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July 20, 2005

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**ETOPOSIDE INDUCES CHROMOSOMAL ABNORMALITIES IN SPERMATOCYTES
AND SPERMATOGONIAL STEM CELLS**

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Running title: Etoposide-induced chromosomal abnormalities in mouse sperm

Prepared for publication in Cancer Research

ABSTRACT

Etoposide (ET) is a chemotherapeutic agent widely used in the treatment of leukemia, lymphomas and many solid tumors, such as testicular and ovarian cancers, that affect patients in their reproductive years. The purpose of the study was to use sperm FISH analyses to characterize the long-term effects of ET on male germ cells. We used a mouse model to characterize the induction of chromosomal aberrations (partial duplications and deletions) and whole chromosomal aneuploidies 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. 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. These results demonstrate that male meiotic germ cells are considerably more sensitive to ET than spermatogonial stem-cell and that increased frequencies of sperm with structural aberrations persist after spermatogonial stem-cell treatment. These findings predict that patients who undergo chemotherapy with ET may have transient elevations in the frequencies of aneuploid sperm, but more importantly, may have persistent elevations in the frequencies of sperm with chromosomal aberrations, placing them at higher risk for abnormal reproductive outcomes long after the end of their chemotherapy.

Keywords: chemotherapy, male germ cells, FISH, aneuploidy, 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 (1). However, chemotherapy regimens commonly include one or more agents that are mutagenic or clastogenic in model systems and are highly toxic to germ cells (2, 3). Thus, as the incidence of survivors of cancer in their reproductive years increases, 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 leukemia, lymphomas and many solid tumors including testicular and ovarian cancers, which are common in children and young adults in their reproductive years (4, 5). ET inhibits topoisomerase II (topo II), which is an enzyme that introduces DNA double strand breaks allowing the passage of one double helix through another, and then reseals the double strand break (5, 6). Topo II removes regions of DNA catenation during DNA replication, chromosome condensation (7) and prior to chromosome segregation during mitosis (8-10) and meiosis (11, 12). ET inhibits topo II activity by forming a ternary complex, DNA-topo II-ET, that prevents the strand religation reaction (13-15) resulting in the creation of double strand DNA breaks and formation of chromosomal aberrations (16, 17).

Over 80% of the cancer patients who are treated with ET regain fertility within a few years after the end of chemotherapy (18, 19), therefore, it is 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 (14, 15, 20, 21) and in female and male germ

cells (22-26). Male meiotic cells are a major target of ET resulting in dominant lethality (27), specific locus mutations (28) and chromosomal damage in metaphase II spermatocytes and zygotes (29). 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 (30-34). Several chemotherapeutic regimens, including those involving ET, have already been shown to induce transient increases in the frequencies of aneuploid sperm using FISH (3, 31, 35-37) suggesting that treated patients are at higher risk for fathering aneuploid 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 significant increases in the frequencies of stable translocations in patients several years after the end of chemotherapy have been reported (38). More recently, we have developed several sperm FISH assays for detecting structural chromosomal aberrations in human sperm (39-41), but they have not yet been applied to evaluate 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 (42). 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: 1) characterize the relative induction of whole chromosome aneuploidy and chromosomal aberrations in sperm after exposure to chemotherapeutic doses of ET; 2) characterize the relative sensitivities of meiotic cells versus

spermatogonial stem cells; and, 3) validate the sperm FISH analyses against conventional cytogenetic analyses of meiotic cells and zygotes (29).

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 hr light/10 hr dark photoperiod at room temperature of 21-23° C and 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 dimethylsulfoxide (DMSO, Sigma). This dose is within the dose range used for human chemotherapy (43, 44). ET was administered intraperitoneally (i.p.) at the final volume of 0.1 ml/30 g 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 analyzing the effects in human sperm about 45 and 110 days after treatment. As in the previous study (29), about 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

Epididymal sperm were isolated from 10 treated (9 at 25 days) and 10 concurrent control animals at 25 and 49 days after treatment according to a standard protocol (33). Briefly, both epididymides were surgically removed, placed into 300 μ l of 2.2% sodium citrate at 32°C and several partial incisions were made with iris scissors (keeping the adjoining tissue intact). After 5 minutes to allow sperm to swim out into the solution, both epididymides were removed from the cell suspension. Seven μ l of sperm suspension from each mouse were pipetted onto dry glass slides precleaned with 100% ethanol for at least 24 hours. The cells were smeared over an area of about 22 x 22 mm using a pipette tip and air-dried overnight. The smears were then used for hybridization or stored at -20°C in N₂ gas. Sperm pretreatment and hybridization conditions were as previously described (42).

Scoring criteria and statistical analysis

A single scorer analyzed coded slides using a Zeiss Axioplan epifluorescence microscope equipped with single, dual and triple bandpass filters for rhodamine, fluorescein and DAPI as previously described (33). Each slide was coded a first time and 2,500 sperm were analyzed. After recoding, a second set of 2,500 sperm was analyzed on a different area of the slide. Only hook-shaped nuclei were scored. All data were recorded using the CYTOscore© computer program developed at LLNL. Sperm scored as having a normal C-T-8 pattern contained a single red (2cen, C), green (2tel, T) and yellow (8) fluorescent signal. Strict scoring criteria were developed and employed for assigning sperm to four classes of structural aberrations and five classes of numerical abnormalities depending on the number and color of each fluorescent domain contained within each sperm nucleus (42). Sperm nuclei were scored as having two

domains of the same loci (e.g., CC-T-8, C-TT-8, C-T-88) if the two fluorescent domains were of similar size, color and separated by a distance larger than that corresponding to the diameter of one domain. Sperm with no detectable fluorescence domains were recorded to assess the efficiency of the FISH procedure.

Statistical analysis

Cochran's test for equal proportions (45) was used to compare the results of the 1st and 2nd sets of 2,500 sperm scored for each mouse. If no significant differences were found, the data sets were combined and the frequencies per 5,000 sperm were calculated. Otherwise, the slide was recoded and reintroduced into a pool of slides to be scored. No slide needed to be rescored. Comparisons among treated and untreated groups were made using the Mann-Whitney *U*-test.

RESULTS

The results of the CT8 analysis of sperm after treatment of meiotic cells (9 animals at 25 days; 45,320 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 1 and 2. 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 to its corresponding concurrent control group.

Sperm effects after etoposide treatment of meiotic cells

After ET treatment of pachytene spermatocytes (Table 1) all classes of chromosomal abnormalities were highly increased with respect to control values (27 to 578-fold, $p < 0.001$, Mann-Whitney *U*-test). The frequency (per 5,000 sperm \pm S.D.) of sperm with chromosomal aberrations increased ~250 fold (127.5 ± 69.1 vs. 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, $p < 0.001$, Mann-Whitney *U*-test). 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 (Figure 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 suggesting 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 etoposide 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 ($p < 0.001$, Mann-Whitney *U*-test) after treatment of spermatogonial stem cells (Table 2). 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 2, the 95% confidence intervals 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 to those obtained using the conventional cytogenetic analyses of metaphase I (MI) and II (MII) spermatocytes and first cleavage (1-CI) zygotes after identical treatment (29). Assuming that chromosome 2 represents ~6.4% of the male mouse haploid genome (46) 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 figure 2A, this is in strong agreement between the frequencies of chromosomal structural aberrations measured in MI and MII spermatocytes by conventional cytogenetic analyses (29), but it is significantly higher than those reported in zygotes (29).

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 figure 2B, this frequency is in agreement with the frequencies of aneuploid cells observed in MII spermatocytes and 1-CI zygotes after fertilization (29).

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 lead 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 time 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 (3, 31, 35, 47). Evidence for stem cell effects is available for a few patients who had received chemotherapy involving ET (36, 37). Long-term effects of chemotherapy exposure on the genetic constitution of human sperm have also been documented using the human sperm/hamster oocyte assay (38). 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 (2, 3, 48). The possibility exists that chemotherapy-induced stem cell effects may have been underestimated because of the focus on

numerical abnormalities of 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 49). Only one study has reported a fold increase higher than 10 (35). 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 (11, 12). 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 (9, 12, 25). As for the induction of aneuploidy, topo II is needed to remove regions of catenations or intertwining 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 (50).

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 consequence of the binding of ET to the DNA-topo II complex and its stabilization. This may have two effects leading to abnormal sperm: 1) it prevents the rejoining of the double strand breaks; and, 2) 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, during anaphase I and II, topo II is not available for resolving regions of catenation between homologs, resulting in nondisjunction or total segregation failure. Under these assumptions, the more topo II is sequestered in the cleavable complex during prophase I, the less will be available for solving regions of catenation among 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 (29) demonstrated the validity of the CT8 assay for 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 d) carry structural aberrations and aneuploidy, respectively (Figure 2). The estimate of aneuploid sperm was in agreement with the frequencies found before and after fertilization (29). 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 (29), PIAN/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 deletion 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 is available for comparison.

In conclusion, we showed 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. Studies of chemotherapy patients with FISH assays that can detect chromosomal structural aberrations, such as the ACM assay (41), 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.

ACKNOWLEDGEMENTS.

We thank Erin Tapley for her valuable contribution to this project. Work performed under the auspices of the U.S. DOE by the University of California, LLNL under contract W-7405-ENG-48 with funding support from NIEHS IAG Y01-ES-8016-5.

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Table 1. Frequencies of sperm carrying structural and numerical abnormalities in B6C3F1 mice after ET treatment of meiotic cells*

Genotype	Fluorescent pattern	Controls			80 mg/kg Etoposide		
		Total	Per 5K \pm S.D.†	95% CI	Total	Per 5K \pm S.D.†	95% CI
No mice		10			9		
No. cells scored		50,278			45,100		
Normal sperm	CT8‡	50,237			42,320		
Sperm with structural aberrations							
Centromeric duplication	CCT8	1 (0, 1)	0.1 \pm 0.3	0 - 0.3	37 (0, 10)	4.1 \pm 3.3§	2.0 - 6.2
Centromeric deletion	OT8	0 (0, 1)	0	0	24 (1, 7)	2.7 \pm 2.0§	1.4 - 4.0
Telomeric duplication	CTT8	1 (0, 1)	0.1 \pm 0.3	0 - 0.3	521 (17, 102)	57.8 \pm 28.1§	39.4 - 76.2
Telomeric deletion	CO8	3 (0, 1)	0.3 \pm 0.5	0 - 0.6	568 (19, 140)	63.0 \pm 38.1§	38.1 - 87.9
Total structural		5 (0, 1)	0.5 \pm 0.5	0.2 - 0.8	1150 (48, 207)	127.5 \pm 69.1§	82.4 - 172.6
Sperm with numerical abnormalities							
Disomy 2	CCTT8	3 (0, 2)	0.3 \pm 0.7	0 - 0.7	80 (8, 23)	8.9 \pm 5.9§	5.1 - 12.7
Nullisomy 2	OO8	1 (0, 1)	0.1 \pm 0.3	0 - 0.3	96 (3, 18)	10.6 \pm 5.3§	7.1 - 14.1
Disomy 8	CT88	2 (0, 1)	0.2 \pm 0.4	0 - 0.5	49 (2, 13)	5.4 \pm 4.2§	2.7 - 8.1
Nullisomy 8	CTO	6 (0, 2)	0.6 \pm 0.8	0.1 - 1.1	195 (8, 38)	21.6 \pm 10.5§	14.7 - 28.5
Total numerical		12 (0, 3)	1.2 \pm 1.1	0.5 - 1.9	420 (20, 84)	46.6 \pm 21.8§	32.7 - 60.5
Diploid sperm	CCTT88	24 (1, 6)	2.4 \pm 1.8	1.3 - 3.5	834 (44, 169)	92.5 \pm 38.4§	67.4 - 117.6
Sperm with complex abnormalities		0	0	0	376 (26, 63)	41.7 \pm 13.6§	32.8 - 49.6

*25 days after treatment with etoposide as detected by the CT8 assay.

†Frequencies per 5,000 sperm \pm Standard Deviation.

‡“O” indicates the absence of an expected domain.

In parentheses are shown the lowest and the highest value among the 10 animals.

§P<0.001 (Mann-Whitney *U*-test)

Table 2. Frequencies of sperm carrying structural and numerical abnormalities in B6C3F1 mice after treatment of spermatogonial stem cells*

Genotype	Fluorescent pattern	Controls			80 mg/kg Etoposide		
		Total	Per 5K ± S.D.†	95% CI	Total	Per 5K ± S.D.†	95% CI
No mice		10			10		
No. cells scored		50,444			50,296		
Normal sperm	CT8‡	50,387			50,182		
Sperm with structural aberrations							
Centromeric duplication	CCT8	1 (0, 1)	0.1 ± 0.3	0 - 0.3	0	0	0
Centromeric deletion	OT8	17 (0, 6)	1.7 ± 1.8	0.6 - 2.8	13 (0, 3)	1.3 ± 1.2	1.4 - 4.0
Telomeric duplication	CTT8	1 (0, 1)	0.1 ± 0.3	0 - 0.3	16 (1, 3)	1.6 ± 0.8§	1.1 - 2.1
Telomeric deletion	CO8	2 (0, 1)	0.2 ± 0.4	0 - 0.5	16 (0, 4)	1.6 ± 1.3§	0.8 - 2.4
Total structural		21 (0, 6)	2.1 ± 1.8	1.0 - 3.2	45 (1, 7)	4.5 ± 1.9	3.3 - 5.7
Sperm with numerical abnormalities							
Disomy 2	CCTT8	1 (0, 1)	0.1 ± 0.3	0 - 0.3	3 (0, 1)	0.3 ± 0.5	0 - 0.6
Nullisomy 2	OO8	3 (0, 1)	0.3 ± 0.5	0 - 0.6	3 (0, 1)	0.3 ± 0.5	0 - 0.6
Disomy 8	CT88	2 (0, 1)	0.2 ± 0.4	0 - 0.5	2 (0, 1)	0.2 ± 0.4	0 - 0.5
Nullisomy 8	CTO	2 (0, 1)	0.2 ± 0.4	0 - 0.5	14 (0, 4)	1.4 ± 1.2	0.5 - 2.3
Total numerical		8 (0, 2)	0.8 ± 1.8	0.4 - 1.2	22 (0, 4)	2.2 ± 1.7	1.2 - 3.2
Diploid sperm	CCTT88	25 (0, 6)	2.5 ± 2.1	1.2 - 3.8	40 (1, 8)	4.0 ± 2.3	2.5 - 5.5
Sperm with complex abnormalities		3 (0, 1)	0.3 ± 0.5	0 - 0.6	7 (0, 3)	0.7 ± 1.1	0 - 1.4

*49 days after treatment with etoposide as detected by the CT8 assay.

†Frequencies per 5,000 sperm ± Standard Deviation.

‡“O” indicates the absence of an expected domain.

In parentheses are shown the lowest and the highest value among the 10 animals.

§P<0.001 (Mann-Whitney *U*-test)

||P<0.05 (Mann-Whitney *U*-test)

Figure Legends

Figure 1 – Regression analyses of the correlation among the frequencies of the various classes of sperm abnormalities within each animal induced by etoposide treatment of pachytene spermatocytes.

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.

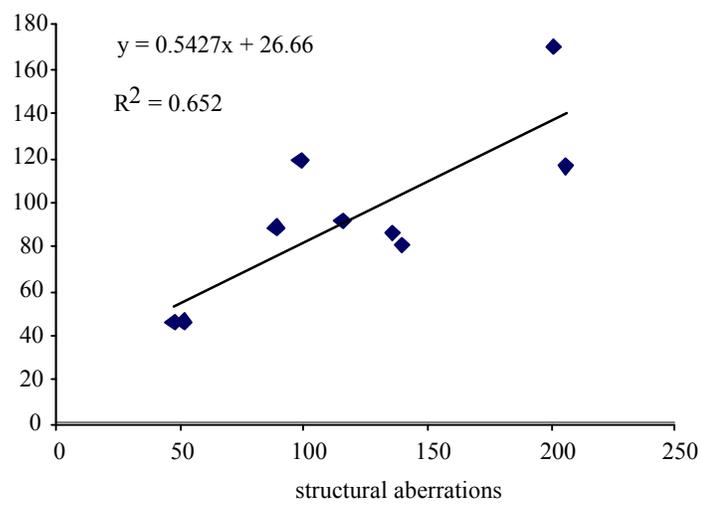
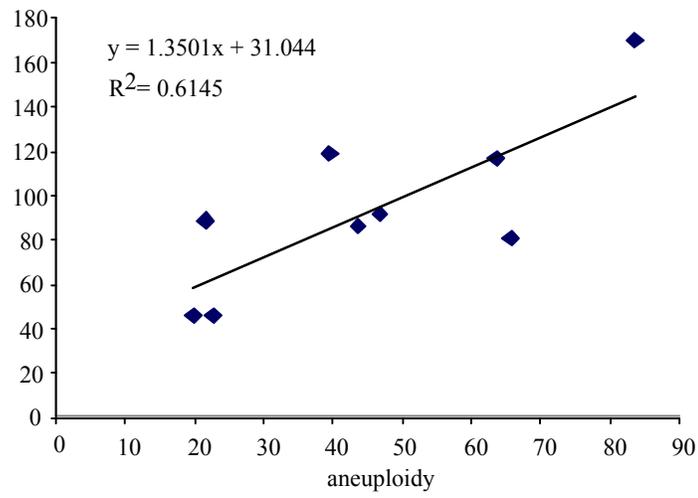
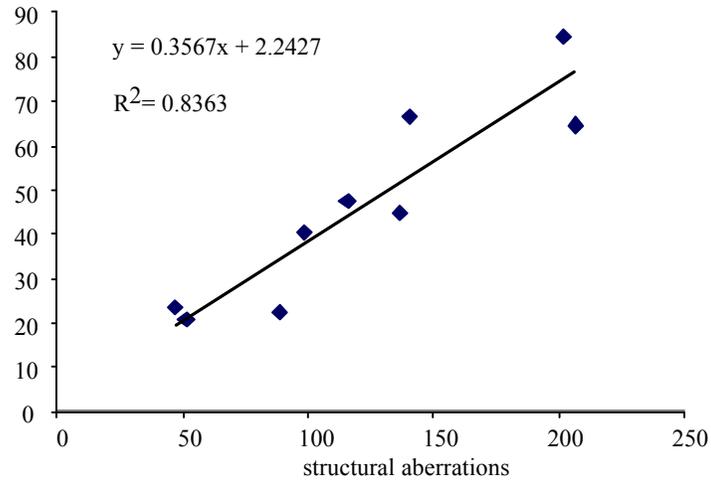


Figure 1

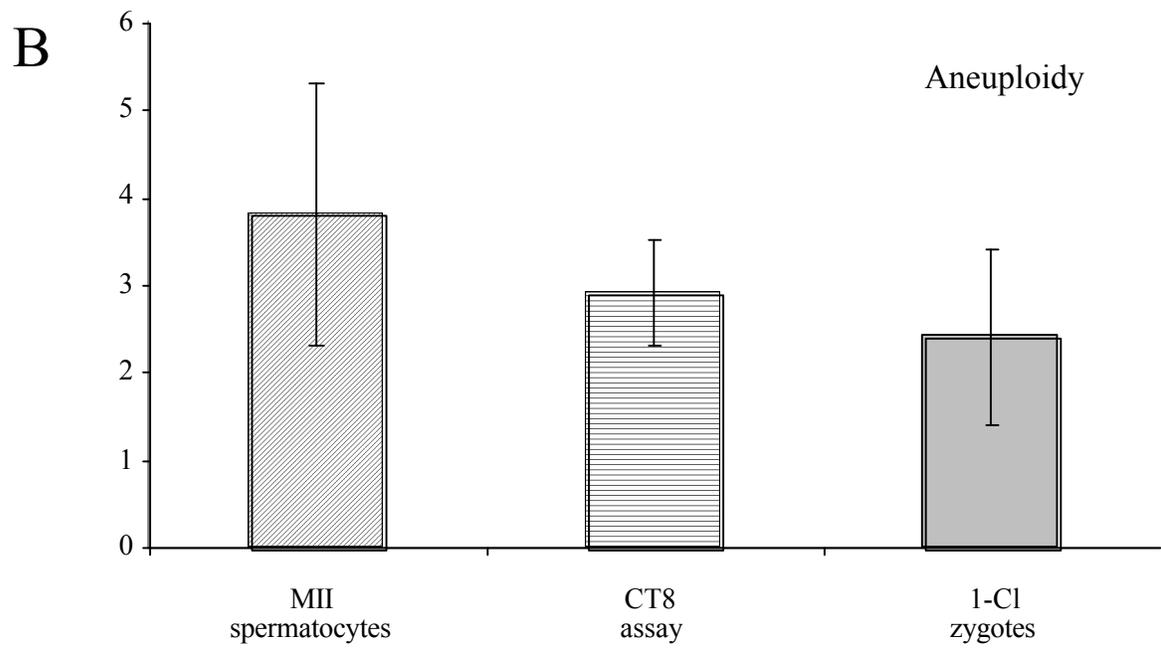
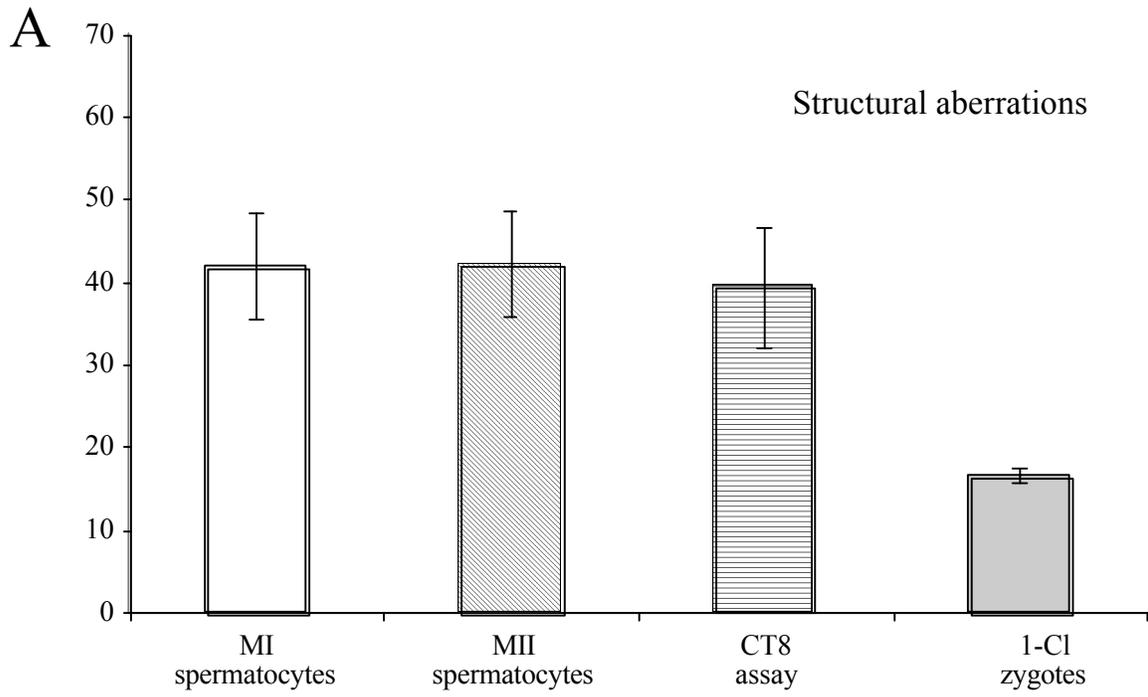


Figure 2