Association between sperm DNA integrity and seminal plasma antioxidant levels in health workers occupationally exposed to ionizing radiation

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There is a paucity of data regarding the association between occupational radiation exposure and risk to human fertility. Recently, we provided the first evidence on altered sperm functional characteristics, DNA damage and hypermethylation in radiation health workers. However, there is no report elucidating the association between seminal plasma antioxidants and sperm chromatin integrity in occupationally exposed subjects. Here, we assessed the seminal plasma antioxidants and lipid peroxidation level in 83 men who were occupationally exposed to ionizing radiation and then correlated with the sperm chromatin integrity. Flow cytometry based sperm chromatin integrity assay revealed a significant decline in αt value in the exposed group in comparison to the non-exposed group (P < 0.0001). Similarly, both total and reduced glutathione levels and total antioxidant capacity in the seminal plasma were significantly higher in exposed group than the non-exposed group (P < 0.01, 0.001 and 0.0001, respectively). However, superoxide dismutase level and malondialdehyde level, which is an indicator of lipid peroxidation in the seminal plasma, did not differ significantly between two groups. The total antioxidant capacity (TAC) and GSH level exhibited a positive correlation with sperm DNA integrity in exposed subjects. To conclude, this study distinctly shows that altered sperm chromatin integrity in radiation health workers is associated with increase in seminal plasma antioxidant level. Further, the increased seminal plasma GSH and TAC could be an adaptive measure to tackle the oxidative stress to protect genetic and functional sperm deformities in radiation health workers.

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1. Introduction

Reproductive function is sensitive to changes in the physical and chemical environment (Younglai et al., 2005). However, there is a paucity of data regarding the association between occupational radiation exposure and risk to human fertility (Schull, 1984; Baranski, 1993; Bonde, 1999; Doyle et al., 2001; Sinno-Tellier et al., 2006; Lin et al., 2010). Recently, we provided the first evidence on the detrimental effects of occupational radiation exposure on functional, genetic and epigenetic integrity of spermatozoa in health workers where a significant decline in sperm functional characteristics, DNA damage and hypermethylation was observed in radiation exposed subjects (Kumar et al., 2013). Sperm DNA is an independent measure of sperm quality that may have better diagnostic and prognostic capabilities than standard sperm parameters like concentration, motility and morphology (Zini et al., 2001). Sperm DNA integrity analysis of radiation health workers using well established techniques such as single cell gel electrophoresis (comet assay) and flow cytometry based DNA fragmentation analysis has revealed an increased level of sperm DNA fragmentation (Kumar et al., 2013).

Cellular exposure to ionizing radiation has been known to produce reactive oxygen species (ROS) which display high reactivity toward macromolecules (Azzam et al., 2012). In order to protect against ROS mediated damage, body has evolved a variety
of defense mechanisms involving antioxidant systems. Antioxidant systems include enzymes such as superoxide dismutase (SOD), catalase and non-enzymatic systems such as glutathione and ascorbic acid. The seminal plasma components of the human ejaculate also have an array of enzymatic and non-enzymatic antioxidant systems (Alvarez et al., 1987) which protect the spermatozoa from reactive oxygen species (ROS) mediated damage (Kankofer et al., 2005). One of the ROS mediated cellular damage is the membrane lipid peroxidation results in the formation of malondialdehyde (MDA) which has been widely used as marker to monitor the degree of peroxidative damage sustained by the spermatozoa. Previous studies have observed the enhanced antioxidant levels in the blood plasma of chronically exposed radiation health workers (Durovic et al., 2008; Russo et al., 2012). However, there is no data elucidating the association between seminal plasma antioxidants and sperm DNA integrity in occupationally exposed subjects. In light of these considerations, this study was planned to quantify the seminal plasma antioxidants and lipid peroxidation level to determine their association with the sperm chromatin integrity in the subjects who are chronically exposed to radiation at workplace.

2. Materials and methods

2.1. Study populations

This retrospective cohort study conducted between January 2010 and March 2012 comprised of 134 male volunteers, of whom 83 were occupationally exposed to ionizing radiation and 51 were non-exposed control subjects. The occupationally exposed volunteers were selected from various hospitals having diagnostic or therapeutic radiation (X/β rays) facilities. The non-exposed volunteers were employees of the same hospitals but were not exposed to above mentioned radiation sources. The volunteers were of the age group 21 to 50 years who are operating instruments having radiation sources for diagnostic or therapeutic purposes for more than a year. All the subjects were considered as chronically exposed to low dose radiation. Subjects suffering from chronic diseases such as diabetes, hypertension, asthma and known genetic disorders were excluded. Similarly, subjects with hypogonadotrophic hypogonadism, testicular dysfunction, testicular atrophy and history of fever during previous three months were excluded from both the groups. Volunteers who fulfilled the criteria were given a questionnaire to obtain information about the duration of stay at their workplace, type of radiation source they were exposed to, their lifestyle, history of illnesses, and problems related to reproduction such as incidences of infertility and miscarriage/s in their partners. The questionnaire also included other confounding factors influencing semen quality and sperm DNA integrity such as, smoking, alcohol, diet. No specific time interval was considered between the time of last irradiation and sample collection. Processing and evaluation of the samples of the two groups were performed in the university infertility research laboratory. The study was approved by the Institutional Ethical Committee, Kasturba Medical College and Hospital, Manipal and a written consent was taken from all the volunteers.

2.2. Exposure monitoring

The occupational exposure levels of the subjects was routinely monitored by thermoluminescent dosimeter (TLD) device. The cumulative exposure level of each subject was collected from the radiation safety officer of the respective hospital where the subject was enrolled.

2.3. Semen analysis and separation of seminal plasma

Semen samples were obtained between 3 and 5 days of sexual abstinence by masturbation in sterile containers. Semen analysis was performed within 1 h of collection under sterile conditions. Upon completion of liquefaction, the sample was mixed well and evaluated for physical and microscopic characteristics according to World Health Organization, 1999. Seminal plasma free from spermatozoa was obtained by centrifuging the semen sample at 2000 rpm for 10 min for antioxidants and lipid peroxidation assessment. All the analyses were carried out blindly.

2.4. Flow cytometry based sperm chromatin integrity assay

The sperm chromatin integrity was assessed as described in our earlier study (Kumar et al., 2013). A minimum of 5000 events were collected per sample. SCSS quantifies the shift from double-stranded to single-stranded DNA following acid denaturation. The extent of denaturation is quantitatively denoted by the term alpha (α), a value that can range from 0 to 1. The α value is a ratio of red fluorescence to total (green and red) fluorescence, and is calculated as follows: mean channel of red fluorescence/mean channel of red fluorescence + mean channel of green fluorescence. A higher shift signifies greater DNA denaturability and reduced/loss of fertility (Evenson and Jost, 1994).

2.5. Assessment of total and reduced glutathione (GSH) in seminal plasma

Total GSH concentration was determined according to method described by Anderson (1985) with minor modification. Briefly, 50 μL of seminal plasma were added to 100 μL of reaction buffer (1.5 mM phosphate buffer, 2.4 mM NADPH, 10 mM DTNB, 0.5U GSH reductase, pH 7.5). The optical density (OD) was recorded exactly after 1 min at 405 nm using Bio-photometer (Eppendorf, Germany). A standard graph was prepared by using various concentration of GSH standard. The total GSH levels were determined by plotting the OD values of the seminal plasma against the standard graph and dropping a perpendicular to X-axis. Estimation of reduced GSH was done according to Beutler et al. (1963) with minor modification. Briefly, 50 μL of seminal plasma were mixed with 50 μL phosphate buffer, 25 μL of precipitating reagent and 25 μL of fresh DTNB. The optical density of the test solution was determined at 405 nm exactly after 1 min. A standard graph was prepared by using various concentration of reduced GSH. The reduced GSH levels in seminal plasma were determined by plotting the optical density (OD) values of the seminal plasma against the standard graph and dropping a perpendicular to X-axis.

2.6. Assessment of total antioxidant capacity (TAC) in seminal plasma

The ability of the seminal plasma to scavenge ABTS + [2,2′-azinobis-[3-ethyl-benzothiazoline-6-sulfonic acid]] radical cation was compared to trolox standard (Re et al., 1999). Briefly, the ABTS + radical cation were pre-generated by mixing 7 mM ABTS (Cat. no. A1888; Sigma Aldrich Inc. USA) stock solution with 2.45 mM potassium persulfate and incubating for 12-16 h in dark condition at room temperature. The absorbance of the ABTS + solution was equilibrated to 0.70 (± 0.02) OD value by diluting with phosphate buffered saline (PBS) at room temperature, then 1 mL was mixed with 10 μL of the seminal plasma followed by measurement of absorbance at 650 nm after 1 min. The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of standard and sample to determine the trolox equivalent antioxidant concentration (TEAC). To calculate the TEAC, the gradient of the plot for the sample were divided by the gradient of the plot for trolox.

2.7. Assessment of seminal plasma superoxide dismutase (SOD)

Estimation of SOD was done according to Marklund and Marklund (1974) with minor modifications. One unit of SOD is defined as the amount of enzyme which inhibits auto-oxidation of pyrogallol by 50%. Briefly, 100 μL seminal plasma was added to 2.85 mL of 0.1 M phosphate buffer (pH 8.4) and 50 μL of 100 mM pyrogallol (Cat No. RM170; HiMedia Laboratories) prepared in 20 mM HCl. The absorbance of the reaction mixture was recorded at 0 and 3 min. Similarly, the auto-oxidation is measured by adding 2.95 mL phosphate buffer (pH 8.4) and 50 μL pyrogallol at 0 and 3 min using a Bio-photometer at a wavelength of 405 nm. SOD activity was measured using calibration curve of percentage inhibition and expressed as enzyme units/μL of seminal plasma.

2.8. Measurement of lipid peroxidation by malondialdehyde (MDA) assay in seminal plasma

The lipid peroxidation levels in the seminal plasma was measured using a thiobarbituric acid reactive substances (TBARS) assay, which monitors MDA production based on the method of Buege and Aust (1978). Briefly, 100 μL of seminal plasma were added to 1.8 mL of 3% phosphoric acid (Cat. no. 215104; Sigma Aldrich Inc. USA) and 0.6 mL of 0.6% TBA (Cat. No. T5500; Sigma Aldrich Inc. USA). These mixtures were heated in boiling water for 45 min. After cooling, the intensity was measured at 590 nm using Bio-photometer and the level was determined using the molar absorption coefficient at 550 nm.

2.9. Statistical analysis

The data was analyzed using Statistical Package for Social Sciences (SPSS 15.0). Data has been summarized using mean and standard error (Mean ± SEM) for
were not significantly different from that of non-exposed subject’s spouses. The subject characteristics are presented in Table 1.

3.2. Evaluation of sperm DNA integrity

Flow cytometer based sperm chromatin assay provides independent measurement of sperm DNA integrity, hence, it is considered as a useful tool for epidemiological studies (Spanò et al., 1998). The DNA denaturation as denoted by $t$ value in the exposed group ($0.2844 \pm 0.0015$) was significantly higher compared to non-exposed group ($0.2766 \pm 0.0012$; $P < 0.0001$). The $t$ value in the subjects with absorbed dose of $> 0.05$ mSv (hereafter referred as high absorbed dose, HAD) was $0.2879 \pm 0.0023$ and this was significantly higher than subjects with absorbed dose of $< 0.05$ mSv (hereafter referred as low absorbed dose, LAD group) ($0.2793 \pm 0.0018$; $P < 0.01$) (Fig. 1).

3.3. Seminal plasma glutathione (GSH) concentration

Total GSH concentration in the exposed group was significantly higher than non-exposed group ($P < 0.01$) (Fig. 2A). Though, subjects with HAD had elevated levels of total GSH in comparison to the subjects with LAD, the differences were not statistically significant ($P = 0.06$). Similar to total GSH level, there was about 1.5 fold increase in reduced GSH level in the exposed subjects which is statistically significant ($P < 0.001$) (Fig. 2B). Though there was a minimal decrease in the oxidized GSH level in exposed subjects in comparison to non-exposed group, the differences were not significant (Fig. 2C). However, the ratio of reduced to oxidized GSH was significantly higher in the exposed group compared to non-exposed ($P < 0.05$) (Fig. 2D).

3.4. Total antioxidant concentration in seminal plasma (TAC)

Total antioxidant capacity was determined to find out the effect of chronic workplace radiation exposure on the total enzymatic and non-enzymatic antioxidant activity in the seminal plasma. Interestingly, seminal GSH level, TAC level was also significantly higher in exposed subjects compared to non-exposed ($P < 0.0001$) (Fig. 3A). Moreover, TAC level in the HAD group was significantly higher than exposed group ($P < 0.05$).

3.5. Seminal plasma superoxide dismutase (SOD)

The concentration of SOD in the exposed and non-exposed group was $0.0364 \pm 0.0007$ and $0.0356 \pm 0.0009$ Units/$\mu$L, respectively, and the difference was not significant. Moreover, SOD level between HAD and LAD were not significantly different (Fig. 3B).

3.6. Lipid peroxidation in the seminal plasma

We compared the extent of lipid peroxidation in both non-exposed and exposed subjects by the assessment of malondialdehyde (MDA) level in the seminal plasma. The level of MDA in the exposed group was $2058.24 \pm 106.31$ nmol/L which was not significantly different from the non-exposed group ($2054.70 \pm 170.65$ nmol/L). Similarly, no significant difference was observed between HAD and LAD groups (Fig. 3C).

3.7. Association between sperm DNA denaturation/damage and GSH, TAC, SOD and lipid peroxidation in the seminal plasma

An attempt was made to determine the association between the extent of sperm DNA fragmentation and the seminal plasma antioxidants level. The $t$ value of the exposed subjects showed a positive correlation with total GSH ($R = 0.48$), oxidized GSH ($R = 0.46$) and TAC level ($R = 0.57$). However, the relationship between alpha $t$ value with antioxidants such as reduced GSH, SOD and MDA level failed to demonstrate any correlation (Fig. 4A–F).

3.8. Influence of confounding factors

To rule out the effects of confounding factors on the outcome, a cross tabulation of the factors such as smoking, alcoholism and diet pattern was performed in both the exposed and non-exposed groups. The number of subjects with smoking, alcohol consumption and diet pattern was found to be similar in both the groups (Table 1). Bivariate analysis using analysis of covariance (ANCOVA) to find out the effect of confounding factors such as alcohol, smoking, diet and interaction of these factors with radiation exposure did not reveal any effect except the total antioxidant capacity (TAC) and Malonaldehyde (MDA) level. Alcohol consumption has decreased the level of TAC ($P = 0.03$) in the radiation exposed subjects (Table 2). Similarly, a marginal increase in MDA level was observed ($P = 0.02$) in the subjects who were on non-vegetarian diet (Table 2) compared to vegetarians whereas radiation exposure did not modulate the MDA concentration in the subjects. In contrast, only occupation radiation exposure was responsible for increase in the sperm DNA damage ($P = 0.005$) and TAC ($P < 0.0001$) in the exposed subjects (Table 2). In addition, the direct comparison of exposed and non-exposed subjects has shown an increase in the total and reduced GSH whereas...
multivariate analysis has not shown any difference between the groups studied (Table 2).

4. Discussion

While our earlier study has clearly demonstrated the detrimental effects of occupational radiation exposure on semen characteristics and genetic/epigenetic integrity of the spermatozoa (Kumar et al., 2013) the key findings in this study were: (1) an adaptive response to chronic occupational exposure as the level of antioxidants are higher and no significant correlation with MDA level and (2) direct correlations between seminal plasma antioxidant status and sperm chromatin integrity in the radiation health workers. These findings indicated the functioning of antioxidant defense mechanism in the seminal plasma to protect the functional and genetic integrity of the spermatozoa.

It has been shown that seminal oxidative stress can be induced by over-production of ROS through blood cells, dead and immature spermatozoa which is one of the important causes of male infertility. The seminal plasma is well equipped with an array of antioxidant defense mechanisms to protect the spermatozoa against oxidants. Antioxidant rich seminal plasma probably compensates the deficiency in cytoplasmic antioxidant enzymes and molecules in the spermatozoa (Agarwal et al., 2003). Specifically, free radical scavengers in seminal plasma such as SOD and GSH have found to be very crucial in reducing the oxidative stress to the spermatozoa (Potts et al., 2000). These considerations represent the rationale for the investigation of antioxidant defenses in the seminal plasma of occupationally exposed subjects presented in this study. Our data suggest an efficient antioxidant response as shown by increased production of GSH, the major antioxidant, in the exposed population which is probably helping to protect the spermatozoa from the deleterious effects of long-term radiation exposure.

Prolonged exposure to high levels of testicular toxicant such as ionizing radiation may produce testicular atrophy thereby shutting off spermatogenesis. However, at lower levels, the adverse effect may be limited to only changes in the motility pattern without affecting fertility significantly (Perreault and Cancel, 2001). The International Commission on Radiological Protection (ICRP), in its 2007 recommendations based on occupational classification, limits artificial irradiation of the public to an average of \(<1\) mSv of effective dose per year, and \(\leq 6\) or \(\leq 20\) mSv/year in the case of occupationally exposed subjects (ICRP 2007). Since occupational radiation exposure levels now strictly fall well within the accepted limits (Maffei et al. 2002), the long-term consequence of low level exposure is possibly observed as altered seminal plasma.
subjects had mean absorbed dose of < 0.05 mSv. In addition, the sperm chromatin structure was also found affected in the subjects who had < 0.05 mSv absorbed dose. Hence the subjects were segregated to two groups based on the cutoff limit of 0.05 mSv to elucidate the changes in seminal plasma antioxidant levels.

A number of compensatory and preparatory mechanisms are activated in response to the damage caused by ionizing radiation. In the present study, we have observed an increase in the seminal plasma GSH and TAC concentrations. Our results are in agreement with the previous finding by Durović et al. (2008) who observed an increase in antioxidant enzymes as a protection against the increased production of ROS during occupational exposure. Recently, enhanced cellular antioxidant defense to chronic radiation exposure in the blood plasma of the interventional cardiologists has been considered as an adaptive response (Russo et al., 2012).

GSH is an abundant tripeptide antioxidant molecule present in the cytoplasm and body fluids. The scavenging properties of GSH are mainly due to its function as cofactor for antioxidant enzyme glutathione peroxidase (GPX) and also its ability to react directly with ROS by donating hydrogen ion (H⁺) from its free sulfhydryl groups. It neutralizes the cytotoxic aldehydes produced during lipid peroxidation and thus protects the sperm plasma membrane (Sørensen et al., 1999). Higher levels of total GSH (tGSH) and reduced GSH (rGSH) levels in seminal plasma indicate that GSH plays a significant role in protection of spermatozoa against oxidative damage and also improves the sperm motility and morphology (Atig et al., 2012). Whenever the tissues are challenged with oxidative stress, the body tries to overcome the deleterious effect by elevating the expression of antioxidant enzymes and antioxidant molecules as an adaptive response (Limón-Pacheco and Gonsebatt, 2009). Similar response has been observed in the occupationally exposed group in the present study. Radiation is known to induce oxidative stress, mainly through hydrolysis of water. Further, the level of tGSH and rGSH were significantly higher in high absorbed dose group which further justifies the argument that elevated oxidative stress induced by chronic low dose radiation exposure induces adaptive response in biological fluid such as seminal plasma. Similar findings are reported in the literature which has demonstrated high antioxidant level in blood of radiation health workers (Durović et al., 2008; Russo et al., 2012).

The detection of TAC in the seminal plasma is considered to be one of the most reliable and simple tests for the diagnosis of male infertility (Mahfouz et al., 2009) as lower TAC levels were observed in seminal plasma of subfertile men as compared with fertile men (Lewis et al., 1995, 1997; Smith et al., 1996). Interestingly, our data has showed a significant increase in TAC in the seminal plasma of the exposed subjects especially between HAD and LAD group which suggests an association between antioxidant defense and high radiation absorbed dose in the health workers.

Studies have demonstrated negative correlation between seminal plasma TAC or GSH levels and sperm quality such as motility and head morphology (Smith et al., 1996; Rajmakkers et al., 2003). In contrast, our study has demonstrated a positive correlation between seminal plasma tGSH, TAC and sperm chromatin integrity. Since sperm chromatin integrity is known to be affected by ROS, it is possible that as a survival strategy, antioxidant defense functions effectively activated to counteract ROS mediated detrimental effects to sperm functions in occupationally exposed subjects to radiation. Superoxide dismutase enzymes are present in both spermatozoa (Alvarez et al., 1987) as well as seminal plasma (Mennella and Jones, 1980). In the present investigation the assessment of SOD activity and MDA level in seminal plasma, did not show strong correlation with chromatin integrity. It is possible that since spermatozoa come in contact with the seminal plasma only after...
Fig. 4. Association between sperm chromatin integrity and seminal plasma GSH, TAC, SOD and malondialdehyde. (A) Correlation analysis of αt values and seminal plasma total GSH level in non-exposed (R=0.22) and exposed group (R=0.48). (B) Correlation analysis of αt values and seminal plasma reduced GSH level in non-exposed (R=0.1) and exposed group (R=0.23). (C) Correlation analysis of αt values and seminal plasma oxidized GSH level in non-exposed (r=0.09) and exposed group (R=0.46). (D) Correlation analysis of αt values and seminal plasma total antioxidant capacity in non-exposed (R=0.32) and exposed group (R=0.57). (E) Correlation analysis of αt values and seminal plasma superoxide dismutase in non-exposed (R=0.1) and exposed group (R=0.17). (F) Correlation analysis of αt values and malondialdehyde in non-exposed (R=0.08) and exposed group (R=0.1).
ejaculation and the oxidative stress induced by occupational radiation exposure for prolonged period are expressed in male germ cells, estimation of SOD and lipid peroxidation in spermatozoa itself might have given a better correlation with DNA integrity of spermatozoa.

In conclusion, this study distinctly shows that there is altered sperm chromatin integrity in radiation health workers which is also associated with elevated level of seminal plasma antioxidants, especially GSH. Further, the increased seminal plasma GSH and TAC could be an adaptive measure to tackle the oxidative stress to protect genetic and functional sperm deformities in radiation health workers.

Declaration of interest

DK has nothing to disclose, SR has nothing to disclose, SK has nothing to disclose, SU has nothing to disclose, NJ has nothing to disclose, GK has nothing to disclose, HK has nothing to disclose, SC has nothing to disclose, SGC has nothing to disclose, PK has nothing to disclose, SKA has nothing to disclose.

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