Paternal DNA damage suppresses in vitro proliferation of mouse inner cell mass

Paternal DNA hasarı fare embriyolarında iç hücre kitlesinin in vitro çoğalmasını baskılar

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Abstract

Objective: Sperm DNA damage is known to cause developmental failure and reduction in the numbers of live offspring and the effects of damages range as diverse as embryonic death and cancer susceptibility in the offspring. Here we report the in vitro proliferation ability of the inner cell mass of the mouse embryos derived from the DNA damaged sperm and its association with post implantation developmental potential.

Material and Method: Day 3.5 mouse embryos derived from the DNA damaged sperm were cultured on MEF feeder layer and proliferation ability of the inner cell mass was assessed for six days. The post implantation developmental competence was studied by fetoplacental analysis on day 18 of gestation.

Results: The development of embryos derived from 6 Gy irradiated sperm demonstrated heterogeneous growth on day 3.5 as approximately 1/3rd of the embryos failed to undergo compaction and demonstrated high frequency of micronuclei. In addition embryos showing developmental delay on day 3.5 failed to form any outgrowth during 6 day of in vitro culture. The fetoplacental analysis on day 18 of gestation showed a 50% reduction in the number of fetus derived from the DNA damaged sperm although the number of implantations was not affected.

Conclusions: Our study demonstrates that DNA damage in sperm can lead to preimplantation embryonic developmental delay resulting in defective ICM proliferation possibly due to increased genomic instability and such embryos die in utero.

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Key words: DNA damage, implantation potential, inner cell mass proliferation

Özet

Amaç: Sperm DNA hasarının gelişimsel bozukluğa ve sağlıklı döl sayısının azalmasına neden olduğu bilinir ve DNA hasarının etki kapsamı embriyonik ölümden kanser yatkınlığına kadar çeşitlilik gösterir. Hasarlanmış sperm DNA'sından elde edilen fare embriyolarının iç hücre yığınının in vitro üreme kabiliyetini ve implantasyon sonrası gelişme potansiyeliyle olan ilişkisini raporladık.

Gereç ve Yöntemler: Hasarlanmış sperm DNA'sından elde edilen 3.5 günlük fare embriyolarının kültürü yapıldı ve 6 gün boyunca iç hücre yığınının üreme kabiliyeti değerlendirildi. Implantasyon sonrası gelişme yeteneği gestasyonun 18. gününde fetoplasental analiz yoluyla çalışıldı.

Bulgular: 6 Gy ışınlama sonrası elde edilen spermle oluşturulan embriyonun gelişiminde 3.5 günde heterojen büyüme gözlendi (heterojen büyüme: embriyoların sıkışmaya doğru giderken yaklaşık 1/3'nün kaybedilmesi ve mikronukleus sayısında artışın gözlenmesi). Buna ek olarak embriyoların gelişiminin 3.5 gününde gelişimsel gecikme sonucu kaybedildiği gözlendi (in vitro kültürünün 6 gün boyunca devam eden doğal şekillenme). Gestasyonun 18. gününde yapılan fetoplasental analizde implantasyon oranı etkilenmemiş olmasına rağmen hasarlanmış sperm DNA'sından elde edilen fetus sayısının %50'sinin redükte olduğu görüldü.

Sonuç: Çalışmamız; spermdeki DNA hasarının, ICM üremesinin defektif olması (genomik instabilite artışı ve inutero ölüm gibi) sonucunda muhtemelen meydana gelen preimplantasyon embriyonik gelişim gecikmesinde yol gösterici olabileceği gösterildi.

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Anahtar kelimeler: DNA hasarı, implantasyon potansiyeli, iç hücre yığının çoğalması

Introduction

The preimplantation embryos are quite sensitive to DNA damages (1) including those introduced by fertilization with irradiated sperm (2). In mouse, sperm DNA damage was associated with developmental failure and reduction in the numbers of live offspring (3) and the effects of damages range as diverse as embryonic death and cancer susceptibility in the offspring (4-8). Although, the detailed mechanisms are yet to be elucidated, it is already known that the cell cycle checkpoint and the regulatory pathway in early embryos differ significantly from those in differentiated somatic cells (9-11).

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, the level of DNA damage that embryos tolerate during their development is not clear (12).We have recently shown that the zygotic stage of mouse embryogenesis lacks G1/S arrest since fertilization of DNA damaged sperm does not delay the entry of the zygotes to S phase although DNA synthesis in

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such embryos was severely suppressed. (13). In addition, the oocytes fertilized by the DNA damaged sperm progressed to 2 cell stage without any significant delay at the G2/M border even though some of them carried sub-optimal amounts of DNA due to the p53 dependent S checkpoint (14).

Although, our earlier observation confirmed p53 dependent checkpoint (13-15) and increased apoptosis in the ICM compartment of the embryos (16) derived from the DNA damaged sperm, until now there is no information on the in vitro proliferation ability of the inner cell mass of the embryos derived from the DNA damaged sperm and its association with in vivo implantation potential. Our result demonstrated a strong association between in vitro ICM proliferation and in vivo developmental competence of the embryos fertilized with DNA damaged sperm.

Materials and Methods

Animals

The animal care and handling were done according to the Kyoto University Guidelines for animal experimentation. Eight to ten week old ICR mice were used in this study and they were maintained under the controlled conditions of temperature (23±2°C), and light (12 h light/dark cycle) with standard diet and water ad libitum. The strain ICR usually has a large ovulation numbers and a minimum of 50 embryos from more than 5 pregnant females were used for analysis per data point.

Induction of sperm DNA damage:

DNA damage to the spermatozoa was introduced by partial body X-irradiation to the testicular area of males covering the rest of the body with lead shields. A dose of 6 Gy was given at a dose rate of 2 Gy/min (250 KVp, 15 mA with 1 mm thick aluminum filter, Rigaku Radioflex 350 X-ray Generator).

In vivo fertilization, embryo recovery and assessment:

Male mice were mated within 24 hr after irradiation to unirradiated females to obtain sperm irradiated embryos (embryos derived from the DNA damaged sperm). Mating was allowed for 1 hr between 6 to 7 am and successful mating was confirmed by the presence of vaginal plug which then was designated as day 0. Animals were killed humanely on day 3.5 and embryos were collected from the uterine horn by gentle flushing with warm ES cell medium. Control embryos were those fertilized with unirradiated sperm and sperm irradiated embryos were those fertilized with 6 Gy irradiated sperm. Embryos were examined under the phase contrast microscope for their morphology and assessed for embryonic staging.

In vitro culture of day 3.5 embryos:

Embryos recovered on day 3.5 were cultured in vitro on mouse embryonic fibroblast (MEF) feeder layer using embryonic stem (ES) cell medium supplemented with 15% fetal bovine serum, 0.1 mM non-essential amino acids, 2 mM glutamine and 1000 units/ml leukemia inhibitory factor (LIF). All manipulations were done at 37°C on a heating stage. Proliferation ability of the embryos fertilized with DNA damaged sperm was assessed regularly for six days and photographed.

Analysis of in vivo implantation potential:

The pregnant mice were killed humanely on day 18 of gestation and the numbers of fetuses and placentas were scored. All fetuses even with morphological anomalies were scored as live fetuses.

Statistics

Statistical significance was assessed using Chi-square test and $p < 0.05$ was considered significant.

Results

Microscopic assessment of the embryos fertilized with DNA damaged sperm was carried out on day 3.5 after fertilization. Although, the initial cleavages in the sperm irradiated embryos were comparable to those of control embryos, morphological features of the two were heterogeneous as we observed approximately 1/3rd of the sperm irradiated embryos failed to undergo compaction on day 3.5 (Fig 1).

To further investigate the embryos derived from the DNA damaged sperm, they were fixed onto slides and stained with DAPI. The results indicated that a substantial number of sperm irradiated embryos demonstrated delay on day 3.5 had a high level of micronuclei (Fig. 2). In contrast, the incidence of micronuclei among normally developed sperm irradiated embryos were similar to the control embryos. When the relative number of embryos with micronucleated cells was ascertained in terms of developmental stage, an elevated micronuclei frequency was shown to be associated with embryos that demonstrated developmental delay on day 3.5 (Fig 2 b,c). This result provided evidence that fertilization with DNA damaged sperm induces genomic instability in some of the embryos which eventually causing developmental delay in their preimplantation development.

A reduction in cell number associated with increased incidence of micronuclei in sperm irradiated embryos made us to determine the growth capability and survival potential in these embryos. We cultured the day 3.5 embryos derived from the DNA damaged sperm individually on MEF feeder layer in vitro for 6 days. The embryo hatching, attachment to feeder layer and formation of out growth was monitored daily. Within two days,

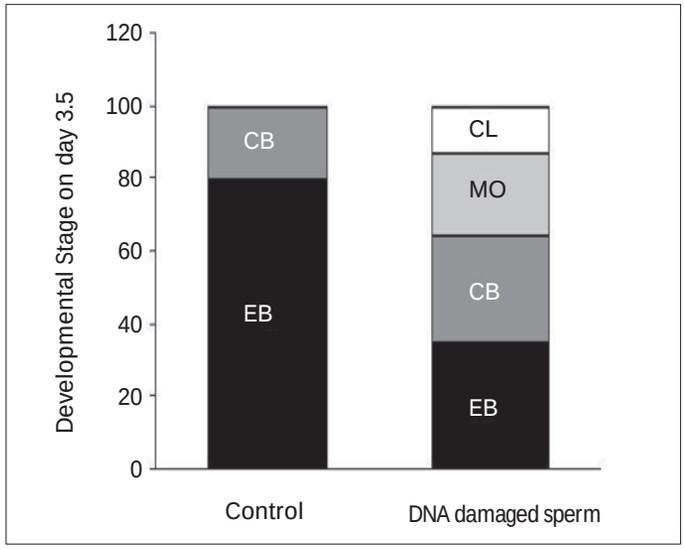

Figure 1. Development of mouse embryos derived from the DNA damaged sperm on day 3.5

EB: expanded blastocyst, CB: cavitating blastocyst, MO: morula, CS: cleavage stage embryos

control embryos attached to the feeder layer, hatched from the zona pellucida, started proliferation and continued to expand throughout the in vitro culture for 6 days (Fig 3 a-c). In contrast, only 55% of the sperm irradiated embryos, normally developed on day 3.5, proliferated and were indistinguishable from the control embryos in which proliferating ICM surrounded by trophoblast was observed during 2-4 days (Fig 3d-f). The remaining embryos showed reduced ICM proliferation during their development in vitro (data not shown). The sperm irradiated delayed embryos have attached to the feeder layer between day 1-3, but failed to expand ICM (Fig 3g-i) suggesting that, normally developed blastocysts from the DNA damaged sperm on day 3.5 were capable of undergoing implantation. In contrast, sperm irradiated delayed embryos developed until blastocyst stage when cultured on MEF feeder layer and then attached to the feeder layer. But none of the delayed embryos formed any outgrowth during 6 day in vitro culture.

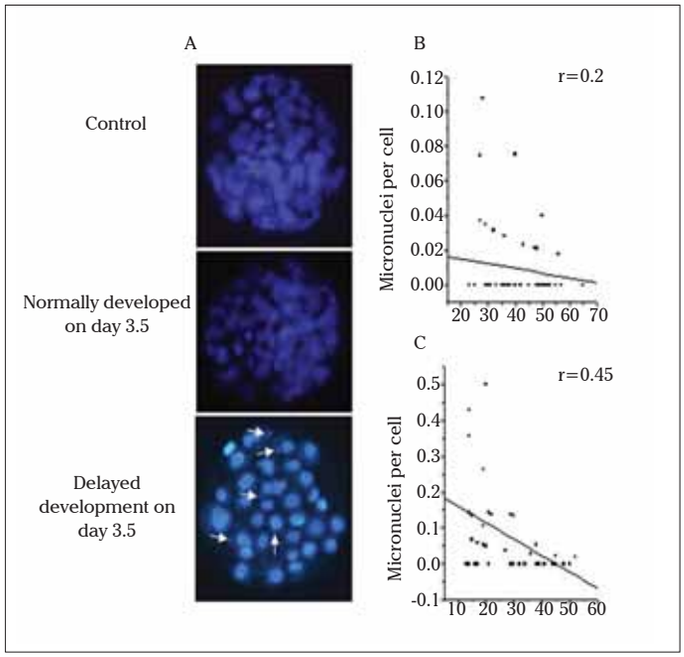

Figure 2. Genetic instability in the embryos on day 3.5

A. The embryo derived from the unirradiated sperm (top), 6 Gy sperm irradiation (middle) and delayed development in embryo derived from 6 Gy sperm irradiation (below) showing numerous micronuclei (arrow head). B. Association between total cell number in the embryos and micronuclei per cell in control and C. sperm irradiated embryos

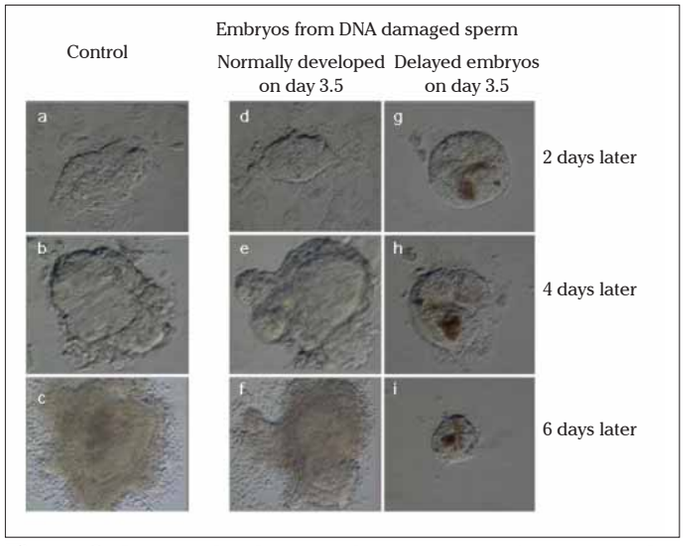

Figure 3. In vitro developmental competence of sperm irradiated embryos

Embryos (day 3.5) derived from 0 and 6 Gy sperm irradiation were cultured on MEF feeder layer. (a-c), Control. (d-f), The Sperm irradiated normally developed (at day 3.5) embryos progressed similar to control embryos during in vitro culture, however, some of them (g-i), Sperm irradiated delayed embryos on day 3.5 (c,f and i: photographed under same magnification). The data were representative of three independent experiments

Discussion

Sperm DNA is tightly packed and decondensation takes place in ooplasm at fertilization. Hence, DNA lesion carried by irradia-

The feto-placental analysis was performed on day 18 of gestation to find out the association between in vitro ICM proliferation and in vivo implantation and post implantation developmental capabilities of the sperm irradiated embryos. The average number of fetus and placenta were comparable in pregnant mice where embryos were derived from the unirradiated sperm. Similarly, the number of placenta observed in the pregnant mice carrying sperm irradiated embryos was almost same as control group suggesting the implantation potential of the sperm irradiated embryos was not affected. In contrast, the fetal number was reduced in these animals by approximately fifty percent suggesting the death of the sperm irradiated embryos/fetus during the course of their postimplantation development (Fig 4).

Taken together, the results from both in vitro and in vivo observations suggest that both sperm irradiated normally developed and delayed embryos still have the implantation potential but many of them lack the in vitro proliferation ability or their capability to undergo differentiation in vivo and die during gestation possibly due to inadequate number of normal ICM cells or increased genomic instability in the embryonic cells.

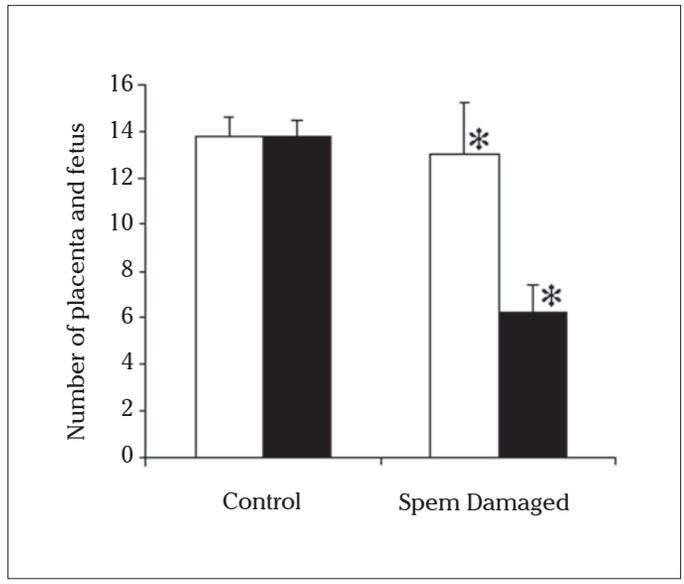

Figure 4. Feto-placental analysis in pregnant mice on day 18 of gestation

■Number of placenta (mean±SEM). □Number of fetus (mean±SEM). ® $p<0.005$

ted sperm induces a series of damage responses in the zygotes and in developing embryos. It has been found in various animal studies that the damage response in early embryos are stage specific (9-11). 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 (17). Similar transition period is widespread among many metazoan species. In mice, the midblastula transition period corresponds to the morula-blastocyst stage when a burst of transcription takes place (18-20).

The survival strategies in the early embryos are stage specific. It was reported that mouse embryos become especially sensitivity to DNA damage after the postimplantation stage and they undergo Atm- and p53-dependent apoptosis (21). Sperm irradiation failed to induce any cleavage delay until four cell stages in mouse embryos (14). In the present observation, some of the sperm irradiated normally developed embryos on day 3.5 failed to demonstrate ICM proliferation possibly due to genomic instability resulting in inadequate number of healthy ICM cells to form viable pregnancy. The sperm irradiated delayed embryos also had numerous micronuclei suggestive of increased genomic instability in these embryos and this could be one reason for the altered postimplantation development in the sperm irradiated embryos. Trophoectoderm is highly radioresistant and it is refractory to radiation induction of apoptosis (1,21). Since the trophoectoderm is the sole requirement for the attachment of the blastocysts to the endometrium and the radioresistance of the extraembryonic cells is the sole reason for the insensitivity of the number of implantation in the sperm irradiated embryos observed in our study. The inner cell mass, including embryonal stem cells are highly sensitive to apoptosis (22), and fetal development of sperm irradiated embryos is reduced to about 50% of the control possibly due either existence of genomic instability after implantation or elimination of inner cell mass by apoptosis resulting in inadequate number of cells required for further differentiation and fetal development.

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