

Association between the extent of DNA damage in the spermatozoa, fertilization and developmental competence in preimplantation stage embryos

Spermatozoada DNA hasarının boyutu ile fertilizasyon ve implantasyon öncesi embriyoların gelişimsel kompetansı arasındaki ilişki

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Abstract

Objective: To examine the fertilizing ability and DNA damage response of preimplantation stage embryos derived from the γ -irradiated mouse sperm carrying varying amounts of DNA strand-breaks.

Material and Methods: The DNA damage in the sperm was induced by exposing the testicular area to different doses of γ -radiation. After mating with healthy female mice, sperm zona binding, fertilizing ability of DNA damaged sperm and developmental competence of embryos derived from the DNA damaged sperm were assessed.

Results: The *in vivo* zona binding ability and fertilizing ability of DNA damaged sperm was significantly affected in the 5.0 and 10.0 Gy sperm irradiation groups. Although the development of the embryos derived from the DNA damaged sperm was not significantly affected until day 2.5 post-coitus, further development was significantly altered, as evidenced by the total cell number in the embryos.

Conclusion: The sperm carrying DNA strand breaks still has the ability to fertilize the oocyte normally. However, the events like zona-binding and successful fertilization depend on the extent of sperm DNA fragmentation. The study has also showed a great heterogeneity in embryonic development at peri-implantation period with respect to the degree of sperm DNA damage.

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Key words: Sperm DNA damage, fertilization, preimplantation development

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Özet

Amaç: Gama ışınları ile ışınlanmış ve değişen miktarda DNA zincir kırığı taşıyan fare sperminin dölleme yeteneğini ve bu spermelerden oluşan implantasyon öncesi embriyoların DNA hasarı yanıtını incelemek.

Gereç ve Yöntemler: Spermde DNA hasarı, testiküler bölgenin farklı dozlarda gama ışınlarına maruz bırakılmasıyla indüklendi. Sağlıklı dişi fareler ile çiftleşmeden sonra, sperm-zona bağlanması, DNA hasarlı sperm dölleme yeteneği ve DNA hasarlı spermden oluşan embriyoların gelişimsel kompetansı değerlendirildi.

Bulgular: 5.0 ve 10.0 Gy ışın uygulanan gruplarda, DNA hasarlı sperm *in vivo* zona bağlanma yeteneği ve dölleme yeteneği anlamlı olarak etkilendi. DNA hasarlı spermden oluşan embriyoların gelişimi koitus sonrası 2.5 güne kadar anlamlı ölçüde etkilenmemekle beraber, sonraki gelişim embriyolardaki toplam hücre sayısından anlaşılacağı gibi anlamlı olarak değişti.

Sonuç: DNA zincir kırıkları taşıyan sperm hala oositi normal bir şekilde dölleme yeteneğine sahiptir, bununla beraber, zona-bağlanması ve başarılı fertilizasyon gibi olaylar sperm DNA parçalanmasının boyutuna bağlıdır. Çalışma ayrıca peri-implantasyon döneminde embriyonik gelişimde sperm DNA hasarının derecesine ilişkin olarak büyük bir heterojenlik göstermiştir.

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Anahtar kelimeler: Sperm DNA hasarı, fertilizasyon, implantasyon öncesi gelişim

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Introduction

Ionizing radiation induces DNA double-strand breaks (DSB) and inflicts a variety of DNA damage responses which include induction of cell cycle checkpoint and apoptosis (1). Paternal irradiation in experimental animals sometimes induces genomic instability in somatic and germ cells, which is detected in the offspring (2). Female mammals undergo a cycle of oogenesis once in their lifetime. In contrast, cycles of spermatogenesis persist throughout the life of sexually mature males.

Therefore, males are more convenient for analyzing the effect of radiation at various stages of spermatogenesis (3).

Although the chromatin is highly condensed in the mature spermatozoa, it is susceptible to various genetic insults, producing large numbers of single and double strand breaks (4). The DNA lesion carried by the sperm induces a series of damage responses in the zygotes and in developing embryos (5-7). However, none of these studies have addressed the response in early embryos with respect to the extent of DNA strand breaks in the spermatozoa present at the time of ferti-

lization. Hence, in this study, an attempt was made to quantify the degree of DNA strand breaks in γ -irradiated spermatozoa and then assess fertilization and subsequent pre-implantation developmental potential of the embryos using a mouse model.

Material and Methods

Animals and irradiation

Eight to twelve week old healthy male and female Swiss Albino mice were used for the experiments. The DNA damage to the spermatozoa was introduced by partial body irradiation (0, 2.5, 5.0 and 10.0 Gy) to the testicular area of males using ^{60}Co teletherapy unit.

Sperm extraction and quantification of DNA strand breaks

Eighteen hours after irradiation, animals were sacrificed and spermatozoa were extracted from the caudae epididymis in one milliliter of pre-warmed Earle's Balanced Salt Solution (EBSS). The sperm suspension was analyzed for DNA integrity by alkaline comet assay as described by Singh *et al.* (8), with minor modifications. Briefly, 10 μl of sperm suspension was mixed with 200 μl of 0.8% low melting agarose and layered on a slide precoated with 1% normal agarose. A third coat of 0.8% agarose was layered over the second layer, followed by overnight incubation in lysing solution (2.5M NaCl, 100mM disodium EDTA, 10mM Trizma base, pH 10, 1% Triton X-100, 10mM GSH and 100 μM heparin) under alkaline conditions (pH 10) at 4°C. After allowing the sperm DNA to unwind in electrophoresis buffer (300mM NaOH, 1mM EDTA, pH >13) for 20 minutes, electrophoresis was carried out at 25V for 20min. The slides were stained with ethidium bromide (2 $\mu\text{g}/\text{ml}$) and observed under a fluorescent microscope (Imager-A1, Zeiss, Germany). The comet evaluation of the captured images was done using Kinetic Imaging software (Komet 5.5, UK). The percent tail DNA was calculated in at least 50 spermatozoa per slide and a minimum of five animals were used per data point.

Assessment of sperm zona binding

The irradiated male mice were mated with normally cycling healthy female mice for one hour. The successful mating was confirmed by the presence of vaginal plug. The oocytes collected at 10h after mating were washed using M16 medium, placed onto clean glass slides and observed using a 40X objective. The data on the number of sperm bound to zona in the different radiation groups was collected. To test the *in vitro* sperm binding ability to zona pellucida, oocytes with a intact cumulus were inseminated in 100 μl sperm suspension containing 1x10⁶ motile spermatozoa/ml incubated at 37°C and 5% CO₂. After 2 hours, oocytes were denuded and observed under the 40X objective for the evaluation of the number of sperm bound to the zona pellucida.

Evaluation of fertilization

The oocytes were collected at 10 hours after mating and successful fertilization was confirmed by the appearance of two pronuclei and two polar bodies.

Assessment of embryonic development potential

Preimplantation embryos were collected from the oviduct on day 1.0, 1.5, 2.0 and 2.5 and from the uterine horn on day 3.0 and 3.5

by gentle flushing with EBSS under the stereomicroscope (Nikon SMZ-10, Japan) and examined under the phase contrast microscope (Olympus IX70, Japan) for morphological assessment. Cell numbers were counted under a fluorescent microscope after treating the embryos with 0.9% trisodium citrate for 15 min, followed by fixation in Carnoy's fixative, and staining with propidium iodide (0.1mg/ml) on a clean glass slide.

Statistical analysis

The results were expressed as mean \pm SEM. The level of significance was determined by One Way Analysis of Variance (ANOVA) and Unpaired 't' test using Graph pad software Inc. USA.

Results

Successful induction of DNA breaks by γ -radiation

The percent tail DNA in spermatozoa exposed to the lowest dose of radiation (2.5Gy) was 7.95 \pm 0.42, which was significantly ($P<0.01$) higher than the control group (5.44 \pm 0.35). Approximately 1.8 and 2.2 fold increase in percent tail DNA was observed in 5 and 10.0Gy group respectively in comparison to the control group. An attempt was made to analyze the distribution of DNA damaged sperm in relation to the tail DNA and the amount of radiation dose received. The number of DNA strand breaks induced by γ -radiation consistently increased with higher doses of radiation. In the control group, the majority of spermatozoa had intact DNA (0-5% tail DNA) and less than 5% were severely damaged. However, in the case of irradiated spermatozoa, the percentage of spermatozoa with intact DNA sharply decreased with a concomitant increase in the spermatozoa with moderate (5-15% tail DNA), high (15-20% tail DNA) and severe (>20% tail DNA) damage.

Sperm-zona binding

The mean number of spermatozoa attached to the zona pellucida in the control group was approximately 1.2. The number of spermatozoa bound to zona pellucida in different sperm irradiated groups was reduced in a dose-dependent manner. Even though no significant decline was observed in the 2.5.0Gy group, 5.0 and 10.0Gy groups exhibited a significant decline in the number of spermatozoa bound to the zona pellucida compared to the control group ($p<0.05$ and $p<0.001$ respectively). In addition, the number of sperm bound in 2.5.0Gy and 10.0Gy groups were significantly different ($p<0.001$) (Fig 1A). When the number of spermatozoa bound to the zona pellucida of fertilized oocytes and unfertilized oocytes were compared, no significant difference was observed in the control and 2.5.0Gy groups. However, in the the 5.0Gy and 10.0Gy groups, the spermatozoa bound to unfertilized oocytes were almost 50% of the spermatozoa that were bound to fertilized oocytes. The ability of sperm binding to zona *in vitro* did not show any significant difference (Fig 1B).

Influence of sperm DNA fragmentation on fertilization

The assessment of fertilization was made in the oocytes collected from the normally cycling healthy females mated with irradiated males. A minimum of 50 oocytes were assessed for each data point. The fertilization rate observed in the control group was approximately 95%, whereas an inverse relationship was observed between the dose of sperm irradiation and fertilization rate. Although, the fertilization rate in the 2.5.0Gy group

was not significantly affected, 5.0Gy sperm irradiation had a significantly reduced fertilization rate ($p < 0.01$). Further, in the 10.0Gy group, approximately 50% of the oocytes failed to demonstrate successful fertilization ($p < 0.001$) (Fig. 2A, Table 1).

Influence of sperm DNA fragmentation on pre-implantation development

The first cleavage of sperm-irradiated embryos (embryos derived from the irradiated sperm) was not affected except in the 10.0Gy group, where approximately 42% embryos showed delayed development. In addition, the embryonic fragmentation was evident in approximately 14% of the 10Gy sperm-irradiated, delayed embryos on day-1 of development. On day-1.5 the embryo fragmentation rate in this group was further increased to 27% (Fig. 3 upper panel). The cleavage rate was further reduced in the 10.0Gy group on day-2 of development where only 71% embryos successfully completed two cleavage divisions and the remaining embryos were arrested at 2-3 cell stage. On day-3 of development, the cleavage delay was also evident in 10.0Gy as well as in 5.0Gy sperm-irradiated embryos. Approximately 40% of 5.0Gy sperm-irradiated embryos and 50% of the 10.0Gy group failed to complete compaction, which was significantly lower than the control group ($p < 0.05$). However, on day-3.0, no morphological

abnormalities were detected in the sperm-irradiated embryos of all the groups. On day-3.5, approximately 65% and 35% embryos reached blastocyst stage in the 5Gy and 10Gy groups respectively, whereas in the control group, all the embryos successfully reached blastocyst stage (Fig. 3 lower panel).

Total cell number in preimplantation stage embryos:

The TCN in the sperm-irradiated embryos was not significantly different on day 1.0 and 1.5. However, on day-2.5, embryos derived from 5.0Gy sperm irradiation demonstrated a significant decline in TCN, which was approximately 9% compared to the control group ($p < 0.05$). Although TCN in this group increased during subsequent developmental periods, it was approximately 23% and 25% lower than control group on day-3.0 and 3.5 respectively. However, 10.0Gy sperm irradiation resulted in lower TCN as early as on day-2.0 of development, which subsequently resulted in the significant decline of TCN throughout their pre-implantation development ($p < 0.05-0.001$). This decline in TCN was approximately 12%, 25% and 32% on day 2.0, 2.5 and 3.0 respectively. On day-3.5, the reduction in TCN in this group was approximately 50% compared to the control group (Fig. 2B).

Discussion

The present study clearly demonstrated that sperm carrying varying levels of DNA strand breaks still has the ability to fertilize the oocyte normally. However, successful fertilization depends on the extent of DNA strand breaks in the sperm at the time of fertilization. In addition, the study has also shown that development of the sperm-irradiated embryos exhibit great heterogeneity at the peri-implantation period with respect to the degree of sperm DNA damage. This work also demonstrate the fact that the effects of sperm DNA fragmentation are apparently visible only when embryonic genome becomes completely functional at the peri-implantation period of development. Several *in vitro* studies demonstrated the decline in fertilization rate with increasing amounts of sperm DNA damage (9, 10). In contrast, other *in vitro* studies in human (11, 12), bovine (13), and mice (5) found no decline in fertilization rates. However, the novel observation in our *in vivo* study has demonstrated that spermatozoa carrying a high level of DNA strand breaks still has the ability to reach the oviduct and fertilize the oocyte successfully. It is possible that a significant number of DNA fragmented spermatozoa might have been retained/removed prior to/during the oviductal passage. This was supported by the fact that the number of spermatozoa bound to the zona pellucida

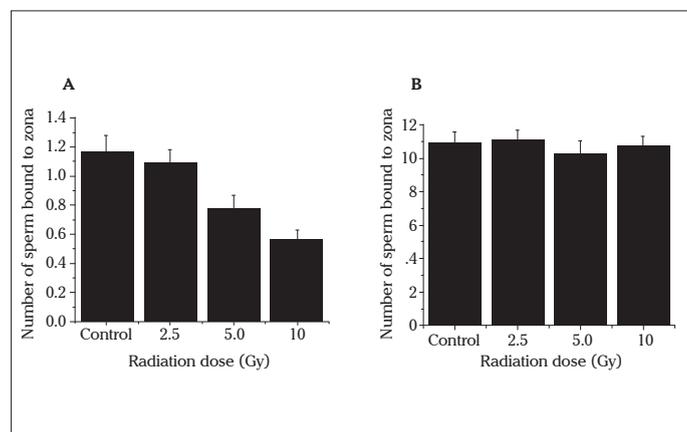


Figure 1. A. *In vivo* zona binding ability of male mice exposed to different doses of γ -radiation (the data includes both fertilized and unfertilized oocytes). $p < 0.05$ for 5.0Gy v/s control; $p < 0.001$ for 10.0Gy v/s control and 2.5.0Gy B. *In vitro* zona binding ability of epididymal sperm collected 18h after exposure to different doses of γ -radiation. The data were indicative of three independent experiments. A minimum of 50 oocytes were assessed in each group to determine the sperm-zona binding

Table 1. Comet analysis showing the distribution of DNA fragmented spermatozoa exposed to various doses of γ -radiation

Group	Spermatozoa evaluated	Distribution of spermatozoa with respect to percent tail DNA (Mean \pm SEM)				
		0-5	5-10	10-15	15-20	>20
Control	274	62.54 \pm 2.7	17.6 \pm 3.0	14.38 \pm 1.9	3.5 \pm 1.8	1.96 \pm 0.6
2.5Gy	275	44.53 \pm 3.5	24.75 \pm 5.0	14.98 \pm 2.8	8.46 \pm 0.4	7.27 \pm 1.2
5.0Gy	262	32.06 \pm 2.2	25.64 \pm 2.1	19.46 \pm 3.5	13.06 \pm 2.1	9.76 \pm 2.9
10.0Gy	271	27.18 \pm 4.6	21.96 \pm 2.5	19.92 \pm 2.5	13.7 \pm 2.0	17.26 \pm 3.3

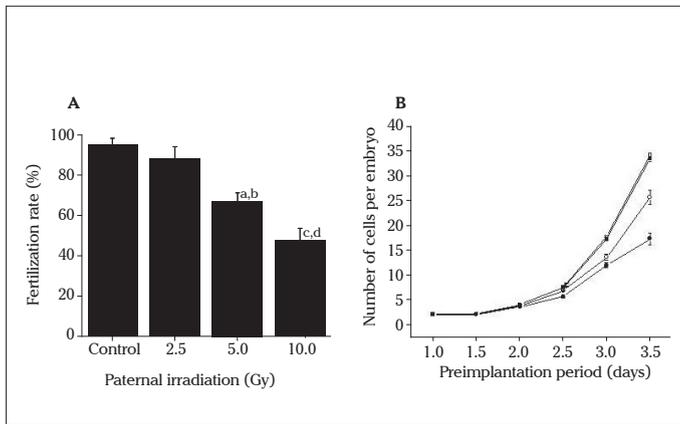


Figure 2. A. Fertilizing ability of sperm exposed to different doses of γ -radiation. ^a $p < 0.001$ for 5.0Gy v/s control; ^b $p < 0.05$ for 5.0Gy v/s 2.5.0Gy; ^c $p < 0.001$ for 10.0Gy v/s control, and 2.5.0Gy; ^d $p < 0.05$ for 10.0Gy v/s 5.0Gy. **B) Total cell numbers (number of cells/embryo) in sperm irradiated embryos at different preimplantation days** (□ Control; ■ 2.5.0Gy; ○ 5.0Gy; ● 10.0Gy). $P < 0.001$ for 10.0Gy v/s control, and 2.5.0Gy on day 2.0; 10.0Gy v/s control, 2.5.0Gy, and 5.0Gy on day 2.5; 5.0Gy v/s control, and 2.5.0Gy on day 3.0; 10.0Gy v/s control, and 2.5.0Gy on day 3.0; 5.0Gy v/s control, and 2.5.0Gy on day 3.5; 10.0Gy v/s control, 2.5.0Gy, and 5.0Gy on day 3.5 $p < 0.05$ for 10.0Gy v/s 5.0Gy on day 2.0; 5.0Gy v/s control on day 2.5

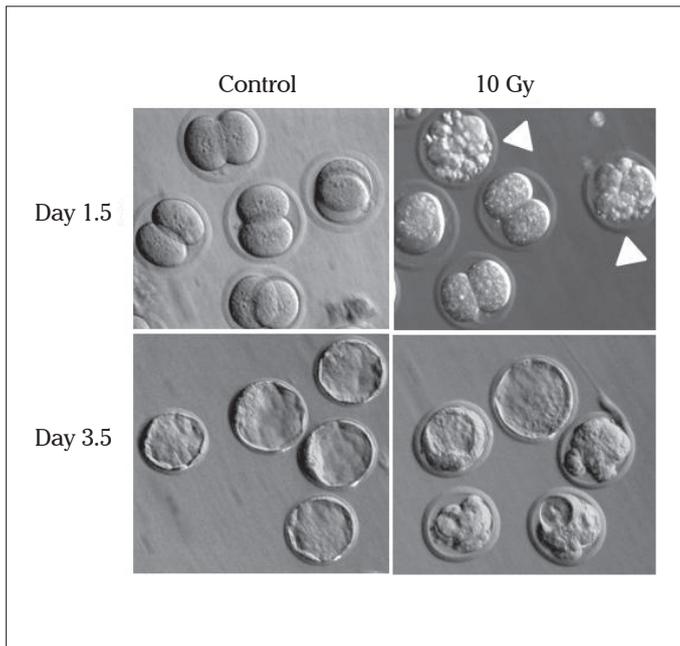


Figure 3. Developmental potential of the sperm irradiated embryos (10.0Gy) and control on day 1.5 and 3.5. Arrowhead in day 1.5 indicates fragmented one-cell embryos and arrowhead in day 3.5 shows cleavage stage embryos in 10.0Gy group

in the 10Gy sperm irradiation group was significantly lower. In addition, the number of spermatozoa bound to unfertilized oocytes was low in the 10.0Gy sperm irradiation group, indicating that a significant number of spermatozoa failed to reach the oocyte. Earlier, it was shown that human spermatozoa bound to oviduct cells had better DNA integrity than those of unbound

sperm, suggesting that sperm selection may occur during sperm transportation in the female reproductive tract *in vivo* (14). This indicates that mostly defective spermatozoa were withheld in the uterus or uterotubal junction and such males showed drastically reduced fertility (15). Thirdly, there might be a decline in zona binding ability of the DNA damaged spermatozoa since it has been shown that sperm with single stranded or denatured DNA generally do not bind to the zona pellucida (16). However, the present study demonstrated no significant difference in zona-binding ability of the spermatozoa with varying levels of DNA strand breaks, when inseminated *in vitro*. This suggests that DNA fragmentation in sperm does not impair the zona binding ability. However, a significant number of damaged sperm are eliminated during their passage in the reproductive tract, which eventually resulted in the reduced number of sperm available for zona binding at the site of fertilization.

The reduced fertilization rate observed in the present study could be possibly due to one of the following factors. Primarily, a high incidence of fragmented oocytes was observed in the 5.0 and 10.0Gy groups. Fertilization with DNA damaged sperm leading to failed sperm head decondensation and pronuclear formation might have resulted in subsequent fragmentation. This is supported by an earlier study where the high incidence of fragmented oocytes resulted from mutagen treated spermatozoa, which is considered to be due to a result of fertilization with highly DNA damaged sperm (17). Thus, decline in fertilization rate is possibly associated with fragmentation caused by fertilization with DNA damaged sperm. Further, reduced oocyte availability in this group could be ruled out since the sum of the fertilized and fragmented oocytes in the 5.0Gy and 10.0Gy groups was almost similar to fertilized oocytes in the control group, indicating that an equal number of oocytes was available for fertilization in all the groups. Thus, the decline in fertilization rate might be either due to the high incidence of oocyte fragmentation caused by DNA damaged sperm which reached the oocyte after escaping the barriers of female reproductive tract, or a decreased number of spermatozoa reaching the oocyte due to selective barriers of the female reproductive tract or a collective defect. In addition, there is a possibility that spermatozoa with gross damage might have been retained while those with unidentified damage might have reached the oocyte since motility of the DNA damaged spermatozoa is not altered. However, the mechanism behind this phenomenon is not known.

The effects of sperm DNA integrity are apparent on the cleavage stage embryos when the paternal genome becomes activated and its transcriptional activity begin to play a contributory role in embryo function (18). It has been found in various animal studies that the damage response in early embryos is stage specific (7, 19). *Xenopus* embryogenesis has been characterized by a period called midblastula transition in which a burst of transcription takes place concomitant with prolongation of cell cycle time (20). A similar transition period is widespread among many metazoan species. In mice, even though mouse embryonic gene transcription starts at the late one-cell stage, the apparent effects become visible during the midblastula transition period, which corresponds to the morula-blastocyst stage when a burst of transcription takes place (21). In the present study, while developmental abnormalities were observed on day 2.0 onwards, it was marked after day 3.0, which corresponds to the morula blastocyst transition stage.

Hence, the embryonic response to genetic insult introduced by the sperm is stage specific, which is possibly associated with changes in chromatin conformation or activation of the embryonic genome (22). Further studies are required to elucidate the mechanism associated with sperm DNA damage response during embryonic genome activation.

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Conflict of interest

No conflict of interest is declared by authors.

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