Etoposide induces chromosomal abnormalities in mouse spermatocytes and stem cell spermatogonia

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BACKGROUND: Etoposide (ET) is a chemotherapeutic agent widely used in the treatment of leukaemia, lymphomas and many solid tumours such as testicular and ovarian cancers, all of which are common in patients of reproductive age. The purpose of the study was to characterize the long-term effects of ET on male germ cells using sperm fluorescence in situ hybridization (FISH) analyses. METHODS: Chromosomal aberrations (partial duplications and deletions) and whole chromosomal aneuploidies were detected in sperm of mice treated with a clinical dose of ET. Semen samples were collected at 25 and 49 days after dosing to investigate the effects of ET on meiotic pachytene cells and spermatogonial stem-cells, respectively. RESULTS: ET treatment resulted in major increases in the frequencies of sperm-carrying chromosomal aberrations in both meiotic pachytene (27- to 578-fold) and spermatogonial stem-cells (8- to 16-fold), but aneuploid sperm were induced only after treatment of meiotic cells (27-fold) with no persistent effects in stem cells. CONCLUSION: These results show that ET may have long-lasting effects on the frequencies of sperm with structural aberrations. This has important implications for cancer patients undergoing chemotherapy with ET because they may remain at higher risk for abnormal reproductive outcomes long after the end of chemotherapy.

\textit{Key words:} aneuploidy/chemotherapy/FISH/male germ cells/structural aberrations

Introduction

Advances in our understanding of cancer biology are producing chemotherapies that have significantly increased cancer survival, especially for cancers before and during the reproductive years (American Cancer Society, 2005). However, chemotherapy regimens commonly include one or more agents that are mutagenic or clastogenic in model systems and are highly toxic to germ cells (Witt and Bishop, 1996; Wyrobek et al., 2005a). Thus, as there are more survivors of cancer in their reproductive years, there are associated concerns that chemotherapy may have induced germ-line mutations that increase the risks of spontaneous abortions, birth defects, genetic diseases or cancer among the children of cancer survivors.

Etoposide (ET) is one of the most commonly used agents in cancer chemotherapy for the treatment of leukaemia, lymphomas and many solid tumours including testicular and ovarian cancers, which are common in children and young adults in their repro ductive years (Liu, 1989; Smith, 1990). ET inhibits topoisomerase II (topo II), an enzyme that introduces DNA double strand breaks allowing the passage of one double helix through another, and then reseals the double strand break (Smith, 1990; Wang et al., 1990). Topo II removes regions of DNA catenation during DNA replication, chromosome condensation (Poljak and Kas, 1995) and prior to chromosome segregation during mitosis (DiNardo et al., 1984; Rose et al., 1990a; Downes et al., 1991) and meiosis (Moens, 1990; Cobb et al., 1997). ET inhibits topo II activity by forming a ternary complex, DNA-topo II-ET, which prevents the strand religation reaction (Anderson and Berger, 1994; Ferguson and Baguley, 1994, 1996), resulting in the creation of double strand DNA breaks and formation of chromosomal aberrations (Suzuki et al., 1995, 1997).

Over 80\% of the cancer patients who are treated with ET regain fertility within a few years after the end of chemotherapy (Pectasides et al., 2004; Howell and Shalet, 2005). It is therefore important to understand the potential long-term effects on germ cell genetic integrity. ET is known to induce both numerical and structural chromosome aberrations in somatic cells (Anderson and Berger, 1994; Ferguson and Baguley, 1996; Cimini et al., 1997; Baguley and Ferguson, 1998) and in female and male germ cells (Kallio and Lähdetie, 1993; Mailhes et al., 1994, 1996; Tateno and Kamiguchi, 2001; Attia et al., 2002). Male meiotic cells are a major target of ET resulting in dominant lethality (Bishop et al., 1997), specific locus mutations (Russell et al., 1998) and chromosomal damage in metaphase II spermatocytes and zygotes (Marchetti et al., 2001). No effects have been reported in spermatogonial stem cells, but these assays often lack the sensitivity and statistical power to detect small effects.
Sperm fluorescence in situ hybridization (FISH) has been adapted to detect aneuploidy in both human and rodent sperm (Martin et al., 1995; Lowe et al., 1996, 1998; Robbins et al., 1997; Rubes et al., 1998; Wyrobek et al., 2005b). A number of chemotherapeutic regimens, including those involving ET, have already been shown to induce transient increases in the frequencies of aneuploid sperm using FISH (Robbins et al., 1997; Martin et al., 1999; De Mas et al., 2001; Frias et al., 2003; Wyrobek et al., 2005a), suggesting that treated patients are at higher risk for fatering aneuploidy conceptions only within the immediate few months following the end of chemotherapy.

Cancer chemotherapies can also induce chromosomal aberrations in sperm. Using the human sperm/hamster oocyte assay, Genesa et al. (1990) reported significant increases in the frequencies of stable translocations in patients several years after the end of chemotherapy. More recently, we have developed several sperm FISH assays for detecting structural chromosomal aberrations in human sperm (Van Hummelen et al., 1996, 1997; Sloter et al., 2000), but these have not yet been applied to the evaluation of chemotherapy regimens.

We also developed a mouse sperm FISH assay (CT8 assay) for the detection of both chromosomal structural aberrations and aneuploidy in mouse sperm (Hill et al., 2003). This three-color FISH assay uses two DNA probes specific for the centromeric and telomeric regions of chromosome 2 plus a probe for the subcentromeric region of chromosome 8. This sperm FISH assay can detect all the major types of chromosomal defects that might be expected after ET chemotherapy.

The purpose of this study was to use a mouse model to: (i) characterize the relative induction of whole chromosome aneuploidy and chromosomal aberrations in sperm after exposure to chemotherapeutic doses of ET; (ii) characterize the relative sensitivities of meiotic cells versus spermatogonial stem cells; and (iii) validate the sperm FISH analyses against conventional cytogenetic analyses of meiotic cells and zygotes (Marchetti et al., 2001).

Materials and methods

Animals and chemical treatment of males

B6C3F1 mice (Harlan Sprague-Dawley Inc., Indianapolis, IN, USA) 6–8 weeks of age at the beginning of the experiments were maintained under a 14 h light/10 h dark photoperiod at room temperature of 21–23°C and a relative humidity of 50 ± 5%. Pelleted food and sterilized tap water were provided ad libitum. The use of animals in the present study was reviewed and approved by the LLNL Institutional Animal Care and Use Committee.

Male mice received 80 mg/kg ET (CAS No 33419–42–0, Sigma Chemical Co, St Louis, MO, USA) dissolved in dimethylsulphoxide (DMSO, Sigma). This dose is within the dose range used for human chemotherapy (Broun et al., 1997; Einhorn, 1997). ET was administered i.p. at a final volume of 0.1 ml/30 g body weight (b.w.). The mice assigned to the two control groups received similar amounts of DMSO only.

Animals were euthanized at 25 and 49 days after treatment to investigate the effects on pachytene spermatocytes and spermatogonial stem cells. This is equivalent to analysing the effects in human sperm ~45 and 110 days after treatment. As in the previous study (Marchetti et al., 2001), ~30% of the ET-treated mice showed signs of morbidity and were euthanized. The average time for the manifestation of morbidity was 19.1 days after treatment (range: 6–41 days).

Analysis of sperm

The kinetics of spermatogenesis are well established for men and several mammalian species, and are remarkably constant within species (Oakberg, 1956; Adler, 1996). This allows the sensitivity of the various spermatogenic cell types to the induction of genetic abnormalities to be investigated by controlling the time between exposure and sperm collection (Witt and Bishop, 1996; Marchetti and Wyrobek, 2005). Sperm collection within the first 3 weeks after exposure measures mutagenic effects on post-meiotic germ cells, while collections occurring 3–5 weeks, 5–7 weeks and >7 weeks after exposure measure effects on spermatocytes, spermatogonia and stem cells, respectively. Therefore, isolation of epididymal sperm 25 and 49 days after exposure measure effects on pachytene spermatocytes and stem cells spermatogonia, respectively.

Epididymal sperm were isolated from 10 treated (nine at 25 days) and 10 concurrent control animals at 25 and 49 days after treatment according to a standard protocol (Lowe et al., 1996). Briefly, both epididymides were removed surgically, placed in 300 μl of 2.2% sodium citrate at 32°C and several partial incisions were made with iris scissors (keeping the adjoining tissue intact). After 5 min to allow sperm to swim out into the solution, both epididymides were removed from the cell suspension. Seven μl of sperm suspension from each mouse were pipetted onto dry glass slides precleaned with 100% ethanol for at least 24 h. The slides were smeared over an area of ~22 × 22 mm using a pipette tip and air-dried overnight. The smears were then used for hybridization or stored at ~20°C in nitrogen gas.

Sperm pretreatment and hybridization conditions were as described previously (Hill et al., 2003). Briefly, sperm smears were fixed with 3:1 methanol: acetic acid, air-dried and incubated in 10 mM dithiothreitol (Sigma) for 30 min on ice followed by a dip in double-distilled water at room temperature. The slides were air-dried and denatured at 78°C for 8 min in 70% formamide (IBI, New Haven, CT, USA) and 2 × SSC, pH 7.0, and then dehydrated in an ice-cold ethanol series (70%, 85% and 100%), for 2 min each. Slides were then air-dried prior to hybridization.

Yeast artificial chromosomes (YACs) for the centromeric and telomeric probes for chromosome 2 were purchased from Research Genetics (Huntsville, MD, USA; mouse chromosome 2 centromere clone ID 460-H-4, Research Genetics Cat. #98022; and mouse chromosome 2 telomere clone ID 121-E-1, Research Genetics Cat. #98022). The clones were cultured according to the supplier’s vendor recommendations and the DNA was isolated via the ‘smash and grab’ method (Rose et al., 1990). The probe for chromosome 8 was a combination of two repetitive sequences near the centromere of chromosome 8 (A4-B1), Chrom. 84 and Chrom. 85 (Boyle and Ward, 1992). Plasmid DNA for the chromosome 8 probes was made using a Qiagen Plasmid Kit (Qiagen, Chatsworth, CA, USA). Probes were labelled with biotin-14-dCTP (2-tel and 8) and/or digoxigenin-11-dUTP (2-cent and 8) by random priming using the BioPrime DNA Labelling System (Invitrogen, Carlsbad, CA, USA).

The probe mixture contained 30 μl of mouse Cot-1 DNA (Invitrogen), 2 μl of herring sperm (Invitrogen) and 4 μl each of centromere and telomere probes for chromosome 2, and 2 μl of chromosome 8 probes. After ethanol precipitation, the DNA was resuspended in 7 μl of CEP hybridization buffer (Vysis, Downers Grove, IL, USA) and 3 μl of distilled water. The probe mixture was denatured at 78°C for 10 min and pre-annealed for 30 min at 37°C. The mixture was applied to the denatured slide, sealed under a cover slip and incubated at 37°C for ~48 hrs. The slides were then washed three times in 50% formamide, 2×SSC, pH 7.0, once in 2 × SSC and once in PN buffer.

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Frequencies of sperm carrying structural and numerical abnormalities in B6C3F1 mice after etoposide (ET) treatment of meiotic cells

Table I. Frequencies of sperm carrying structural and numerical abnormalities in B6C3F1 mice after etoposide (ET) treatment of meiotic cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fluorescent pattern</th>
<th>Controls</th>
<th>80 mg/kg ET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>per 5K ± SDb 95% CI</td>
</tr>
<tr>
<td>No mice</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>No. cells scored</td>
<td></td>
<td>50,278</td>
<td></td>
</tr>
<tr>
<td>Normal sperm</td>
<td>CT8c</td>
<td>50,237</td>
<td></td>
</tr>
</tbody>
</table>

### Sperm with structural aberrations

- **Centromeric duplication**
  - CCT8: 1 (0, 1) 0.1 ± 0.3 0–0.3 37 (0, 10) 4.1 ± 3.3* 2.0–6.2
  - CT8: 0 (0, 1) 0 0 24 (1, 7) 2.7 ± 2.0* 1.4–4.0

- **Telomeric duplication**
  - CTT8: 1 (0, 1) 0.1 ± 0.3 0–0.3 521 (17, 102) 57.8 ± 28.1* 39.4–76.2
  - CT8: 3 (0, 1) 0.3 ± 0.5 0–0.6 568 (19, 140) 63.0 ± 38.1* 38.1–87.9

- **Total structural**
  - 5 (0, 1) 0.5 ± 0.5 0.2–0.8 1150 (48, 207) 127.5 ± 69.1* 82.4–172.6

### Sperm with numerical abnormalities

- **Disomy 2**
  - CCT8: 3 (0, 2) 0.3 ± 0.7 0–0.7 80 (8, 23) 8.9 ± 5.9* 5.1–12.7
  - CT8: 0 (0, 1) 0.1 ± 0.3 0–0.3 96 (3, 18) 10.6 ± 5.3* 7.1–14.1

- **Nullisomy 2**
  - OO8: 1 (0, 1) 0.1 ± 0.3 0–0.3 49 (2, 13) 5.4 ± 4.2* 2.7–8.1
  - OT8: 2 (0, 1) 0.2 ± 0.4 0–0.5 195 (8, 38) 21.6 ± 10.5* 14.7–28.5

- **Total numerical**
  - 12 (0, 3) 1.2 ± 1.1 0.5–1.9 420 (20, 84) 46.6 ± 21.8* 32.7–60.5

- **Diploid sperm**
  - CCT8: 24 (1, 6) 2.4 ± 1.8 1.3–3.5 834 (44, 169) 92.5 ± 38.4* 67.4–117.6

### Results

The results of the CT8 analysis of sperm after treatment of meiotic cells (nine animals at 25 days; 45,100 sperm), spermatogonial stem cells (10 animals at 49 days; 50,296 sperm) and the two concurrent controls of 10 animals each (25 day controls, 50,278; 49 day controls, 50,444) are shown in Tables I and II. No significant differences were found in the frequencies of sperm with structural or numerical abnormalities between the two control groups except for a slight difference in the frequencies of sperm with a deletion of the centromeric region of chromosome 2. Thus, each group of ET-treated mice was compared with its corresponding concurrent control group.

### Sperm effects after ET treatment of meiotic cells

After ET treatment of pachytene spermatocytes (Table I), all classes of chromosomal abnormalities were highly increased with respect to control values (27 to 578–fold, *P* < 0.001). The frequency (per 5,000 sperm ± SD) of sperm with chromosomal aberrations increased ~250 fold (127.5 ± 69.1 versus 0.5 ± 0.5). In the treated group, duplications and deletions of the telomeric region of chromosome 2 were between 14- and 23-fold higher than duplications and deletions of the centromeric region of chromosome 2. For both chromosomal regions, the frequencies of sperm carrying duplications and deletions were not different from a 1:1 ratio.

Numerical abnormalities were also significantly increased after ET exposure (27- to 106-fold, *P* < 0.001). The frequencies of disomic sperm were 8.9 ± 5.9 for chromosomes 2 and...
10.6 ± 5.3 for chromosome 8, respectively, corresponding to 29- to 106-fold increases above controls. Sperm nullisomic for chromosomes 2 or 8 were 5.4 ± 4.2 and 21.6 ± 10.5, respectively, corresponding to 27- and 30-fold increases above controls. Disomy and nullisomy for chromosome 2 occurred at similar frequencies, but nullisomy for chromosome 8 was 4-fold higher than disomy for chromosome 8. Nullisomic sperm for the chromosome 8 may have been overestimated for technical reasons because, unlike the centromeric probe for chromosome 2, the probe for chromosome 8 is located below the centromere (A4-B1) and it is possible that some of the nullisomic sperm may have contained a chromosomal fragment with the centromeric region of chromosome 8.

Diploid sperm were increased 39-fold above controls and represented the most common type of numerical abnormality induced by ET in sperm. Finally, sperm with complex fluorescent genotypes (i.e. due to at least two events such as CTTT88) were not observed in controls, but were detected at a frequency 41.7 ± 13.6 in treated mice.

Significant animal-to-animal variation was found within the treated group (P < 0.001). The frequencies of sperm with duplications of the telomeric region of chromosome 2 showed the most variation among treated animals and varied by a factor of 7-fold, possibly reflecting variations in the effective ET dose to target cells. Regression analyses (Fig. 1) showed that animals with high frequencies of sperm with chromosomal structural aberrations also had high frequencies of sperm with other types of chromosomal abnormalities. This suggested that a common mechanism may be responsible for inducing the various types of chromosomal defects. The best correlation was obtained between sperm with structural aberrations and sperm with aneuploidy ($R^2 = 0.84$).

### Sperm effects after ET treatment of spermatogonial stem cells

Among the chromosomal abnormalities detected by the CT8 assay, only duplications and deletions of the telomeric region of chromosome 2 were elevated ($P < 0.001$) after treatment of spermatogonial stem cells (Table II). Specifically, the average frequencies of sperm with duplications and deletions of the telomeric region of chromosome 2 were 1.6 ± 0.8 and 1.6 ± 1.3 in treated mice versus 0.1 ± 0.3 and 0.2 ± 0.4 in controls and, as shown in Table II, the 95% confidence intervals (CIs) for treated and control values did not overlap. These results suggest that spermatogonial stem cells were affected by ET and, therefore, these increased levels of chromosome structural aberrations may persist with time.

Unlike treatment of meiotic cells, ET treatment of spermatogonial stem cells did not show significant animal-to-animal variation.

### Extrapolation of sperm CT8 data to the whole genome and comparison with cytogenetic data

The data obtained with the CT8 assay after ET treatment of meiotic cells were compared with those obtained by Marchetti et al. (2001). In this study, the induction of chromosomal aberrations was investigated by conventional cytogenetic analyses of metaphase I (MI) and II (MII) spermatocytes and first cleavage (1-CI) zygotes after identical ET treatment. Assuming that chromosome 2 represents ~6.4% of the male mouse haploid genome (Disteche et al., 1981) and that ET induces a random distribution of breaks across the genome, our data suggest that ~40% of mouse sperm carry chromosomal structural aberrations after ET treatment of meiotic cells. As shown in Fig. 2A,
this is in strong agreement with the frequencies of chromosomal structural aberrations measured in MI and MII spermatocytes by conventional cytogenetic analyses (Marchetti et al., 2001), but it is significantly higher than those reported in zygotes (Marchetti et al., 2001).

We then used the frequencies of sperm disomic for chromosome 2 and 8 as detected by the CT8 assay to estimate a genome-wide frequency of disomic sperm assuming that the rate of ET-induced nondisjunction was similar for all chromosomes. This calculation predicted that 2.9% of sperm were disomic after ET exposure of meiotic cells. As shown in Fig. 2B, this frequency is in agreement with the frequencies of aneuploid cells observed in MI spermatocytes and 1-Cl zygotes after fertilization (Marchetti et al., 2001).

Discussion

As more cancer survivors regain their fertility after cancer therapy, there is growing concern about the possibility that therapy may be inducing genomic alterations in their germ cells that might result in an increased risk in abnormal reproductive outcomes and genetic diseases among their offspring. This study was designed to investigate the potential long-term effects of ET on germ cell genetic integrity using an animal model. The data showed that ET exposure of pachytene spermatocytes induced major increases in the frequencies of sperm with chromosome structural and numerical abnormalities, while exposure of spermatogonial stem cells led to significant increases in the frequencies of sperm chromosomal structural aberrations but not numerical abnormalities. These results confirm that ET is a potent inducer of chromosomal abnormalities in male germ cells and that sperm with chromosomal aberrations may be continually produced by the affected spermatogonial stem cells resulting in persistent elevations of chromosomally abnormal sperm long after exposure to ET. An important implication of these findings is that cancer survivors may be at a significantly higher risk of fathering abnormal reproductive outcomes throughout their reproductive life and not only during the first few months following chemotherapy as currently thought.

Prior studies with chemotherapy patients have so far focused on numerical abnormalities and have reported only transient effects with no persistent effects on stem cells. (Martin et al., 1997; Robbins et al., 1997; Frias et al., 2003; Wyrobek et al., 2005a). Evidence for stem cell effects is available for a few patients who had received chemotherapy involving ET (Martin et al., 1999; De Mas et al., 2001). Long-term effects of chemotherapy exposure on the genetic constitution of human sperm have also been documented using the human sperm/hamster oocyte assay (Genesca et al., 1990). Germ cell mutagenicity studies in rodents have shown that commonly used chemotherapeutic agents (i.e. melphalan, mitomycin C, procarbazine) are among the few agents that induced heritable mutations in spermatogonial stem cells (Shelby, 1996; Witt and Bishop, 1996; Wyrobek et al., 2005a). The possibility exists that chemotherapy-induced stem cell effects may have been underestimated because of the focus on numerical abnormalities in the sperm FISH studies that have been conducted so far with chemotherapeutic patients.

Our study demonstrates the exquisite sensitivity of male meiotic cells to ET. Previous studies using FISH assays to detect chromosomal abnormalities in sperm of rodents or humans have reported increases in the 2 to 6-fold range (reviewed in Wyrobek et al., 2005a,b). Only one study has reported a fold increase higher than 10 (Frias et al., 2003). Therefore, the frequencies of sperm with chromosomal structural
and numerical abnormalities found after exposure of pachytene spermatocytes to ET in the present study are by far the highest ever reported using a FISH assay.

Male meiotic cells may be particularly susceptible to ET because topo II activity is highest in meiosis and peaks during pachytene (Moens, 1990; Cobb et al., 1997). Chromosomal structural aberrations may be caused during chromosome condensation by disruption of the cleavable complex formed by the binding of topo II to the DNA and prevention of the DNA-strand rejoining activity of topo II (Rose et al., 1990a; Cobb et al., 1997; Tateno and Kamiguchi, 2001). As for the induction of aneuploidy, topo II is needed to remove regions of catenations or interwining of DNA duplexes that originate during replication and/or meiotic recombination of homologous chromosomes. Because ET interferes with the role of topo II during this decatenation process, aneuploidy may be induced because of failures in decatenation of homologous chromosome arms prior to anaphase I and/or due to disturbance in resolution of sister centromeres at MII (Kallio and Lähdetie, 1997).

Regression analyses showed that mice with high frequencies of sperm with structural aberrations also had high frequencies of aneuploid sperm suggesting a common mechanism for the induction of these types of chromosomal abnormalities. We propose that this is a consequence of the binding of ET to the DNA-topo II complex and its stabilization. This may have two effects leading to abnormal sperm: (i) it prevents the rejoining

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**Figure 2.** Comparison of the percentages of cells with chromosomal structural aberrations (A) and aneuploidy (B) as detected by the cytogenetic analysis of spermatocytes and first-cleavage zygotes (Marchetti et al., 2001) versus the estimates of abnormal sperm as determined by the CT8 assay.
of the double strand breaks; and (ii) it sequesters topo II to the site of the break. Chromosomal structural aberrations would then arise during chromosome condensation at the transition from prophase to metaphase I when the unrejoined strand breaks result in chromosomal fragmentation. Subsequently, topo II is not available during anaphase I and II for resolving regions of catenation between homologous or sister chromatids during anaphases. Therefore, high levels of structural aberrations will correlate with high levels of numerical abnormalities. This may also explain the high frequencies of sperm with multiple chromosomal abnormalities found after exposure of pachytene spermatocytes.

Comparisons with the cytogenetic data in spermatocytes and zygotes (Marchetti et al., 2001) demonstrated the validity of the CT8 assay for detecting chromosomal and numerical abnormalities. Extrapolation of the CT8 data to the entire genome indicated that ∼40% and 3% of sperm collected after exposure of meiotic cells (25 days) carry structural aberrations and aneuploidy, respectively (Fig. 2). The estimate of aneuploid sperm was in agreement with the frequencies found before and after fertilization (Marchetti et al., 2001). The estimate of sperm with structural aberrations was in agreement with the frequencies of metaphase I and II spermatocytes with chromosomal aberrations and was significantly higher than the frequencies reported in zygotes. As discussed by Marchetti et al. (2001), PAINT/DAPI analysis may have underestimated the frequencies of chromosomally abnormal zygotes due to the nature of the aberrations induced by ET (i.e. terminal deletions), so that only zygotes with extensive terminal deletions would have been classified as abnormal. Extrapolation of the CT8 data obtained 49 days after ET exposure suggests that ∼2% of mouse sperm carry chromosomal structural aberrations after exposure of spermatogonial stem cell; however, no cytogenetic data are available for comparison.

In conclusion, we showed that ET may have long-lasting effects on the frequencies of sperm with structural aberrations in the mouse. This has important implications for cancer patients undergoing chemotherapy with ET because they may remain at higher risk for abnormal reproductive outcomes long after the end of chemotherapy. Studies of chemotherapy patients with FISH assays that can detect chromosomal structural aberrations, such as the ACM assay (Sloter et al., 2000), are recommended to confirm these murine findings and to determine whether other chemotherapeutic agents have the potential to induce persistent chromosomal lesions in spermatogonial stem cells.

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