

**DNA REPAIR DECLINE DURING MOUSE SPERMIOGENESIS RESULTS IN THE
ACCUMULATION OF HERITABLE DNA DAMAGE**

Francesco Marchetti and Andrew J. Wyrobek

Biology and Biotechnology Research Program, Lawrence Livermore National Laboratory,
Livermore, CA 94550, USA; Life Sciences Division, Lawrence Berkeley National Laboratory,
Berkeley, CA 94720

Corresponding author:

Dr. Francesco Marchetti,

Life Sciences Division, MS74R0157,

Lawrence Berkeley National Laboratory,

1 Cyclotron Road, Berkeley CA 94720

Telephone: (510) 486-7352;

Telefax: (510) 486-6619

e-mail: fmarchetti@lbl.gov

Key word: diepoxybutane, zygote, chromosomal aberrations

Prepared for submission to DNA Repair

ABSTRACT

The post-meiotic phase of mouse spermatogenesis (spermiogenesis) is very sensitive to the genomic effects of environmental mutagens because as male germ cells form mature sperm they progressively lose the ability to repair DNA damage. We hypothesized that repeated exposures to mutagens during this repair-deficient phase result in the accumulation of heritable genomic damage in mouse sperm that leads to chromosomal aberrations in zygotes after fertilization. We used a combination of single or fractionated exposures to diepoxybutane (DEB), a component of tobacco smoke, to investigate how differential DNA repair efficiencies during the three weeks of spermiogenesis affected the accumulation of DEB-induced heritable damage in early spermatids (21-15 days before fertilization, dbf), late spermatids (14-8 dbf) and sperm (7- 1 dbf). Analysis of chromosomal aberrations in zygotic metaphases using PAINT/DAPI showed that late spermatids and sperm are unable to repair DEB-induced DNA damage as demonstrated by significant increases ($P < 0.001$) in the frequencies of zygotes with chromosomal aberrations. Comparisons between single and fractionated exposures suggested that the DNA repair-deficient window during late spermiogenesis may be less than two weeks in the mouse and that during this repair-deficient window there is accumulation of DNA damage in sperm. Finally, the dose-response study in sperm indicated a linear response for both single and repeated exposures. These findings show that the differential DNA repair capacity of post-meiotic male germ cells has a major impact on the risk of paternally transmitted heritable damage and suggest that chronic exposures that may occur in the weeks prior to fertilization because of occupational or lifestyle factors (i.e, smoking) can lead to an accumulation of genetic damage in sperm and result in heritable chromosomal aberrations of paternal origin.

INTRODUCTION

Spermatogenesis is a complex differentiating system that is initiated from stem cells through division of spermatogonia to form spermatocytes (meiotic cells), which after two meiotic divisions give rise to haploid spermatids (postmeiotic cells) [1]. During the last phase of spermatogenesis, also known as spermiogenesis, haploid spermatids undergo major morphological changes to form mature spermatozoa [2, 3]. The somatic and meiotic histones of spermatids are replaced ~14 days before ejaculation in the mouse (~21 days in humans) with basic transition proteins [4] and then with protamines, which are arginine-rich proteins that condense the chromatin to a level that is 6 times more compacted than that observed in mitotic chromosomes [5] and causes the DNA to become transcriptionally inactive and inaccessible to DNA repair proteins [6]. The process requires a profound change in DNA topology that involves the introduction of many single and double strand breaks to eliminate negative supercoiling [2, 7]. Transition proteins are thought to play an important role in assuring the proper repair of these breaks by bringing broken DNA fragments into close proximity [5] and there is evidence that they can stimulate DNA repair following genotoxic insults and contribute to maintaining the integrity of the male genome [5, 8-10].

It is well established that postmeiotic male germ cells are extremely sensitive to the induction of heritable genomic damage. Over fifty years of germ cell mutagenesis have shown that the majority of mutagens induce their highest effect during the last few weeks of spermatogenesis before fertilization [11, 12]. The high sensitivity of the postmeiotic period to mutagenic exposure has been associated with the reduced DNA repair capacity of late spermatids and sperm as compared to early spermatids and other spermatogenic cell types [13-17]. All major DNA repair pathways seem to be less functional in late spermatids and sperm [17-20].

This inability of sperm to repair DNA lesions as they occur may make them particularly susceptible to repeated exposures that take place because of occupational or life style (e.g. tobacco smoking) reasons. These observations suggest that genomic damage induced in late spermatids and sperm may accumulate in the fertilizing sperm and be transmitted to the embryo.

The analysis of paternal chromosomes at the metaphase plate of the first-cleavage division (1-CI) provides the earliest time after fertilization where paternal chromosomes can be investigated by classic cytogenetic methods [12, 21]. At least 16 male germ cell mutagens have been shown to increase chromosomal aberrations in mouse zygotes after exposure of postmeiotic male germ cells [12]. These mutagens have a variety of mechanisms of actions, including DNA alkylation, protein alkylation, and DNA cross-linking, yet, they all produced almost exclusively chromosome-type aberrations, i.e., affecting both sister chromatids, at 1-CI metaphase. Although it is still unclear why this is the case, these consistent observations of chromosome-type aberrations prove that double strand breaks (DSBs) are an obligatory step in the processing of sperm lesions into chromosomal aberrations at 1-CI metaphase. It was postulated that protamine adducts may be the primary type of damage induced in postmeiotic cells by alkylating agents and that adducted protamines create physical stresses in the chromatin structure leading to DSBs before fertilization [22]. Alternatively, adducted protamines may be refractory to removal from DNA during pronuclear formation in the fertilized egg, and thus may indirectly function as “bulky DNA adducts”. Other lesions, such as single strand breaks, base damages and apurinic or apyrimidinic sites could be converted into DSBs by misrepair before zygotic S-phase leading chromosome-type aberrations at 1-CI metaphase [23]. Studies with DNA repair inhibitors in mouse zygotes after sperm treatment with X-rays and chemical agents have provided compelling evidence that chromosomal aberrations were formed after fertilization rather than before [23-25].

Finally, we recently obtained strong evidence that DSBs persisted unrepaired in the sperm for at least 7 days before fertilization and that improper repair of transmitted DSBs by the egg leads to increases frequencies of zygotes with aberrations in paternal chromosomes at 1-C1 metaphase [26].

Substantial evidence indicates that tobacco smoking by either parent is associated with increased risk of abnormal pregnancy outcomes. However, little is known of the mechanisms by which tobacco smoking may damage germ cells and affect the developing embryo. Tobacco smoke contains numerous carcinogenic, mutagenic, and reproductive toxicants [27-30]. 1,3-butadiene (BD) is one of the few constituents of tobacco smoke tested for heritable and developmental effects in laboratory animals [31-34]. BD is classified as a probable human carcinogen [35] and is present in mainstream tobacco smoke at a concentration of 16-75 $\mu\text{g}/\text{cigarette}$ and at higher concentrations (205-361 $\mu\text{g}/\text{cigarette}$) in sidestream smoke [36], the main constituent of environmental tobacco smoke or second-hand smoke. BD has marked species differences in susceptibility to the carcinogenic effect, possibly due to differences in metabolism [37-40]. BD is metabolized by the cytochrome P-450-dependent monooxygenases to 1,2-epoxybutene-3 (EB), which is further metabolized by oxidation to diepoxybutane (DEB) [39-41]. DEB is a bifunctional alkylating agent that induces interstrand and intrastrand DNA-DNA crosslinks by alkylating two adjacent bases within the major groove of a DNA duplex [42, 43] and DNA-protein crosslinks [44-46]. DEB is both a somatic and germ cell mutagen in mammals [47-49]. Exposure of male rodents to DEB induces cytogenetic damage in meiotic cells [50] and in zygotes [50, 51], as well as dominant lethality and heritable translocations in the offspring [51].

The specific goals of this study were to determine: i) whether the ability of repairing DEB-induced DNA damage declines as male germ cells progress through spermiogenesis; ii)

whether DEB-induced sperm lesions accumulate during spermiogenesis; iii) the effective duration of the DNA repair-deficient phase of spermiogenesis after DEB exposure; and, iv) the dose-response curve for the induction of chromosomal aberrations in zygotes after either single or fractionated DEB exposure of male germ cells.

MATERIAL AND METHODS

Animals and treatments

The use of vertebrate animals in these experiments was approved by both the Lawrence Livermore National Laboratory and Lawrence Berkeley National Laboratory Institutional Animal Care and Use Committees. B6C3F1 mice (Harlan Sprague-Dawley Inc., Indianapolis, IN, USA) between 8 and 12 weeks of age were maintained under a 12 hr light/12 hr dark photoperiod (light from 7:00 am to 7:00 pm) at room temperature of 21-23° C and relative humidity of 50 ± 5%. Food and water were provided *ad libitum*. Male mice were randomly assigned to treated and control experimental groups.

All experimental treatment regimens are shown in Figure 1. The highest acute DEB dose utilized in this study was selected based on published data [51] and initial experiments in which mice were treated with a dose of 42 mg/kg DEB (CAS No. 1464-53-5, Sigma) and mated with untreated females seven days later. This DEB dose produced severe morbidity as demonstrated by a significant reduction in the percentages of males that mated (30% vs 80% in controls), therefore, its use was discontinued. DEB was dissolved in PBS and administered intraperitoneally (i.p.) at the final volume of 0.01 ml/g body weight. For fractionated exposure studies, males received daily doses of 4 mg/kg DEB for 1, 2, or 3 weeks before mating and allowed to mate 0, 7 or 14 days after the end of treatment (experiments 9 through 14 in Fig. 1). To compare fractionated vs. single exposures, males were treated with 28 mg/kg DEB and allowed to mate with untreated females 7, 14 or 21 days later (experiments 6, 7 and 8 in Fig. 1). Finally, for the dose-response studies, male mice were treated with 2, 3, or 4 mg/kg DEB for seven days and allowed to mate with untreated females on the day of the last injection

(experiments, 2, 4 and 9 in Fig. 1) or to 14, 21 and 28 mg/kg DEB administered acutely seven days before mating (experiments 3, 5 and 6 in Fig. 1).

Preparation of zygotic metaphases and FISH

Female mice received an i.p. injection of 7.5 I.U. of pregnant mare's serum (PMS, Sigma Chemical Co., St. Louis, MO, USA) to increase the number of maturing ovarian follicles, followed 48 hr later by an i.p. injection of 5.0 I.U. of human chorionic gonadotrophin (hCG, Sigma Co.) to induce ovulation. After the hCG injection, females were caged with males (1:1) and checked for vaginal plugs 8 hr later. Mated females received an i.p. injection of 0.08 mg/kg colchicine (CAS No. 64-86-8, Sigma Co.) dissolved in 0.2 ml of distilled water 24 hr after HCG to prevent the union of the two parental pronuclei and arrest zygotic development at the metaphase stage of the first cleavage division [21]. Mated females were euthanized by CO₂ inhalation 6 hr after colchicine injection, zygotes were flushed out from isolated oviducts and processed according to the mass harvest procedure [52]. The prepared slides were air-dried for at least 24 hr at room temperature, then kept in nitrogen atmosphere at -20°C until hybridization.

First-cleavage (1-CI) metaphases were hybridized with a probe mixture containing ten DNA composite painting probes: five biotin-labeled probes, each specific for chromosomes 1, 3, 5, X or Y and five FITC-labeled probes, each specific for chromosomes 2, 4, 6, X or Y (CAMBIO, Cambridge, UK). Amplification of the signals was obtained using the CAMBIO Dual Color Painting Kit (Biotin-Texas Red and FITC) as previously described [53]. A Zeiss Axioplan2 fluorescent photomicroscope was used for cytogenetic analysis. The microscope was equipped with a double-bandpass excitor (81P102, Chroma Technology, Brattleboro, VT) for visualizing the red (Texas red) and green (FITC) signals; a triple-bandpass filter set (61002, Chroma Technology) for the simultaneous detection of the red, green, and blue (DAPI) signals;

and another DAPI filter set (487901, Zeiss) for visualizing DAPI fluorescence only. Images of normal and aberrant metaphases were captured using the CytoVision™ Imaging Analysis System (Applied Image Biosystems Inc., Santa Clara, CA) and assembled using the Adobe Photoshop 5.0 software (Adobe System Incorporated, San Jose, CA). Metaphase analysis and scoring was done as previously described [53, 54].

Statistical analysis

For each experimental group, three-four independent matings each using 12 males and 12 females were used. The data from the three-four repetitions were pooled and the mean plus the standard error of the mean was calculated for all the endpoints measured. A chi-square was performed to assure that the observations within each group followed a Poisson distribution. If this was not the case, a chi-square with adjustment for overdispersion [55] was used to test for differences in the various endpoints between controls and treated groups.

RESULTS

A total of 13 regimens of single or fractionated exposures to DEB plus controls (Figure 1) were used to investigate how the differential abilities of early spermatids, late spermatids and sperm to repair DEB-induced damage affected the accumulation of DNA damage in male germ cells and its transmission to the zygote. As shown in Figure 1, the duration between the end of DEB exposure and fertilization was used to assess the relative sensitivities of sperm (7-1 days before fertilization, bdf), late spermatids (14-8 dbf) and early spermatids (21-15 dbf) to the induction of heritable DNA damage as detected using PAINT/DAPI analysis of chromosomal aberrations at first metaphase after fertilization [53, 54, 56, 57].

Chromosomal aberrations detected in zygotes after repeated exposures of male germ cells to DEB

The cytogenetic results for single and fractionated DEB exposures are shown in Table 1. Seven daily exposures of 4 mg/kg DEB significantly increased the frequencies of zygotes with chromosomal aberrations when sperm and late spermatids were treated ($p < 0.001$; Experiments 9 and 10) but not when round spermatids were exposed (Experiment 11). These results show that the last two weeks of mouse spermiogenesis, which corresponds to the period of spermatogenesis that is thought to be DNA repair-deficient in prior studies [13-20], is also the sensitive window for induction of chromosomal aberrations in sperm after paternal exposure to DEB, and that both sperm and late spermatids are unable to repair DEB-induced damage.

To determine whether there was accumulation of heritable DNA damage in sperm, the results of the one-week exposures (experiments 9, 10 and 11) were used to predict the frequencies of zygotes with chromosomal aberrations that would be expected in the multi-week

exposures (experiments 12, 13 and 14). For example, experiments 9 and 10 together covered the same window of spermiogenesis as experiment 12. Based on the results of experiments 9 and 10 (Table 1), we expected that 20% (11.8 plus 9.2) of the zygotes would have chromosomal aberrations in experiment 12. The observed frequency of zygotes with chromosomal aberrations in experiment 12 (16.7%) was not different for the expected ($P=0.3$). Similarly, the frequencies of zygotes with chromosomal aberrations in experiments 13 (8.7%) and 14 (16.4%) were not different ($P=0.5$ and $P=0.2$, respectively) from those expected (10.3% and 21.1%) based on the results of the one-week exposures that covered the same window of spermiogenesis, (experiments 10 plus 11 for experiment 13; experiments 9 plus 10 plus 11 for experiment 14). The concordance between the estimates of zygotes with chromosomal aberrations based on the one-week exposures (experiments 9, 10 and 11, Table 1) and those observed in multi-week exposures (experiments 12, 13 and 14, Table 1) suggest that DEB-induced heritable damage accumulates in late spermatids and sperm.

Identification of the DNA repair-deficient window of mouse spermiogenesis

To investigate the efficiency of repairing DEB-induced heritable damage during the three weeks of spermiogenesis, we compared the results among the experiments in which the same dose of DEB was given either as a single daily dose or fractionated over seven days (Table 1 and Figure 3). Administration of 28 mg/kg DEB as seven daily doses of 4 mg/kg each during the last week before mating (experiment 9) or as a single dose seven days before mating (experiment 6) produced similar frequencies of zygotes with chromosomal aberrations (11.8 vs. 9.7%; $P=0.4$). This suggests that there was no detectable repair of DEB lesions during the last week before mating and that DEB-induced damage accumulated during this time. However, when 28 mg/kg

DEB was given as a single dose 14 days before mating (experiment 7), the frequency of zygotes with chromosomal aberrations was significantly lower than that found when DEB was administered over 7 days (experiment 10; 4.2 vs. 9.2%, $P < 0.05$; Figure 3A), but still higher than control values ($P < 0.01$). This indicates that some repair occurred for lesions induced on day 14 before mating. Administration of 28 mg/kg DEB 21 days before fertilization (experiment 8) did not increase the frequency of zygotes with chromosomal aberrations with respect to the spontaneous frequency as it was observed after repeated exposures of early spermatids (experiment 11). Collectively, these results indicate that early spermatids are fully competent to repair DEB-induced lesions, that late spermatids become less proficient in lesion repair with some DNA repair still occurring on day 14 before mating, and that sperm are completely repair deficient and prone to accumulation of heritable DNA damage.

Shape of the dose-response curve after single or repeated exposures to DEB

To characterize the shape of the dose-response curve for the induction of germ cell lesions that lead to heritable chromosomal aberrations during the most sensitive window of spermiogenesis, male mice were treated with seven daily injections of 2, 3 or 4 mg/kg DEB (experiments 2, 4 and 9) and mated to untreated females immediately after the last injection (fractionated exposures). For single exposures, male mice were treated with 14, 21 or 28 mg/kg DEB (experiments 3, 5 and 6) and mated to untreated females seven days later. The results of the dose-response study showed a linear-dose response ($r^2 = 0.95$; Figure 3B) and that the frequencies of zygotes with chromosomal aberrations were dependent on the DEB dose but not on whether it was given as a single dose or fractionated over seven days. The increases ($P > 0.1$) in the frequencies of zygotes with chromosomal aberrations after treatment with 14 mg/kg DEB as a

single dose or over seven days before mating did not reach statistical significance because the number of metaphases that were analyzed did not provide enough power to detect such a small effect.

These results provide further evidence that during the DNA repair-deficient period of spermiogenesis, there is accumulation of DNA lesions in sperm that are transmitted to the zygote where they originate chromosomal aberrations.

Subpopulation of sperm with highly damaged chromosomes

The analysis of zygotic metaphases using PAINT/DAPI highlighted the presence of a subset of zygotes with extremely high levels of cytogenetic damage in the paternal chromosomes (Figure 2B and Tables 1). These highly damaged paternal chromosomal complements were observed only in those experiments (4, 5, 6, 9, 12, and 14) that exposed male germ cells to DEB as sperm (i.e., during the last week before mating) and represented about 2% of all metaphases analyzed (31/1595) and ~17% of the abnormal metaphases found (31/182). The spectrum of chromosomal aberrations observed in these highly damaged metaphases did not differ from that observed in those with less chromosomal damage. Chromosomal exchanges and acentric fragments represented the most common aberrations in both types of metaphases. These results indicate that there is a subpopulation of sperm that is particularly sensitive to the effects of DEB and that highly damaged sperm are able to fertilize mouse eggs and support development through at least the first cell cycle after fertilization.

DISCUSSION

We show that male postmeiotic germ cells differ in their abilities to repair DEB-induced lesions and that this has a significant impact on the amount of heritable genetic damage that is transmitted to the zygote and converted into chromosomal structural aberrations at 1-C1 metaphase. Comparisons between single and fractionated exposure regimens over the three weeks of mouse spermiogenesis showed that: i) the last two weeks before fertilization are the critical time-window for the induction of heritable lesions in sperm after paternal exposure to DEB; ii) heritable lesions accumulate in fertilizing sperm after exposure of late spermatids and sperm; and iii) the DNA repair-deficient window of mouse spermiogenesis for DEB-induced lesions is less than two weeks long. Our findings are consistent with a three-window model for the differing sensitivity of spermiogenesis to DEB-induced damage. These three-windows differ in their chromatin organization and DNA repair competency (Figure 4). During the repair-proficient window (I), characterized by efficient DNA repair capacity and DNA complexed with histones, DEB-induced lesions are repaired by the spermatid DNA repair machinery and little DNA damage is transmitted to the zygote. During the transition window (II), characterized by declining DNA repair capacity and the replacement of histones with transition proteins and protamines, some lesions are not repaired and transmitted to the zygote. During the repair deficient window (III), characterized by the absence of DNA repair and the compaction of DNA by protamines, unrepaired DEB-induced lesions are transmitted to the zygote. Unrepaired sperm DNA lesions can be misrepaired by the egg repair machinery into chromosomal aberrations during G1 and become visible at 1-C1 metaphase.

It is well known that chromosomal defects transmitted through male and female germ lines are associated with a variety of abnormal reproductive outcomes [58] and that the parental

origin of *de novo* genetic and chromosomal defects is not random: e.g., autosomal aneuploidy has a preferential maternal origin [59], while point mutations and structural chromosomal rearrangements have a preferential paternal origin [60]. It has been proposed that post-meiotic male germ cells are susceptible to the accumulation of DNA lesions in the fertilizing sperm because the DNA repair capacity declines during the latter part of spermiogenesis [17]. Our results provide two lines of evidence in support for the accumulation of heritable lesions during the last two weeks of spermiogenesis: (i) the frequencies of zygotes with chromosomal aberrations found after exposing male mice to DEB for two or three weeks were not different from those expected based on the results of the one-week exposures that covered the same period of spermiogenesis (Table 1); (ii) DEB administration as either single dose seven days before mating or distributed over the last seven days immediately before mating produced similar frequencies of zygotes with paternally-derived chromosomal aberrations (Table 3). These results demonstrate that DNA lesions induced during the last phase of spermiogenesis are not repaired and that continuous exposure during this sensitive window results in the accumulation of heritable lesions. Once the sperm fertilizes the egg, these lesions generate DSBs before zygotic S-phase and are misrepaired into the chromosomal exchanges or acentric fragments that are observed at 1-C1 metaphase (Figure 1). This unique feature of male germ cell biology indicates that the male is particularly vulnerable to exposure to environmental mutagens during this sensitive window because such DNA lesions will persist unrepaired in sperm prior to fertilization and once in the egg have the potential to generate chromosomal aberrations which have detrimental effects on normal embryonic development [53].

Our results confirm and extend prior studies of the DNA repair-deficient window during spermiogenesis [13-20]. The results of our experiments showed that the frequencies of zygotes

with chromosomal aberrations were significantly higher when a total dose of 28 mg/kg DEB was given over days 14 through 8 before mating than when it was given as a single dose 14 days before mating (Table 2 and Figure 3A). We interpret these results as evidence that DNA repair is still occurring on day 14 before mating when the spermatid DNA is still complexed with histones, and continues during the next few days as transition proteins replace histones. The replacements of histones by transition proteins may provide DNA repair enzymes access to the damaged DNA and allows its repair. This is consistent with the observation that transition proteins stimulate DNA repair [8]. Overall, our results show that late spermatids lose their DNA repair capacity gradually over a period of several days as they complete the replacement of histones with transition proteins and protamines and that the window of no detectable DNA repair during spermiogenesis may be less than the two weeks that are currently thought.

The inability of late spermatids and sperm to repair heritable lesions as they occur make these cells particularly sensitive to exposures that persist over time. This could be particularly important for chronic exposures that occur because of occupational or lifestyle factors (i.e., tobacco smoking) that may affect the genetic constitution of male germ cells in the weeks prior to fertilization. Indeed, several studies have reported elevated levels of DNA base damage [61], DNA adducts [62, 63] and DNA strand breaks [64] in sperm of smokers. Studies using FISH have also shown that smokers have increased levels of chromosomal defects in their sperm [65-67]. It is tempting to speculate that daily tobacco smoke may induce accumulation of DNA damage in sperm especially during the last two or three weeks before fertilization and that even moderate smoking may lead to accumulated detrimental effects on the genetic integrity of the fertilizing sperm.

We noted that a few of the fertilizing sperm had unusually high levels of chromosomal damage (Figure 2B). Data from previous studies from our laboratory using B6C3F1 mice (Figure 5) showed that this is a common occurrence in male exposures that produced chromosomal aberrations in the majority of the zygotes. As shown in Figure 4, zygotes with structural aberrations involving 7 or more paternal chromosomes were found only in treatments that produced chromosomal aberrations in >45% of the zygotes analyzed. In addition, zygotes with highly damaged paternal chromosomal complements represented <10% of the abnormal zygotes. However, in the present study, although DEB induced chromosomal aberrations in only 10-15% of the zygotes analyzed, zygotes with highly damaged paternal chromosomal complements represented ~30% of the abnormal zygotes. A similar observation was previously reported in mouse zygotes after paternal exposure to 26 mg/kg DEB [51] and suggests that there is a subpopulation of sperm that is highly susceptible to DEB. We speculate that this subpopulation of sperm may have a more relaxed chromatin conformation that allows greater numbers of DNA intra- and inter-strand DEB adducts to be formed and hence greater numbers of chromosomal aberrations in zygotes. Regardless of the molecular mechanism underlying the exquisite sensitivity of this subpopulation of sperm to DEB, our results demonstrate that high levels of heritable damage in sperm do not affect fertilization and development during the first cell cycle after fertilization.

Comparisons of our results with the literature [49, 51], confirm the higher sensitivity of postmeiotic male germ cells to DEB as compared to female germ cells. The yield of zygotes with chromosomal structural aberrations is at least 3-fold higher after exposing male gametes to DEB seven days before mating than after treating female gametes with a similar dose ~2 days before mating, which represent treatment of dictyate oocytes [49]. The difference is even higher when

the amount of chromosomal damage is considered [49]. Although dictyate oocytes are characterized by a diffuse chromatin state that is thought to make them particularly sensitive to DNA-interacting chemical agents [68-70], oocytes have fully functional DNA repair mechanisms throughout oogenesis and provide gene products that are responsible for repairing DNA damage in both parental genomes after fertilization [26, 71, 72]. Therefore, it is likely that DNA lesions induced by DEB in oocytes are repaired before fertilization and before pronuclear DNA synthesis takes place. Also, after oocyte exposure, chromatid-type breaks and exchanges represented half of the aberrations that were observed at zygotic metaphase [49]; in contrast, exposure of male germ cells induced almost exclusively chromosome-type aberrations. Because chromosome-type aberrations require the presence of a DNA double strand break before DNA synthesis begins, these results suggest that DEB-induced sperm DNA lesions produced double strand breaks before the oocyte repair machinery had the chance of repairing the original DEB lesion, most likely during the profound chromatin reorganization that occurs in the male pronucleus immediately after fertilization and before pronuclear S-phase [73]. These findings indicate that chromatin organization and, more importantly, DNA repair status in gametes have a high impact on both the types and frequencies of chromosomal aberrations generated in zygotes.

In conclusions, our results show that late postmeiotic male germ cells are extremely susceptible to the accumulation of DNA lesions because of diminished DNA repair capacity and that these lesions are transmitted to the fertilized egg where they are converted into chromosomal aberrations during the first cell cycle after fertilization. Our results also show that continuous low dose exposures during the postmeiotic phase of spermatogenesis are as detrimental as acute exposures. This has important implications for males in their reproductive

years who are exposed to low levels of chemicals because of chronic occupational or life style (e.g. tobacco smoking) exposures in the weeks prior to successful fertilization.

ACKNOWLEDGEMENTS

Work performed under the auspices of U.S. DOE by the Lawrence Livermore National Laboratory under contract W-7405-ENG-48 and the Lawrence Berkeley National Laboratory under contract DE-AC02-05CH11231 with funding support from California TRDRP 7RT-0073 (AJW) and 13RT-0140 (FM).

REFERENCES

- [1] K.C. Kleene, A possible meiotic function of the peculiar patterns of gene expression in mammalian spermatogenic cells, *Mech. Dev.* 106 (2001) 2-23.
- [2] D. Wouters-Tyrou, A. Martinage, P. Chevaillier, P. Sautiere, Nuclear basic proteins in spermiogenesis, *Biochimie* 80 (1998) 117-128.
- [3] M.L. Meistrich, W.A. Brock, S.R. Grimes, R.D. Platz, L.S. Hnilica, Nucleoprotein transitions during spermatogenesis, *Fed. Proc.* 37 (1978) 2522-2525.
- [4] M.L. Meistrich, B. Mohapatra, C.R. Shirley, M. Zhao, Roles of transition nuclear proteins in spermiogenesis, *Chromosoma*, 111 (2003) 483-488.
- [5] G. Boissonneault, Chromatin remodeling during spermiogenesis: a possible role for the transition proteins in DNA strand break repair, *FEBS Lett*, 514 (2002) 111-114.
- [6] A.L. Kierszenbaum, L.L. Tres, RNA transcription and chromatin structure during meiotic and postmeiotic stages of spermatogenesis, *Fed. Proc.* 37 (1978) 2512-2516.
- [7] K. Steger, T. Klonisch, K. Gavenis, B. Drabent, D. Doenecke, M. Bergmann, Expression of mRNA and protein of nucleoproteins during human spermiogenesis, *Mol. Hum. Reprod.* 4 (1998) 939-945.
- [8] N. Caron, S. Veilleux, G. Boissonneault, Stimulation of DNA repair by the spermatidal TP1 protein, *Mol. Reprod. Dev.* 58 (2001) 437-443.
- [9] R.M. Laberge, G. Boissonneault, Chromatin remodeling in spermatids: a sensitive step for the genetic integrity of the male gamete, *Arch. Androl.* 51 (2005) 125-133.
- [10] R.M. Laberge, G. Boissonneault, On the nature and origin of DNA strand breaks in elongating spermatids, *Biol. Reprod.* 73 (2005) 289-296.

- [11] M.D. Shelby, Selecting chemicals and assays for assessing mammalian germ cell mutagenicity, *Mutat. Res.* 325 (1996) 159-167.
- [12] F. Marchetti, A.J. Wyrobek, Mechanisms and consequences of paternally-transmitted chromosomal abnormalities, *Birth Defects Res. C. Embryo Today* 75 (2005) 112-129.
- [13] G.A. Sega, Unscheduled DNA synthesis in the germ cells of male mice exposed in vivo to the chemical mutagen ethyl methanesulphonate, *Proc. Natl. Acad. Sci. U.S.A.* 71 (1974) 4955-4959.
- [14] G.A. Sega, Unscheduled DNA synthesis (DNA repair) in the germ cells of male mice: its role in the study of mammalian mutagenesis, *Genetics* 92 (1979) s49-s58.
- [15] R.E. Sotomayor, G.A. Sega, Unscheduled DNA synthesis assay in mammalian spermatogenic cells: an update, *Environ. Mol. Mutagen.* 36 (2000) 255-265.
- [16] W.M. Baarends, R. van der Laan, J.A. Grootegoed, DNA repair mechanisms and gametogenesis, *Reproduction* 121 (2001) 31-39.
- [17] A.K. Olsen, B. Lindeman, R. Wiger, N. Duale, G. Brunborg, How do male germ cells handle DNA damage? *Toxicol. Appl. Pharmacol.* 207 (2005) 521-531.
- [18] A.A. van Loon, E. Sonneveld, J. Hoogerbrugge, G.P. van der Schans, J.A. Grootegoed, P.H. Lohman, R.A. Baan, Induction and repair of DNA single-strand breaks and DNA base damage at different cellular stages of spermatogenesis of the hamster upon in vitro exposure to ionizing radiation, *Mutat. Res.* 294 (1993) 139-148.
- [19] G. Hamer, H.L. Roepers-Gajadien, A. van Duyn-Goedhart, I.S. Gademan, H.B. Kal, P.P. van Buul, D.G. de Rooij, DNA double-strand breaks and gamma-H2AX signaling in the testis, *Biol. Reprod.* 68 (2003) 628-634.

- [20] G. Xu, G. Spivak, D.L. Mitchell, T. Mori, J.R. McCarrey, C.A. McMahan, R.B. Walter, P.C. Hanawalt, C.A. Walter, Nucleotide excision repair activity varies among murine spermatogenic cell types, *Biol. Reprod.* 73 (2005) 123-130.
- [21] .R.P. Donahue, Cytogenetic analysis of the first cleavage division in mouse embryos (fertilization-pronuclei-T163H translocation), *Proc. Natl. Acad. Sci. U S A.* 69 (1972) 74-77.
- [22] G.A. Sega, R.P. Alcota, C.P. Tancongco, P.A. Brimer, Acrylamide binding to the DNA and protamine of spermiogenic stages in the mouse and its relationship to genetic damage, *Mutat. Res.* 216 (1989) 221-230.
- [23] Y. Matsuda, N. Seki, T. Utsugi-Takeuchi, I. Tobari, Changes in X-ray sensitivity of mouse eggs from fertilization to the early pronuclear stage, and their repair capacity, *Int. J. Radiat. Biol.* 55 (1989) 233-256.
- [24] Y. Matsuda, N. Seki, T. Utsugi-Takeuchi, I. Tobari, X-ray- and mitomycin C (MMC)-induced chromosome aberrations in spermiogenic germ cells and the repair capacity of mouse eggs for the X-ray and MMC damage, *Mutat. Res.* 211 (1989) 65-75.
- [25] Y. Matsuda, I. Tobari, M. Maemori, N. Seki, Mechanism of chromosome aberration induction in the mouse egg fertilized with sperm recovered from postmeiotic germ cells treated with methyl methanesulfonate, *Mutat. Res.* 214 (1989) 165-180.
- [26] F. Marchetti, J. Essers, R. Kanaar, A.J. Wyrobek, Disruption of maternal repair increases sperm-derived chromosomal aberrations, *Proc. Natl. Acad. Sci. U S A.* (2007) Epub October 31, 2007.

- [27] L.H. Kuller, L. Garfinkel, P. Correa, N. Haley, D. Hoffmann, S. Preston-Martin, D. Sandler, Contribution of passive smoking to respiratory cancer, *Environ. Health Perspect.* 70 (1986) 57-69.
- [28] G. Lofroth, Environmental tobacco smoke: overview of chemical composition and genotoxic components, *Mutat. Res.* 222 (1989) 73-80.
- [29] L.D. Claxton, R.S. Morin, T.J. Hughes, J. Lewtas, A genotoxic assessment of environmental tobacco smoke using bacterial bioassays, *Mutat. Res.* 222 (1989) 81-99.
- [30] S. Nesnow, M. Argus, H. Bergman, K. Chu, C. Frith, T. Helmes, R. McGaughy, V. Ray, T.J. Slaga, R. Tennant, Chemical carcinogens. A review and analysis of the literature of selected chemicals and the establishment of the Gene-Tox Carcinogen Data Base. A report of the U.S. Environmental Protection Agency Gene-Tox Program, *Mutat. Res.* 185 (1987) 1-195.
- [31] R.E. Morrissey, B.A. Schwetz, P.L. Hackett, M.R. Sikov, B.D. Hardin, B.J. McClanahan, J.R. Decker, T.J. Mast, Overview of reproductive and developmental toxicity studies of 1,3-butadiene in rodents, *Environ. Health Perspect.* 86 (1990) 79-84.
- [32] M.S. Christian, Review of reproductive and developmental toxicity of 1,3-butadiene, *Toxicology* 113 (1996) 137-143.
- [33] I.D. Adler, J. Filser, H. Gonda, G. Schriever-Schwemmer, Dose response study for 1,3-butadiene-induced dominant lethal mutations and heritable translocations in germs cells of male mice, *Mutat. Res.* 397 (1998) 85-92.
- [34] D. Anderson, Butadiene: species comparison for metabolism and genetic toxicology, *Mutat. Res.* 405 (1998) 247-258.

- [35] S.Z. Cagen, R.L. MacDonald, G. Van Gelder, Review of risk assessments on 1,3-butadiene (1985-1991), *Toxicology* 113 (1996) 215-220.
- [36] K.D. Brunneemann, M.R. Kagan, J.E. Cox, D. Hoffmann, Analysis of 1,3 butadiene and other selected gas-phase components in cigarette mainstream and sidestream smoke by gas chromatography-mass selective detection, *Carcinogenesis* 11 (1990) 1863-1868.
- [37] G. Johanson, J.G. Filser, PBPK model for butadiene metabolism to epoxides: quantitative species differences in metabolism, *Toxicology* 113 (1996) 40-47.
- [38] J. Thornton-Manning, Dahl, AR, Bechtold, WE, Henderson RF, Gender and species differences in the metabolism of 1,3-butadiene to butadiene monoepoxide and butadiene diepoxide in rodents following low-level inhalation exposure., *Toxicology* 113 (1996) 322-325.
- [39] G.A. Csanady, P.E. Kreuzer, C. Baur, J.G. Filser, A physiological toxicokinetic model for 1,3-butadiene in rodents and man: blood concentrations of 1,3-butadiene, its metabolically formed epoxides, and of haemoglobin adducts--relevance of glutathione depletion, *Toxicology* 113 (1996) 300-305.
- [40] A.A. Elfarrar, R.J. Krause, R.R. Selzer, Biochemistry of 1,3-butadiene metabolism and its relevance to 1,3-butadiene-induced carcinogenicity, *Toxicology* 113 (1996) 23-30.
- [41] C. Maniglier-Poulet, X. Cheng, J.A. Ruth, D. Ross, Metabolism of 1,3-butadiene to butadiene monoxide in mouse and human bone marrow cells, *Chemico-Biological Interact.* 97 (1995) 119-129.
- [42] S. Park, N. Tretyakova, Structural characterization of the major DNA-DNA cross-link of 1,2,3,4-diepoxybutane, *Chem. Res. Toxicol.* 17 (2004) 129-136.

- [43] S. Park, C. Anderson, R. Loeber, M. Seetharaman, R. Jones, N. Tretyakova, Interstrand and intrastrand DNA-DNA cross-linking by 1,2,3,4-diepoxybutane: role of stereochemistry, *J. Am. Chem. Soc.* 127 (2005) 14355-14365.
- [44] J.E. Cochrane, T.R. Skopek, Mutagenicity of butadiene and its epoxide metabolites: I. Mutagenic potential of 1,2-epoxybutene, 1,2,3,4-diepoxybutane and 3,4-epoxy-1,2-butanediol in cultured human lymphoblasts, *Carcinogenesis* 15 (1994) 713-717.
- [45] M. Costa, A. Zhitkovich, M. Harris, D. Paustenbach, M. Gargas, DNA-protein cross-links produced by various chemicals in cultured human lymphoma cells, *J. Toxicol. Environ. Health.* 50 (1997) 433-449.
- [46] R. Loeber, M. Rajesh, Q. Fang, A.E. Pegg, N. Tretyakova, Cross-linking of the human DNA repair protein O6-alkylguanine DNA alkyltransferase to DNA in the presence of 1,2,3,4-diepoxybutane, *Chem. Res. Toxicol.* 19 (2006) 645-654.
- [47] D. Jacobson-Kram, S.L. Rosenthal, Molecular and genetic toxicology of 1,3-butadiene, *Mutat. Res.* 339 (1995) 121-130.
- [48] M. Spano, C. Bartoleschi, E. Cordelli, G. Leter, L. Segre, A. Mantovani, P. Fazzi, F. Pacchierotti, Flow cytometric and histological assessment of 1,2:3,4-diepoxybutane toxicity on mouse spermatogenesis, *J. Toxicol. Environ. Health* 47 (1996) 423-441.
- [49] C. Tiveron, R. Ranaldi, B. Bassani, F. Pacchierotti, Induction and transmission of chromosome aberrations in mouse oocytes after treatment with butadiene diepoxide, *Environ Mol. Mutagen.* 30 (1997) 403-409.
- [50] Y. Xiao, A.D. Tate, Clastogenic effects of 1,3-butadiene and its metabolites 1,2-epoxybutene and 1,2,3,4-diepoxybutane in splenocytes and germ cells of rats and mice in vivo, *Environ. Mol. Mutagen.* 26 (1995) 97-108.

- [51] I.D. Adler, U. Kliesch, C. Tiveron, F. Pacchierotti, Clastogenicity of diepoxybutane in bone marrow cells and male germ cells of mice, *Mutagenesis* 10 (1995) 535-541.
- [52] J.B. Mailhes, Z.P. Yuan, Cytogenetic technique for mouse metaphase II oocytes, *Gamete Res.* 18 (1987) 77-83.
- [53] F. Marchetti, J.B. Bishop, L. Cosentino, D. Moore II, A.J. Wyrobek, Paternally transmitted chromosomal aberrations in mouse zygotes determine their embryonic fate, *Biol. Reprod.* 70 (2004) 616-624.
- [54] F. Marchetti, X. Lowe, D.I. Moore, J. Bishop, A.J. Wyrobek, Paternally inherited chromosomal structural aberrations detected in mouse first-cleavage zygote metaphases by multicolor fluorescence in situ hybridization painting, *Chromosome Res.* 4 (1996) 604-613.
- [55] D. Collett. Modeling binary data. London: Chapman and Hall; 1991.
- [56] F. Marchetti, X. Lowe, J. Bishop, J. Wyrobek, Induction of chromosomal aberrations in mouse zygotes by acrylamide treatment of male germ cells and their correlation with dominant lethality and heritable translocations, *Environ Mol. Mutagen.* 30 (1997) 410-417.
- [57] F. Marchetti, J.B. Bishop, X. Lowe, W.M. Generoso, J. Hozier, A.J. Wyrobek, Etoposide induces heritable chromosomal aberrations and aneuploidy during male meiosis in the mouse, *Proc. Natl. Acad. Sci. U.S.A.* 98 (2001) 3952-3957.
- [58] D. McFadden, J. Friedman, Chromosome abnormalities in human beings, *Mutat. Res.* 396 (1997) 129-140.
- [59] T. Hassold, P. Hunt, To err (meiotically) is human: the genesis of human aneuploidy, *Nature Rev. Genet.* 2 (2001) 280-291.
- [60]. J.F. Crow, The origins, patterns and implication of human spontaneous mutations, *Nature Rev. Genet.* 1 (2001) 40-47.

- [61] H.M. Shen, S.E. Chia, Z.Y. Ni, A.L. New, B.L. Lee, C.N. Ong, Detection of oxidative DNA damage in human sperm and the association with cigarette smoking, *Reprod. Toxicol.* 11 (1997) 675-680.
- [62] S. Horak, J. Polanska, P. Widlak, Bulky DNA adducts in human sperm: relationship with fertility, semen quality, smoking, and environmental factors, *Mutat. Res.* 537 (2003) 53-65.
- [63] S. Horak, J. Polanska, P. Widlak, High levels of bulky DNA adducts in human sperm correlate with impaired fertility, *Acta Biochim. Pol.* 50 (2003) 197-203.
- [64] R.J. Potts, C.J. Newbury, G. Smith, L.J. Notarianni, T.M. Jefferies, Sperm chromatin damage associated with male smoking, *Mutat. Res.* 423 (1999) 103-111.
- [65] W.A. Robbins, M.F. Vine, K.Y. Truong, R.B. Everson, Use of fluorescence in situ hybridization (FISH) to assess effects of smoking, caffeine, and alcohol on aneuploidy load in sperm of healthy men, *Environ. Mol. Mutagen.* 30 (1997) 175-183.
- [66] J. Rubes, X. Lowe, D. Moore, 2nd, S. Perreault, V. Slott, D. Evenson, S.G. Selevan, A.J. Wyrobek, Smoking cigarettes is associated with increased sperm disomy in teenage men, *Fertil. Steril.* 70 (1998) 715-723.
- [67] Q. Shi, E. Ko, L. Barclay, T. Hoang, A. Rademaker, R. Martin, Cigarette smoking and aneuploidy in human sperm, *Mol. Reprod. Dev.* 59 (2001) 417-421.
- [68] P.D. Sudman, W.M. Generoso, Female-specific mutagenic response of mice to hycanthone, *Mutat. Res.* 246 (1991) 31-43.
- [69] P.D. Sudman, J.C. Rutledge, J.B. Bishop, W.M. Generoso, Bleomycin: female-specific dominant lethal effects in mice, *Mutat. Res.* 296 (1992) 143-156.
- [70] M.A. Katoh, K.T. Cain, L.A. Hughes, L.B. Foxworth, J.B. Bishop, W.M. Generoso, Female-specific dominant lethal effects in mice, *Mutat. Res.* 230 (1990) 205-217.

- [71] B. Brandriff, R.A. Pedersen, Repair of the ultraviolet-irradiated male genome in fertilized mouse eggs, *Science* 211 (1981) 1431-1433.
- [72] M.J. Ashwood, R. Edwards, DNA repair by oocytes, *Mol. Hum. Reprod.* 2 (1996) 46-51.
- [73] D.W. McLay, H.J. Clarke, Remodelling the paternal chromatin at fertilization in mammals, *Reproduction* 125 (2003) 625-633.

Table 1 – Chromosomal aberrations in first-cleavage (1-Cl) zygote metaphases after repeated or single exposures of male mice to DEB at various times prior to mating with untreated females.

Exp. No.	Dose mg/kg	No. of doses	Total dose mg/kg	Mating time ^a	No. of 1-Cl analyzed	1-Cl with aberrations	
						No.	% ± S.E. ^b
1	0	7	0	0	288	2	0.7 ± 0.3
2	2	7	14	0	225	5	2.2 ± 1.1 ^g
3	14	1	14	7	270	5	1.9 ± 1.0 ^g
4	3	7	21	0	226	20 (4) ^c	8.8 ± 4.4 ^d
5	21	1	21	7	288	16 (3) ^c	5.6 ± 1.0 ^d
6	28	1	28	7	257	25 (4) ^c	9.7 ± 2.3 ^d
7	28	1	28	14	213	9	4.2 ± 0.6 ^e
8	28	1	28	21	227	3	1.3 ± 0.2
9	4	7	28	0	323	38 (4) ^c	11.8 ± 4.5 ^d
10	4	7	28	7	262	24	9.2 ± 3.2 ^{e, f}
11	4	7	28	14	278	3	1.1 ± 0.8
12	4	14	52	0	294	49 (11) ^c	16.7 ± 4.1 ^d
13	4	14	52	7	275	24	8.7 ± 2.7 ^d
14	4	21	84	0	207	34 (5) ^c	16.4 ± 1.4 ^d

^aDays between last DEB injection and day of mating.

^bStandard error.

^cZygotes with structural aberrations affecting more than 6 paternal chromosomes.

^dP<0.001 vs. controls (Chi-square).

^eP<0.01 vs. controls (Chi-square).

^fP<0.05 vs. experiment 7 (Chi-square).

^gP>0.14 vs. controls (Chi-square).

Figure Legends

- Figure 1.** Exposure regimens for the 14 experiments of the present study. A schematic of the last three weeks of mouse spermatogenesis is shown at the top. Below the schematic, the duration of each treatment and the administered daily dose of DEB are given.
- Figure 2.** Photomicrographs of mouse first-cleavage (1-CI) zygotes collected after exposing male mice to DEB. Analysis of metaphases was done using multicolor FISH painting probes with a probe combination that detects ~60% of all chromosomal exchanges. With this combination, chromosomes 1, 3, and 5 appear red, chromosomes 2, 4, and 6 appear green and the sex chromosomes appear yellow. **A.** Normal zygote with the Y-bearing paternal chromosomes on the right. **B.** Paternal chromosomes with extensive damage in a zygote fertilized by a sperm from a male treated with DEB. Examples of chromosomal exchanges (arrows) and of acentric fragments (arrowheads) are indicated.
- Figure 3.** Comparison of the frequencies of zygotes with chromosomal aberrations after either single or fractionated exposures to DEB. **A.** For the time-response study, male mice were treated with 28 mg/kg DEB either as a single dose or as seven daily doses of 4 mg/kg and mated with untreated females. The numbers in parentheses refer to the experiment numbers of Figure 1. Bars represent the standard error. * $P < 0.05$ vs. single exposure. **B.** For the dose-response study, male mice were treated with 14, 21 or 28 mg/kg DEB either as single doses seven days before mating or distributed over seven days. The numbers in parentheses refer to the experiment numbers of Figure 1. Bars represent the standard error.
- Figure 4.** Three-window model for the sensitivity of spermiogenesis to DEB-induced damage. A schema of spermiogenesis is shown with the timing of appearance of transition proteins (TP) and protamines (based on Meistrich et al 2003). See Discussion for explanation.

Figure 5. Comparisons of the distribution of damaged zygotes after paternal DEB exposure with other paternal exposure studies. The chemicals are ranked by increasing percentages of abnormal zygotes. For DEB, the data from experiments 4, 5, 6, 9, 12 and 14 were used (182/1595). As the frequencies of zygotes with chromosomal aberrations increase so do the proportions of zygotes with more than 2 chromosomal aberrations. DEB has a profile that is similar to those produced by exposures that generate much higher frequencies of zygotes with chromosomal aberrations. C: controls; ET: Etoposide; DEB: diepoxybutane; AA: acrylamide; IR: ionizing radiation; CP: cyclophosphamide; MMS: methyl methanesulphonate; MLP: melphalan. Data from (41) for acrylamide; (42) for etoposide; and (38) for the other chemicals.

days	21	14	7	0		
	Spermatocytes		Spermatids		Sperm	Total dose (mg/kg)
	Meiosis		Postmeiosis			
			Repair deficient			
Experiment 1						0
Experiment 2				2 2 2 2 2 2 2		14
Experiment 3			14			14
Experiment 4			3 3 3 3 3 3 3			21
Experiment 5			21			21
Experiment 6			28			28
Experiment 7		28				28
Experiment 8	28					28
Experiment 9				4 4 4 4 4 4 4		28
Experiment 10			4 4 4 4 4 4 4			28
Experiment 11	4 4 4 4 4 4 4					28
Experiment 12			4 4 4 4 4 4 4	4 4 4 4 4 4 4		56
Experiment 13	4 4 4 4 4 4 4		4 4 4 4 4 4 4			56
Experiment 14	4 4 4 4 4 4 4	4 4 4 4 4 4 4	4 4 4 4 4 4 4	4 4 4 4 4 4 4		84

Figure 1

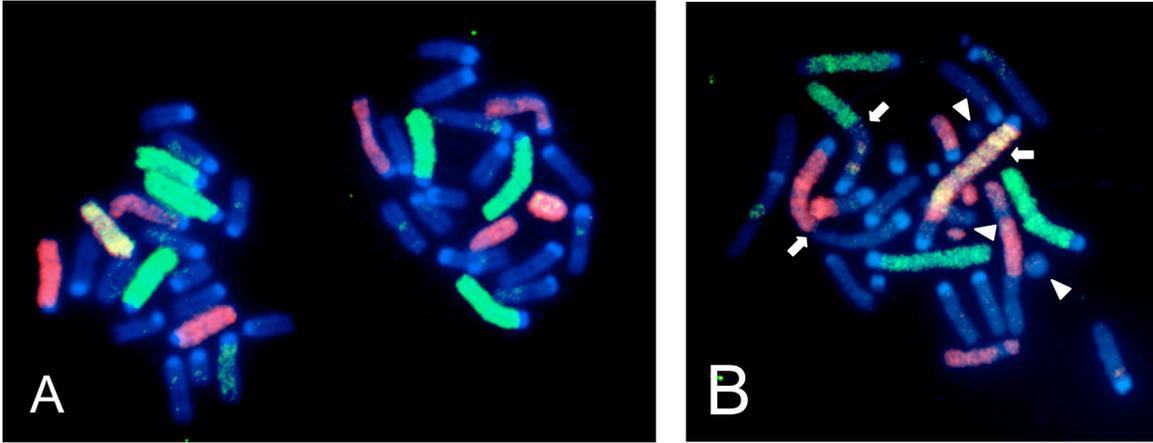


Figure 2

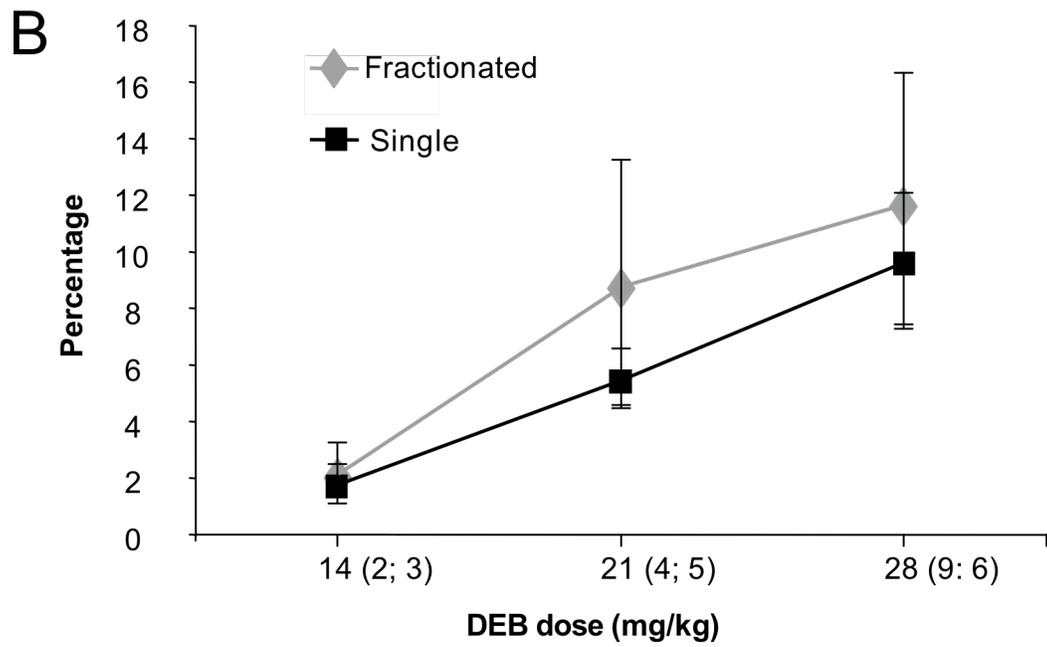
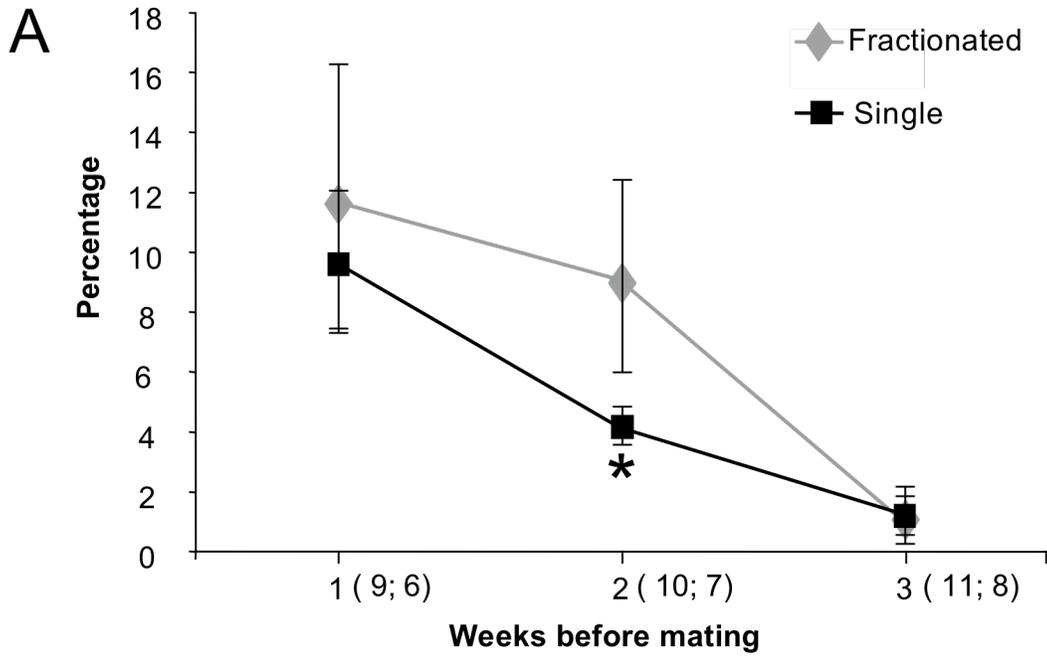


Figure 3

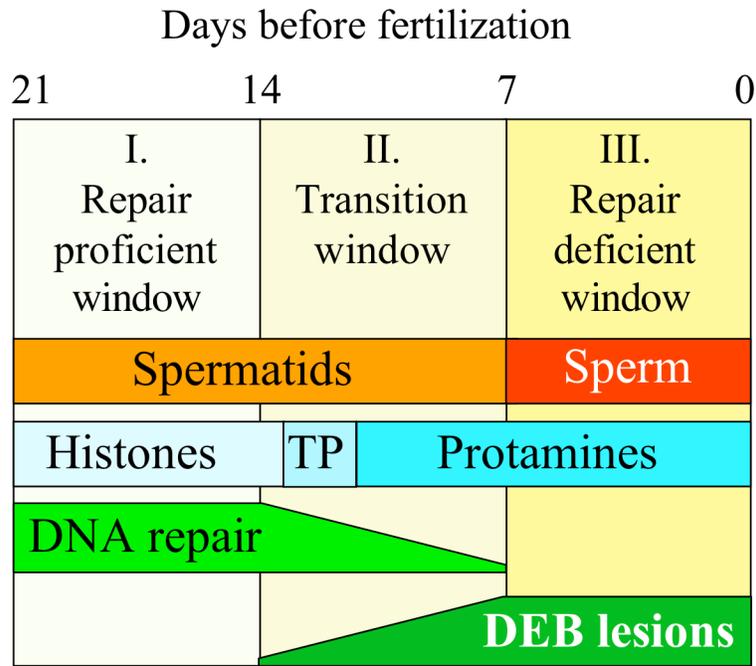


Figure 4

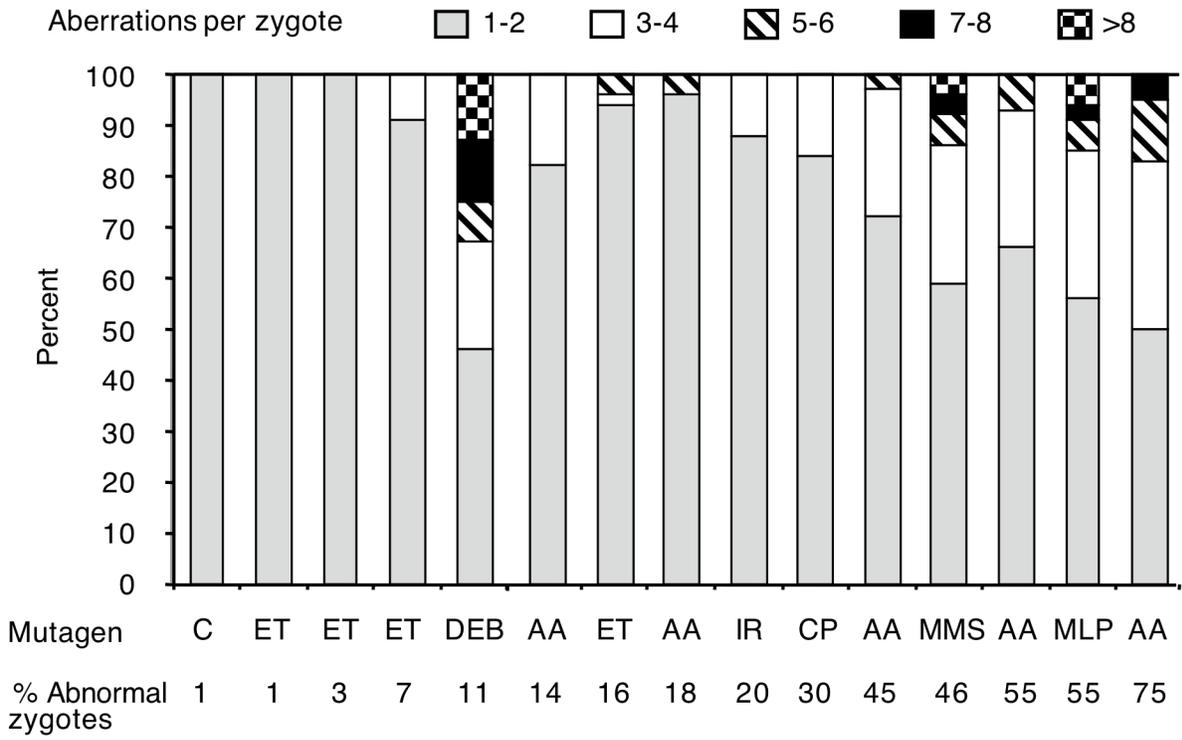


Figure 5