Transgenerational changes in somatic and germ line genetic integrity of first-generation offspring derived from the DNA damaged sperm

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Objective: To report a more quantitative approach to study the influence of varying levels of sperm DNA damage on transgenerational changes in genomic instability in a mouse model.

Design: Experimental prospective study.

Setting: Embryology research laboratory.

Animal(s): Swiss albino mice.

Intervention(s): The sperm DNA damage was induced by different doses of γ-irradiation to male mice followed by mating with healthy female mice.

Main Outcome Measure(s): Genomic integrity in embryos, fetus, and spermatozoa of F1 mice derived from the DNA-damaged sperm.

Result(s): The transgenerational changes in genetic integrity were attributed by a dose-dependent increase in the frequency of micronuclei in preimplantation embryos and a concomitant increase in genomic instability in fetal liver cells and sperm chromatin modifications in F1 males. A strong positive correlation was observed between the extent of sperm DNA damage and somatic and germ-line genomic instability.

Conclusion(s): Sperm-mediated transgenerational genomic instability is dependent on the amount of DNA damage present in the sire’s sperm at the time of fertilization. (Fertil Steril 2010;93:2486–90. ©2010 by American Society for Reproductive Medicine.)

Key Words: Sperm DNA damage, preimplantation embryos, genomic instability, transgenerational effect

The DNA lesion carried by the sperm induces a series of damage responses in the zygotes and in developing embryos (1–5). The germ-line instability caused by various toxicants is able to persist in populations for several generations, and this instability would lead to a significant increase in mutation load (6).

The survival strategies used by the preimplantation embryos in response to sperm DNA damage are not completely understood. Although embryos do show some DNA repair capacity (7), the level of DNA damage that embryos tolerate during their development is not clear (8). It has been shown that the zygotic stage of mouse embryogenesis lacks G1/S arrest because fertilization of DNA-damaged sperm does not delay the entry of the zygotes to S-phase, although DNA synthesis in such embryos was severely suppressed (2). Transgenerational changes in mutation rates and DNA damage originates from paternal DNA damage (9), and the damage responses come in a stage-specific manner during preimplantation development (5).

Here we present data on transgenerational changes in genetic integrity in preimplantation embryos in response to fertilization with DNA-damaged sperm and a concomitant increase in the genomic instability of fetal liver cells and sperm chromatin abnormalities in F1 males. The data provide important information on genetic risk associated with fertilization with DNA-damaged sperm.

MATERIALS AND METHODS

Animals

The animal care and handling were done according to the Institutional Guidelines for animal experimentation. Eight- to 12-week-old Swiss albino mice were used for the experiments. A minimum of 50 embryos/fetuses from more than five pregnant females were used for analysis per data point.

Induction of Sperm DNA Damage

DNA damage to the spermatozoa was introduced by partial body irradiation (0–10 Gy) to the testicular area of males using 60Co teletherapy unit.

Sperm Extraction and Quantification of DNA Damage

Animals were killed 18 hours after irradiation, and spermatozoa were extracted from the cauda epididymis. The sperm suspension was analyzed for DNA integrity by acridine orange (AO) bindability and comet assay.
AO staining of sperm  Smears were prepared from the sperm suspension on a clean glass slide and stained with 0.1% AO in citrate buffer (pH 2.5) as described earlier (10). At least 500 spermatozoa were evaluated from each slide under fluorescent microscope (Imager-A1, Zeiss, Germany) and the percentage of normal and abnormal chromatin conformation was calculated.

Comet assay  The DNA fragmentation in spermatozoa was assessed by alkaline comet assay as described by Singh et al. (11), with minor modifications. Briefly, the suspension containing approximately 1 x 10^6 sperm was mixed with low melting agarose and layered in between normal agarose layers. Overnight incubation in lysing solution was done under alkaline conditions (pH 10) at 4°C. After sperm DNA unwinding in electrophoresis buffer (pH > 13), the slides were stained with ethidium bromide (2 mg/mL) and observed under a fluorescent microscope. The Kinetic Imaging software (Komet 5.5, UK) was used to calculate the percent tail DNA in at least 50 spermatozoa per slide.

In Vivo Fertilization, Embryo Recovery, and Evaluation for Genomic Instability  Male mice were mated 18 hours after irradiation with unirradiated females to obtain embryos derived from the DNA damaged sperm. After successful mating (day 0), the animals were humanely killed at days 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5, and embryos were collected to assess genomic instability by micronucleus assay. Briefly, embryos were treated with 0.9% trisodium citrate for 15 minutes, followed by fixation in Carnoy’s fixative, and then transferred on to a clean glass slide, dried, and stained with propidium iodide (0.1 mg/mL). The total cell number of the embryo and frequency of micronuclei were assessed under fluorescent microscope as described earlier (12). The data was expressed as number of micronuclei per embryonic cell.

Micronucleus Analysis in Fetal Liver Cells  The fetuses derived from the DNA-damaged sperm were collected from the pregnant mice on day 18 of gestation and the liver was removed. The single cell suspension of liver was treated with 0.56% KCl for 1 minute and then fixed using Carnoy’s fixative. The cells were dropped on to precleansed slides and stained with 0.002% AO in Sorensen’s buffer (pH 6.8). The micronuclei scoring was done under fluorescent microscope in at least 1,000 cells.

Sperm DNA Integrity in F1 Males  The spermatozoa collected from 10-week-old F1 generation males derived from the DNA damaged sperm were analyzed for their nuclear integrity by AO bindability and comet assay.
Statistical Analysis
The results were expressed as mean ± SEM. The level of significance was determined by one-way analysis of variance using GraphPad Software (GraphPad Software Inc., San Diego, CA).

RESULTS
Induction of Sperm DNA Damage by Testicular Irradiation
The number of spermatozoa with denatured DNA as estimated by AO bindability assay increased in a dose-dependent manner and significance was evident in the 5- and 10-Gy group compared with the 0-Gy group ($P < .001$) (Fig. 1A).

The comet analysis clearly demonstrated a significant increase in the percent tail DNA even in spermatozoa exposed to the lowest dose of radiation (2.5 Gy), which was not evident by AO bindability method. However, approximately 1.7- and 2.23-fold increase in percent tail DNA was observed in the 5-Gy and 10-Gy group, respectively (Fig. 1B).

Genomic Instability in Preimplantation Embryos derived from DNA-Damaged Sperm
Analysis of the embryos derived from the DNA-damaged sperm revealed an increase in the micronuclei frequency that was dependent on the amount of radiation delivered to induce sperm DNA damage. The increase in micronuclei frequency was not significantly different in any of the groups until day 3.0; however, between days 3.0 and 3.5, an approximately fourfold increase in the micronuclei frequency was observed in the 2.5-Gy group, and this was not significantly different from the micronuclei frequency observed in the 0-Gy group on day 3.5. At a higher dose of 5 Gy and 10 Gy, the micronuclei frequency was enhanced by 13- and 25-fold, respectively, on day 3.5, which was significantly different ($P < .001$) from the 0-Gy group (Fig. 2). Thus, the damage introduced to the sperm by 5 Gy and 10 Gy γ-irradiation significantly induced micronuclei only after four to five cleavage divisions, which is corresponding to day 3.5 of development, although the sperm-induced DNA damage was persistent in these embryos from the time of fertilization.

Transgenerational Genetic Instability in Somatic and Germ Cell Compartment
To test whether genomic instability is equally elevated in the somatic and germ cell compartment of first generation (F1) offspring and the increase is dependent on the extent of the DNA damage carried by the sperm, micronuclei analysis in fetal liver cells and chromatin analysis of spermatozoa was performed. The micronuclei frequency in fetal liver cells was not significantly different between the 0-Gy and the 2.5-Gy groups; however, there was approximately 3- and 10-fold increase in the micronuclei frequency in the 5-Gy and 10-Gy groups, respectively ($P < .001$) (Fig. 3A). Furthermore, the number of viable fetuses in the 5-Gy and 10-Gy groups was significantly lower than the 0-Gy group (data not shown).

The AO bindability analysis of F1 generation spermatozoa has demonstrated a significant enhancement in percent denatured spermatozoa in the 10-Gy group ($P < .01$) when compared with the 0-Gy group. However, other groups did not show any significant
increase in sperm denaturation (Fig. 3B). Similarly, the comet analysis of F1 spermatozoa has also demonstrated a significant amount of tail DNA in the 10-Gy group ($P < .001$), which was approximately 1.5-fold higher than the 0-Gy group. However, other groups failed to show any significant enhancement in tail DNA (Fig. 3C). The tail DNA of F1 offspring’s spermatozoa was approximately 30% lower than paternal spermatozoa exposed to 10-Gy radiation (8.64 ± 0.43 vs. 12.14 ± 0.52), and this decline was statistically significant ($P < .05$). Similarly, the 2.5-Gy and 5-Gy groups also showed a significant decline in percent tail DNA compared with that of paternal spermatozoa in the corresponding groups.

The regression analysis was performed to determine the association between the number of micronuclei observed in day 3.5 preimplantation stage embryos and micronuclei in fetal liver cells. Although no significant association was found in the 2.5-Gy group ($R = .09$), the 5-Gy and 10-Gy groups showed a significant positive correlation ($R = .51$ and .74, respectively) (Fig. 4).

**DISCUSSION**

The data presented in this study revealed high level of unstable genome in preimplantation embryos with an increase in sperm DNA damage load as evident by the micronucleus analysis. To establish whether genetic instability is equally elevated in the germ line and somatic tissues of F1 offspring, we have used micronucleus analysis in liver cells and chromatin analysis of spermatozoa. Interestingly, the genetic instability is transmitted to both somatic and germ-line compartments of the F1 offspring derived from the DNA-damaged sperm. However, the extent of instability observed in embryos, somatic, and germ line are dependent on the amount of DNA damage in the paternal spermatozoa. The sperm-mediated damage in the embryos triggered response only after day 3.5 resulted in a substantial increase in micronuclei frequency, suggesting a delay in the activation of pathways inducing instability. In the embryos, DNA double-strand breaks were introduced at the
zygotic stage, yet micronuclei frequency clearly increased only at day 3.5 in the 5- and 10-Gy group. Thus, the micronuclei must have been induced by newly generated double-strand breaks. Indeed, delayed DNA double-strand breaks were evident as the foci of γ-H2AX overlapped with micronuclei in the embryos derived from the DNA damaged sperm (5). It has been shown that radiation-induced chromosome instability in vitro could be attributed to the long-term delay in chromosome replication (13). It is also suggested that one of the initial steps of damage response is inefficient in early preimplantation stages of mouse embryos because of altered chromatin conformation at these stages (7), and these could be the possible reasons for delayed onset of genetic instability in preimplantation embryos derived from the DNA damaged sperm.

Our earlier observation demonstrated increased frequency of micronuclei in the embryos derived from 6-Gy X-irradiated sperm that activated p21 mediated cell-cycle checkpoint in these embryos (5). However, in the present study, 10-Gy irradiation to spermatozooa induced more than twofold increase in the detectable DNA damage, and these sperm could fertilize approximately 48% oocytes. Furthermore, the resulting embryos successfully implanted and approximately 50% of these embryos reached the term (personal observation), although the micronuclei frequency at the time of implantation was significantly high in this group. In contrast, embryos derived from the DNA damaged sperm in p21−/− mice had very high frequency of micronuclei at the time of implantation, and these embryos died during midgestation (5). However, in the present study, some of the implanted embryos derived from 10-Gy paternal irradiation successfully reached term, but the genetic instability was significantly high in both the somatic and germ-line compartment of the F1 offspring, although the extent of instability detected in the germ line is significantly lower than the level of sperm damage in the sire.

To date, there has been little experimental evidence for transgenerational effect of sperm DNA damage. Although the present study is unique in its examination of the possible transgenerational influence of varying level of sperm DNA damage in both the somatic and germ-line compartment of the F1 offspring, there are few drawbacks in extrapolating these observations to human sperm. Even though the irradiation is a good model where exact dose can be delivered to induce a specific amount of damage, there could be differences in the chromatin architecture of human and mouse spermatozoa with respect to protamine mass and DNA mass ratio, which eventually cause differences in the embryonic response to genetic insult. In addition, there are many other causes for DNA damage in human sperm where factors from human seminal plasma are known to play an important role in modulating the damage. Second, it is not possible to state that all the embryos were derived from the DNA damaged sperm as there is no method available to select the DNA damaged sperm to fertilize the egg. Hence, further studies would be needed in humans to validate our data on transgenerational genetic risk associated with the DNA damaged sperm.

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