

# Effects of the Chemotherapy Cocktail Used to Treat Testicular Cancer on Sperm Chromatin Integrity

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**ABSTRACT:** The incidence of testicular cancer has increased dramatically over the past 50 years. Advances in treatment, which include the coadministration of bleomycin, etoposide, and cisplatin (BEP), have brought the cure rate to over 90%. After treatment, most patients go through a temporary period of azoo/oligozoospermia. Although the sperm concentration in approximately 80% of the patients returns to at least 10 million/mL, little is known about the integrity of the chromatin of their germ cells. Using an animal model, we assessed DNA integrity in the spermatozoa of male rats treated for 3, 6 or 9 weeks with BEP at doses, adjusted for surface area, equivalent to 0X, 1/3X, 2/3X, or 1X of the human dose. We did not observe any difference in protamination content, as assessed by the chromomycin A3 (CMA3) assay. After 9 weeks of 1X treatment, the susceptibility of DNA to denaturation evaluated by

the sperm chromatin structure assay (SCSA®)/acridine orange assay (AO) was increased, as well as the number of single and double DNA strand breaks measured by the TUNEL and COMET assays. Parameters obtained from the AO and TUNEL assays were highly correlated with the motility of the spermatozoa, suggesting that conventional sperm analysis parameters can serve as a good indicator of chromatin integrity and vice versa. Correlation studies also suggested that the parameters obtained with the different assays do not overlap, but complement each other. Thus, BEP treatment altered spermatozoal chromatin quality, and these alterations may impact adversely on progeny outcome.

Key words: Sperm DNA, DNA strand breaks.

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Advances in cytotoxic chemotherapy have improved the overall 5-year survival for all stages of testicular germ cell tumors to more than 90% (Huddart and Birtle, 2005; Kopp et al, 2006). Because of the young age of testis cancer patients, most commonly between 15 and 35 years of age (Segal, 2006), consideration of the effects of the treatment on fertility and the reproductive function of these men is an issue of particular importance. Several studies described fertility in men before and after cisplatin-based chemotherapy (bleomycin-etoposide-cisplatin, BEP). This cocktail of chemotherapeutic agents combines 3 different drugs having 3 different effects on cells, ultimately leading to the death of the cancerous cells: bleomycin causes DNA breaks (Vanderwall et al, 1997), etoposide inhibits topoisomerase II (Russell et al, 2000), and cisplatin is an alkylating agent cross-linking DNA (Wang and Lippard, 2005). Shortly after treatment, patients show

a drastic decrease in the number and motility of spermatozoa produced, as well as an increase in morphologically abnormal spermatozoa (Stephenson et al, 1995). An evaluation of the number of spermatozoa reveals that their production has been reinitiated in most men after 5 years (Lampe et al, 1997). Nevertheless, this analysis does not provide information with respect to the quality of the spermatozoa present, in terms of their ability to fertilize or their genomic integrity. Spermon et al (2003) showed that patients who were treated for testicular cancer have a higher risk of infertility; however, there is no evidence for an increase in congenital malformations among progeny (Senturia et al, 1985; Byrne et al, 1988). Very recent data have shown the presence of an abnormally high percentage of DNA-damaged sperm in samples from men after BEP chemotherapy (Spermon et al, 2006).

The demonstration that the spermatozoon can bring genetic damage into the oocyte at fertilization and contribute to the development of abnormal pregnancy outcome has led to the development of many techniques to assess sperm function and DNA integrity (Agarwal and Allamaneni, 2005; Marchetti and Wyrobek, 2005; O'Brien and Zini, 2005). Multiple assays have been developed to measure sperm chromosomal aberrations, abnormal chromatin packaging, chromatin structural integrity, and DNA breakage (for review, see Perreault

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et al, 2003). The chromatin structure of the sperm is very different from that of somatic cells. Indeed, during spermiogenesis, histones are replaced first by transition proteins followed by protamines (Braun, 2001), resulting in a very condensed structure of sperm DNA. While alteration of this structure or the induction of DNA strand breaks during spermatogenesis may not affect the fertilizing ability of spermatozoa, it may induce definitive changes in the genomic information transmitted to the progeny. The relative value of the tests to assess sperm integrity has been the subject of discussion due to the fact that it is not clear whether they provide overlapping or distinct information. Furthermore, our current understanding of the predictive value of these tests for abnormal reproductive outcome and effects on progeny is limited. Therefore, animal models are very useful in allowing a direct linkage between sperm chromatin integrity and effects on progeny outcome.

Previous reports from our laboratory demonstrated that subchronic exposure to the chemotherapeutic agent cyclophosphamide induced DNA strand breaks in the sperm (Codrington et al, 2004) in addition to affecting fertility by increasing preimplantation and postimplantation loss and abnormal progeny (Trasler et al, 1985). We recently developed and characterized an animal model in which male rats are exposed to the chemotherapeutic cocktail used to treat testicular cancer, BEP (Bieber et al, 2006). These animals showed decreases in reproductive organ weights (testis, epididymis, seminal vesicle, and prostate), sperm count and motility, and defects in the structure of the flagella of the spermatozoa (Bieber et al, 2006). Interestingly, these rats sired progeny which were apparently normal until the end of gestation without any change in preimplantation or postimplantation loss, but most of the pups died between birth and postnatal day 2 (Bieber et al, 2006). We hypothesize that BEP treatment induced sperm DNA damage responsible for the effect on progeny outcome; to test this hypothesis, we analyzed the chromatin integrity of sperm from these rats.

## Materials and Methods

### *Animals and Treatment*

Adult male Sprague-Dawley rats (300–350 g) were purchased from Charles River Canada (St Constant, Canada) and housed under controlled light conditions (14:10 hours light:dark) in the Animal Resources Centre of McGill University. Animals were provided with food and water ad libitum. All animal studies were conducted in accordance with the principles and procedures outlined in the Guide to the Care and Use of Experimental Animals prepared by the Canadian Council on Animal Care (McGill Animal Resources Centre protocol 4699).

Rats were treated as previously described (Bieber et al, 2006). Briefly, rats were treated for 1, 2, or 3 cycles of 3 weeks. The rats from the control group were gavaged on days 1 through 5 of each week with 1 mL of 7:3 saline (Roche, Laval, Canada): DMSO (Fisher Scientific, Ottawa, Canada). On day 2 of each week, control rats were given 1 mL of saline by intraperitoneal injection. The drug-treated animals received BEP regimens based on therapeutically relevant doses: specifically, a 1X dose equivalent to the human treatment regime, and 2/3X and 1/3X. The 1X dose-treated rats were gavaged on days 1 through 5 of each week with 3.0 mg/kg cis-platinum (LKT Laboratories, St Paul, Minn) and 15.0 mg/kg etoposide (LKT Laboratories) dissolved in 7:3 saline:DMSO. On day 2 of each week, male rats were given an intraperitoneal injection of 1.5 mg/kg bleomycin (LKT Laboratories) dissolved in saline. Cauda epididymal spermatozoa were collected during the week following of the end of the treatment.

### *Cauda Sperm Collection*

Cauda epididymides were excised, trimmed free of fat, and finely minced in PBS (1 mmol/L  $\text{KH}_2\text{PO}_4$ , 10 mmol/L  $\text{Na}_2\text{HPO}_4$ , 137 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.0) to release spermatozoa. The spermatozoa were filtered (93  $\mu\text{m}$ , SETAR, Canada) and washed twice with hypotonic buffer (0.45% NaCl) to lyse any contaminating cells; spermatozoa were then washed 2 times further with PBS and immediately frozen at  $-80^\circ\text{C}$ .

### *Chromomycin A3 Staining*

The flow cytometry-based chromomycin A3 (CMA3) staining assay was adapted from the slide-based method (Bianchi et al, 1993), as previously described (Zubkova et al, 2005). Briefly, spermatozoa were stained in CMA3 staining solution (0.25 mg/mL in McIlvaine buffer [17 mL of 0.1 mol/L citric acid mixed with 83 mL of 0.2 mol/L  $\text{Na}_2\text{HPO}_4$  and 10 mmol/L  $\text{MgCl}_2$ , pH 7.0]) for 20 minutes at  $25^\circ\text{C}$  in the dark, washed twice in PBS, sonicated, and stored at  $4^\circ\text{C}$  in the dark until analysis. A positive control was obtained by preincubating the spermatozoa with 200 mmol dithiothreitol at  $37^\circ\text{C}$  for 10 minutes. Flow cytometry analysis was done at the Institut de Recherche Clinique de Montréal (IRCM), using a MoFlo High Performance Cell Sorter (DakoCytomation, Fort Collins, Colo) equipped with a 460/10 filter and an I90 argon ion laser tuned to 457-nm line excitation. The resulting fluorescence was detected with a 580/30 band-pass filter and quantified using Summit v.3.1 software (DakoCytomation). A minimum of 5000 spermatozoa per sample were analyzed.

### *Acridine Orange Assay*

To measure the susceptibility of sperm nuclear DNA to low pH-induced denaturation in situ, the acridine orange (AO) assay was applied, using the method previously described as the SCSA<sup>®</sup> (Evenson et al, 2002). Briefly, 200- $\mu\text{L}$  sperm samples ( $4 \times 10^6$  cells/mL in PBS) were thawed for 2 minutes at  $37^\circ\text{C}$ , sonicated on ice, and mixed with 400  $\mu\text{L}$  of denaturation buffer (0.08N HCL, 0.15 mol NaCl, and 0.1% Triton X-100, pH 1.4) for 30 seconds at  $4^\circ\text{C}$  to denature

uncondensed sperm DNA. After 30 seconds, 1.2 mL of AO staining solution (0.126 mol  $\text{Na}_2\text{HPO}_4$ , 0.037 mol citric acid buffer, 1 mmol EDTA, 0.15 mol NaCl, pH 6.0 containing 6  $\mu\text{g}/\text{mL}$  AO [Sigma Chemical Company, St Louis, Mo]) was added. Exactly 3 minutes after the addition of the denaturation buffer, spermatozoa were analyzed using a FACScan flow cytometer (BD Biosciences, Mississauga, Canada) fitted with an argon ion laser (488-nm line excitation). A positive control was obtained by preincubating the spermatozoa with 20 mmol  $\text{H}_2\text{O}_2$  at room temperature for 1 hour. Green fluorescence emission of AO was measured at 515–530 nm with a band-pass filter and red fluorescence of AO was detected through a 630–650-nm long-pass filter. The raw data were processed using WinList Software (Verity Software House, Topsham, Me). The DNA fragmentation index (DFI = mean red fluorescence/total of red and green fluorescence) was analyzed according to 3 different variables, as previously described (Evenson and Wixon, 2005): the mean DFI, the standard deviation of DFI (SD DFI), and the percentage of cells outside the main population (% DFI). A minimum of 5000 events were analyzed for every sample.

### TUNEL Assay

The quantity of DNA free 3'-OH ends was assessed in spermatozoa using the TUNEL assay coupled with flow cytometric analysis using the Apo-Direct™ kit (Said et al, 2005) with the following modifications. Frozen sperm samples were thawed, sonicated, and resuspended overnight in 70% ethanol at  $-20^\circ\text{C}$  to a concentration of  $1-2 \times 10^6$  cells/mL. Samples were then centrifuged for 5 minutes at  $5000 \times g$ , washed twice in 1 mL of wash buffer, and incubated in 100  $\mu\text{L}$  staining solution (containing the reaction buffer, terminal deoxytransferase (TdT) enzyme, FITC-tagged deoxyuridine triphosphate nucleotides in distilled water, according to kit instructions) in the dark at  $37^\circ\text{C}$  for 1 hour. The reaction was stopped by washing twice with rinse buffer. Spermatozoa were then resuspended in 500  $\mu\text{L}$  propidium iodide (PI)/RNAse and stored in the dark overnight at  $4^\circ\text{C}$ . Positive controls were obtained by pretreating the cells with deoxyribonuclease I (100 U/ $\mu\text{L}$ ) for 20 minutes at room temperature; negative controls consisted of sperm incubated in the staining solution lacking the TdT enzyme. FITC staining was analyzed using the BD FACSAria Cell Sorting System (BD Bioscience, San Jose, Calif) fitted with a 488-nm laser. For FITC detection, light emission was filtered through a 502-nm long-pass filter as well as a 530/30-nm band-pass filter, while PI was detected using a 556-nm long-pass filter followed by a 575/26-nm band-pass filter. Fluorescence was quantified by the BD FACSDiva software (BD Biosciences). A minimum of 5000 events were analyzed for every sample.

### COMET Assay

DNA strand breaks in spermatozoa were evaluated using the alkaline comet assay, as previously described (Codrington et al, 2004). Frozen sperm samples were thawed on ice and resuspended in PBS to a concentration of  $1-3 \times 10^5$  cells/mL. Fifty microliters of the cell suspension were added to 500  $\mu\text{L}$  of molten agarose (0.5% low-melting-point grade in  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  free PBS, pH 7.4, at  $42^\circ\text{C}$ ). Fifty microliters were

immediately pipetted and evenly spread onto slides (Trevigen Inc, Gaithersburg, Md) in duplicate, and the gel was allowed to solidify at  $4^\circ\text{C}$  in the dark for 10 minutes. Slides were immersed in prechilled ( $4^\circ\text{C}$ ) lysis buffer (2.5 mol NaCl, 100 mmol EDTA, and 10 mmol Tris-HCl; final pH 10) containing 10% DMSO, 1% Triton X-100, and 40 mmol dithiothreitol for 1 hour on ice, washed in distilled water for 5 minutes, and incubated for 3 hours at  $37^\circ\text{C}$  in lysis buffer containing 0.1 mg/mL Proteinase K. Slides were then washed in distilled water, kept 10 minutes at  $4^\circ\text{C}$ , and immersed in freshly prepared alkaline solution (1 mmol EDTA and 0.05 mol NaOH, pH 12.1) for 45 minutes in the dark. Slides were washed twice in 1X Tris-Borate-EDTA buffer (TBE, pH 7.4) for 5 minutes, and electrophoresis was done at 14 V (0.7 V/cm) for 10 minutes (Mini-Sub Cell GT; Bio-Rad Laboratories, Inc, Mississauga, Canada). Slides were then fixed in ice-cold 70% ethanol for 5 minutes and stored at room temperature. DNA was stained with 50  $\mu\text{L}$  of SYBR Green solution (Trevigen) (1:10 000 in Tris-EDTA buffer, pH 7.5) and immediately analyzed using a DAGE-MTI CCD300-RC camera (DAGE-MTI Inc, Michigan City, Ind) attached to an Olympus BX51 epifluorescence microscope. Fifty cells per slide were randomly analyzed, for a total of 100 cells per animal, and fluorescent images were scored for comet parameters. Tail length, percent tail DNA, and tail extent moment (tail length/fraction of tail DNA) were measured using the KOMET 5.0 image analysis system (Kinetic Imaging Ltd, Liverpool, United Kingdom).

### Statistical Analysis

Statistical analyses were done using the SigmaStat 2.03 software package (SPSS Inc, Chicago, Ill). Significant differences due to the treatment were determined using a 1-way analysis of variance followed by the Bonferroni test ( $P < .05$ ). Correlation analyses were done by either Pearson's (parametric) or Spearman's (nonparametric) test, as appropriate.

## Results

### Effects of BEP Treatment on Spermatozoal Chromatin Structure

Chromatin structure was assessed in spermatozoa after 3, 6, or 9 weeks of BEP treatment using 2 different assays: CMA3 and AO staining. CMA3 mean fluorescence (Figure 1) was not altered following any of the treatment regimens, suggesting that BEP does not affect the protamine content in spermatozoal chromatin. Using the AO assay, we measured the susceptibility of the spermatozoa to low-pH denaturation. The mean DNA fragmentation index (mean DFI) represents the mean fluorescence observed in the population; mean DFI was not affected by any time or dose of treatment (Figure 2A). The standard deviation of the DFI (SD DFI), reflecting the width of the sample population or the extent of damage in the sample, was significantly increased after 9 weeks of treatment with the highest

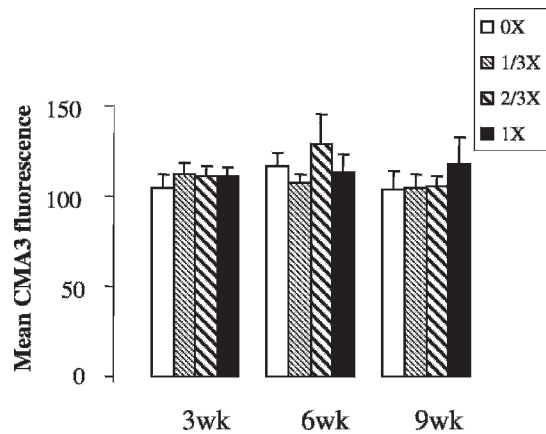


Figure 1. Protamination levels, indirectly determined by CMA3 staining, of spermatozoa from rats treated for 3, 6, or 9 weeks with BEP at 0X, 1/3X, 2/3X, and 1X doses. Spermatozoa were collected from the cauda epididymides, sonicated and labelled with CMA3. Mean fluorescence was determined by flow cytometry. Values are mean  $\pm$  SEM,  $n = 5$ . No significant differences were observed.

dose, whereas other treatment lengths and doses did not significantly affect this parameter (Figure 2B). The % DFI, which reflects the percentage of cells outside the main population or the percentage of damaged cells, showed a nonsignificant increase after 9 weeks of treatment with the highest dose (Figure 2C). At this time point, spermatozoa from rats treated with the 2/3X and 1X BEP doses showed a very high variability, with 1 and 3 animals respectively, showing a greater than fourfold increase compared to the control (data not shown). As our rat model is outbred, this wide variation could be the result of genetic susceptibility to DNA alteration or sensitivity to the BEP treatment, reflecting what may also be the case in humans.

#### Effects of BEP Treatment on DNA Strand Breaks

DNA strand breaks were measured in spermatozoa after 3, 6, or 9 weeks of BEP treatment using 2 different assays: the TUNEL and COMET assays. Using the TUNEL assay coupled to flow cytometry, we did not observe any effect of 3 or 6 weeks of BEP treatment. In contrast, after 9 weeks of treatment we observed a significant dose-dependent increase in DNA strand breaks with the 2/3X and 1X doses (Figure 3). Single and double DNA strand breaks were also evaluated after 9 weeks of treatment using the COMET assay; 3 parameters were analyzed. The percentage of DNA present in the tail (% tail DNA) (Figure 4A), the tail length (Figure 4B), and the tail extent moment (Figure 4C) were significantly increased after the 1X dose treatment. Interestingly, spermatozoa from rats treated with the 1/3X and 2/3X doses showed an intermediate increase, albeit nonsignificant (Figure 4A through C).

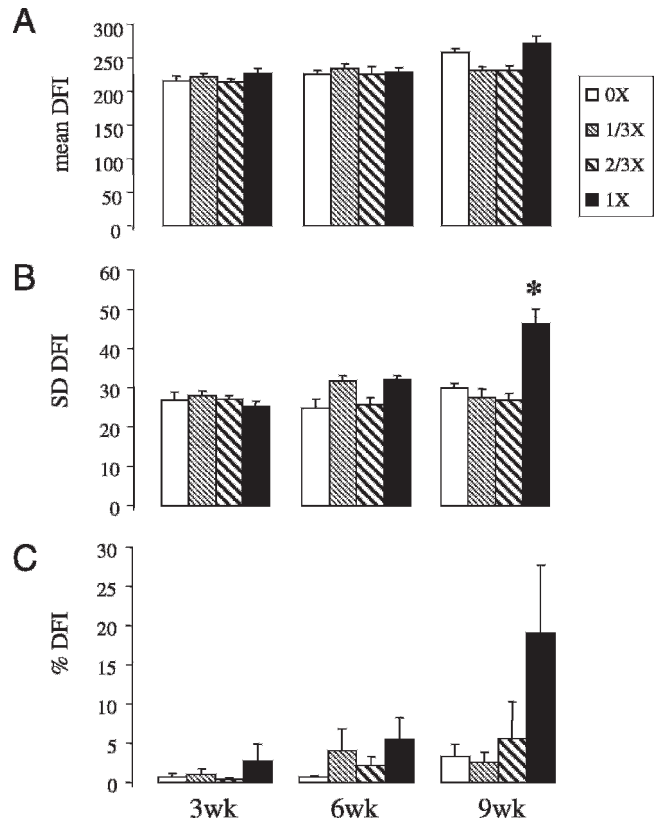


Figure 2. Sperm chromatin structure assay (SCSA<sup>®</sup>, AO assay) for spermatozoa of rats treated for 3, 6, or 9 weeks with BEP at 0X, 1/3X, 2/3X, and 1X doses. Spermatozoa were collected from the cauda epididymides and sonicated, the SCSA<sup>®</sup> assay was done, and samples were analyzed by flow cytometry. Values for (A) mean DNA fragmentation index (DFI), (B) SD of the DFI, and (C) % DFI are shown as mean  $\pm$  SEM. Values at 3, 6, or 9 weeks are for 5, 4, and 6 animals, respectively. \* $P < .005$  compared to the time-matched controls in a 1-way ANOVA test (with the Bonferroni correction).

#### Correlation Between Spermatozoal DNA Integrity Assay Parameters

In order to compare the different assays, the correlation among the different assay parameters was evaluated. We observed that the standard deviation of DFI (SD DFI) obtained in the AO assay correlated with the TUNEL assay (Figure 5A), but not with the COMET assay (Figure 5B). The TUNEL assay was also correlated to the mean DFI ( $r = .405$ ;  $P = .05$ ;  $n = 23$ ), but surprisingly, this correlation did not exist with the % DFI ( $r = .254$ ;  $P = .24$ ,  $n = 23$ ). This lack of correlation was probably due to the extreme variability of the % DFI (see SEM bar in Figure 2C). We also observed a correlation between the TUNEL assay and the COMET assay results (Figure 5C). This finding was expected, as these 2 assays detect DNA strand breaks in sperm DNA. The distribution of the different doses (Figure 5C) clearly demonstrates that even if these 2 parameters are correlated, the distribution is not well

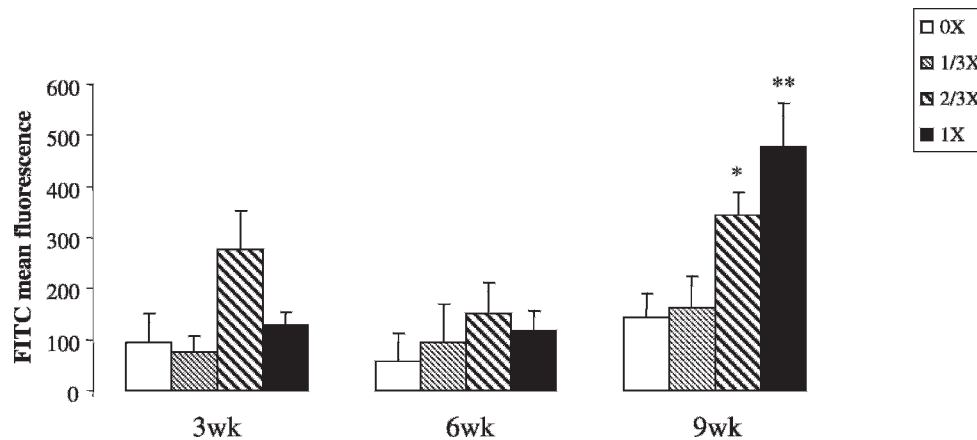


Figure 3. DNA strand breaks, determined by the TUNEL assay, in spermatozoa from rats treated for 3, 6, or 9 weeks with BEP at 0X, 1/3X, 2/3X, and 1X doses. Spermatozoa were collected from the cauda epididymides and sonicated, the TUNEL assay was performed, and samples were analyzed by flow cytometry. Values for dUTP-FITC mean fluorescence are shown as mean  $\pm$  SEM. Values at 3, 6, or 9 weeks are for 5, 5, and 6 animals, respectively. \* $P < .05$ , \*\* $P < .01$  compared to time-matched controls in a 1-way ANOVA test (with the Bonferroni correction).

defined along the TUNEL axis, whereas it is clear along the COMET axis. We observed that the 0x animals are tightly grouped on the lower part of both axes and that the 1X animals always give high COMET values. The distribution of the different rats within the 1X group is tight on the COMET axis, while there is a wide spread on the TUNEL axis, suggesting a lower variability with the COMET assay. Thus, the COMET assay is more sensitive and less variable than the TUNEL assay for the extreme values; treatment with intermediate doses may have induced intermediate levels of DNA strand breaks that were not well detected by these techniques.

#### Correlation Between Spermatozoal DNA Integrity and Spermatozoa Motility

The sperm motility from the 9 weeks 1X BEP-treated rats has been described previously by our lab to be decreased compared to controls (Bieber et al, 2006). We observed that the percent sperm motility parameter (ratio of cells that are moving at or above the minimum determined speed to total cells) was negatively correlated with the TUNEL results (Figure 6A) and the AO assay parameters, SD DFI (Figure 6B), mean DFI ( $r = -.779$ ;  $P = .022$ ;  $n = 8$ ) and % DFI ( $r = -.833$ ;  $P = .005$ ;  $n = 8$ ). Interestingly, conventional sperm motility parameters (path velocity, progressive velocity, track speed, lateral amplitude, beat frequency, straightness, and linearity) did not correlate with the sperm DNA integrity parameters described in this paper (data not shown).

## Discussion

This study shows that the BEP chemotherapy cocktail used to treat testicular cancer affects sperm chromatin

integrity in a rat model. BEP treatment increased the number of DNA strand breaks, as assessed by the TUNEL and the COMET assays. BEP exposure also increased the susceptibility of DNA to low pH-buffer denaturation, as measured by the AO assay, suggesting that the chromatin structure has been altered. However, we did not observe any effect of this cocktail of drugs on the protamine content linked to sperm DNA, suggesting that the compaction of chromatin during the spermiogenesis process was not affected.

BEP treatment altered sperm chromatin integrity only after 9 weeks of treatment with the highest dose, which is equivalent to the human treatment after adjustment for surface area (Einhorn and Donohue, 1998). Spermatogenesis is a highly time-regulated process (Clermont, 1972); 3, 6, or 9 weeks of chronic treatment reflect the effects of first exposing spermatids, spermatocytes and spermatogonia, respectively, to BEP. Our results suggest that BEP treatment induces damage in spermatogonia that cannot be repaired and is subsequently detected in epididymal spermatozoa. The nature of the damage induced in spermatogonia needs to be further investigated. Previous work suggested that differentiating spermatogonia (Meistrich, 1986) and postmeiotic germ cells (Trasler et al, 1986) are more sensitive to chemotherapeutic agents, but mitotic cells are also vulnerable (Trasler et al, 1987). Chronic treatment with the single agent cyclophosphamide resulted in a decrease in the expression of the stress-response genes in pachytene spermatocytes and round spermatids (Aguilar-Mahecha et al, 2002), but, to the best of our knowledge, changes in the gene expression profile of spermatogonia after exposure to individual or combined chemotherapeutic agents have not been reported.

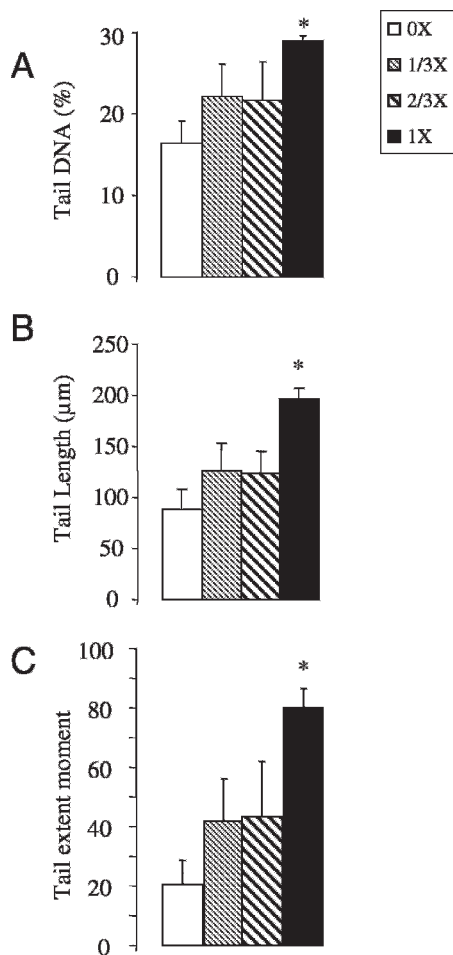


Figure 4. DNA strand breaks, determined by the COMET assay, of spermatozoa from rats treated for 9 weeks with BEP at 0X, 1/3X, 2/3X, or 1X doses. Spermatozoa were collected from the cauda epididymides, the COMET assay was performed, and samples were analyzed on slides. Values for (A) % tail DNA, (B) tail length, and (C) tail extent moment are shown as mean  $\pm$  SEM. Values are for 5 animals/group. \* $P < .01$  compared to controls in a 1-way ANOVA test (with the Bonferroni correction).

In humans, the effects of BEP treatment on the DNA integrity of surviving spermatozoa have been studied using fluorescence in situ hybridization (FISH), and the results are conflicting. One study found an increase in aneuploidy (De Mas et al, 2001), another found no change (Thomas et al, 2004), and yet a third found a decrease in aneuploidy (Martin et al, 1997). The discrepancies between these 3 studies are probably due to the different clinical backgrounds and posttherapeutic delays in each patient group. Very recently, Spermon et al (2006) have assessed sperm chromatin integrity pre-BEP and post-BEP chemotherapy in humans. They observed an improvement in DNA condensation after the treatment as measured by the CMA3 assay, but the values before or after treatment remained lower than those of normal donors. In our study, we did not

observe any difference in the CMA3-mean fluorescence between treated and control animals. This discrepancy may be explained by the higher compaction and lower accessibility of the sperm DNA in rat than in human (Bench et al, 1996). In addition, unlike treated patients, the animals used in our study did not have testicular cancer, nor did they undergo orchiectomy prior to the treatment. The disease itself leads to a decrease in the sperm count and abnormalities in sperm prior to the treatment (Baker et al, 2005); it is not clear whether testicular cancer also induces changes in the genomic integrity of spermatozoa. An increase in DNA strand breaks, determined by the TUNEL assay, has been described pre-BEP and post-BEP treatment in patients compared to normal donors (Spermon et al, 2006). Surprisingly, no increase has been observed when comparing the prechemotherapy and postchemotherapy sperm samples (Spermon et al, 2006), suggesting that the damage is due to the disease and not to the treatment. Nevertheless, our results strongly suggest that the drug treatment increases the number of DNA strand breaks in spermatozoa.

Semen quality tests have long been restricted to the number and motility parameters of the sperm. We observed an inverse correlation between sperm motility and DNA damage. Such an inverse correlation has been described also in thalassemic patients (Perera et al, 2002) and in men with varicocele (Smith et al, 2006). These results imply that conventional sperm parameters may be indicators of chromatin integrity and vice versa. However, BEP treatment induces oligozoospermia and a decrease in motility of the sperm (Baker et al, 2005). Further studies are needed to determine if motility and sperm chromatin integrity are linked, or if they result from distinct effects of the treatment on sperm maturation.

It is now well established that the integrity of sperm DNA and chromatin correlate with fertility (Sakkas et al, 2003). The present study suggests that the altered chromatin quality in BEP-exposed sperm will adversely impact on progeny outcome. Nevertheless, the ultimate goal of directly linking one form of sperm chromatin damage to a specific progeny outcome has not yet been achieved. In our model, BEP treatment did not affect fertility, preimplantation and postimplantation loss, or litter size on gestation day 21 (Bieber et al, 2006), suggesting that the chromatin damage observed in the sperm of these males is not involved in these processes. Nevertheless, most of the pups sired by treated males (1X BEP for 9 weeks) died before postnatal day 2 (Bieber et al, 2006). The sperm samples used in this study were collected from the animals used for the previous progeny outcome study, allowing us to make a direct correlation between these parameters. Only the

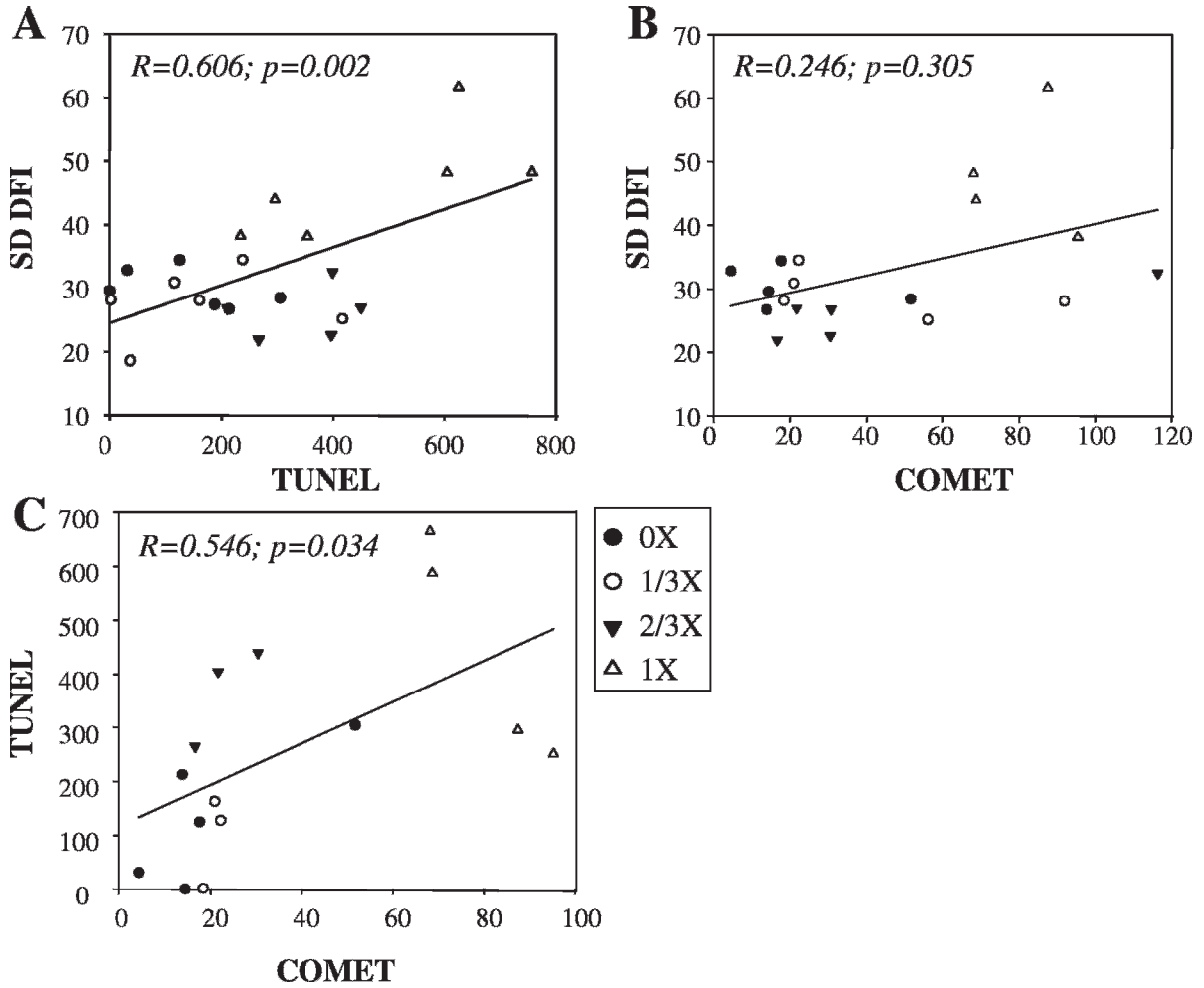


Figure 5. Correlation between: (A) the AO assay SD DFI and the TUNEL assay, (B) the AO assay SD DFI and the COMET assay, and (C) the TUNEL and COMET assays. Values from rats treated for 9 weeks with BEP at 0X (dark circles), 1/3X (open circles), 2/3X (dark triangles), and 1X (white triangles) doses are reported.

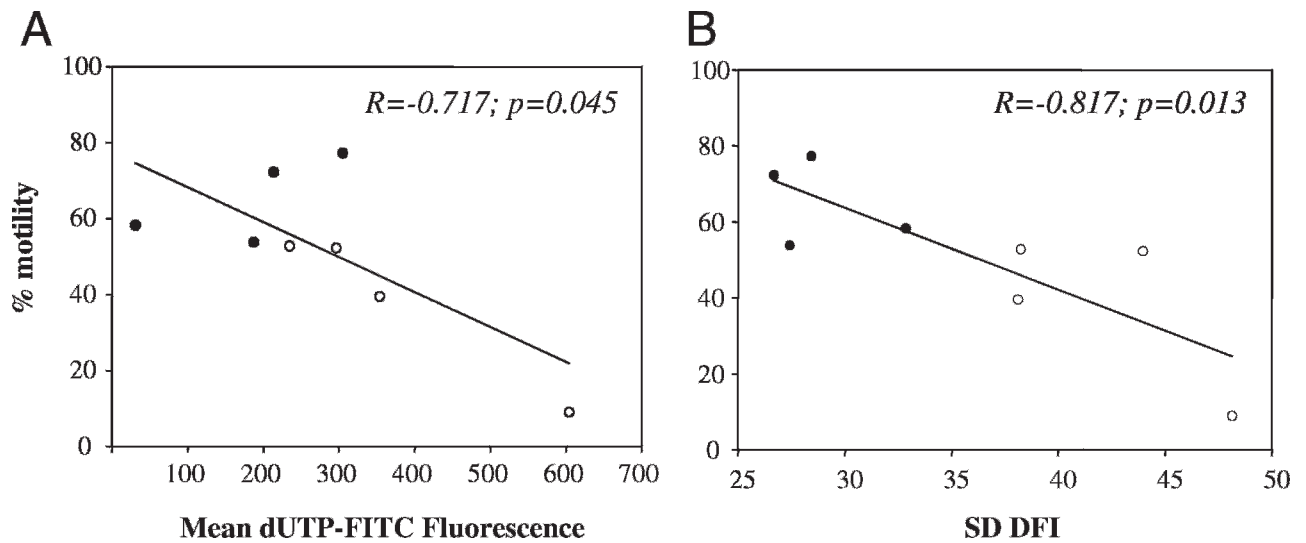


Figure 6. Correlation between sperm motility and (A) the TUNEL assay or (B) the AO assay parameters. Values from rats treated for 9 weeks with BEP at 0X (dark circles) or 1X (open circles) doses are reported.

TUNEL assay showed a trend of an inverse correlation with the number of surviving pups ( $r = -.66$ ,  $P = .07$ ,  $n = 8$ ), suggesting that an increase in the number of DNA strand breaks in the sperm can lead to an increase in the death rate after birth. The use of assisted reproduction techniques, such as IVF or ICSI, allows the correlation of sperm chromatin integrity with the ability to fertilize, steps in early embryo development, and the implantation success ratio (Razavi et al, 2003; Agarwal and Allamaneni, 2004; Lewis and Aitken, 2005). Animal models may also be useful for such studies. Comparison of treatments with different effects on sperm chromatin structure and different progeny outcome defects may help to elucidate the relationship between specific sperm chromatin structure defects and progeny outcomes.

The role of sperm chromatin structure testing in routine semen analysis has been discussed (Perreault et al, 2003). The choice of assays to be included is important. In this study, we did 4 assays, giving 3 different measures of sperm chromatin integrity: its maturity and compaction, determined by the level of protamine bound to the DNA; the chromatin structure, measured by susceptibility to low-pH denaturation; and the extent of single and double DNA strand breaks, as assessed by the TUNEL and the COMET assays. While the TUNEL assay coupled with flow cytometry analysis on rat sperm was done previously (Zubkova and Robaire, 2006), it had not been compared to the COMET assay performed on slides. Using the same samples for both assays, we have shown that these 2 tests do correlate in the rat as they do in the human (Donnelly et al, 2000). Interestingly, the COMET assay seemed to be less variable and more sensitive to dose response than the TUNEL assay. We have also shown that the results of the AO assay correlate with the TUNEL assay, consistent with a recent study comparing fertile and infertile patients (Chohan et al, 2006). Surprisingly, we did not find any correlation between the AO results and the COMET assay parameters, in contrast to the findings of Aravindan et al (1997) for human spermatozoa. Differences in correlation level for the AO assay and the TUNEL or COMET assays have been described previously (Perreault et al, 2003), suggesting that each test addresses a different parameter involved in sperm chromatin integrity. Thus, the CMA3, COMET, and AO assays complement each other in predicting fertility problems; each is relevant and likely to be needed for comprehensive clinical sperm analysis.

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