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Original Article

Sperm DNA damage in non-familial sporadic heritable retinoblastoma (NFSHRb)

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ABSTRACT

Background/objectives: Loss of sperm DNA integrity hampers accurate transmission of genetic information to offspring, which may lead to childhood morbidity or even cancer. Sperm DNA damage (SDD) is chiefly oxidative in nature. Retinoblastoma (Rb) is the most common childhood cancer. The study was planned with an aim to analyze sperm DNA quality in the fathers of children with non-familial sporadic heritable retinoblastoma (NFSHRb) and determine if SDD may result in *de novo* germline RB1 mutations.

Methods: 40 fathers of children with Rb and 30 controls were enrolled and categorized into 4 groups based on their social habits. Blood of parents and affected child was PCR amplified and sequenced to identify presence of any sequence variation in RB1 gene. Only those cases were enrolled where parents did not have somatic RB1 gene mutations; 20 children were heterozygous for RB1 gene mutation and 6 children did not harbour any somatic mutation. DFI, ROS and 8-OHdG were analyzed.

Results: Semen analysis showed no significant differences in cases and controls. Seminal mean ROS levels and mean DFI levels were significantly higher in cases as compared to controls ($p < 0.005^*$). ROS and DFI levels in smokers and alcohol users were higher as compared to non-smokers and alcohol non-users.

Conclusions: Oxidative SDD may lead to *de novo* germ line mutations and may be the underlying aetiology of NFSHRb. Oxidative damage to sperm DNA was observed in alcohol consumers and smokers and may increase incidence of Rb in this population subset. Thus poor lifestyle and social habits adversely affect paternal genome integrity.

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1. Introduction

Non-familial sporadic heritable retinoblastoma (NFHRb) arises by bi-allelic inactivation of the retinoblastoma tumour suppressor gene (RB1). The retinoblastoma protein (pRb) is a key regulator of cell cycle progression by controlling the G1/S phase transition via its binding to the E2F family of transcription factors and chromatin remodelling proteins.¹

Despite significant research on human sperm DNA integrity over the last decade, our understanding on the mechanisms of sperm DNA damage (SDD) and their effect on reproductive outcome is still incomplete. The structure of sperm chromatin is highly specialized and condensed, which is essential for maintaining genomic integrity, but the sperm nuclear periphery harbours telomeric DNA and promoters of genes of critical developmental importance. This portion of sperm genome is histone bound and less compact as compared to the central protamine-bound fraction and thus susceptible to oxidative stress (OS) and other environmental insults. Thus, OS targets the peripheral part of sperm genome (telomeric DNA), causes the oxidation of guanine repeats in telomeric DNA and leads to transversion, single and double strand breaks, shortening of telomere and making the sperm genome unstable, and is prone to aberrant recombination events and mutations.

Social habits of father, such as nicotine consumption (oral/smoking) and excessive alcohol intake, may lead to systemic and seminal OS, and hence elevated reactive oxygen species (ROS) levels cause oxidative DNA damage to sperm. As a consequence of DNA damage, there is accumulation of mutagenic oxidized bases due to deficient DNA repair mechanism, sperm accumulates mutations at a much higher rate than the oocyte. A high mutagenic load may overburden the oocyte repair mechanism post-fertilization and result in persistence of DNA damage/mutation in the zygote genome.² This may increase the incidence of cancers and other congenital anomalies in the offspring³ and may be a possible etiological factor in NFHRb. In this study, we included only those cases where parents had no somatic mutation in RB gene and thus the mutation arose *de novo* in germline or was a post-zygotic event due to presence of highly mutagenic 8-hydroxy-2'-deoxyguanosine (8-OHdG). Accumulation of DNA damage secondary to unhealthy social habits highlights the role of lifestyle and its impact on genomic integrity, which impacts lifelong health of offspring.

2. Methods

2.1. Study design and subjects

Of 40 cases, i.e. fathers of children with Rb that were recruited from Retinoblastoma clinic of Dr. Rajendra Prasad Centre for Ophthalmic Sciences, AIIMS, New Delhi, only 26 fathers, who met the inclusion criteria, were included in the study. Parents with somatic mutation in RB1 gene were excluded ($n = 14$). Control group consisted of 30 age-matched men with a healthy child. Written informed consent was obtained from both cases and controls. The study was approved by Institutional Ethics Committee (IESC/T-80/28.02.2014).

A structured questionnaire was used to obtain information on demographic characteristics, birth-related factors, childhood medications, residential history and familial and genetic factors, as well as on maternal and paternal medications, occupation and other environmental and lifestyle factors. Fathers were asked about their smoking habit, tobacco consumption and alcohol use, specifically the number of cigarettes usually smoked per day, tobacco usage and alcohol consumption over the last 10 years before providing the sample.

They were then categorized into 4 groups: Group A: Nicotine consumers, Group B: Nicotine non-consumers, Group C: Alcohol users and Group D: Alcohol non-users. The Group A, i.e. nicotine consumers, included the subjects who were smokers and tobacco consumers whereas the Group B, i.e. nicotine non-consumers, included the subjects who were non-smokers and non-tobacco consumers. The Group C, i.e. Alcohol users, included the subjects who consumed alcohol over 10 years before giving the sample whereas the Group, D i.e. Alcohol non-users, included the subjects who were not taking alcohol. Blood and semen samples were collected from all the subjects.

2.2. Semen analysis

Semen analysis was performed (concentration, motility, morphology) as per WHO 2010 guidelines.⁴ Semen was analyzed for ROS and DNA fragmentation index (DFI).

2.3. Chemiluminescence assay

ROS production in 400 μ l of liquefied neat semen was measured after addition of 10 μ l of 5 mM solution of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma Chemical Co., St. Louis, MO, USA) in dimethylsulphoxide (DMSO). Chemiluminescence was measured for 10 min using the Berthold detection luminometer (USA). The relative light units per minute (RLU/min) obtained by luminometer were then recalculated according to the original spermatozoa concentration in semen sample and expressed as RLU/s/million spermatozoa.

2.4. Sperm chromatin structure assay (SCSA)

The SCSA was performed using a FAC Scan flow cytometer (BD Biosciences). 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), excluding debris and high DNA stainability (HDS) cells. Extent of DNA damage was expressed as DFI, which is the ratio of mean red fluorescence to total mean fluorescence (red + green) i.e., the level of denatured DNA over the total DNA. The DFI of the subjects were analyzed in duplicate by a single operator, and the mean value was used for comparison.

2.5. Enzyme-linked immunosorbent assay (ELISA)

8-OHdG measurements were made using ELISA (Cayman Chemical, USA) following manufacturer's protocol. Absorbance was measured at 450 nm using a micro-plate reader (Biotech, USA).⁵

2.6. Statistical analysis

Data were represented in mean $\pm$ SD as well as in median (minimum range, maximum range) and was analyzed by using statistical software Stata 12.1. The association between two categorical variables was seen by using Chi-square/Fisher's exact test. The mean differences for continuous variables between two groups were seen by using Student's t-test (for normally distributed data) and Mann–Whitney test (for non-normally distributed data). All the *p*-values less than 0.05 were taken as significant.

3. Results

Only 26 fathers were included as these parents were negative for somatic RB1 gene mutation. 20 children were heterozygous for the somatic mutation in RB1 gene and 6 children did not have any somatic mutation. The mean age of the cases and controls was 29.5 ± 5.4 years and 28.5 ± 4.3 years, respectively. The mean age of children of the cases was 1.6 ± 0.8 years and that of the controls was 1.5 ± 0.5 years. Of 26 cases, 22 (84.62%) were nicotine consumers – Group A, 4 (15.38%) were nicotine non-consumers – Group B, 15 (57.69%) were alcohol users – Group C and 11 (42.31%) were alcohol non-users – Group D, whereas in 30 controls, 16 (53.33%) were nicotine consumers – Group A, 14 (46.67%) were nicotine non-consumers – Group B, 10 (33.33%) were alcohol users – Group C and 20 (66.67%) were alcohol non-users – Group D. There were no significant differences in the seminal volume, pH, liquefaction time, viscosity, progressive motility (A + B), sperm count and morphology between cases in different groups and in controls (*p* > 0.05).

There was a significant difference (*p* < 0.0001*) observed in mean neat semen ROS levels in cases, i.e. 56.7 ± 46.3 RLU/s/million sperm, and controls, i.e. 21.6 ± 7.9 RLU/s/million sperm (the cut-off value of ROS was ≤ 27 RLU/s/million sperm). The mean DFI was significantly higher (*p* = 0.0017*) in cases, it was $30.3 \pm 5.9\%$ compared to $23.2 \pm 9.4\%$ in controls (the cut-off level established in our laboratory was 28% DFI). Among total subjects, HDS levels were significantly higher in cases as

compared to controls [7.1% (2.7, 18.2) vs 6.4% (3.6, 17.8); *p* = 0.05*]. The mean levels of oxidative mutagenic base, 8-OHdG, was significantly higher in cases [72.5 (12.8–631.1) pg/ml] as compared to controls [32.7 (5.6–89) pg/ml] and its cut-off value was 33.25 pg/ml [Table 1]. Table 2 shows the effects of lifestyle habits on experimental parameters (ROS, DFI, 8-OHdG) between the groups.

3.1. Lifestyle habits and experimental parameters

3.1.1. Lifestyle habits vs ROS

Among total subjects, the ROS values were higher in nicotine consumers as compared to nicotine non-consumers and the difference was significant, i.e. ROS [29.1 (11.4, 191.5); 23.8 (6.7, 38.6); *p* = 0.046*] [Fig. 1(a)]. Among total subjects, alcohol users had higher values of ROS in comparison with alcohol non-users but the difference was not significant, i.e. ROS [31.4 (8.2, 191.5); 24.9 (6.7, 97.5); *p* = 0.152] [Fig. 1(b)].

3.1.2. Lifestyle habits vs DFI

Among total subjects, the values of DFI were higher in nicotine consumers as compared to nicotine non-consumers, i.e. DFI [28.6 (8.5, 43.2); 24.5 (3.3, 32.8); *p* = 0.020*], and the difference was significant [Fig. 2(a)]. Among total subjects (cases + controls), alcohol users had higher values of DFI in comparison with alcohol non-users but the difference was not significant, i.e. DFI [27.8 (4.3, 43.2); 27.5 (3.3, 42); *p* = 0.541] [Fig. 2(b)].

The association of combinations of ROS and DFI variable was found to be significantly associated with each other (*p* = 0.017*). The odds ratio could not be calculated in the combination (ROS*DFI = 01) as there were no cases (*n* = 0) found in this combination, i.e. ROS ≤ 27 RLU/s/million sperm and DFI > 28%. The risk of occurrence of retinoblastoma in the children of alcohol users was 6.7 times greater, whose ROS > 27 RLU/s/million sperm and DFI $\leq 28\%$, i.e. OR 6.7, 95% CI: (0.5–91.3), and was statistically not significant (*p* = 0.155). But the odds of occurrence of retinoblastoma in children were 13.3 times in combination of ROS > 27 RLU/s/million sperm and DFI > 28%, i.e. OR 13.3, 95% CI: (1.1–166.4), and was statistically significant (*p* = 0.044*) [Table 3]. Table 4 shows the various key for combinations of ROS and DFI.

Table 1 – Comparison of experimental parameters in cases and controls (For ROS and DFI data shown in mean $\pm$ S.D; whereas for 8-OHdG data are shown in median (lower limit–upper limit); **p* < 0.05 was considered as significant.

Variables	Cut-off values	Cases (<i>n</i> = 26)	Controls (<i>n</i> = 30)	<i>p</i> -Value
ROS (RLU/s/million sperm)	27	56.7 ± 46.3	21.6 ± 7.9	<0.0001*
DFI (%)	28	30.3 ± 5.9	23.2 ± 9.4	0.0017*
8-OHdG (pg/ml)	33.25	72.5 (12.8–631.1)	32.7 (5.6–89)	<0.0001***

Table 2 – Effect of lifestyle habits on experimental parameters (ROS, DFI and 8-OHdG). Data are shown in median (lower limit–upper limit) for ROS and DFI; whereas for 8-OHdG data are shown in mean $\pm$ S.D; **p* < 0.05 was considered as significant.

Variables	Nicotine consumers	Nicotine non-consumers	<i>p</i> value	Alcohol users	Alcohol non-users	<i>p</i> value
ROS (RLU/s/million sperm)	29.1 (11.4, 191.5)	23.8 (6.7, 38.6)	0.046*	31.4 (8.2, 191.5)	24.9 (6.7, 97.5)	0.152
DFI (%)	28.6 (8.5, 43.2)	24.5 (3.3, 32.8)	0.020*	27.8 (4.3, 43.2)	27.5 (3.3, 42)	0.541
8-OHdG (pg/ml)	54.4 ± 4.5	32.3 ± 2.3	0.050*	102.8 ± 3.7	26.6 ± 2.5	0.001*

Fig. 1 – (a) Box- and whisker-plots showing the median and range of ROS in the native semen samples of nicotine consumers and nicotine non-consumers. A significantly higher ROS could be found in nicotine consumers in comparison to nicotine non-consumers ($p = 0.046^*$). (b) Box- and whisker-plots showing the median and range of ROS of the semen samples of alcohol users and alcohol non-users. No significant difference was found between the groups ($p = 0.152$).

Fig. 2 – (a) Box- and whisker-plots showing the median and range of DFI in the semen samples of nicotine consumers and nicotine non-consumers. A significantly higher DFI could be found in nicotine consumers in comparison to nicotine non-consumers ($p = 0.020$). (b) Box- and whisker-plots showing the median and range of DFI of the semen samples of alcohol users and alcohol non-users. No significant difference was found between the groups ($p = 0.541$).

4. Discussion

Children with Rb either inherit a mutation (Familial Rb) or a de novo germline mutation (NF5HRb) occurs in the RB1 gene resulting in the development of Rb.⁶ The mutations in RB1 promoter region can be identified in 92% of suspected RB1 alleles either as heterozygous germline mutations in heritable cases or as bi-allelic somatic mutation in retinoblastoma tumours. In around 8% of RB1 alleles, no RB1 inactivating event has been identified leaving open the possibility that some cases of Rb may be caused by alternative genetic or epigenetic events that modify pRb function.⁷ In this study, 20 children harboured heterozygous somatic mutation while 6 children were negative for these changes in blood. It is possible that in these cases both the mutations arose in retinal tissue or there was decreased expression of RB1 gene. Recent studies have documented that hypermethylation of RB1 gene may also be a

cause of Rb.⁸ Also, OS has been documented to lead to hypermethylation of tumour suppressor genes and hypomethylation of repetitive elements in the genome.⁹ Thus, OS lowers expression levels of RB transcripts and may explain for the aetiology in cases negative for RB1 gene mutations, but methylation studies were not a part of this study.

OS has been established as a major cause of DNA damage and is linked to poor lifestyle habits of parents, especially fathers, as sperm is highly vulnerable to oxidative damage due to minimal cytosolic anti-oxidants.¹⁰ In previous studies from our lab we have documented that a high level of oxidatively damaged sperm DNA is associated with male infertility and recurrent spontaneous abortions,¹¹ but its role in childhood cancers has not been addressed and there is no awareness of potential effect in these disorders and thus the critical need to document the effect of loss of sperm DNA integrity. Persistence of DNA damage and mutagenic oxidative adducts

Table 3 – Analysis with combination of parameters (ROS and DFI) and lifestyle habits showing odds ratio; *p values <0.05 were considered as significant.

Variables	ROS*DFI	Cases	Controls	p-Value	Odds ratio (95%CI)	p-Value
A: Nicotine consumers	00	5	6	0.273	1.0	–
	01	2	4		0.6 (0.1, 4.7)	0.629
	10	5	3		2 (0.3, 12.8)	0.465
	11	10	3		4 (0.7, 23.1)	0.121
B: Nicotine non-consumers	00	1	7	0.272	1.0	–
	01	0	3		–	–
	10	1	3		2.3 (0.1, 50.9)	0.590
	11	2	1		14 (0.6, 338.8)	1.62
C: Alcohol users	00	3	5	0.017*	1.0	–
	01	0	3		–	–
	10	4	1		6.7 (0.5, 91.3)	0.155
	11	8	1		13.3 (1.1, 166.4)	0.044*
D: Alcohol non-users	00	3	8	0.674	1.0	–
	01	2	4		1.3 (0.2, 11.5)	0.794
	10	2	5		1.1 (0.1, 8.7)	0.952
	11	4	3		3.5 (0.5, 26.9)	0.214
Combined (A + B + C + D)	00	6	13	0.028*	1.0	–
	01	2	7		0.6 (0.1, 3.9)	0.097
	10	6	6		2.1 (0.5, 9.6)	0.488
	11	12	4		6.5 (1.4, 28.8)	1.466

Table 4 – Key for combinations in Table 3.

ROS*DFI	ROS (RLU/s/million sperm)	DFI (%)
00	ROS ≤ 27	DFI ≤ 28
01	ROS ≤ 27	DFI > 28
10	ROS > 27	DFI ≤ 28
11	ROS > 27	DFI > 28

post-fertilization may adversely affect embryonic development¹² and may lead to childhood cancer.

The results of this study highlight that elevated ROS levels and DFI, both in conjunction and independently, can increase the risk of development of Rb, and this emphasizes the need for adoption of a healthy lifestyle to prevent oxidative DNA damage. This DNA damage with mutagenic oxidative base adducts may lead to accumulation of mutations in both sperm DNA (germline) or in zygote (somatic), which constitutes the first hit as per Knudson's two-hit hypothesis. Majority of mutations arise during cell replication (spermatogenesis) and thus sperm is highly susceptible to accumulation of mutations as compared to oocyte.¹³ Non-smokers/non-alcohol consumers have lower ROS levels. Recent studies from our lab have documented that yoga and meditation result in significant decline in ROS levels and improvement in sperm DNA integrity following 6 months practice of yoga and meditation.^{14–16} This may not only significantly positively impact DNA integrity but also lowers incidence of male factor infertility, recurrent spontaneous abortions, several neuro-psychiatric disorders and childhood cancers. Paternal unhealthy lifestyle habits are positively associated with the risk of Rb and simple lifestyle modifications may improve DNA health, and thus decrease incidence of childhood morbidity and even cancer.

Conflicts of interest

The authors have none to declare.

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