Published in final edited form as:

J Androl. 2012 ; 33(2): 229–238. doi:10.2164/jandrol.111.013532.

Paternal DNA damage resulting from various sperm treatments persists after fertilization and is similar prior and after DNA replication¹

Yasuhiro Yamauchi, Jonathan M. Riel, and Monika A. Ward

Institute for Biogenesis Research, John A. Burns School of Medicine, University of Hawaii, 1960 East-West Rd, Honolulu, HI, 96822

Abstract

In spite of its highly condensed state sperm DNA is vulnerable to damage that can originate from oxidative stress and/or the activity of sperm-specific nucleases. After fertilization, in the oocyte, paternal chromatin undergoes dramatic changes and during this extensive remodeling it can be both repaired and degraded, and these processes can be linked to DNA synthesis. Here, we analyzed sperm response to damage-inducing treatments both before and after fertilization, and prior or after zygotic DNA replication.

Epididymal mouse spermatozoa were either frozen without cryoprotection (FT) or treated with detergent Triton X-100 coupled with dithiothreitol (TX+DTT) to induce DNA damage. Fresh, untreated sperm served as control. Immediately after preparation spermatozoa from three groups were taken for comet assay, or for intracytoplasmic sperm injection (ICSI) into prometaphase I (proMI) oocytes to visualize prematurely condensed single chromatid chromosomes, or into mature metaphase II (MII) oocytes to visualize chromosomes after DNA replication. Comet assay revealed increased DNA fragmentation in treated sperm when compared to control, with FT sperm more severely affected. Chromosome analysis demonstrated paternal DNA damage in oocytes injected with treated but not with fresh sperm, with FT and TX+DTT groups now yielding similar damage. There were no differences in the incidence of abnormal paternal karyotypes prior and after DNA synthesis in all examined groups.

This study provides evidence that subjecting sperm to DNA damage inducing treatments results in degradation of highly condensed sperm chromatin when it is still packed within the sperm head, and that this DNA damage persists after fertilization. The difference in DNA damage in sperm subjected to two treatments was ameliorated in the fertilized oocytes suggesting that some chromatin repair might have occurred. This process, however, was independent of DNA synthesis, and took place during oocyte maturation.

Keywords

sperm; ICSI; sperm chromosomes; sperm DNA damage; DNA synthesis; oocyte

Introduction

The postmeiotic male germ cells are sensitive to the induction of heritable genomic damage due to progressively declining ability to repair DNA lesions (Ahmed, et al., 2010, Marchetti

¹This material is based on work supported by NIH HD048845 and NIH RR024206 (Project 2) grant to M.A.W.

Corresponding author: Monika A. Ward, Institute for Biogenesis Research, University of Hawaii, 1960 East-West Rd, Honolulu, HI, 96822, Tel: 808-956-0779, Fax: 808-956-7316, mward@hawaii.edu, URL: <http://www.ibr.hawaii.edu/faculty/mward.html>.

and Wyrobek, 2008, Sotomayor and Sega, 2000). Sperm DNA damage is one of the major causes of male infertility and is of much concern in relation to the paternal transmission of mutations and cancer (Adiga, et al., 2010, Aitken, et al., 2009, Fernandez-Gonzalez, et al., 2008), especially considering that sperm with damaged DNA can fertilize oocytes (Marchetti and Wyrobek, 2008, Zenzes, 2000). It has been shown that although embryos resulting from ICSI with sperm harboring DNA damage can develop to blastocyst in vitro and can also implant, this development is severely impaired with significant post-implantation loss (Ahmadi and Ng, 1999, Ahmadi and Ng, 1999) and drastic consequences observed in produced offspring (Fernandez-Gonzalez, et al., 2008).

We have previously shown that sperm DNA can become degraded by various treatments in vitro and suggested that this damage can originate from the activity of sperm-specific nucleases (Szczygiel, et al., 2003, Szczygiel and Ward, 2002, Ward and Ward, 2004). In these past experiments we evaluated sperm DNA integrity via zygote chromosome analysis after intracytoplasmic sperm injection (ICSI), revealing high incidence of paternal chromosome breaks. In the oocyte, paternal chromatin undergoes dramatic changes and during this extensive remodeling it can be repaired (Derijck, et al., 2008, Marchetti, et al., 2007). It has also been suggested that damaged sperm DNA can undergo further degradation in the oocyte, and that this process is associated with and may be enhanced by DNA replication (Yamauchi, et al., 2007, Yamauchi, et al., 2007). Thus, the paternal chromosome damage observed by us before (Szczygiel, et al., 2003, Szczygiel and Ward, 2002, Ward and Ward, 2004) might have represented sperm chromatin damage augmented by the oocyte, and DNA synthesis in particular.

Here we examined the role of oocyte and of zygotic DNA synthesis in repairing or degrading previously damaged sperm DNA. We subjected mouse epididymal sperm to treatments that induce chromatin breaks and examined DNA integrity prior to fertilization, and after ICSI before and after DNA synthesis. We report that the bulk of sperm DNA damage persists after fertilization and its severity does not change in the course DNA replication.

Methods

Chemicals

Mineral oil was purchased from Squibb and Sons (Princeton, NJ) and pregnant mares' serum gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) from Calbiochem (San Diego, CA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO) unless otherwise stated.

Animals

Female B6D2F1 oocyte donors for ICSI were obtained at 6 weeks of age National Cancer Institute (Raleigh, NC) and allowed to mature up to 8-10 weeks prior to hormonal stimulation and oocyte retrieval. The mice were fed ad libitum with a standard diet and maintained in a temperature and light-controlled room (22°C, 14h light/10h dark), in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and guidelines presented in National Research Council's (NCR) "Guide for Care and Use of Laboratory Animals, 8th Edition" published by Institute for Laboratory Animal Research (ILAR) of the National Academy of Science, Bethesda, MD, 2011. The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii.

Media

Sperm and oocyte collection and subsequent manipulation, including microinjections were done in HEPES-buffered CZB medium (HEPES-CZB, (Kimura and Yanagimachi, 1995)). Culture of sperm-injected oocytes and embryos was done in CZB medium (Chatot, et al., 1989).

Sperm preparation

Sperm samples were pretreated in two ways: 1) freeze thawing, and 2) 0.5% Triton X-100 + 2 mM DTT treatment, which were previously shown to induce DNA damage (Szczygiel et al, 2003, Szczygiel and Ward, 2002). Live motile swim-up spermatozoa were used as controls. For freeze thawing, cauda epididymides were removed from one male mouse and placed in 300 μ l of HEPES-CZB in an organ tissue culture dish (Falcon, Bedford, MA). The epididymal contents were expressed from the cauda epididymides with needles, and the tissue was discarded. The spermatozoa were allowed to disperse for 2-5 min at room temperature and then 20 μ l of the sperm suspension was transferred to a 250 μ l tube. The tube was then plunged into liquid nitrogen (1 min), thawed at room temperature (1 min), and frozen in liquid nitrogen again (1 min). After a second thawing, a portion of sperm suspension was immediately taken for ICSI or for comet assay. For TX+DTT treatment, the epididymal fluid was squeezed out from cauda epididymides and placed in a tube with 1 ml of chilled HEPES-CZB with Triton X-100 and DTT added. Spermatozoa were vigorously pipetted to disperse evenly in the solution, incubated for 15 min at room temperature, and then suspension was carefully layered over a 0.5 ml cushion of 1 M sucrose, 25 mM Tris, pH 7.4, in a microfuge test tube. The step gradient was centrifuged at 3000 *g* in a swing bucket rotor for 10 min at 4°C. The pellet was resuspended in 100 μ l of HEPES-CZB, and used immediately for ICSI and comet assay. Swim-up spermatozoa were prepared by gently placing a drop of epididymal fluid on the bottom of a 1.5 ml microfuge tube containing 0.5 ml of HEPES-CZB and incubating the suspension for 15 min at 37°C. Next, 10-30 μ l were gently taken off the top of the suspension and transferred to another tube, and used immediately for ICSI and comet assay.

Preparation of mature and immature oocytes

To obtain mature (MII) oocytes, female mice were induced to superovulate by consecutive injections of 5 iu eCG and 5 iu hCG, 48 h apart. Oocytes collected from oviducts 12–14 h after hCG injection were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (300 USP units/ng) in HEPES-CZB. The oocytes were washed thoroughly and used immediately for ICSI.

To obtain immature (proMI) oocytes females were induced to superovulate with injections of 5 iu eCG. Oviducts were removed 48 h after the injection of eCG and placed in HEPES-CZB in a Petri dish. The biggest follicles were punctured to release germinal vesicle stage (GV) oocytes. GV oocytes surrounded by cumulus cells were placed in CZB drops under mineral oil and cultured. Cumulus cells were removed by pipetting after 2 h of culture. The oocytes that underwent germinal vesicle breakdown (GVBD) were selected for further 2 h culture to reach prometaphase I (proMI). After that the oocytes were taken immediately for injection.

Comet assay

Comet assay was performed using the Trevigen Kit (Trevigen, Gaithersburg, MD, cat no 4250-050-K) under neutral conditions as described by us before (Yamauchi, et al., 2007). For each sample tested, 50 DNA tails were photographed and analyzed. The length of each tail was measured from the center of the comet to the end of the tail using Image J software

(Rasband, 2007), and each tail was categorized into one of four tail types reflecting the severity of DNA damage (Fig. 1C). The severity of DNA damage increases proportionately with tail length and with tail type, from 1 to 4. Three experimental replicates were done for each examined group.

Intracytoplasmic sperm injection (ICSI)

Injections were performed as described before (Szczygiel and Yanagimachi, 2003) within 1-2 h from oocyte and sperm collection. Sperm-injected oocytes were transferred into CZB medium and cultured at 37°C. The survival of ICSI oocytes was scored 1-2 h after the commencement of culture.

BrdU staining

BrdU staining was performed as described by us before (Ajduk, et al., 2006). Briefly, MII and ProMI oocytes injected with sperm were incubated in presence of 10 mM 5-bromo-2-deoxyuridine (BrdU), and 7 and 20 h post ICSI, respectively, were fixed, stained with anti-BrdU antibody conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR) and propidium iodide, and examined using fluorescence.

Sperm chromosome analysis

Chromosome preparation and analysis were performed as described by us before (Riel, et al., 2007, Yamauchi, et al., 2007). After ICSI into MII oocytes, the oocyte activation was scored 6 hours after the commencement of culture; the oocytes with two well-developed pronuclei and extruded 2nd polar body were considered activated. Activated oocytes were transferred into CZB containing 0.006 mg/ml vinblastine, which was added to inhibit spindle formation and syngamy. The proMI oocytes are able to complete meiotic maturation after sperm injection but do not undergo subsequent activation (Clarke and Masui, 1986) so it was not necessary to incubate them in presence of syngamy inhibitor.

Between 19 and 21 h (MII) or after 18 h (proMI) after ICSI, oocytes were treated with 1% pronase (1000 tyrosine units/mg; Kaken Pharmaceuticals, Tokyo, Japan) for 5 min at room temperature to soften the zonae pellucidae. Oocytes were then treated with hypotonic solution (1:1 mixture of 1% sodium citrate:30% fetal bovine serum) for 5 min at 37°C or 10 min at 25°C. Chromosomes were spread on glass slides by the gradual fixation/air-drying method (Mikamo and Kamiguchi, 1983). The preparations were stained with 2% Giemsa (Merck, Darmstadt, Germany) in PBS (pH 6.8) for 10 min for conventional chromosome analysis. The percentages of zygotes with normal and abnormal chromosomes were determined. In addition to scoring normal vs. abnormal karyoplasts, we also calculated the incidence of chromosome aberrations, i.e. aberration rate, which represents the total number of aberrations divided by the number of oocytes examined. This aberration rate reflects the intensity of DNA damage.

Statistical analyses

Fisher's Exact Test was applied for ICSI data, t-test for comparison of average comet tail lengths and aberration rates, and Mantel-Haenszel chi square test for comparison of comet tail lengths and tails distribution. For all statistical tests, differences with a *P* value of < 0.05 were regarded as significant.

Results

Freeze thawing and TX+DTT treatments induce comet assay detectable DNA fragmentation in sperm

Sperm chromatin is very highly condensed (Ward, 1994, Ward and Coffey, 1991) making it difficult to examine. Comet assay is one of the few methods that allow assaying for chromosome breaks in intact sperm. Here, we used this approach to verify that two sperm manipulations, freeze thawing and treatment with TX+DTT induce DNA fragmentation. The results are shown as the analysis of the frequency of comets with different tail lengths and with different tail types (Fig. 1). Both tail length and tail type reflect the severity of DNA damage.

The average comet tail length was significantly higher in frozen-thawed sperm when compared to control (115.78 ± 41.39 vs. 153.37 ± 64.55 , $P < 0.001$). Although the difference between TX+DTT group (128.81 ± 59.50) and control did not reach statistical significance in analysis of the average comet tail lengths, significant differences were noted when examining the distributions of comet tail lengths and comet tail types. In controls, the majority of comet tail lengths were within a 50 - 150 μ m range. In both freeze thawed and TX+DTT groups there were significantly less comets with this tail length range, and more comets with tails greater than 150 μ m ($P < 0.001$, Fig. 1A). In respect to comet tail type distribution, the majority of the comets in the control group was classified as tail type 1 (>80%), reflecting the lowest level of DNA fragmentation. Both frozen-thawed and TX +DTT treated sperm showed significantly less comets with tail type 1, and more with tail types 2-4, reflecting higher level of damage ($P < 0.001$, Fig. 1B). Freeze thawing was overall more harmful to sperm DNA integrity than treatment with TX+DTT but statistically significant difference between those two sperm treatments was noted only in comparison of average tail lengths ($P < 0.001$).

Overall, comet assay analysis documented that sperm subjected to freeze thawing or to TX +DTT treatment have chromatin fragmentation.

Behavior of proMI oocytes during and after ICSI

Freeze thawed, TX+DTT treated, and fresh sperm were then injected into the cytoplasm of ProMI and MII oocytes. ProMI oocytes were obtained after in vitro culture of GV oocytes for 4 h (Fig. 2A-C) and used immediately for injection. These oocytes were fragile and died easily during micromanipulation (Table 1). The oocyte survival was lower after injection into proMI oocytes compared to MII oocytes (48%, 251/523 vs. 80%, 305/381; $P < 0.001$; pooled data from all examined groups). Those oocytes that survived injection continued to mature and 18-20 h later reached MII stage, evidenced by extrusion of the first polar body (PB1). Extruded PB1 was often larger than expected, deformed, and accompanied by additional 'pseudo polar bodies' (Fig. 2D), presumably containing sperm chromatin. Although the great majority (>80%) of oocytes reached MII at 18-20 h after injection, some arrested at MI (Table 2, Fig. 2E). The ratio of oocytes that reached MII and those that arrested at MI was first scored with viable oocytes based on PB1 extrusion, and then after chromosome preparation; the results of these scorings matched (Table 2). When non-injected proMI oocytes were cultured in vitro under the same conditions and for the same period of time, the incidence of MI arrest was higher than that of injected oocytes (Table 2, Fig. 2F) suggesting that this developmental impairment was not due to sperm injection. In few experiments the oocytes that did not reach MII stage after 18-20 h of culture were incubated longer to exclude the possibility of developmental delay rather than arrest; one of these oocytes reached MII.

ProMI oocytes maturing in vitro after sperm injection do not undergo DNA synthesis

The principle behind this study was that proMI oocytes injected with sperm would allow visualization and examination of sperm chromosomes before the DNA synthesis. To verify that proMI oocytes did not undergo replication after ICSI, we performed BrdU staining of sperm injected MII and proMI, and non-injected proMI oocytes. When MII oocytes were injected with sperm, 67% (20/30) of oocytes were BrdU positive (Fig. 3A-B) 7 h after injection, which is the incidence similar to that reported by us before (Ajduk, Yamauchi and Ward, 2006). None of the injected (0/19) and non-injected (0/17) proMI oocytes that were allowed to mature in vitro to MII stage expressed BrdU staining (Fig. 3C-D).

DNA replication in the oocyte has no effect on DNA fragmentation of paternal chromosome complement after injection

MI and proMI oocytes injected with sperm were subjected to chromosome analysis. The majority of chromosome plates prepared after ICSI into MII chromosomes had analyzable chromosomes (90-94%). On the contrary, the majorities of chromosome plates prepared after ICSI into proMI oocytes yielded chromosomes that were clumped, and as such were unsuitable for analysis (Fig. 4A, Table 1).

After MII ICSI, the paternal chromosomes were considered normal when an egg contained 40 structurally normal metaphase chromosomes (Fig. 4D). It was not always possible to distinguish between chromosomes of paternal and maternal origin based on their morphology. However, because oocyte chromosomes rarely show structural aberrations at the first cleavage metaphase after parthenogenetic activation (Yamauchi, Doe, Ajduk and Ward, 2007), abnormal chromosomes (chromosome and chromatid breaks and exchanges) within fertilized oocytes were considered of sperm origin. After proMI ICSI, the paternal and maternal chromosomes could be easily distinguished. In oocytes that reached MII stage each maternal chromosome comprised of joined two chromatids (Fig. 4B and 4F-H maternal). Paternal chromosomes comprised of single chromatids and were longer and thinner than the maternal chromosomes (Fig. 4E, paternal). Maternal chromosomes in oocytes that arrested at MI were also easily distinguishable with recognizable bivalents and chiasmata (Fig. 4C and 4E maternal).

Chromosome analysis revealed more chromosome damage in sperm subjected to TX+DTT and freeze thawing when compared to fresh sperm, regardless oocyte type (Table 1, Fig. 3E-F). Paternal DNA damage observed after injection into proMI oocytes was similar to that obtained after injection into mature oocytes in all examined groups. Contrary to what was observed in comet assay, there were no differences in the incidence of normal karyotypes and in the aberration rates between freeze thawed and TX+DTT treated sperm.

Discussion

In this study we took the advantage of the ability of immature oocytes to transform sperm DNA into chromosomes without preceding DNA synthesis to compare paternal DNA damage in the oocytes before and after DNA replication.

The oocyte cytoplasm has long been known to be able to transform interphase nuclei into metaphase chromosomes and maturing oocytes also possess this ability (Balakier, 1978, Gurdon, 1968). Sperm chromosomes in immature oocytes were visualized for the first time by Clarke and Matsui (Clarke and Masui, 1986), who demonstrated that time needed for transformation of sperm chromatin was much longer than that required for transformation of somatic cell DNA. Here we followed this time regime and successfully visualized sperm chromosomes before DNA synthesis.

We subjected sperm to DNA damaging treatments and injected them into immature prometaphase I (ProMI) oocytes. ProMI oocytes were sensitive to micromanipulation and only ~50% survived the injection. However, the subsequent 'behavior' of these oocytes was similar to that reported before for oocytes fertilized with conventional insemination. More than 80% of oocytes injected with sperm at ProMI reached metaphase II (MII) demonstrating that injection does not interfere with oocyte ability to mature in vitro. On the contrary, it seems that injection and/or presence of sperm nuclei within ooplasm facilitated oocyte maturation as only 50-60% of control, non-injected oocytes matured to MII, with the remaining arrested at metaphase I (MI). This MI arrest might have been due to oocytes being only partially competent to resume meiosis. Partially competent oocytes are able to undergo germinal vesicle breakdown, condense their chromosomes, form metaphase spindle and progress to metaphase I, at which stage they arrest (Wickramasinghe, et al., 1991), and this arrest was shown to be associated with the lack of p34^{cdc2} kinase inactivation and restriction of the degradation of cyclin B (Hampl and Eppig, 1995).

Immature oocytes injected with sperm were cultured allowing for sperm chromatin transformation, and were then subjected to chromosome preparation and analysis. Low number of oocytes yielded analyzable chromosome plates; more than half of the oocytes contained paternal chromatin in the form of a condensed clump, or an incomplete paternal chromosome set, or lacked the entire paternal complement. It has been previously shown that maturing oocytes may eliminate paternal chromatin by extruding 'pseudo polar bodies' or forming abnormally large polar bodies (Jedrusik, et al., 2007). Here, we observed various polar body anomalies, including presence of 'pseudo polar bodies' (Fig. 2DE) so this can explain missing or lack of sperm chromosomes. Because ICSI was used as the fertilization method in both cases (Jedrusik, et al., 2007) (and this study) it remains to be tested whether this phenomenon occurs also in immature oocytes that are inseminated. The condensed chromatin clumps that we also observed might represent paternal chromatin arrested in its remodeling. Sperm nucleus in immature oocytes undergoes remodeling starting with initial chromatin decondensation, then recondensation into a tightly packed mass from which eventually discrete metaphase chromosomes become resolved (Clarke and Masui, 1986). The clumps that we observed often included single protruding chromosome arms (see Fig. 4A) so it seems that the impairment was at the final stage of this remodeling, chromosome formation.

DNA damage in this study was assessed by comet assay on sperm and by chromosome analysis after fertilization. Our data indicate that sperm DNA damage assayed with the comet assay does predict paternal chromatin breaks measured by chromosome analysis, so the two approaches correlate well. However, we cannot directly mechanistically relate DNA damage before and after fertilization because of the different nature of the two assays. In the comet assay all protamines and histones are extracted by high salt and disulfide reducing agents rendering DNA fully accessible for analysis; under neutral conditions, which we have used, mostly double strand DNA breaks (DSB) are detected. The comet assay requires a significant number of DNA breaks to yield detectable results. In contrast, sperm chromosome analysis can potentially detect even a single DSB, which if accompanied by a break in chromosome scaffold, results in a formation of a chromosome fragment. As such, chromosome analysis is more sensitive.

The main goal of our study was to compare the integrity of sperm chromosomes before and after DNA synthesis in order to test if this process plays a role in DNA repair/degradation. The relationship between DNA synthesis and DNA damage recognition and repair in the oocytes and other cells is well known (Menezo, et al., 2010). DNA damage can arrest DNA replication to allow the cell repair the damage or, if repair does not take place, to direct cell towards apoptosis (Jurisicova and Acton, 2004). Some DNA repair proteins, for example

RecA, require the initiation of DNA synthesis before they respond to damage (Simmons, et al., 2007). Replication arrest in response to UV can lead to formation of DSB (Limoli, et al., 2002). DNA replication can also propagate DNA damage (Roos and Kaina, 2006). It has been shown before that partially damaged sperm DNA can undergo complete degradation in the oocytes after injection, and that this degradation was dependent on DNA synthesis and could be inhibited by DNA synthesis inhibitor aphidicolin (Yamauchi, et al., 2007). Here, when we injected sperm with damaged DNA into the oocytes, a high incidence of paternal chromosome breaks was observed after injection into both immature and mature oocytes suggesting that DNA synthesis did not affect already damaged sperm chromatin in any way, neither its repair nor its degradation.

The two sperm treatments, freeze thawing and TX+DTT, yielded variable DNA damage in comet assay, with freeze thawing being more detrimental for chromatin integrity. However, the difference between the treatments was not observed after ICSI, as assayed by chromosome analysis, suggesting that some of DNA damage in freeze thawed sperm could become repaired in the oocyte.

It is known that mammalian oocytes have active DNA repair machinery throughout oogenesis, and can repair both paternal and maternal genomes after fertilization (Ashwood-Smith and Edwards, 1996, Brandriff and Pedersen, 1981, Marchetti, et al., 2007). The primary aberrations observed in this study were chromosome-type aberrations (DSB), which are also the primary type of aberrations found in the zygotes after sperm exposure to ionizing radiation and chemical mutagens (Marchetti and Wyrobek, 2005) or after subjecting sperm to various physical and chemical manipulations (Szczygiel et al., 2003, Szczygiel and Ward, 2002). Repair of DSB can occur along two different pathways, non-homologous end joining (NHEJ) and homologous recombination (HR). In the zygote, NHEJ functions in G1 phase while HR operates during S-G2 transition; both pathways were shown to be involved in the repair of radiation-induced sperm DNA damage in normal mature oocytes, with the NHEJ playing a greater role than the HR pathway (Derijck, et al., 2008, Marchetti, et al., 2007). Because the incidence and severity of DNA damage in this study were similar after ICSI into immature and mature oocytes, the repair process, if any, must have taken place early during oocyte maturation, and was not dependent on DNA synthesis. This indicates that the mechanism responsible could be NHEJ. However, as far as we are aware presence of NHEJ in maturing oocytes has not been demonstrated and future studies with NHEJ deficient mice will be necessary to prove that this pathway is indeed involved in repair of paternal DNA during oocyte maturation.

To summarize, by comparing paternal chromosome breakage in sperm and in the oocytes after fertilization before and after first round of DNA synthesis we demonstrated that (1) most but not all of DNA breaks detected in sperm persist after fertilization and that (2) DNA synthesis does not increase or repair paternal DNA damage. Our study adds to the understanding of the processes occurring in the fertilized oocytes, with particular relevance to transmission of paternal DNA defects.

References

- Adiga SK, Upadhy D, Kalthur G, Bola Sadashiva SR, Kumar P. Transgenerational changes in somatic and germ line genetic integrity of first-generation offspring derived from the DNA damaged sperm. *Fertil Steril.* 2010; 93:2486–90. [PubMed: 19591998]
- Ahmadi A, Ng SC. Developmental capacity of damaged spermatozoa. *Hum Reprod.* 1999; 14:2279–85. [PubMed: 10469696]
- Ahmadi A, Ng SC. Fertilizing ability of DNA-damaged spermatozoa. *J Exp Zool.* 1999; 284:696–704. [PubMed: 10531556]

- Ahmed EA, de Boer P, Philippens ME, Kal HB, de Rooij DG. Parp1-XRCC1 and the repair of DNA double strand breaks in mouse round spermatids. *Mutat Res*. 2010; 683:84–90. [PubMed: 19887075]
- Aitken RJ, De Iuliis GN, McLachlan RI. Biological and clinical significance of DNA damage in the male germ line. *Int J Androl*. 2009; 32:46–56. [PubMed: 19076252]
- Ajduk A, Yamauchi Y, Ward MA. Sperm chromatin remodeling after intracytoplasmic sperm injection differs from that of in vitro fertilization. *Biol Reprod*. 2006; 75:442–51. [PubMed: 16775225]
- Ashwood-Smith MJ, Edwards RG. DNA repair by oocytes. *Mol Hum Reprod*. 1996; 2:46–51. [PubMed: 9238657]
- Balakier H. Induction of maturation in small oocytes from sexually immature mice by fusion with meiotic or mitotic cells. *Exp Cell Res*. 1978; 112:137–41. [PubMed: 564777]
- Brandriff B, Pedersen RA. Repair of the ultraviolet-irradiated male genome in fertilized mouse eggs. *Science*. 1981; 211:1431–3. [PubMed: 7466400]
- Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. *J Reprod Fertil*. 1989; 86:679–88. [PubMed: 2760894]
- Clarke HJ, Masui Y. Transformation of sperm nuclei to metaphase chromosomes in the cytoplasm of maturing oocytes of the mouse. *J Cell Biol*. 1986; 102:1039–46. [PubMed: 3949872]
- Derijck A, van der Heijden G, Giele M, Philippens M, de Boer P. DNA double-strand break repair in parental chromatin of mouse zygotes, the first cell cycle as an origin of de novo mutation. *Hum Mol Genet*. 2008; 17:1922–37. [PubMed: 18353795]
- Fernandez-Gonzalez R, Moreira PN, Perez-Crespo M, Sanchez-Martin M, Ramirez MA, Pericuesta E, Bilbao A, Bermejo-Alvarez P, Hourcade Jde D, Fonseca FR, Gutierrez-Adan A. Long-Term Effects of Mouse Intracytoplasmic Sperm Injection with DNA-Fragmented Sperm on Health and Behavior of Adult Offspring. *Biol Reprod*. 2008; 78:761–72. [PubMed: 18199884]
- Gurdon JB. Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. *J Embryol Exp Morphol*. 1968; 20:401–14. [PubMed: 5750105]
- Hampl A, Eppig JJ. Analysis of the mechanism(s) of metaphase I arrest in maturing mouse oocytes. *Development*. 1995; 121:925–33. [PubMed: 7743936]
- Jedrusik A, Ajduk A, Pomorski P, Maleszewski M. Mouse oocytes fertilised by ICSI during in vitro maturation retain the ability to be activated after refertilisation in metaphase II and can generate Ca²⁺ oscillations. *BMC Dev Biol*. 2007; 7:72. [PubMed: 17584490]
- Juriscova A, Acton BM. Deadly decisions: the role of genes regulating programmed cell death in human preimplantation embryo development. *Reproduction*. 2004; 128:281–91. [PubMed: 15333779]
- Kimura Y, Yanagimachi R. Intracytoplasmic sperm injection in the mouse. *Biol Reprod*. 1995; 52:709–20. [PubMed: 7779992]
- Limoli CL, Giedzinski E, Bonner WM, Cleaver JE. UV-induced replication arrest in the xeroderma pigmentosum variant leads to DNA double-strand breaks, gamma -H2AX formation, and Mre11 relocalization. *Proc Natl Acad Sci U S A*. 2002; 99:233–8. [PubMed: 11756691]
- Marchetti F, Essers J, Kanaar R, Wyrobek AJ. Disruption of maternal DNA repair increases sperm-derived chromosomal aberrations. *Proc Natl Acad Sci U S A*. 2007; 104:17725–9. [PubMed: 17978187]
- Marchetti F, Wyrobek AJ. Mechanisms and consequences of paternally-transmitted chromosomal abnormalities. *Birth Defects Res C Embryo Today*. 2005; 75:112–29. [PubMed: 16035041]
- Marchetti F, Wyrobek AJ. DNA repair decline during mouse spermiogenesis results in the accumulation of heritable DNA damage. *DNA Repair (Amst)*. 2008; 7:572–81. [PubMed: 18282746]
- Menezo Y, Dale B, Cohen M. DNA damage and repair in human oocytes and embryos: a review. *Zygote*. 2010; 18:357–65. [PubMed: 20663262]
- Mikamo K, Kamiguchi Y. Primary incidences of spontaneous chromosomal anomalies and their origins and causal mechanisms in the Chinese hamster. *Mutat Res*. 1983; 108:265–78. [PubMed: 6835223]

- Rasband, WS. ImageJ software [Internet]. Bethesda: U.S: National Institute of Health; Available from: <http://rsb.info.nih.gov/ij/>
- Riel JM, Huang TT, Ward MA. Freezing-free preservation of human spermatozoa--a pilot study. *Arch Androl.* 2007; 53:275–84. [PubMed: 18309900]
- Roos WP, Kaina B. DNA damage-induced cell death by apoptosis. *Trends Mol Med.* 2006; 12:440–50. [PubMed: 16899408]
- Simmons LA, Grossman AD, Walker GC. Replication is required for the RecA localization response to DNA damage in *Bacillus subtilis*. *Proc Natl Acad Sci U S A.* 2007; 104:1360–5. [PubMed: 17229847]
- Sotomayor RE, Sega GA. Unscheduled DNA synthesis assay in mammalian spermatogenic cells: an update. *Environ Mol Mutagen.* 2000; 36:255–65. [PubMed: 11152558]
- Szczygiel MA, Moisyadi S, Ward WS. Expression of foreign DNA is associated with paternal chromosome degradation in intracytoplasmic sperm injection-mediated transgenesis in the mouse. *Biol Reprod.* 2003; 68:1903–10. [PubMed: 12606337]
- Szczygiel MA, Ward WS. Combination of dithiothreitol and detergent treatment of spermatozoa causes paternal chromosomal damage. *Biol Reprod.* 2002; 67:1532–7. [PubMed: 12390885]
- Szczygiel, MA.; Yanagimachi, R. Intracytoplasmic Sperm Injection. In: Nagy, A.; Gertsenstein, M.; Vintersten, K.; Behringer, RR., editors. *Manipulating the Mouse Embryo - A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press; 2003. p. 585-597.
- Ward MA, Ward WS. A model for the function of sperm DNA degradation. *Reprod Fertil Dev.* 2004; 16:547–554. [PubMed: 15355763]
- Ward WS. The structure of the sleeping genome: implications of sperm DNA organization for somatic cells. *Journal of Cellular Biochemistry.* 1994; 55:77–82. [PubMed: 8083302]
- Ward WS, Coffey DS. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biology of Reproduction.* 1991; 44:569–74. [PubMed: 2043729]
- Wickramasinghe D, Ebert KM, Albertini DF. Meiotic competence acquisition is associated with the appearance of M-phase characteristics in growing mouse oocytes. *Dev Biol.* 1991; 143:162–72. [PubMed: 1985016]
- Yamauchi Y, Ajduk A, Riel J, Ward MA. Ejaculated and Epididymal Mouse Spermatozoa Are Different in Their Susceptibility to Nuclease-Dependent DNA Damage and in Their Nuclease Activity. *Biol Reprod.* 2007
- Yamauchi Y, Doe B, Ajduk A, Ward MA. Genomic DNA damage in mouse transgenesis. *Biol Reprod.* 2007; 77:803–12. [PubMed: 17652664]
- Yamauchi Y, Shaman JA, Boaz SM, Ward WS. Paternal pronuclear DNA degradation is functionally linked to DNA replication in mouse oocytes. *Biol Reprod.* 2007; 77:407–15. [PubMed: 17494913]
- Yamauchi Y, Shaman JA, Ward WS. Topoisomerase II-Mediated Breaks in Spermatozoa Cause the Specific Degradation of Paternal DNA in Fertilized Oocytes. *Biol Reprod.* 2007; 76:666–72. [PubMed: 17182890]
- Zenzes MT. Smoking and reproduction: gene damage to human gametes and embryos. *Hum Reprod Update.* 2000; 6:122–31. [PubMed: 10782570]

Fig. 1. DNA fragmentation in sperm subjected to freeze thawing and treatment with TX+DTT assessed by comet assay

A: Distribution of comet tail lengths; B: Distribution of comet tail types; C: Examples of comet tail types (1, short tail; 2, long tail, with majority of DNA still in the head; 3, long tail with DNA evenly distributed through out; 4, long tail, with most of the DNA at the distal portion). The severity of DNA damage increases proportionately with tail length and with tail type, from 1 to 4. In A and B each graph bar represents an average percentage of sperm $\pm$ standard deviation of $n=3$ replicates, with 50 sperm scored per replicate. Bar in C = 50 μm .

Fig. 2. Morphological changes of the oocytes maturing in vitro

A: GV oocytes collected 48 h after eCG injection; B: GV oocytes denuded from cumulus cells after 2 h of culture; C: ProMI oocytes after 4 h culture; D: oocytes that were injected with sperm at proMI stage and subsequently cultured for 18-20 h, note that these are MII oocytes but in addition to PB1 they extruded additional 'pseudo polar bodies'; E: as in D but note that some of the oocytes did not reach MII stage and arrested at MI; F: control oocytes cultured in vitro for the same time but without sperm injection either developed to MII and extrude PB1 or remained arrested at MI stage. Scale = 100 μ m

Fig. 3. BrdU staining of sperm injected immature oocytes

MI (A&B) and ProMI (C&D) oocytes were injected with sperm and cultured for 7 and 20 h, respectively, in the presence of BrdU. The oocytes were then fixed and stained with anti-BrdU antibody (green, B&D) and with propidium iodide to visualize total DNA (red, A&C). No BrdU staining was noted in oocytes injected with sperm at proMI. Bar = 50 μ m.

Fig. 4. Chromosome analysis of oocytes matured in vitro

A: a clump of paternal chromatin that failed to form separate chromosomes after ICSI into proMI oocyte; B: chromosomes of oocyte that matured in vitro reaching MII (n=20); C: chromosomes of oocyte that matured in vitro but arrested at MI (n=20); D: normal maternal (m, n=20) and paternal (p, n=20) zygote chromosome complements after fresh sperm ICSI into ovulated MII oocyte; the assignment of complement origin is arbitrary in this case, based previous experience that maternal chromosomes tend to be shorter than paternal, note characteristic morphology of zygotic chromosomes different than that of MI, MII maternal and prematurely condensed paternal chromosomes shown in other figure panels; E: normal maternal (m, n=20) and paternal (p, n=20) chromosome complement after fresh sperm ICSI into proMI oocytes; note that paternal chromosomes have distinctly different morphology that allows differentiating them from maternal chromosomes and that maternal chromosomes arrested at MI; F: normal maternal (n=20) and paternal (n=20) chromosome complements after ICSI into proMI oocytes, with maternal chromosomes reaching MII; G: normal maternal (n=20) and abnormal paternal chromosome complements after ICSI with TX+DTT treated sperm into proMI oocyte; note many abnormalities of the paternal complement such as associated chromosomes (long arrows), small chromosome fragments (short arrows) and ring chromosomes (arrowheads); H: normal maternal (n=20) and abnormal paternal (n=19 + 2 large chromosome fragments shown with arrows and 1 small

fragment shown by arrowhead) chromosome complements after with frozen-thawed sperm ICSI of TX+DTT treated sperm into proMI oocytes. Scale = 20 μ m.

Table 1

Sperm DNA damage before and after DNA synthesis.

Sperm treatment	Group	No oocytes injected [No replicates]	No oocytes survived (%) ¹	No chromosome plates prepared	No analyzable chromosome plates (%) ²	No normal chromosome plates (%) ³	Aberration rate
Fresh	Maturing	221 [5]	102 (46) ^a	93	27 (29) ^a	23 (85) ^b	0.19 ^b
	MII	101 [5]	77 (76)	60	54 (90)	52 (96) ^b	0.06 ^b
Frozen-thawed	Maturing	124 [4]	69 (56) ^a	68	21 (31) ^a	10 (48)	1.14
	MII	111 [3]	82 (74)	35	33 (94)	17 (52)	1.40
TX+DTT	Maturing	178 [4]	80 (45) ^a	76	36 (47) ^a	19 (53)	0.89
	MII	169 [3]	146 (86)	78	72 (92)	37 (51)	1.35

Percentage calculated from:

¹ oocytes injected,

² chromosome plates prepared,

³ analyzable chromosome plates.

Statistical significance (P<0.05):

^a different than MII within treatment,

^b different than other examined groups (Fisher's Exact Test and t-test)

Table 2

Maturation ability of oocytes subjected to ICSI at ProMI stage and cultured in vitro.

Sperm treatment prior to ICSI	Live oocytes prior to chromosome preparation ¹		Oocytes found on the slide and MII/MI analyzed		Oocytes found on the slide with analyzable karyotypes	
	No scored	MIH (%)	No scored	MIH (%)	No scored	MIH (%)
Fresh	73	90	84	92	27	93
Frozen-thawed	69	81	63	83	21	95
TX+DTT	21	81	74	88	36	81
No ICSI control	92	63 ^a	57	53 ^b	35	49 ^b

¹ Live oocytes were scored in some but not all experimental replicates (n=3/5, n=4/4, n=1/4 for Fresh, Frozen-thawed and TX+DTT, respectively), which explains the higher number of oocytes scored on the slide in Fresh and TX+DTT groups.

Statistical significance (P<0.05):

^a different than Fresh and Frozen-Thawed,

^b different than other examined groups (Fisher's Exact Test).