

# Effects of Chemotherapeutic Agents for Testicular Cancer on the Male Rat Reproductive System, Spermatozoa, and Fertility

ADRIENNE M. BIEBER,\*† LUDOVIC MARCON,\*† BARBARA F. HALES,\* AND BERNARD ROBAIRE\*‡

*From the Departments of \*Pharmacology & Therapeutics and †Obstetrics & Gynecology, McGill University, Montréal, Québec, Canada.*

**ABSTRACT:** Testicular cancer is the most common cancer affecting men of reproductive age. Advances in treatment of the disease, which include the coadministration of bleomycin, etoposide, and cisplatin (BEP), have brought the cure rate to over 90%. This high cure rate, coupled with the young age of patients, makes elucidation of the impact of the treatment on reproductive function, fertility, and progeny outcome increasingly important. The goal of this study was to determine the effects of BEP, in doses analogous to those given to humans, on the male reproductive system, spermatozoa, fertility, and progeny outcome in an animal model. Male Sprague-Dawley rats were treated daily with BEP for 3 cycles of 3 weeks each, for a total of 9 weeks. After 6 and 9 weeks, males were mated to 2 groups of untreated females. BEP treatment resulted in decreases in testicular and epididymal weights of 52% and 28%, respectively, when compared to control. Decreased testis and epididymis weights were accompanied by impairment of spermatogenesis and by a decrease in spermatozoal count of nearly 90% ( $11.9 \times 10^7$  spermatozoa per caput epididymidis in control vs  $1.65 \times 10^7$  in BEP-treated rats). The percent of motile spermatozoa in the treated rats was more than

30% lower than in controls. Defects in the flagella of spermatozoa increased by more than twofold in the midpiece, and by more than sixfold in the principal piece. Paternal BEP treatment, for either 6 or 9 weeks, did not affect fertility, pre- or postimplantation loss, litter size, or sex ratio among progeny on gestation day 21. In contrast, among the pregnancies allowed to proceed to delivery, a significant number of pups sired by males treated with BEP for 9 weeks died between birth and postnatal day 2; this was not observed in pups sired by males treated for 6 weeks. Markers of postnatal development were not affected in the surviving offspring from either group. Thus, despite the dramatic effects of the testicular cancer drug regimen on spermatogenesis, the numbers of spermatozoa, and their motility and morphology, male rats were fertile. While fetal development was apparently normal, early postnatal mortality, which may be associated with a delay in parturition, was elevated among the progeny sired by males exposed to BEP for 9 weeks.

Key words: cis-Platinum, etoposide, bleomycin, testis, epididymis, progeny outcome, flagellar defect.

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Testicular cancer is the most common cancer affecting men of reproductive age. While the incidence of testicular cancer has been rising steadily for several decades (Adami et al, 1994), mortality due to the disease has been declining (Forman and Moller, 1994). Advances in the treatment of the disease, which include the coadministration of bleomycin, etoposide, and cis-platinum (BEP), have brought the 5-year survival rate to over 90% for those patients who are considered to be at good risk (Mead and Stenning, 1997). This high cure rate makes the posttreatment quality of life of testicular cancer patients a concern, and because of the young age of the patients, consideration of the impact of the treatment on

fertility and reproductive function has become increasingly important.

After treatment for testicular cancer, patients experience a decrease in the number of spermatozoa produced and in their motility, as well as an increase in morphologically abnormal spermatozoa (Stephenson et al, 1995). Spermatogenesis recovers in most men after 5 years (Lampe et al, 1997). However, reports on the chromatin quality of the surviving spermatozoa are conflicting: one study found an increase in aneuploidy (De Mas et al, 2001), another found no change (Thomas et al, 2004), and yet another found a decrease in aneuploidy (Martin et al, 1997). The sperm chromatin structure assay revealed no increase in DNA fragmentation (Stahl et al, 2004); however, one case report described a man whose sole semen abnormality after treatment with BEP was an increase in DNA denaturation (Deane et al, 2004).

While studies of the effects of BEP on spermatogenesis in human testicular cancer patients provide valuable information, these data are confounded by the fact that the subjects have a diseased testis and have undergone orchidectomy. It has been shown that the semen quality of

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Correspondence to: Bernard Robaire, Department of Pharmacology and Therapeutics, 3655 Promenade Sir-William-Osler, Montréal, Québec, Canada H3G 1Y6 (e-mail: Bernard.robair@mcgill.ca).

† Both authors contributed equally to this work.

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testicular cancer patients is already decreased at diagnosis (Petersen et al, 1999b; Jedrzejczak et al, 2004). The semen quality further decreases following orchidectomy, even before the initiation of chemotherapy (Petersen et al, 1999a). Furthermore, the patients in these studies have received various numbers of cycles of BEP, and the time from treatment to semen collection is variable. It is thus extremely difficult to conclude whether the effects on fertility are due to chemotherapy, orchidectomy, or the cancer itself.

The effects of BEP treatment on progeny outcome are difficult to examine in humans. It has been suggested that fertility in patients with testicular cancer was decreased by 30% after treatment (Huyghe et al, 2004). Several reports have suggested that there is no increase in congenital malformations among the progeny of testicular cancer patients who were treated with BEP (Senturia et al, 1985; Byrne et al, 1988). Because of small sample sizes, however, these reports do not have the power to detect an increase in relative risk of less than threefold to fivefold (Lahdetie et al, 1994). Animal studies indicate that paternal treatment with cis-platinum alone can affect progeny outcome: chronic cis-platinum treatment in the rat resulted in increased pre- and postimplantation loss, a change in sex-ratio of the offspring, as well as an increase in malformed and growth-retarded fetuses (Seethalakshmi et al, 1992). The effects of etoposide or bleomycin alone, or of the BEP combination, on progeny outcome have not been examined in an animal model. Furthermore, postnatal development of pups sired by male rats treated with any of the 3 drugs has yet to be examined.

We hypothesized that even in the absence of testicular cancer and orchidectomy, the chemotherapeutic regimen used to treat testicular cancer is deleterious to the production of spermatozoa, to their morphology and motility, and can affect fertility and progeny outcome. We tested this hypothesis in the rat model.

## Materials and Methods

### Chemicals

All chemicals were purchased from Sigma Chemical Co (St Louis, Mo), unless otherwise noted.

### Animals, Treatment, and Mating Protocol

Adult male (300–350 g) and virgin female (200–225 g) Sprague-Dawley rats 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 Research Centre protocol 4699).

Males were randomly divided into 2 groups of 10 rats each. 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 (Fischer Scientific, Fair Lawn, NJ). On day 2 of each week, control rats were given 1 mL of saline by intraperitoneal injection. The animals from the drug-treated group 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, they were given an intraperitoneal injection of 1.5 mg/kg bleomycin (LKT Laboratories) dissolved in saline. This dose regimen was chosen based on the standard dose given to humans (Benedetto, 1999), adjusted for surface area according to the following formula:  $f \times \text{mg/kg} = \text{mg/m}^2$ , where  $f$  equals 6.0 for the rat (Bachmann et al, 1996). This dosing regimen differs from that used in humans in that humans are treated for 1 week per cycle of 3 weeks; each course of treatment generally consists of 2 or 4 cycles.

Males were mated after 6 weeks of treatment and at the completion of the 9-week treatment. On day 6 of the week (a non-treatment day), males were placed in a cage overnight with 2 naturally cycling females in proestrus, as determined by vaginal smears. The progeny of these females were used for the analysis of fetal development on gestation day 21. Four days later, on day 2 of the subsequent week, the males were mated again. The progeny of these females were used for analysis of postnatal growth and development. Females were checked for sperm by vaginal smear on the morning after mating. This was considered to be gestation day 0.

### Tissue Collection and Histology

At the end of the 9-week treatment, males were anesthetized and the ventral prostate, seminal vesicles, left testis, and left epididymis were removed and weighed. The contralateral testis and epididymis were cleared with saline and perfused with Bouin fluid as a fixative through the abdominal aorta. The tissues were then excised, postfixed for an additional 24 hours in the same fixative, dehydrated, and embedded in paraffin. To evaluate spermatogenesis, 5- $\mu\text{m}$  testis sections were cut and stained with periodic acid-Schiff, according to the manufacturer's instructions (Sigma). Sections were viewed with a Leica AS LMD microscope (Leica, Wetzlar, Germany) equipped with a RS Photometrics CoolSNAP fx digital camera (Roper Scientific, Tucson, Ariz).

The left epididymides were sectioned into caput-corporis and cauda regions. The caput-corporis epididymides were frozen in liquid nitrogen for the determination of spermatozoal counts; spermatozoa from the cauda epididymidis were used for motility and morphology analyses, as described below.

### Spermatozoal Counts

The previously frozen caput-corporis epididymides were homogenized in 5 mL of 0.9% saline, 0.1% merthiolate, and 0.05% Triton X-100 (VWR International, Mississauga, Canada), for 2 intervals of 15 seconds separated by a 30-second interval. Heads of spermatozoa were counted using a hemocytometer to assess the absolute number of sperm per caput-corporis epididymidis (Robb et al, 1978).

### *Spermatozoal Motility*

Spermatozoa from the cauda epididymides were used immediately for computer-assisted sperm analysis (CASA), as previously described (Slott et al, 1993), with the exception that the medium used was as follows: Hanks balanced salt solution (Gibco Invitrogen Co, Grand Island, NY), supplemented with 4.2 mg/mL HEPES, 0.35 mg/mL sodium bicarbonate, 2.0 mg/mL bovine serum albumin, 0.9 mg/mL D-glucose, and 0.025 mg/mL soybean trypsin inhibitor, pH 7.3–7.4, at 37°C (Klinefelter et al, 1991). Briefly, the epididymis was trimmed free of fat, rinsed in medium, clamped at the corpus-cauda junction, and severed at the corpus side of the clamp. Several tubules of the distal cauda were pierced with a #11 scalpel, and the tissues were transferred to a Petri dish containing 10 mL of medium, allowing spermatozoa to disperse into the medium. The tissues were removed, and the spermatozoa were left to disperse for several minutes. An aliquot of 10  $\mu$ L of the spermatozoa-containing medium was transferred to a prewarmed 80- $\mu$ m-deep glass cannula for CASA analysis using the HTM-IVOS system (Hamilton Thorne Research, Beverly, Mass) and version 12 of the Toxicology software (analysis speed = 60 Hz; minimum track duration = 30 frames at 60 Hz; minimum average-path velocity [VAP] = 50; minimum straightness [STR] = 80). For each animal, 4 slides, each with two 80- $\mu$ m-deep chambers, were analyzed. At least 100 spermatozoa per slide were analyzed. The following parameters were determined: percent of motile spermatozoa, percent of progressively motile spermatozoa, curvilinear velocity (VCL), straight line velocity (VSL), VAP, amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN = VSL/VCL  $\times$  100), and straightness (STR = VSL/VAP  $\times$  100).

### *Spermatozoal Morphology*

The cauda epididymides were minced into 7 mL of phosphate-buffered saline (PBS) (Roche). The suspension was filtered, washed several times with PBS, fixed for 1 hour in 1% glutaraldehyde (Mecalab LTD, Montreal, Canada) in PBS, and washed again with PBS. The resulting pellet was suspended in 1% agarose (Gibco Invitrogen) and embedded for electron microscope analysis as follows. The samples were washed 2 times in 0.1 M sodium cacodylate buffer containing 3% sucrose, pH 7.4; post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide; and embedded in epoxy resin. Spermatozoal ultrastructure was analyzed on the electron microscope (Philips 410 Electron Microscope, Eindhoven, The Netherlands). At least 100 midpiece cross-sections and 100 principal-piece cross-sections per sample were photographed. The number of midpiece cross-sections and principal-piece cross-sections with any abnormality, as well as the number with a cytoplasmic droplet, were recorded.

### *Analysis of Pregnancy Outcome*

On gestation day 21, sperm-positive females were sacrificed by CO<sub>2</sub> asphyxiation and laparotomized. The ovaries were removed, and the numbers of corpora lutea were recorded. The uteri were removed and examined for any abnormalities, including resorption moles. The numbers of implantation sites in the uteri were recorded. The extent of preimplantation loss per litter was calculated as follows: (number of corpora lutea – number of implantation sites)/(number of corpora lutea). The number of post-

implantation losses was calculated as follows: (number of implantation sites – number of live fetuses)/(number of implantation sites). The fetuses were removed successively, blotted, weighed, examined for external malformations, sacrificed by hypothermia, and immersion-fixed in Bouin fixative. After fixation, the fetuses were dissected and the major organs examined for internal malformations. The sex-ratio was calculated as the ratio of the number of male fetuses to the total litter size.

### *Postnatal Growth and Development*

Pregnant females were placed alone in a cage on gestation day 20. From day 21 onward they were monitored regularly for signs of labor. Labor was considered complete when all pups were cleaned and nursing and when the vaginal area was free of blood. Upon completion of labor, pups were removed from the cage, weighed, sexed, examined for malformations, labeled by toe-clipping, and returned to the mother. The pups were weighed biweekly until weaning, after which time they were weighed once per week until postnatal day 62. Pups were weaned on postnatal day 21, at which time 4 males and 4 females from each litter were placed in separate cages, and the remaining pups were sacrificed. The pups were monitored daily for any changes, and the days of eye opening, vaginal opening, and preputial separation were recorded. Female pups were sacrificed by CO<sub>2</sub> asphyxiation at 10 weeks of age, and the spleen, kidneys, and ovaries were weighed. Male pups were sacrificed at 13 weeks, and the spleen, kidneys, left testis, left epididymis, seminal vesicles, and ventral prostate were weighed.

### *Statistical Analysis*

The number of postnatal deaths was analyzed using the Fischer's exact test. All other data were analyzed using the Student's *t* test, with Bonferonni correction as appropriate. Data are presented as the mean plus or minus standard error of the mean (SEM). The level of significance was considered *P* < .05.

## **Results**

### *Weights of the Male Rats and Reproductive System Tissues*

During the treatment, one rat from the BEP group became sick and was sacrificed. The remaining BEP-treated rats gained less weight than the control rats, although their body weights did increase over the course of the treatment (Figure 1). We observed a dramatic effect on the weights of the testes and epididymides in the BEP-treated rats. There was a decrease in testis weight of approximately 50% and a decrease in epididymal weight of approximately 30% in the BEP-treated rats compared to controls. The reduction in organ weights remained significant when expressed as relative organ weight (data not shown). There was no significant effect on seminal vesicle or ventral prostate weights (Figure 2).

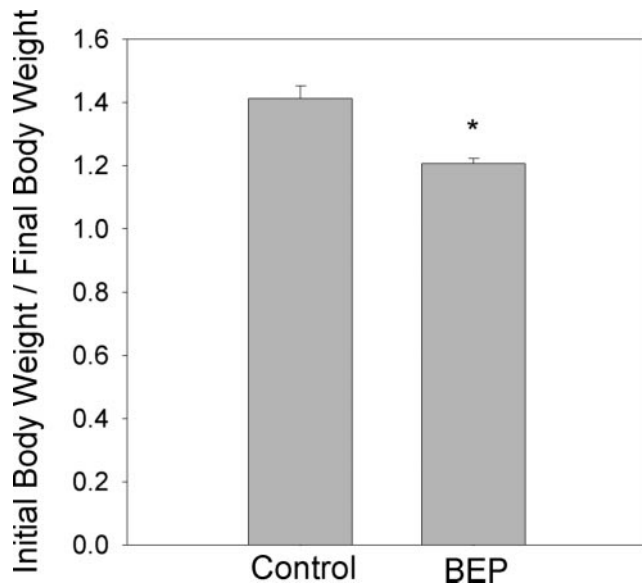


Figure 1. Body weight changes over the course of the 9-week treatment with bleomycin, etoposide, and cis-platinum (BEP), expressed as weight on the last day of treatment divided by weight on the first day of treatment (control,  $n = 8$ ; BEP,  $n = 7$ ). The BEP-treated rats did not gain as much weight as the control rats. \*  $P < .01$ .

### Testis Histology

We examined the histopathology of the testis of rats exposed to BEP for 9 weeks. Testis cross-sections of BEP-treated rats were characterized by severe atrophic and germ cell-depleted seminiferous tubules compared to controls, reflecting the decrease in testis weight (Figure 3A and B). Loss (sloughing) of immature germ cells into the lumen, giant multinucleated cell formation, along with extensive vacuolization of seminiferous tubules and Sertoli cell-only tubules were observed among the sections of treated rats (Figure 3C through F).

### Spermatozoal Numbers, Motility, and Morphology

We observed a striking effect on the number of spermatozoa in the BEP-treated rats. The total number of spermatozoa per caput-corpora epididymides in the BEP-treated rats was reduced by more than 90% when compared to control rats (Figure 4).

The motility characteristics of the BEP-treated spermatozoa were significantly altered (Figure 5). The overall percent of motile spermatozoa was approximately 30% lower in the BEP-treated rats compared to controls. Although the range of values for percent motile spermatozoa was relatively small among the control group (68%–77%), the values for the drug-treated rats varied greatly; one rat had 7.5% motile spermatozoa, while another had 71% motile spermatozoa. The mean percent of progressively motile spermatozoa was unchanged in the BEP-treated group. All 3 of the velocity parameters (VAP, VCL, and VSL) were significantly decreased in the BEP-

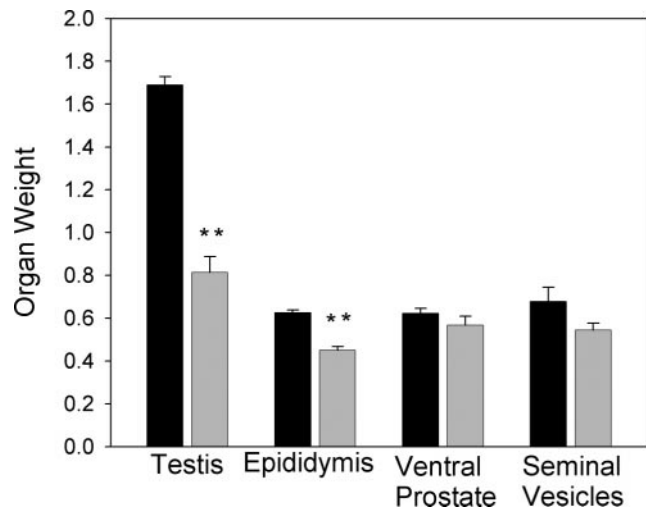


Figure 2. Weights of reproductive organs after 9 weeks of treatment with vehicle or bleomycin, etoposide, and cis-platinum (BEP) (control,  $n = 8$ ; BEP,  $n = 7$ ). There was a significant decrease in the weights of the testes and epididymides of the BEP-treated animals, whereas the weights of the seminal vesicles and ventral prostate remained unchanged. \*\*  $P < .001$ .

treated animals; both the VAP and the VCL decreased by approximately 10%, and the VSL decreased by approximately 13%. In addition, the parameters reflecting beat characteristics were altered in the drug-treated rats; the ALH was approximately 20% lower and the BCF approximately 10% higher when compared to similar values in controls. The parameters that reflect the straightness (STR and LIN) with which the spermatozoa swim were not significantly altered in the drug-treated rats.

We observed an increase in the percent of spermatozoa with morphological abnormalities in the midpiece of the flagella after BEP treatment (Figure 6). The midpiece of a control spermatozoon consists of 9 microtubule doublet pairs surrounding 1 central doublet. Each of the 9 peripheral doublet pairs is associated with 1 outer dense fiber. Surrounding the outer dense fibers is the mitochondrial sheath (Figure 7A). In the control group, the only abnormalities seen were an abnormal spatial arrangement of outer dense fibers and the presence of 2 or more flagellar sections within 1 membrane. Some of the abnormalities observed in the midpiece cross-sections of spermatozoa from BEP-treated rats (Figure 7) were the same as the 2 abnormalities seen in the control group. Interestingly, additional abnormalities, not observed in the control group, were found in spermatozoa from the BEP-treated animals. These included an absence of the mitochondrial sheath, an abnormal number of outer dense fibers (either too many or too few), hemilateral absence of the axoneme and the outer dense fibers, a malformed mitochondrial sheath, the presence of some normal outer dense fibers and some that appear small and malformed, as well as

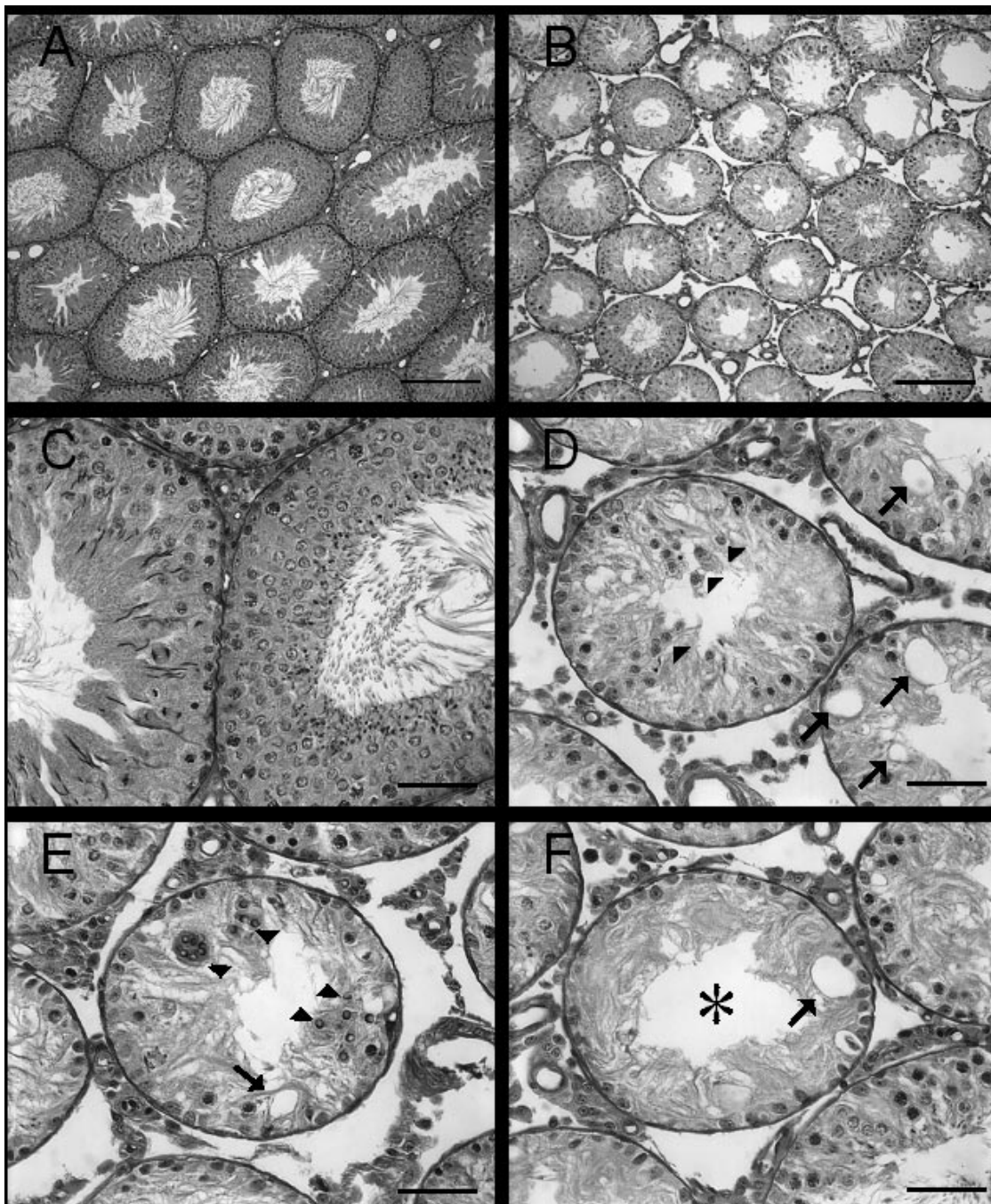


Figure 3. Histopathological examination of testis seminiferous epithelium. Representative testis sections of control (A) and bleomycin, etoposide, and cis-platinum (BEP)-treated rats (B). Sections were stained with periodic acid-Schiff to show histology of seminiferous tubules. (A, B) A high proportion of tubules in the testis of BEP-treated rats (B) presented degenerated seminiferous epithelium and germ cells compared to controls (A). Higher magnification of testis sections from control and BEP-treated rats. (C) Control rats showed normal seminiferous tubule organization and spermatogenesis; (D-F) testis sections from treated rats showed abnormal seminiferous tubules. Nine-week BEP exposure resulted in severe abnormalities, such as tubules with drastic reduction in germ cell content, sloughing of immature germ cells into the lumen (indicated by solid arrowheads), and extensive vacuolization (arrows). Scale bar = 200  $\mu\text{m}$  in A and B; Scale bar = 50  $\mu\text{m}$  in C-F.

sections that were malformed and had a combination of the abnormalities listed above, in addition to excess cytoplasm that contained outer dense fibers and debris (Figure 7). There was no increase in the percent of sperm

with a cytoplasmic droplet in the BEP-treated group (data not shown).

In addition to defects in the midpiece, an increase in the percent of spermatozoa with abnormalities in the prin-

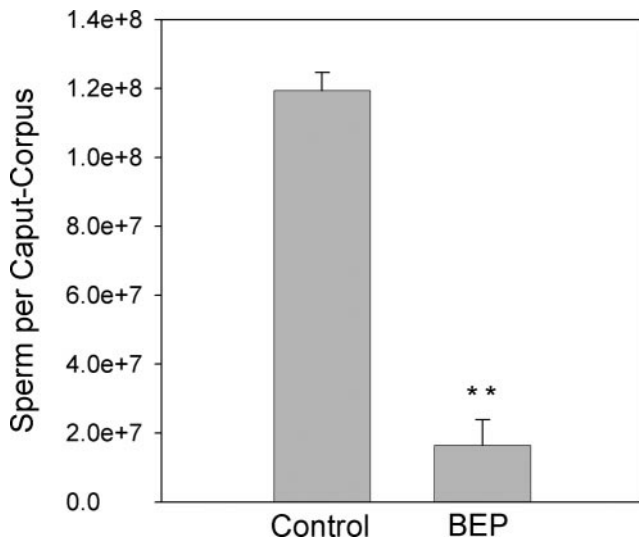


Figure 4. Spermatozoal counts in the caput-corpus epididymidis after 9 weeks of treatment with vehicle or bleomycin, etoposide, and cis-platinum (BEP) (control,  $n = 8$ ; BEP,  $n = 7$ ). Spermatozoal heads were counted with a hemocytometer to determine the reserves of spermatozoa in the caput-corpus epididymidis. BEP-treated rats had a reduction in spermatozoal count of approximately 90%. \*\*  $P < .001$ .

cial piece of the flagella was observed (Figure 6). The principal piece of a spermatozoon from a control rat (Figure 8A) consists of the same 9 plus 2 arrangement of microtubules as the midpiece; however, outer dense fibers 3 and 8 are replaced by the 2 longitudinal columns of the fibrous sheath. Surrounding the outer dense fibers are the circumferential ribs. In this region of the flagella, the abnormalities seen in the BEP group were also seen in the control group, but at significantly higher incidences compared to the control group (Figure 6); these included a hemilateral absence of the outer dense fibers, with or without an intact axoneme, and a missing outer dense fiber (Figure 8).

#### Fertility and Progeny Outcome

In pregnancies sired by males exposed to BEP for 6 or 9 weeks, all sperm-positive females became pregnant; there were no changes in pre- or postimplantation loss, litter size, or sex-ratio (Table). In addition, there were no effects on fetal weights. There were no external malformations in either of the control groups or in the 6- or 9-week BEP-exposed groups. No internal malformations were observed in any of the major organs, such as the liver, kidneys, lungs, or heart.

The second group of females that were mated to males after 6 and 9 weeks of BEP treatment were allowed to proceed through gestation to delivery. All the females mated to males that were treated for 6 weeks with BEP were pregnant and delivered live pups between gestation days 21 and 22. The average number of pups per litter that survived past day 1 was  $14.9 \pm 0.65$  in the 6-week

BEP group and  $13.1 \pm 1.1$  in the control group. Additionally, the growth curves for the pups sired by 6-week BEP-treated males did not differ from the pups sired by control animals (data not shown). Developmental markers (dates of eye opening, vaginal opening, and preputial separation) did not differ between control and BEP-treated groups.

All of the females mated to males exposed to BEP for 9 weeks were also sperm positive. However, unlike the females mated to males after 6 weeks of BEP treatment, only 1 of 10 sperm-positive matings resulted in a litter of a normal size (Figure 9). Of the others, we observed one mother eat all of her pups while they were still alive, one had 1 pup that died within 1 day, 3 had litters of 5 or less, and 4 others that were sperm positive were never observed with any pups. It is noteworthy that the 1 normal-sized litter in the 9-week group was not sired by the 1 male that had normal sperm motility. Interestingly, only 2 of the 10 sperm-positive females mated to BEP-exposed males went into labor on gestation day 22 ( $P \leq .002$ ,  $\chi^2$  analysis), compared with 7 out of 7 females mated to control males. Of the other 8 females mated to BEP-treated males, parturition occurred on gestation day 23 in 4, and for the remaining 4 females, there was no observable evidence of surviving offspring; in contrast, all females mated to control animals went into parturition on day 22 or earlier. Of the surviving pups sired by BEP-treated males, no abnormalities were observed in postnatal growth (Figure 10), developmental markers (data not shown), or organ weights (data not shown).

## Discussion

Although patients treated with BEP for testicular cancer experience significant reproductive problems, no studies to date have confirmed that these symptoms are directly the result of the chemotherapy. Furthermore, the risk to the progeny of testicular cancer patients treated with BEP has yet to be elucidated. The use of an animal model allows us to administer BEP in a dose regimen that is clinically relevant, without the presence of testicular cancer or orchidectomy. This provides the opportunity to elucidate the role of BEP in decreasing semen quality and to determine whether BEP treatment has the potential to adversely affect progeny outcome.

In the current study, male rats were treated continuously for 9 weeks with BEP, and the reproductive organs and sperm quality were analyzed upon the completion of treatment. The BEP-treated animals experienced a substantial decrease in the weights of both the testes and epididymides. Reduction of the testis size (weight) along with disrupted spermatogenesis are well-known side effects of cisplatin-based chemotherapy (Lampe et al, 1997;

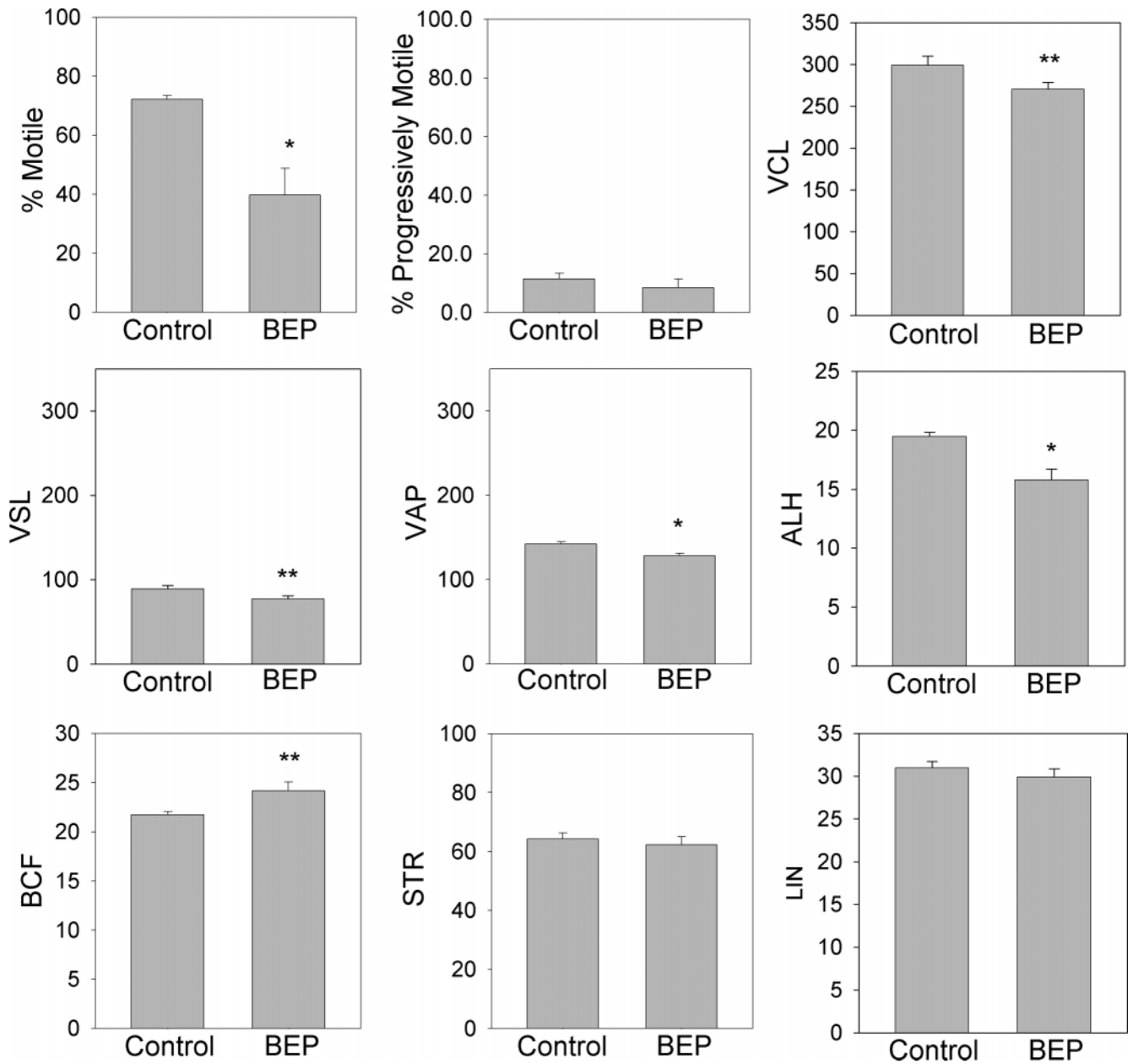


Figure 5. Effects of bleomycin, etoposide, and cis-platinum (BEP) treatment on percent of motile spermatozoa, percent progressively motile spermatozoa, as well as the motility characteristics of spermatozoa obtained from the distal cauda epididymidis of control or BEP-treated rats (Control,  $n = 8$ ; BEP,  $n = 7$ ). VCL indicates curvilinear velocity; VSL, straight line velocity; VAP, average-path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; and LIN, linearity. \*  $P < .01$ ; \*\*  $P < .05$ .

Howell and Shalet, 2001). We observed that exposure to chronic BEP chemotherapy resulted in marked alterations of seminiferous tubule histology, with severely impaired spermatogenesis. At the testicular level, 9-week chronic BEP chemotherapy induced germ cell depletion and tubule atrophy. Abnormalities in the spermatogenesis process may account for the resulting low spermatozoal count observed in that group. We found that 71% of the BEP-treated rats had a spermatozoal count that was decreased by more than 90% when compared to controls.

Relatively few studies have examined the impact of BEP treatment on the number of spermatozoa in men immediately after the completion of chemotherapy. The spermatozoal concentration in men who have undergone BEP gradually improves after completion of treatment (Tomomasa et al, 2002), and thus it is difficult to compare the response in humans with the results of the current study.

In a study by Stephenson et al (1995), 57% of human patients treated with BEP had less than 50% motile sper-

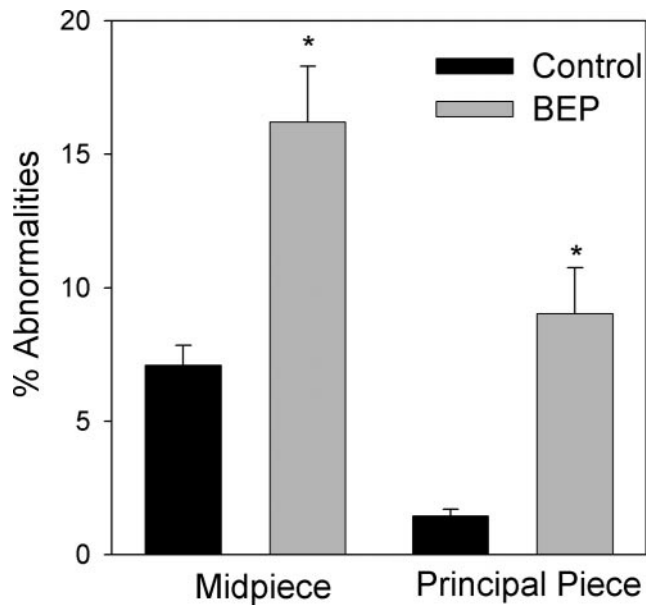


Figure 6. Percent abnormalities in both the midpiece and principal piece of spermatozoa from control or bleomycin, etoposide, and cis-platinum (BEP)-treated rats (control,  $n = 4$ ; BEP,  $n = 4$ ). At least 100 midpiece and principal-piece cross-sections were counted per rat, and the numbers of sections with any abnormality were recorded. The BEP-treated animals experienced an increase in the percent of both midpiece and principal-piece cross-sections with abnormalities. \*  $P < .05$ .

matozoa. In the rat, motility of less than 70% is considered abnormal (Seed et al, 1996). In the current study, 86% of drug-treated rats had spermatozoal motility below 70%. In addition to the percent motility, the VAP, VSL, VCL, BCF, and ALH values were significantly altered in the BEP-treated rats. Of these parameters, VSL, VCL, and BCF have been shown to be correlated with fertility (Toth et al, 1991). This may explain why fertility remains a problem even in patients who are not oligospermic (Hansen et al, 1991).

No studies to date have examined the morphology of spermatozoa from human testicular cancer patients at the electron microscope level. We observed an increase in the incidence of morphologically abnormal spermatozoa, as well as a larger variety of abnormalities, in BEP-treated rats. Interestingly, several of the midpiece abnormalities we observed uniquely in BEP-treated rats have been reported after other drug treatments, such as cyclosporine A (Masuda et al, 2003) or triptolide, a diterpene triepoxide isolated from a Chinese plant (Huynh et al, 2000). These abnormalities, however, are extremely rare in control rats (Syntin and Robaire, 2001). The flagellum is formed during spermiogenesis in the testis, indicating that the increase in abnormalities in BEP-treated rats probably reflects a defect in spermiogenesis, rather than epididymal maturation. This defect may be at the gene expression level and may occur as early as during the pachytene spermatocyte phase of spermatogenesis, as this is when

the first genes required for flagellar formation are expressed (Horowitz et al, 2005). Nevertheless, a number of the flagellar defects found in selenium-deficient rats were detected only after the spermatozoa left the caput epididymidis (Olson et al, 2004), indicating that the defects we observed may also originate during spermatozoal maturation in the epididymis. In murine models, defects in the flagella of spermatozoa are frequently associated with infertility (Huttner et al, 1993; Olson et al, 2005).

To the best of our knowledge, this is the first study to assess the impact in the rat model of concurrent administration of bleomycin, etoposide, and cis-platinum. Each of these drugs, however, has previously been given individually to rats (Seethalakshmi et al, 1992; Russell et al, 2000, 2004). Of the 3 drugs, cis-platinum has been reported to affect the numbers of spermatozoa and their motility (Seethalakshmi et al, 1992). After 9 weeks of daily intraperitoneal treatment with 0.5 mg/kg cis-platinum, a decrease in sperm count of approximately 60% and a decrease in sperm motility of approximately 50% were reported in rats. The effect on motility is much greater than we observed in our study; however, the effect on sperm count is much smaller. The greater effect on motility with cis-platinum alone may be a result of the route of administration, although it is interesting to note that the percent of sperm that were motile in the control rats in that study (56%) is well below what is considered normal (Seed et al, 1996). However, the greater effect on numbers of spermatozoa in our study is likely to be due to an additive effect of the 3 drugs and underscores the value of elucidating the impact of the combination regimen in the rat model.

As a result of the effects of BEP treatment on sperm count, motility, and morphology, we anticipated a decrease in fertility; a reduction in sperm production of more than 90% has been demonstrated to impair fertility in the rat (Robaire et al, 1984). Furthermore, changes in motility and morphology are likely to impair fertility. Interestingly, paternal exposure to cis-platinum alone has been reported to increase the incidence of fetal malformations and deaths (Seethalakshmi et al, 1992). Surprisingly, after either 6 or 9 weeks of paternal BEP treatment, there was no adverse impact on fetal development, as assessed on gestation day 21; fetal morphology was normal, as were the numbers of live fetuses per litter. First, the sperm number required to maintain fertility in BEP-treated rats was less than 10% compared to controls. Second, even though a significant proportion of the remaining 10% have decreased motility and flagellar defects, their function was not affected sufficiently to alter progeny outcome. These results are in stark contrast to experiments with cyclophosphamide, another anticancer drug. After paternal treatment with this drug, at doses that did not alter male reproductive organ weights or sperm counts,

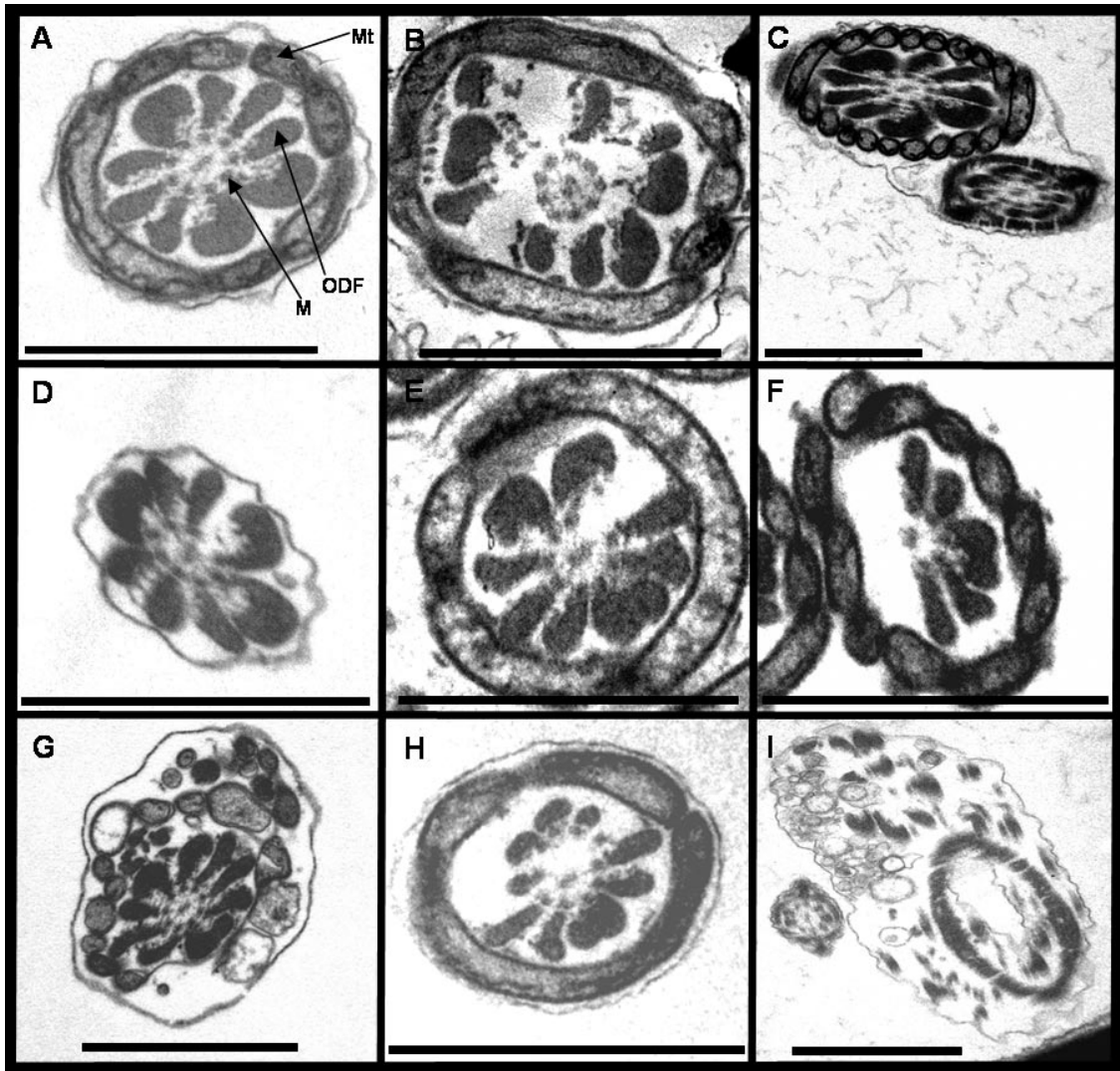


Figure 7. Morphology of the midpiece of a control spermatozoon, displaying the 9 plus 2 arrangement of microtubules (M), the 9 outer dense fibers (ODF), and the mitochondrial sheath (Mt) (A), as well as spermatozoa from rats treated with bleomycin, etoposide, and cis-platinum (BEP) (B-I). Abnormalities include an abnormal spatial arrangement of outer dense fibers (B), the presence of 2 flagellar sections within 1 membrane (C), absence of a mitochondrial sheath (D), an abnormal number of outer dense fibers (E), hemilateral absence of the outer dense fibers and axoneme (F), a malformed mitochondrial sheath (G), malformed outer dense fibers (H), as well as a malformed midpiece (I). Scale bars = 100 μm.

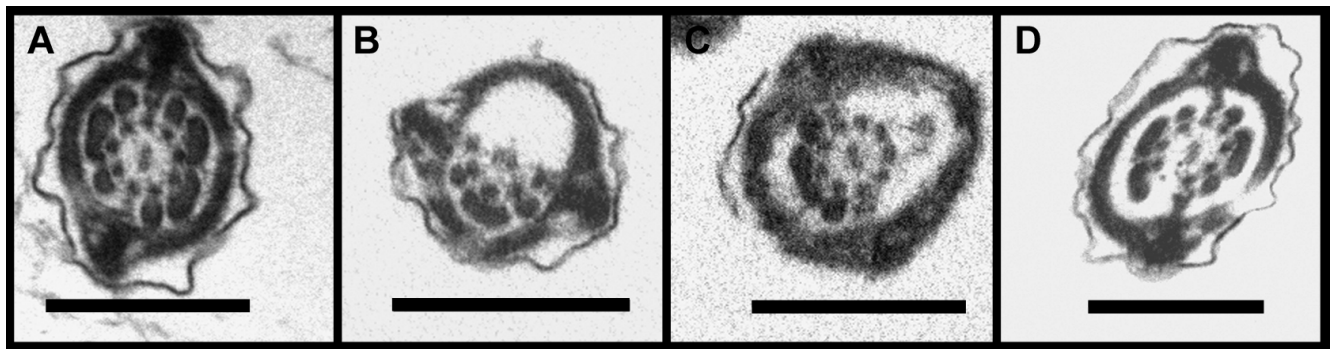


Figure 8. Morphology of the principal piece of a control spermatozoon (A) as well as spermatozoa from rats treated with bleomycin, etoposide, and cis-platinum (BEP) (B-D). Abnormalities include the hemilateral absence of the outer dense fibers, with (B) or without (C) hemilateral absence of the axoneme, as well as an abnormal number of outer dense fibers (D). Scale bars = 50 μm.

Pregnancy outcome in litters sired by males treated for 6 or 9 weeks with BEP\*

	Duration of Treatment			
	6 wk		9 wk	
	Control	BEP	Control	BEP
Litter size	14.61 ± 0.54†	14.50 ± 0.73	13.53 ± 1.19	12.54 ± 0.82
Preimplantation loss	0.05 ± 0.02	0.09 ± 0.03	0.15 ± 0.06	0.17 ± 0.04
Postimplantation loss	0.01 ± 0.02	0.02 ± 0.06	0.07 ± 0.02	0.11 ± 0.04
Sex-ratio	0.48 ± 0.03	0.49 ± 0.04	0.50 ± 0.03	0.39 ± 0.05

\* BEP indicates bleomycin, etoposide, and cis-platinum treatment, as defined in the "Materials and Methods" section. 6 Weeks: control, n = 18; BEP, n = 12; 9 Weeks: control, n = 15; BEP, n = 11. Preimplantation loss is defined as the number of corpora lutea minus the number of implantation sites per litter. Postimplantation loss is defined as the number of implantation sites minus the number of fetus per litter on day 20 of gestation.  
 † All values are expressed as the mean ± SEM.

there were increases in pre- and postimplantation loss and congenital malformations in the progeny (Trasler et al, 1985).

When the females mated to males treated with BEP for 9 weeks were allowed to deliver, a significant proportion of their pups did not survive past postnatal day 1. We observed that of 10 litters, only 1 was of a normal size. There was no decrease in litter size nor was there an increase in fetal deaths when pregnancy was terminated on gestation day 21, indicating that it is likely that many of the sperm-positive mothers that were allowed to proceed through gestation may have delivered full litters of live pups, which subsequently died and were eaten or were eaten alive. No obvious lethal defects were observed

among the fetuses examined on gestation day 21. We speculate that delayed parturition had an adverse impact on postnatal survival in the 4 litters in which this occurred. Prenatal maternal exposures to dexamethasone, diethylstilbestrol, ethylene glycol dimethyl ether, 2-methoxyethanol, or Aroclor 1254 have been reported to delay parturition in rats (White et al, 1983; Chatterjee et al, 1993; Marty and Loch-Caruso, 1998; Lee et al, 2003). Delayed parturition is frequently associated with an increase in perinatal mortality (Rands et al, 1982; Leonhardt et al, 1991; Zimmerman et al, 1991). Alternatively, the

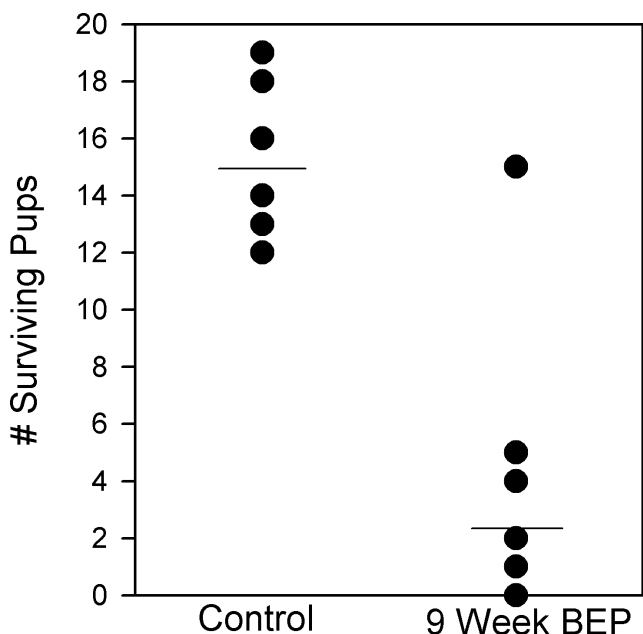


Figure 9. Numbers of pups per litter sired by males treated for 9 weeks with bleomycin, etoposide, and cis-platinum (BEP) or saline that survived past postnatal day 1. All the litters sired by control males had at least 12 pups, all of which survived. Of the litters sired by BEP-treated males, 6 did not have any pups that survived past day 1, 3 had litters of 5 or fewer pups, and 1 had a litter of 15 pups. Chi-square analysis was used to compare the number of litters with pups surviving past day 1.

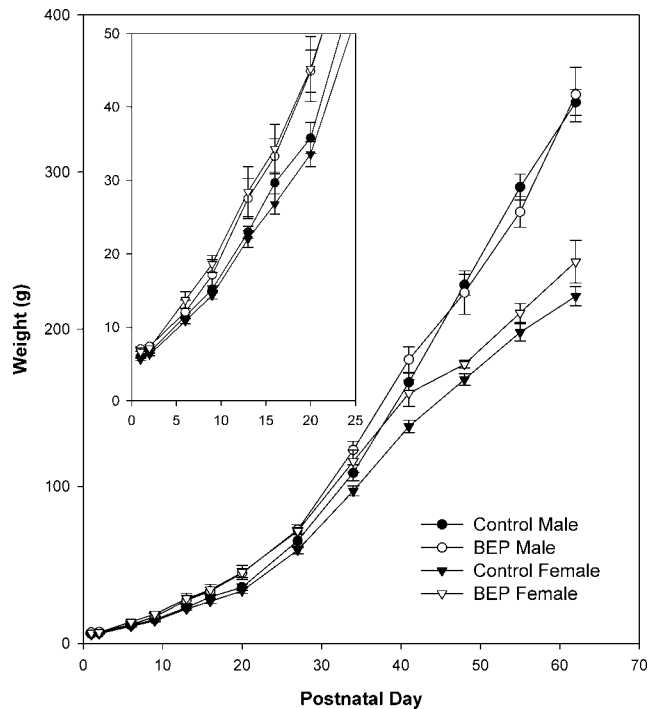


Figure 10. Postnatal growth of surviving pups sired by males treated with bleomycin, etoposide, and cis-platinum (BEP) for 9 weeks. Pups were weighed biweekly until weaning, after which they were weighed weekly. Male and female pups are grouped separately. No difference in growth rate was observed between pups sired by control or BEP-treated males. Pups sired by males treated with BEP for 6 weeks showed a similar pattern. Values represent means ± standard errors of the mean (control, n = 7; BEP, n = 4). Inset is postnatal days 1–25.

pups may have had a fatal functional deficit that we did not observe. To the best of our knowledge, this is the first study to show an effect of paternal chemical exposure on the timing of parturition and postnatal survival of progeny.

These results clearly show that BEP treatment has a deleterious impact on the quality of spermatozoa in the male rat, resulting in a decrease in spermatozoal numbers and motility, an increase in morphologically abnormal spermatozoa, and harmful effects to the postnatal development of the progeny. These data indicate that the chemotherapeutic agents, rather than cancer or orchidectomy, may affect the quality of spermatozoa in patients who have been treated for testicular cancer.

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