Original Article

Paternal Age Influences Human Sperm Chromatin Sensitivity to Ionizing Radiation

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Abstract

Objective : To understand the age related sensitivity of human and mouse sperm chromatin to radiation.

Study design: This prospective study examined the comparative *in vitro* radiosensitivity of sperm DNA from normozoospermic human ejaculate (N= 47) and mouse epididymal spermatozoa (N= 40), from various age groups. Human ejaculates and mouse sperm suspension were exposed to 0 and 5 Gy,-radiation using ^{60}Cobalt tele-therapy unit and sperm DNA damage was assessed by single cell gel electrophoresis (neutral comet assay).

Results: The DNA fragmentation analysis of 5 Gy irradiated spermatozoa has revealed a significant increase in olive tail moment (OTM) in men above 40 years ($P<0.05$). In contrast, the OTM did not vary in mouse spermatozoa in any of the irradiated groups.

Conclusion: Though, the data has emerged from in vitro radiation exposure, from the clinical point of view, the study warns that men who are above 40 years are at higher risk of acquiring sperm DNA fragmentation after therapeutic and accidental radiation exposures.

Key words: anti-cancer agents, comet assay, ionizing radiation, paternal age, sperm DNA integrity.

Introduction

It is now evident that the anticancer treatments such as radiation and chemotherapeutic agents pose a great threat to sperm chromatin integrity.[1,2] Though, DNA repair mechanisms play a pivotal role in mutagenesis, certain repair pathways aberrantly function with the advancement in age.[3,4] It has been shown that mutagenic response to ionizing radiation in germline is modulated by age.[5] Importantly, the persistence of unrepaired DNA lesions in germ cells may have deleterious consequences of transgenerational genomic instability thereby resulting in abnormal reproductive outcome in the offspring.[6-9]

Sperm chromatin is composed of tightly packed DNA with small basic proteins called protamines, which helps in chromatin remodeling process.[10] It is now evident that altered protamine level increases the susceptibility of sperm to undergo DNA fragmentation and eventual poor outcomes in assisted reproduction.[11] The proportion of protamine and nucleosomal structure are different in mouse and human spermatozoa[10-12] in which human sperm nuclear DNA has a heterogeneous structure with some regions and genes remaining associated with histones or with other proteins.[10,13,14] Hence this difference in chromatin structure may demonstrate the differences in sperm DNA damage susceptibility to a genotoxic agent.[15,16]

Although there has been a drastic increase in the incidence of cancer in men, the advancements in therapeutic regimes have significantly improved the survival of the affected individuals. The number of survivors in the reproductive age group is increasing; hence it is important to evaluate the sensitivity of sperm chromatin structure to anticancer agents. The current scenario demands in depth understanding of the differential sensitivity of spermatozoa with respect to the age of the individuals. In this study we investigated the spermatozoal susceptibility to ionizing radiation in vitro. To examine the comparative radiosensitivity of sperm DNA, both mouse and human spermatozoa from various age groups were included in this study.

Materials and methods

Human subjects

This prospective study included 47 men attending University infertility clinic for semen evaluation. Only normozoospermic ejaculates were included in this study as poor quality ejaculates are known to carry higher level of baseline DNA fragmentation.[17] The study was approved by the Institutional Ethical Committee.

Semen samples were obtained between 3-5 days of sexual abstinence by masturbation in sterile nontoxic containers. Semen analysis was performed within one hour of collection under sterile conditions. Upon completion of liquefaction, the sample was mixed well and evaluated for physical and microscopic characteristics according to WHO criteria.[18]

Animals

The animal care and handling were done according to the institutional guidelines for animal experimentation and the proposal was approved by the Institutional Ethical Committee. Healthy Swiss albino male mice between the age of 8-52 weeks maintained under the controlled conditions of temperature (23±2°C) and light (12 h light/dark cycles) with standard diet and water ad libitum were used in this study. The animals were sacrificed and spermatozoa were extracted from the caudae epididymis in 2 mL of pre-warmed Earle's Balanced Salt Solution (EBSS) (Medium 199,Sigma Chemical Co., St. Louis, USA Cat. No. M5017) supplemented with 0.1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, USA Cat. No. A3311). The spermatozoa were centrifuged and the pellet was resuspended in 500 µL of medium for the further experiments

In vitro irradiation

Fixed volume 500 µL of the human ejaculate and mouse sperm suspension were transferred to a petridish and then exposed to 0 and 5 Gy-radiation using ^{60}Co tele-therapy unit with a dose of 1 Gy/ min at room temperature.

Single cell gel electrophoresis (Neutral comet assay)

Within 30 mins after irradiation, the DNA fragmentation in spermatozoa was assessed by the comet assay as described earlier[19] with minor modifications. Briefly, the spermatozoa suspended in sterile Phosphate Buffer Saline (PBS), (pH 7.4) were mixed with equal volume of 1 % low melting point (LMP) agarose (Sisco Research Laboratories, India, Cat. No. 0140151) and layered on a slide pre-coated with 1 % normal melting point (NMP) agarose (Sisco Research Laboratories, India, Cat.No.0144162). Agarose was allowed to gel by placing the slides at -4°Cfor 5 mins. A third coat of agarose was layered over the second layer followed by overnight incubation in lysing solution (2.5 M sodium chloride, 100 mM disodium EDTA, 10 mMTrizma base, 10% DMSO, 1 % Triton X-100, 20 mM DTT (pH 10) at 4°C. Sperm DNA unwinding was carried out by immersing the slides in electrophoresis buffer (300 mM Sodium acetate, 100 mMTris base, pH= 9) for 20 mins followed by electrophoresis at 20 V (VcM= 0.74 V/cm, 100 mA) for 60 mins (performed at 4°C). The slides were neutralized by flooding with neutralization buffer (0.4 M TrisHCl buffer) for 5 minutes, dehydrated by immersing them in chilled absolute alcohol for 10 mins and stored till further processing.

For visualization and scoring, the slides were rehydrated in cold PBS for 10 minutes and stained with 2g/ml Ethidium bromide. The slides were observed under a fluorescent microscope (Imager-A1, Zeiss, Germany) and images were captured using 40 X objective. A minimum of 50 randomly selected images were captured from each slide which was coded to prevent observer's bias. The sperm with DNA damage attained the shape of comet with tail region consisting of fragmented DNA and head region with intact DNA. The representative images have been provided in Supplementary Fig 1. The comet evaluation of the captured images was done using Kinetic Imaging software (Komet 5.5, UK).

Statistical analysis

The data represents mean and standard error (Mean ± SEM) of the values. The statistical significance level was calculated using One Way Analysis of Number of Variance (ANOVA) (Tukey test) using Graph PADInstat software, USA. The graphs were plotted using Origin 6.0 (USA).

Results

Human study

The present study included a total of 47 normozoospermic men from various age groups (Group I, 21-30 years, N=10; Group II, 31-40 years, N=28; Group III, 41-50 years, N=9). The sperm count per milliliter of ejaculate (mean ± SEM) in Group I, II and III was 62.0 ± 10.72; 49.25±5.54 and 35.25±5.92 respectively which was not significantly different between each group. Similarly, total sperm motility also did not show significant difference between any of the groups (Group I: 65.22 ± 5.21, Group II: 63.25 ± 1.68; Group III: 56.37 ± 4.55). Neutral comet assay was performed to determine the extent of DNA fragmentation in the ejaculates of various groups. The DNA fragmentation as measured by OTM did not vary between group I & II. Though, a moderate increase in the baseline OTM was observed in group III, the difference was not statistically significant with other groups studied. Exposure to 5 Gy-radiation induced a marginal, non-significantly higher level of OTM in group I and II within 30 min after irradiation. However, the OTM was increased by approximately 1.7 folds in group III which was significantly higher than the unirradiated control (P<0.05) (Fig 1).

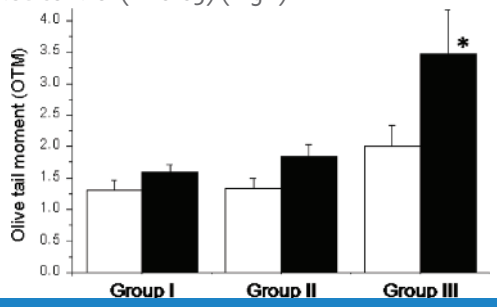
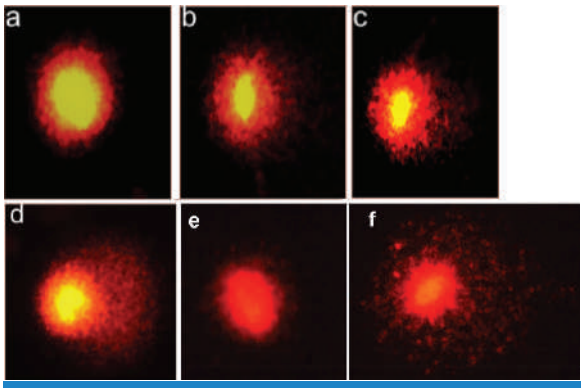


Fig 1: DNA fragmentation in in vitro irradiated human spermatozoa from various age groups. Olive tail moment in unirradiated control and 5 Gy-irradiated human spermatozoa(*P < .05 Vs unirradiated).



Supplementary Fig 1 : Representative images of single cell gel electrophoresis (Comet) a-d) human sperm cells displaying various degrees of DNA fragmentation. e-f) sperm cells from mice showing various degrees of DNA fragmentation.

Mouse study

In order to determine the species specific sensitivity of ionizing radiation in relation to the age, mouse spermatozoa from 8 (Group I), 12(Group II), 26 (Group III), and 52 (Group IV), weeks were used in replicates.[20] The number of animals (N) used in each group was 10. Similar to human ejaculates, the sperm count and motility in mice did not vary significantly between each group (sperm count in millions/ml: 18.08 ± 2.8, 17.13 ± 2.1, 16.41 ± 2.5, 16.61 ± 3.4 and percent sperm motility: 66.9 ± 2.19, 51.9 ± 6.09, 53.2 ± 5.8, 58.4 ± 5.7 in 8, 12, 26, 52 weeks groups respectively). The DNA fragmentation was quantified in at least 50 spermatozoa from each group. The baseline OTM showed a minimum non-significant variation between different age groups studied. *In vitro* -irradiation was performed on mouse spermatozoa and conditions were kept identical to human sperm irradiation. In contrast to human spermatozoa, the OTM level did not vary in any of the irradiated group including the highest age group of 52 weeks which demonstrated only 1.14 fold non significant increases in the OTM level when compared to unirradiated control (Fig 2).

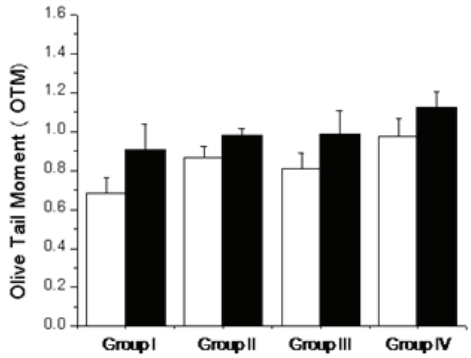


Fig 2: DNA fragmentation in in vitro irradiated mouse spermatozoa from various age groups. Olive tail moment in unirradiatedcontrol and 5 Gy-irradiated mouse spermatozoa.

Discussion

In this study, we compared the sperm chromatin sensitivity to in vitro exposed ionizing radiation from the individuals of varying age group. In vitro irradiation of spermatozoa enabled us to elucidate the direct and untargeted effect of radiation on male germ cells in relation to the age of the individuals. Parallely, mouse spermatozoa were also evaluated to compare the species specific response to ionizing radiation using neutral comet assay. We found that human spermatozoa from the subsets of age between 40-50 years had increased sensitivity to radiation induced DNA fragmentation. In contrast, spermatozoa from mouse up to 52 weeks of age failed to show any significant increase in the radiation induced DNA damage.

Ageing is associated with an overall increase in oxidative stress possibly due to decreased ATP and increased reactive oxygen species (ROS) production.[21] Studies have shown that the effect of age on human spermatozoa is associated with change in motility pattern[22] and DNA integrity[17,23] which are probably due to the compromised maturing efficiency of spermatozoa in older mammals during their epididymal transit.[24] However, we did seemarginal increase in the baseline DNA fragmentation both in mice and human spermatozoa, though studies have shown different protamine and nucleosomal structure in mice and human[10,12] which are responsible for compactness of sperm nuclear DNA. Along with difference in sperm nuclear structure, study subjects were not uniformly distributed in human study; because of this reason may be we were not able to prove statistical difference in baseline DNA damage. Hence, giving the contradictory results to the current literature.

It has been shown that oxidative stress constitutes one of the mechanisms for inducing the DNA damage in spermatozoa, and defective chromatin remodeling renders spermatozoa susceptible to oxidative attack.[25] To investigate whether sperm with dissimilar chromatin architecture exhibit differential sensitivity to genotoxic insult, in this study, both human and mouse spermatozoa from different age groups were exposed to -radiation. The results of our study showed that the direct effect of ionizing radiation on sperm is dependent on the age in human but not in mouse model.

The gonadotoxic effects of cancer therapy especially on sperm DNA integrity is well documented[26,27], yet it remains controversial whether the susceptibility of spermatozoa to commonly used ionizing radiation is related to the age of the patients. In order to test this hypothesis, we included 47 normozoospermic ejaculates from different age groups exposed to radiation. Though, we did see marginal increase in sperm DNA fragmentation until 40 years of age, men who are above 40 years of age had significantly elevated level of sperm DNA fragmentation in response to radiation. The increased genetic abnormalities with age have largely been attributed to genetic changes in the egg and sperm

of the parents.[28] There are reports that DNA damage and chromosomal errors increase with age in sperm[17,29] possibly due to qualitative change in the lineage of sperm producing cells as they age and/or due to defective DNA repair machinery.[28] Since mature sperm is transcriptionally silent, the elevated DNA lesions observed in the present study is unlikely due to aberrant repair pathway but possibly because of age related changes in chromatin architecture. The results also emphasize on the fact that the men who are above 40 years are more prone to acquire sperm DNA damage.

Apart from the differences in the proportion of protamine and nucleosomal structure in mouse and human spermatozoa[10,12,] there were also some differences in the resistance to oxidation between the sperm nuclei of the different species.[30] It has been shown that both mouse and human spermatozoa were quite resistant to in vitro irradiation.[31] However, the distribution of DNA damage among individual sperm cells after irradiation in mice was heterogeneous.[32] In contrast, the human sperm is most susceptible to undergo DNA fragmentation in response to in vitro stimulation when compared to mouse and bull sperm.[16] Our results suggest that sensitivity of mouse spermatozoa to in vitro genotoxic insult is much lower than that of human spermatozoa which agrees with earlier report.[16] The proportion of protamine 2 is 67% and 34% in human and mouse spermatozoa respectively. In addition, some nucleosomal structure is retained in human (up to 15%) and, in smaller quantity(~1%) in mouse spermatozoa[10,12], which could explain the resistance of mouse sperm chromatin to undergo fragmentation in response to in vitro irradiationin comparison to human sperm.

There are limitations in comparing age related sensitivity between two species as mice and humans have a maximum life-span of 4 years and 120 years, respectively which are at two extremes of the longevity range.[33] In spite of their similarities at the molecular, cellular and physiological level, there are dissimilarities in the rates at which they age.[34] However, mouse models are still considered as one of the primary systems in studies of human longevity. Hence, mice model was used in the present study to compare the in vitro radiosensitivity of human spermatozoa of various age groups.

Despite of several observed age related changes, our results demonstrate that irradiation of human spermatozoa results in age dependent increase in the level of DNA fragmentation possibly due to age related differences in chromatin architecture. Therefore,from the clinical point of view, the study demonstrates that men who are above 40 years are at higher risk of acquiring sperm DNA fragmentation after therapeutic and accidental radiation exposures hence proper counseling should be seriously considered as sperm chromatin integrity is of fundamental importance for these subjects to father a child. As the present study is restricted to in vitro irradiation of spermatozoa, further studies are required to confirm our observation in patients who have received radiation therapy.

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