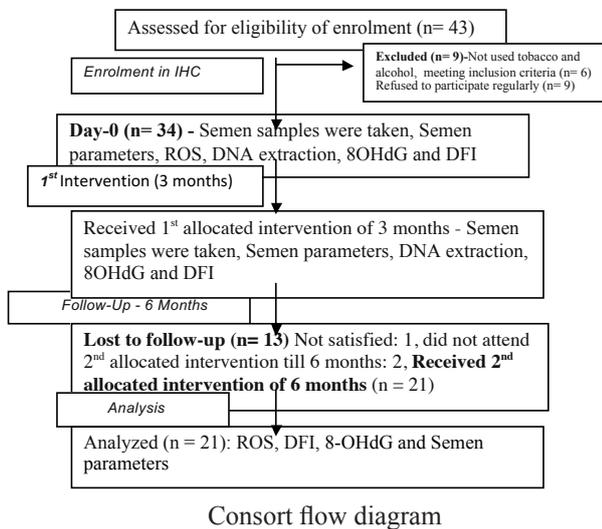
Parents were interviewed independently in person. A structured questionnaire was used to obtain information on demographic characteristics, birth-related factors, familial and genetic factors, as well as on maternal and paternal medications, occupation and other environmental and lifestyle factors, such as smoking and alcohol intake prior to and following the conception of the index child was collected. Other confounding factors (varicocele, history of antioxidant intake etc.) that could affect DNA damage levels were ruled out.

After approval from ethical board of the institute (IESC/T-364), we recruited 43 men reported to the All India Institute of Medical Sciences, New Delhi, India for our study. The informed consent was obtained by each participant.

All participants were provided with a detailed description of the study. Inclusion criteria required that participants be 25 to 45 years old, must have complete one pack year of cigarette smoking (Moizs, 2013) 30, and/or reported 1 or more alcohol "binges" per month. (Davis et al., 2013). A binge was defined as 5 or more drinks each day (Davis et al., 2013). Participants were also excluded if they self-reported a diagnosis of schizophrenia, bipolar or delusional disorder.

The design of interventions were published previously in detail by our group (Yadav et al., 2012). But in short, the intervention program lasted for 2 hours each day and that was for 6 months, comprising theory and practice sessions. Generally, the program starts with an array

of asanas consists of various postures and pranayama which consists of typical breathing exercise. Estimation of all programmed biochemical markers were done in fasting venous blood samples. Blood samples (5mL) were collected in heparin coated vials and centrifuged at 2000g for 15 minutes and plasma was preserved at -80°C. The biochemical markers were assayed by commercial ELISA kits. We validated and pre tested the quality-control for the biochemical markers and the methods used.



Results

The mean age of fathers of children with sporadic RB was 33.17±11.2 yrs as compared to 32.5±4.5 years in the controls. The mean age of RB affected children was 1.6±0.4yrs and of healthy children 1.41±0.75 yrs. The semen parameters such as liquefaction time, pH, viscosity, sperm count and motility in these individuals were within the reference range and were comparable with controls.

ROS

Seminal mean ROS levels were 1.5-times higher (36.086±1.83 vs. 20.51±2.71 RLU/s/million; p>0.01) in fathers of RB children, compared to controls (Table 1). The area under the curve (Figure 2c) was 78% (P<0.01; 95% CI, 0.715 to 0.912), with 64.1% sensitivity and 65.5% specificity (Figure 4). From ROC curve analysis, ROS level of 27.25 RLU/s/million sperm was set as the threshold value to differentiate between fathers of children with RB and controls. Among the cases, 67.925% had high ROS levels above the threshold (27.25 RLU/s/million). This highlights significantly elevated ROS levels in father of RB cases.

Table 1. Comparison of the laboratory parameters between the study groups

Parameters	Controls (n=50)	Cases (n=56)	p-Value*	Cutoff Value	Sensitivity (%)	Specificity (%)	AUC*	p Value*
ROS (RLU/s/million)	20.51±2.71	36.086±1.83	<0.01	27.25	64.1	65.1	78	<0.01
DFI (%)	21.9±9.4	31.50±6.67	<0.01	28	64.1	62.1	74	<0.01
8-OHdG (pg/mL)	23.10±2.71	66.02±2.91	<0.01	33.25	66.7	65.5	74	<0.01
T/S	0.38±0.027	0.35±0.021	>0.01	-	-	-	-	-

*AUC - Area under the curve, ROS- Reactive oxygen species, DFI- DNA fragmentation index, 8-OHdG- 8-hydroxy-2'-deoxy guanosine, T/S- Telomere to single copy gene ratio, * P-value <0.05 was considered to be significant

DFI

There was a significant difference in mean DFI levels (31.50 ± 6.67 vs. 21.9 ± 9.4; p<0.01) in fathers of children with RB, as compared to controls (Table 1). The area under the curve was 74% (P<0.01; 95 % CI, 0.715 to 0.912), with 62% sensitivity and 64.1% specificity. From ROC curve (Figure 2a) analysis, DFI of 28% was set as the threshold

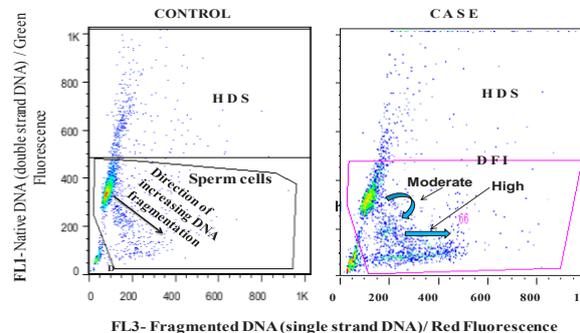


Figure 1. Dot Plot Cytograms of Control and Case Semen Samples Respectively by SCSA. X-axis (FL3) represents fragmented DNA and Y-axis (FL1) represents native DNA showing the percentage of spermatozoa with high levels of DNA fragmentation (DFI%), and high DNA stainability showing the percentage of spermatozoa with high DNA stainability (%HDS). Each cytogram dot represents a single spermatozoon with dual-parameter green and red fluorescence values. The debris (bottom-left corner) was excluded from the analysis.

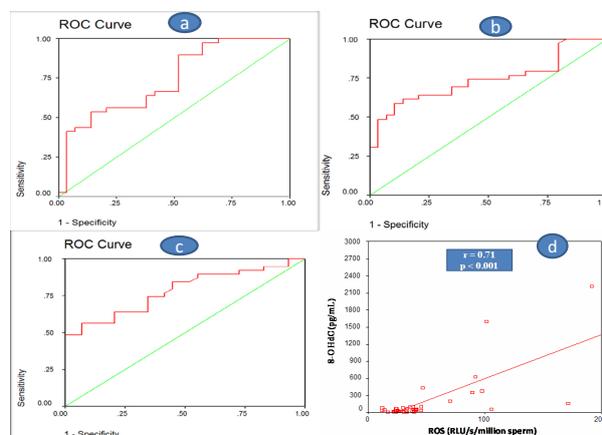
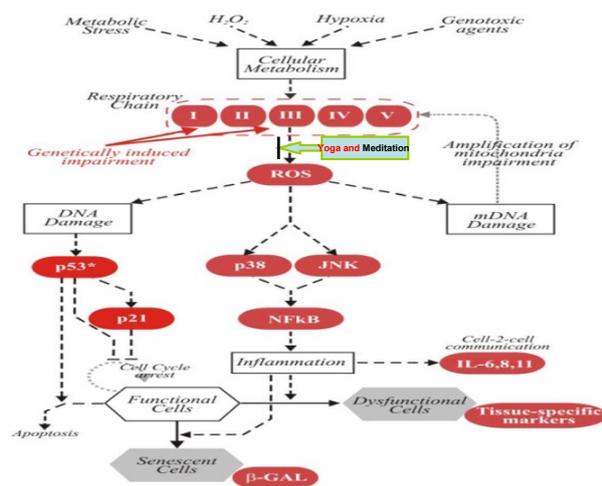


Figure 2. ROC curve for (a)DFI [Sensitivity- 64.1%; Specificity- 62.1%; AUC= 74%; p=0.001], (b)8-OHdG [Sensitivity- 66.7%; Specificity- 65.5%; AUC= 74%; p =0.001], (c) ROS [Sensitivity- 64.15%; Specificity- 65.1%; AUC= 78%; p<0.001] and (d) correlation between levels of 8-OHdG in sperm DNA and seminal ROS in father of children with RB

Table 2. Impact of life style intervention on quantitative levels of various experimental parameters in tobacco users and alcoholics at base level (day 0), after

Experimental Parameters	ROS (RLU/sec/million sperm)					DFI (%)					8-OHdG (pg/mL)				
	Life Style Habits	0 Day	3 Months	6 Months	P-Value (0 Vs 3 Months)	p -Value (0 Vs 6 Months)	0 Day	3 Months	6 Months	P-Value (0 Vs 3 Months)	p -Value (0 Vs 6 Months)	0 Day	3 Months	6 Months	P-Value (0 Vs 3 Months)
Alcoholics (n=6)	35.51 ± 1.87	29.11 ± 1.90	23.81 ± 3.16	>0.05	<0.05	31.74 ± 6.72	30.09 ± 2.11	21.19 ± 2.93	>0.05	<0.05	54.05 ± 4.43	44.61 ± 1.16	34.11 ± 2.59	<0.05	<0.05
Tobacco Users (n=8)	38.20 ± 1.83	31.51 ± 1.97	26.02 ± 2.11	<0.05	<0.05	34.50 ± 6.67	28.09 ± 3.17	25.19 ± 2.93	<0.05	<0.05	66.02 ± 2.91	49.61 ± 1.89	39.48 ± 2.66	<0.05	<0.05
Alcoholics+ Tobacco users (n=7)	43.11 ± 7.23	31.48 ± 4.16	23.13 ± 2.48	<0.05	<0.05	39.57 ± 3.91	33.30 ± 5.30	26.19 ± 2.93	>0.05	<0.05	178.59 ± 19.78	89.67 ± 13.55	36.99 ± 8.51	<0.05	<0.05

**Figure 3. Impact of Various Cellular Stressors on Cellular Systems and Possible Mechanisms of Action of Yoga and Meditation**

value to differentiate between fathers of affected children and those of controls 73.28% of fathers of children with RB had higher DFI than the cut-off value (28%). This highlights that fathers of RB cases had higher levels of DNA damage as compared to controls.

8-OHdG in sperm DNA isolates

The levels of 8-OHdG were significantly higher in fathers of children with RB (66.02 ± 2.91 vs. 23.10 ± 2.71 pg/mL; $p < 0.01$) as compared to controls (Table 1). The area under the curve was 74 % ($P < 0.01$; 95 % CI, 0.715 to 0.912), with 65.5% sensitivity and 66.7 % specificity. Using ROC curve (Figure 2b) analysis, a threshold value of 33.25 pg/mL was obtained to discriminate from the control group. We observed that 69% of fathers of children with RB had higher value 8-OHdG than cut off levels (33.25 pg/mL). This emphasizes that majority of DNA damage in sperm was oxidative in nature

8-OHdG in plasma of affected child and non affected Siblings

8-OHdG plasma levels were found to be high in RB patients (mean= 7579 ± 858 pg/ml) as compare to their siblings (mean= 6479 ± 678 pg/ml).

Sperm telomere length (T/S)

Mean relative sperm telomere length (T/S) of fathers of children with RB was shorter as compared to controls (0.35 ± 0.021 vs. 0.38 ± 0.027 ; $p > 0.01$) though the difference

was not significant (Table1). The correlation of 8-OHdG level with seminal ROS in fathers of children with RB and controls was found to be highly significant among fathers of children with RB ($r = 0.71$, $p < 0.01$) (Figure 5), however, in the control group, although the correlation was positive, but it was not significant ($r = 0.086$, $p > 0.01$).

Impact of Interventions on various experimental markers

The study included 30 participants (32.38 ± 7.19 yrs). We divided the participants in 4 groups, in 1st group we included those men who never used tobacco or alcohol. Second group consists of men who used only tobacco (no alcohol). Similarly, in 3rd group only men who consumed excess alcohol more than 5 drinks at one time per day were included while in 4th group we included men who used both (i.e tobacco and alcohol) (consort flow diagram). The baseline characteristics are presented in Table 2. There was a significant reduction in oxidative stress markers such as ROS, DFI and 8-OHdG after successive interventions.

Discussion

Following sex determination, oogenesis begins with the formation of primary oocytes and the production of female gametes. Oogenesis occurs when the mother herself is in utero. The oocytes are in a mitotically inactive state throughout life and only undergo further (meiotic) cell division prior to ovulation (Von Stetina and Orr-Weaver, 2011). This is in marked contrast to the production of male gametes (spermatogenesis), which are produced continuously after puberty and undergo several cycles of premeiotic replication and is a source of replication errors and thus a source of mutation. Also mutant spermatogonial stem cells have a proliferation advantage over non mutant cells. Mutations especially single nucleotide substitutions accumulate at a much higher rate, when there is DNA damage and about 75% new mutations occur in male germ line and it is reported that 2 mutations are added with each year of advancing paternal age (Kong et al., 2012). Recent report suggests increased incidence of autosomal dominant disorders, complex polygenic conditions (schizophrenia, autism, epilepsy, bipolar disorder), cleft palate, cardiovascular malformations, diaphragmatic hernia in children born to older fathers (Aitken et al., 1998; Crow 2000; Aitken et al., 2003; Aitken et al., 2014, Kumar et al., 2015). In this study also it was usually the last born child in the family who developed cancer and was associated with advanced age of father (19 fathers > 35 years). Advanced paternal age

is associated with increased mutational load in children. This is due to seminal oxidative stress and mt dysfunction associated with aging and imperfect, inefficient and incomplete repair by oocyte which results in *de novo* mutations post fertilization and once the mutational load crosses a threshold level it increases disease burden in children. Children conceived following assisted reproductive technology also have a higher incidence of cancers and birth defects and is usually associated with paternal sperm DNA damage (Aitken et al., 2014; Kumar et al., 2015). Sperm, is a transcriptionally and translationally inert cell and has a limited DNA damage detection and repair mechanism and Sperm exist in a state of oxygen paradox as they require oxygen for ATP production but are also exposed to high ROS levels which damage both mitochondrial and nuclear DNA (Callaway, 2012). Dysfunctional mitochondria produce higher free radical levels and further damage both the mitochondrial and nuclear genome and preferentially target guanine rich telomeres which lie in sperm nuclear periphery. Oxidative stress results in accelerated attrition of telomeres and make genome unstable and prone to aberrant recombination events (Lee and Hsiao, 1996; Thialgavathy et al., 2013a,b). Thus, accumulation of dysfunctional mitochondria, short telomeres, increased levels of mutagenic base (8OHdG), MDA dimers, lipid aldehyde adducts binding to proteins of ETC, leading to excess leakage of electrons and free radical production and single and double strand breaks in nuclear DNA, dysregulated transcripts and altered gene expression and signal transduction secondary to oxidative stress make the sperm genome vulnerable to mutations and epigenetic errors. Oxidative stress also results in aberrant methylation and result in hypermethylation of tumor suppressor gene and genome wide hypomethylation may be underlying mechanism of increased incidence of childhood morbidity and cancers (Lee and Hsiao, 1996). Presence of 8 hydroxy 2 deoxyguanosine not only is mutagenic but it also impacts sperm epigenome by preventing methylation of cytosine in CpG islands and thus results in genome wide hypomethylation and unmasking of repetitive elements and dysregulation of sperm transcripts which results in genomic instability and may also predispose to cancer. This may also be the reason infertile men are predisposed to both gonadal and extragonadal tumours and infertility is believed to be an early marker of cancer and a higher incidence of cancers and autosomal dominant and complex polygenic disorders are reported in children conceived by assisted conception in male factor infertility. Higher levels of 8OHdG were detected in plasma of children with Retinoblastoma as compared to non affected siblings. There is considerable evidence that oxidative stress and oxidative DNA damage plays a major role in the etiology of various types of congenital abnormalities (Keith T. Jones, 2008). The abnormal spermatozoa frequently display typical features of oxidative stress i.e. excessive level of ROS and depleted antioxidant capacity (Guz et al., 2013; Shamsi et al., 2010). Moreover, we have reported in previous studies that a high level of oxidatively damaged sperm DNA is associated with infertility, pre and post implantation losses, congenital malformations and

complex neuropsychiatric disorders (Dada et al., 2016). Despite significant progress in the last decade regarding a possible role of oxidative stress in the etiology of infertility, implantation failure, congenital malformations its role in various childhood cancers has not been addressed. Though a slight increase in cancers in children conceived via ART is reported in infertile couples with male factor infertility (Aitken et al., 2010; Syed et al., 2011). About 80 percent men with idiopathic infertility have defective sperm function due to oxidative DNA damage. We believe that poor sperm DNA quality could be a possible etiological factor for non-familial RB. In this study, we included only those cases where both partners were cytogenetically normal and had no mutation in RB gene and the child had no family history of RB. The mothers were normal on clinical and gynecological examination and also did not have any somatic RB gene mutation and the fathers had normal semen parameters. Since neither parent had RB gene mutation in the blood, the RB mutation was either in the sperm or oocyte DNA or arose early in embryonic development. Once spermatogenic cell division is completed, maturation of the spermatid into the spermatozoon involves condensation of the DNA and cessation of transcriptional activity (Hargreave et al., 2013). Thus, any damaged residues remaining in the sperm DNA are delivered to the oocyte upon fertilization, which may increase the possibility of mutations when the zygote divides. Based on these facts, we propose that *de novo* germline mutations and mutations post fertilization and dysregulation in sperm transcripts and shorter telomeres contribute to genome hypermutability and may contribute to cancer in children. DFI levels were significantly higher in sperm of fathers of children with RB, as compared to the controls since 83% of cases had higher DFI than the cut-off (>28.00%) and a 89% of cases had higher ROS than the cut-off. Although the threshold for sperm DFI in infertile men has been established (Venkatesh et al., 2011; Kumar et al., 2012), the role of sperm factor in early childhood diseases and cancers has not been extensively evaluated and DFI cut-off, 8-OHdG threshold, ROS values have not been established.

Our results suggested that elevated 8-OHdG levels and increased DFI in sperm DNA might be associated with RB, since fathers of children with RB had a statistically significant elevated level of this mutagenic base, as compared to the control group. This confirmed the hypothesis that oxidative stress as one of the major causes of sperm DNA damage (Mishra et al., 2013). In a recent study from our laboratory (Mishra et al., 2014; Mishra et al., 2016), we documented that damage in sperm DNA is mainly oxidative and mutations accumulate due to deficient DNA detection (lower PARP levels) and DNA repair mechanisms (lower OGG1 levels) (Sweta et al., 2014). Sperm has only BER mechanism but lacks APE and XRCC and thus needs to work in consort with the oocyte to remove the damaged DNA. However with advanced parental age and associated genomic fatigue with suboptimal repair capacity in aging oocyte may not completely remove the DNA lesions and their persistence is associated with increased risk of first and second hit in RB gene.

To summarize, to the best of our knowledge, this is the first study to assess the effects of oxidative DNA damage load by estimating ROS, DFI, 8-OHdG and telomere length in sperm of fathers of children with non-familial sporadic RB. Studies in our lab are ongoing to determine if oxidative stress is associated with aberrant methylation and if hypomethylation of oncogenes and hypermethylation of tumor suppressor like RB may be the cause of increased incidence of childhood cancers. Recent study has reported that amplification of MYC oncogene is associated with highly aggressive RB with an earlier age of onset (Astudillo et al., 2014). Oxidative stress results in microsatellite instability and also predisposes to dynamic mutations but the role of oxidative stress in MYC amplification need to be evaluated. Although, genetic causes of RB in children are irreversible, sperm oxidative DNA damage can be minimized by simple life style modifications. If high DNA damage is detected, counseling to such parents to delay the second child till seminal free radical levels and DNA damage decline significantly may prevent retinoblastoma and other cancers in offspring (siblings). This study highlights that oxidative damage to sperm DNA may be causal in childhood cancers but further studies on larger sample size are required. This study also highlights that biological parenting begins much before the birth of a child even before conception and the need to maintain a healthy lifestyle even before one plans to have a child as sperm tends to accumulate genetic aberrations and epigenetic changes right from the father himself is conceived.

Impact of yoga and meditation and life style habits

There is wide acceptance for meditation and yoga-based life style to manage stress, which is a major cause for tobacco use and binge drinking (Khasky and Smith, 1999). Research on the neurobiology of substance abuse disorders indicates that chronic drug use is associated with deregulated prefrontal-dependent cognitive control, which may impact affect and inhibitory pathways (Kravitz, 2015). Neuroimaging data suggest mindfulness practice has the capacity to modify and enhance mechanisms of cognitive control of automatic behaviors (also known as top-down neural activity) which could help reduce reactivity towards nicotine and, consequently, aid smoking cessation (Farb et al., 2007; Allen et al., 2012).

There is urgent need for alternative treatments which underscore the need for smoking/drinking cessation strategies. Mind-body practices are a good candidate (Bock, 2012). Tobacco use and binge drinking elevate the levels of ROS and oxidative mutagenic base 8OHdG (Kumar et al., 2015). Cadmium levels are higher in smokers and cause downregulation in expression levels of OGG1 and are associated with persistence of higher levels of oxidized mutagenic adducts.

Intriguingly enough, as we have also reported in our previous study (Kumar et al., 2015), the results of this study too confirmed that DNA fragmentation was significantly higher in smokers Vs nonsmokers and drinkers Vs non drinkers. With practice of meditation and yoga, DNA fragmentation index reduced gradually over a period of 6 months of intervention, along with a

rapid decrease in seminal ROS and 8OHdG Reduction in DFI also indicates an improvement in fertility, as DFI is considered a good indicator for the same. This finding is highly relevant because though genetic causes are irreversible but oxidative DNA damage can be minimized by adopting healthy lifestyle modification including yoga and meditation and minimizing or stopping substance abuse. Also, oxidative stress has been implicated as the root cause of damage by reproductive toxicants in deterioration of semen quality as well as inducing oxidative and DNA damage in sperm. (Aitkin et al., 2014). However, we have also documented that yoga and meditation can be beneficial in reduction in anxiety, BMI, and improving quality of life and severity of depression associated with these disorders. (Kumar et al., 2015; Dada et al., 2016; Mohanty, 2016). Though several antioxidants are used to manage oxidative stress and may result in improvement of sperm membrane permeability and fluidity their effect on DNA is not known and thus though the sperm motility and oocyte binding capacity may improve but it may actually result in transmission of sperm with damaged DNA at the time of fertilization. Microarray studies from our lab (outside per view of this paper) have shown that expression levels of antioxidant genes, PARP1 and OGG1 increase and there is upregulation in telomerase activity following short term meditation yoga intervention. In addition there was decreased expression levels of proinflammatory and proapoptotic genes and upregulation of genes involved in cellular repair and anti-inflammatory genes. In addition Yoga and meditation also improves cardiorespiratory tone and increases perfusion to hippocampus, cingulate gyrus and cerebral cortex and reduced levels of cortisol which further aids in reduction of oxidative stress (Mittal et al., 2015; Dadat et al., 2016; Mohanty et al., 2016).

This is a clinically important finding since men having children with retinoblastoma might have an ongoing oxidative stress damaging the sperm DNA, and hence at an increased risk of having subsequent children with imprinting disorders and other health issues. Controlling or reducing sperm oxidative DNA damage through yoga and meditation hence might not only preserve their fertility but also reduce the risk of imprinting disorders such as retinoblastoma as oxidative stress is associated aberrant methylation.

Overall, the results of this study show that yoga and meditation-based lifestyle intervention might result in a regulating free radical levels and reducing oxidative DNA damage and improving sperm DNA integrity. It also normalises the levels of sperm transcripts and aids in maintenance of genomic stability through maintenance of telomere length and increased expression of genes involved in DNA repair, down regulation of proinflammatory genes and upregulation in telomerase activity. Practice of yoga and meditation could also be the key to healthy senescence (especially delay testicular aging) as it might have a buffering effect on the smoking and drinking induced oxidative stress, oxidative DNA damage and DNA fragmentation index. Oxidative stress, DNA damage and shorter telomeres are 3 cardinal marks of aging and thus yoga and meditation reverses all these 3 parameters and thus though we cannot alter our biological

age we can definitely slow the rate at which we age (Dada et al., 2015; Kumar et al., 2015b). This study paves the road map for designing more robust studies to assess these variables. To the best of our knowledge, this is the first study to assess the effect of yoga and meditation in management of seminal oxidative stress and oxidative DNA damage in sperm. This may assist the couples in planning future pregnancy, only when seminal ROS levels normalize and DNA damage decline significantly, thus decreasing childhood disease burden and prevent cancer.

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