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Impact of the Chemotherapy Cocktail Used to Treat Testicular Cancer on the Gene Expression Profile of Germ Cells from Male Brown-Norway Rats[1]

Geraldine Delbès,[3] Donovan Chan,[3] Pirjo Pakarinen,[7] Jacquetta M. Trasler,[3,4,5] Barbara F. Hales,[3] and Bernard Robaire[2,3,6]

Departments of Pharmacology and Therapeutics,[3] Pediatrics,[4] Human Genetics,[5] and Obstetrics and Gynecology,[6] McGill University and the Montreal Children's Hospital Research Institute, Montréal, Québec, Canada H3G 1Y6
Department of Physiology,[7] Institute of Biomedicine, University of Turku, Turku 20520, Finland

ABSTRACT

Advances in treatment for testicular cancer that include the coadministration of bleomycin, etoposide, and cisplatin (BEP) have brought the cure rate to higher than 90%. The goal of this study was to elucidate the impact of BEP treatment on gene expression in male germ cells. Brown-Norway rats were treated for 9 wk with vehicle (0×) or BEP at doses equivalent to 0.3× and 0.6× the human dose. At the end of treatment, spermatogenesis was affected, showing altered histology and a decreased sperm count; spermatozoa had a higher number of DNA breaks. After 9 wk of treatment, round spermatids were isolated, and RNA was extracted and probed on Rat230–2.0 Affymetrix arrays. Of the 31 099 probe sets present on the array, 59% were expressed in control round spermatids. BEP treatment significantly altered the expression of 221 probe sets, with at least a 1.5-fold change compared with controls; 80% were upregulated. We observed a dose-dependent increase in the expression of oxidative stress response genes and no change in the expression of genes involved in DNA repair. BEP upregulated genes were implicated in pathways related to *Jun* and *Junb* protooncogenes. Increased mRNA levels of *Jun* and *Junb* were confirmed by quantitative RT-PCR; furthermore, JUN protein was increased in elongating spermatids. Thus, BEP exposure triggers an oxidative stress response in round spermatids and induces many pathways that may lead to the survival of damaged cells and production of abnormal sperm.

chemotherapy, gene expression, gene regulation, Jun protooncogene, round spermatids, spermatogenesis, testicular cancer, toxicology

INTRODUCTION

The worldwide incidence of testicular cancer has increased in the last 50 yr [1, 2], affecting young men of reproductive age, mostly between 20 and 34 yr of age [3]. The current standard treatment consists of unilateral orchidectomy followed by chemotherapeutic treatment using the combination of bleomycin, etoposide, and cisplatin (BEP) [4]: bleomycin induces DNA breaks [5], etoposide inhibits topoisomerase II [6], and cisplatin is an alkylating agent cross-linking DNA [7]. This cocktail of chemotherapeutic drugs has resulted in the improvement of overall survival after 5 yr for all stages of testicular germ cell tumors to higher than 90% [8], but it also has led to transient or permanent loss of fertility [9]. Indeed, after BEP treatment it has been shown that men produce a reduced number of spermatozoa, with low motility and an increased incidence of abnormal forms [10–12]. An evaluation of the numbers of spermatozoa revealed that spermatogenesis recovered in most men after 5 yr [13], but these men reported difficulties in fathering children, with a longer time to pregnancy [14]. The impact of the chemotherapeutic cocktail used to treat testicular cancer on male germ cell quality is not yet fully understood and needs to be further analyzed to understand the potential consequences on progeny outcome.

Previous studies from our laboratory have described the effects of BEP treatment on the Sprague-Dawley rat [15–17]. Male rats were treated with the BEP chemotherapeutic cocktail for 9 wk to mimic the human treatment. Using this animal model it was shown that BEP treatment induced disruption of spermatogenesis, leading to a decreased sperm count [15]. Mature spermatozoa collected from the cauda epididymidis showed abnormal morphology [15], abnormal chromatin structure, and an increased number of DNA strand breaks [16]. Interestingly, despite the reduced number of spermatozoa, paternal BEP treatment did not affect fertility, preimplantation or postimplantation loss, litter size, or sex ratio on Gestational Day 21. Nevertheless, parturition was delayed in some cases, and the pups sired by males treated with BEP for 9 wk showed early postnatal mortality [15] with no obvious developmental abnormalities. To assess the reversibility of the impairment of spermatogenesis by the BEP treatment, a recovery study was published recently in which males were analyzed at 3, 6, and 9 wk after the end of the treatment [17]; consistent with the human scenario [13], spermatogenesis recovered over time after BEP exposure. Interestingly, preimplantation loss remained elevated in litters sired by BEP-treated males, even after 9 wk of recovery [17], suggesting that spermatogonia were affected. Overall, this animal model has allowed for the description of the impact of the BEP regimen on male germ cells, showing that the treatment leads to the production of abnormal male germ cells, potentially inducing abnormal progeny outcome.

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[2]Correspondence: Bernard Robaire, Department of Pharmacology and Therapeutics, McGill University, 3655 Promenade Sir-William Osler, Montréal, QC, Canada H3G 1Y6. FAX: 514 398 7120;
e-mail: bernard.robaire@mcgill.ca

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The chemotherapeutic drugs that are used to treat testicular cancer induce DNA damage [18]. Thus, we can expect that a DNA damage response, characterized by changes in the expression of genes that determine cell fate by activating cell cycle checkpoints and pathways toward cell survival or death, will be activated in the germ cells. We hypothesize that BEP treatment induces gene expression changes that may lead to the survival of damaged cells, and that the production of abnormal sperm is responsible for the adverse impact on progeny. To test this hypothesis, we analyzed the impact of BEP treatment on gene expression in male rat germ cells using a whole rat genome microarray analysis followed by a systematic pathway analysis. In the present study we used an inbred rat model, the Brown-Norway rat, to minimize interindividual genetic variation in the microarray analysis.

MATERIALS AND METHODS

Animals and Treatment

Male Brown-Norway rats (age 4 mo, 16 per group) were obtained from Harlan (Indianapolis, IN) and housed on a 12L:12D cycle. Food and water were provided ad libitum. All animal studies were conducted in accordance with the guidelines outlined in *A 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 (n = 16 per group) as described previously [15] with a few modifications. Briefly, the drug-treated animals received BEP regimens for 9 wk based on therapeutically relevant doses: 0.3× and 0.6×, with a 1× dose being equivalent to the human treatment regimen, as adjusted for weight and surface area. Drugs were purchased from the Royal Victoria Hospital pharmacy (Montreal, QC, Canada). The rats treated with the 0.6× dose were gavaged on Days 1 through 5 of each week with 1.8 mg/kg *cis*-platinum (Mayne Pharma) and 9.0 mg/kg etoposide (Novapharm). On Day 2 of each week, male rats were given an intraperitoneal injection of 0.9 mg/kg bleomycin (Bristol-Meyers) dissolved in 19:1 saline:ethanol; rats in the 0.3× group received half of these doses. The rats from the control group were treated in an identical manner but were given the vehicle only.

Tissue Collection

At the end of the 9-wk treatment period, some rats (n = 8 per group) were anesthetized, and 1 ml blood was collected from the renal vein. The ventral prostate, seminal vesicles, left testis, and left epididymis then were removed, weighed, and frozen in liquid nitrogen. The contralateral testis and epididymis were perfused through the abdominal aorta as described previously [15], first with saline to clear the blood and then with Bouin fluid as a fixative. Perfused tissues were immersed in Bouin solution for at least 24 h, dehydrated, and embedded in paraffin. Other animals (n = 8 per group) were killed by CO_2 asphyxiation and decapitation; their testes were removed for isolation of spermatogenic cells, and cauda epididymal spermatozoa were collected as described previously [16] and stored at −80°C until further analysis.

Histology-Immunohistochemistry

Spermatogenesis was assessed using 5-μm testicular sections that had been stained with periodic acid-Schiff following the manufacturer's instructions (Sigma, Oakville, ON, Canada). Surface areas of the seminiferous tubules were evaluated by measuring at least 100 tubules in one section per animal (n = 4) using the microdissection software (Leica Microsystems, Richmond Hills, ON, Canada). TUNEL-positive cells were revealed using the ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Chemicon International Inc., Temecula, CA) following the manufacturer's recommendations. TUNEL-positive cells per tubule were counted in at least 200 tubules on two different sections for each animal (n = 4).

Sperm Count and Motility

Both the testes and caput-corpus epididymides were frozen and later homogenized in 5 ml of 0.9% saline, 0.1% merthiolate, and 0.05% Triton X-100 (VWR International, Mississauga, ON, Canada), for two intervals of 15 sec separated by a 30-sec interval. Heads of spermatozoa were counted using a hemocytometer to assess the absolute number of sperm per testis [19]. Mature sperm were collected from the cauda epididymidis, and the motility was measured using CASA as described previously [15].

COMET Assay

DNA strand breaks in spermatozoa were evaluated using the alkaline comet assay as described previously [16].

Hormone Measurement

Serum testosterone concentration was measured using a Testosterone ELISA kit (IBL Immuno-Biological Laboratories, Hamburg, Germany) following the manufacturer's protocol. Luteinizing hormone and FSH concentrations were determined as described previously [20].

Cell Separation

Spermatogenic cells were obtained through cell separation by velocity sedimentation using the STA-PUT method as described previously by Bellvé et al. [21] and modified by Aguilar-Mahecha et al. [22]. Briefly, both rat testes were decapsulated and digested by enzymatic treatment under continuous agitation (120 cycles/min) at 34°C, first with 0.5 mg/ml collagenase (C9891; Sigma) for 12 min, followed by 0.5 mg/ml trypsin (type 1; T8003; Sigma) and 1 μg/ml DNase I (type 1; DN-25; Sigma) for 16 min after sedimentation and washing. After dissociation, tubules were filtered through a nylon mesh in the presence of DNase I and washed with RPMI (RPMI medium 160; Invitrogen, Burlington, ON, Canada) containing 0.5% BSA. Cells were centrifuged and filtered; a total of 3.5×10^8 to 6×10^8 cells in 25 ml of 0.5% BSA in RPMI was loaded in the velocity sedimentation apparatus (STA-PUT; Proscience, Don Mills, ON, Canada), followed by a 2%–4% BSA (Roche Diagnostics, Laval, QC, Canada) gradient in RPMI for separation by sedimentation at unit gravity. Fractions of pachytene spermatocytes, round spermatids (steps 1–9), and elongating spermatids (steps 10–19) were identified by phase-contrast microscopy. Briefly, Sertoli cells are large cells recognized easily by their nonhomogeneous shape and a multilobular nucleus. Pachytene spermatocytes are large and round cells with a big cell nucleus, whereas the round spermatids are small, round cells with a smaller, very well-defined nucleus. Fractions with greater than 70% purity were pooled, aliquoted, pelleted, and stored at −80°C until further analysis.

RNA Extraction

Total RNA was extracted from the round spermatid fractions (~5×10^6 cells) using the RNeasy kit (Qiagen, Mississauga, ON, Canada), and residual genomic DNA was eliminated by deoxyribonuclease treatment (DNAse set; Qiagen). The RNA quality was assessed by Genome Quebec using a bioanalyzer (Agilent Technologies, Santa Clara, CA) and was quantified using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Gene Expression Analysis

Gene expression was assessed in the round spermatids using Affymetrix Rat Genome 230 2.0 microarrays in collaboration with Genome Quebec. RNA (3 μg) was reverse transcribed, and 10 μg cRNA was hybridized on the microarray according to the manufacturer's recommendations. Chips considered for further analysis showed a background signal of less than 83 and a *Gapdh* 3′/5′ hybridization rate from 0.91 to 1.37 (n = 3, 4, and 3 for the 0×, 0.3×, and 0.6× treatment groups, respectively). The Robust Multiarray Average algorithm [23] was applied, and only the genes with an expression value of 5 or higher were considered as expressed. The raw data obtained were further normalized per gene to the median (GeneSpring v7.0; Agilent Technologies). Statistical significance between the three groups (0×, 0.3×, and 0.6×) was tested by nonparametric ANOVA using a P value of <0.05, and probe sets from that list then were filtered for those for which expression was upregulated or downregulated by a minimum of 1.5-fold. Clustering of the genes was done using a k-means analysis. Annotation and biological function of these genes were obtained from the NetAffx Analysis Center (Affymetrix) and the Rat Genome Database. Cellular pathways were obtained using Pathway Studio 4.0 (Ariadne Genomics, Rockville, MD) with the ResNet-3.0 database.

Real-Time Quantitative RT-PCR

RNA was diluted to a working concentration of 2 ng/μl, and QuantiTect One-Step SYBR Green quantitative RT-PCR (Qiagen) was completed using the Roche LightCycler (Roche Diagnostics) according to the manufacturer's instructions. Polymerase chain reaction thermal cycling parameters were: 95°C for 15 min (one cycle), 94°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec (50 cycles). Seminiferous tubule cells were used to make 1-, 10-, 50-, and 100-

TABLE 1. Quantitative RT-PCR primers.

Gene name	Symbol	Accession no.	Reverse primer	Forward primer
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	NM_017008	tggaattgtgagggagatgct	agagagaggccctagttgct
Jun oncogene	*Jun*	NM_021835	taacagtgggtgccaactca	cgcaaccagtcaagttctca
Jun B oncogene	*Junb*	NM_021836	cctgacccgaaaagtagctg	atgtgcacgaaaatggaaca

ng/ml RNA stocks for standard curves for quantification. Reverse transcription-PCR primers (Table 1) were designed with Primer3 software (http://frodo.wi.mit.edu) [24] and provided by Alpha DNA (Montreal, QC, Canada). Each sample from six different cell separations was run in duplicate. The measured amount of each cDNA was normalized against *Gapdh* from the same sample.

Protein Extraction and Western Blotting

Round spermatid pellets were thawed on ice and placed in 500 μl of RIPA buffer (150 mM NaCl; 1% Nonidet P-40; 0.5% deoxycholate; 0.1% SDS; and 50 mM Tris, pH 7.5) containing 10 μl/ml protease inhibitor cocktail (Sigma). The samples were homogenized with an ultrasonicator (Sonics & Materials Inc., Newtown, CT) and centrifuged at 10 000 × *g* for 10 min at 4°C. The remaining supernatant from each sample was aliquoted and stored at −20°C for protein assay (Bio-Rad Laboratories) and Western blotting. Protein from each sample (25 μg) was separated with 10% SDS-PAGE and then transferred onto equilibrated Nitrocellulose Hybond-C Super membrane (Amersham Biosciences, Oakville, ON, Canada) by electroblotting. Membranes were blocked in 5% skim milk and then probed with primary antibodies against JUN, JUNB (1:200 in 5% BSA; Cell Signaling Technologies, Danvers, MA), or LAMINB1 (1:2000 in 3% skim milk; Cedarlane, Hornby, ON, Canada). Horseradish peroxidase-coupled anti-rabbit antibody (Amersham Biosciences) was used to detect antigen-antibody interactions by enhanced chemiluminescence. The bands were quantified by densitometric analysis using a Chemi-Imager v5.5 imaging system (Alpha Innotech, San Leandro, CA). The measured value of each band was normalized against LAMIN B1 (LMNB1) from the same sample. Each experiment contained four samples from each group and was run in duplicate. The positive control was protein from HeLa cells.

Immunofluorescence

After separation, 500 000 cells of each germ cell type were spun on slides using the StatSpin Cytofuge (Bio-Rad Laboratories) for 2 min at 600 rpm. Cells were immediately fixed by immersion in methanol at −20°C for 10 min, air dried, and kept at −20°C until further use. On the day of staining, slides were immediately immersed in two successive baths of PBS for 5 min. After 1 h of incubation in the blocking solution (5% BSA, 10% normal goat serum, and 0.1% Triton in PBS) at room temperature, slides were covered overnight with the primary antibody (JUN and JUNB, 1:200 in blocking solution) at 4°C in a humidified chamber. After three washes in PBS for 5 min, a goat fluorescein-coupled anti-rabbit secondary antibody was added (Vector Laboratories Inc., Burlingame, CA) and incubated in the dark for 30 min at room temperature. After three washes in PBS, slides were covered with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories). Negative controls were obtained by incubating the slides overnight with the blocking solution without the primary antibody. Micrographs were taken using a Cool Snap camera attached to a Leica DM LB2 epifluorescence microscope.

RESULTS

Effects of BEP on the Male Rat Reproductive System

During the 9 wk of treatment, all rats gained weight, but the treated animals gained significantly less (data not shown). At the end of the treatment, we observed a dose-dependent decrease in the weight of all reproductive organs (Table 2). Sperm production decreased significantly in a dose-dependent manner, and the mature spermatozoa produced after the high-dose treatment showed a significant increase in the number of DNA strand breaks as measured by the COMET assay (Table 2).

Analysis of serum testosterone revealed a tendency for testosterone levels to decrease after treatment (Table 2), even though this decrease was not significant, perhaps because of high variability in controls. Interestingly, the variability in testosterone levels appeared to be less in treated animals; this may be due to a reduction in the pulsatility of testosterone secretion. However, high-dose BEP treatment induced a significant decrease in serum LH (Table 2) and increase in serum FSH levels (Table 2).

Effects of BEP on Spermatogenesis and Germ Cell Purification

After 9 wk of BEP treatment, spermatogenesis was assessed by examining histology in testicular sections. We observed a disruption of spermatogenesis in the high-dose treatment group (Supplemental Fig. 1, A and B, available online at www.biolreprod.org). Some tubules showed normal spermatogenesis but with a lower number of germ cells (Supplemental Fig.

TABLE 2. Effects of the BEP treatment on the rat reproductive organ weights, sperm parameters, and serum hormone levels.[a]

Parameter	0X	0.3X	0.6X
Reproductive organ weights (g)			
Testis	1.863 ± 0.071	1.441 ± 0.043**	1.038 ± 0.029**
Epididymis	0.539 ± 0.008	0.475 ± 0.014**	0.410 ± 0.020**
Ventral prostate	0.362 ± 0.017	0.262 ± 0.032*	0.223 ± 0.009**
Seminal vesicle	0.312 ± 0.021	0.246 ± 0.035	0.191 ± 0.006**
Sperm parameters			
Testicular sperm count ($\times 10^8$)	1.50 ± 0.09	1.22 ± 0.04*	0.90 ± 0.04**
Sperm DNA strand breaks (tail extent moment [%])	29.09 ± 3.84	51.60 ± 10.23	61.59 ± 10.84*
Serum endocrinology			
LH (ng/ml)	0.222 ± 0.072	0.169 ± 0.081	0.046 ± 0.004*
FSH (ng/ml)	1.088 ± 0.180	1.609 ± 0.112	3.284 ± 0.250*
Testosterone (ng/ml)	1.049 ± 0.291	0.829 ± 0.123	0.711 ± 0.070
Testicular histology			
Tubule surface (μm^2)	73576 ± 4044	53708 ± 1408**	44411 ± 2117**
No. of TUNEL+ cells/tubule	0.119 ± 0.034	0.317 ± 0.058*	0.497 ± 0.061**

[a] Values are means ± SEM of 5 to 16 animals per group.
* $P < 0.05$ compared to the control (0X) group.
** $P < 0.001$ compared to the control (0X) group.

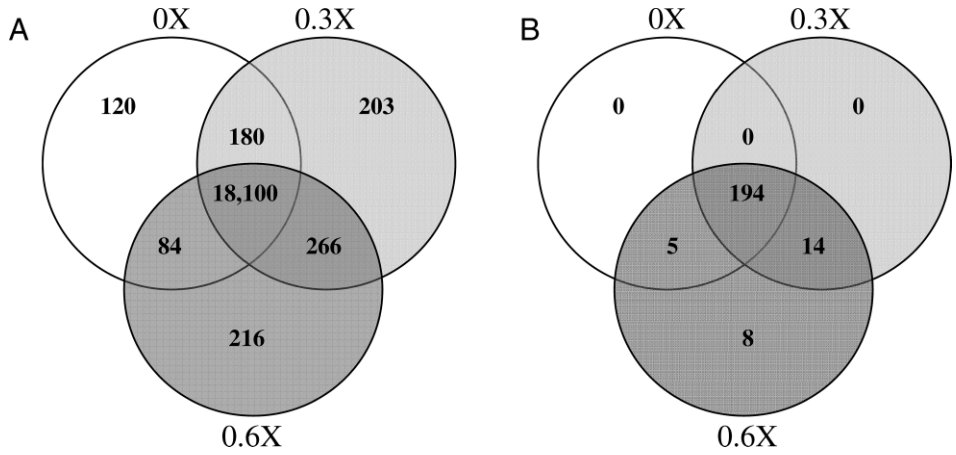

FIG. 1. **A**) Numbers of probe sets expressed in the round spermatids in controls (0×) and after treatment with 0.3× or 0.6× doses of BEP. The control cells expressed ~50% of the probe sets present on the array. **B**) Numbers of probe sets significantly affected in round spermatids after BEP treatment.

1b1), whereas in other tubules there were few, if any, germ cells (Supplemental Fig. 1b2). We observed a dose-dependent decrease in tubule surface area, with one-way ANOVA of the mean and of the median being significant (Table 2). This decrease in the tubule diameter was due to a decrease in the surface areas of both the lumen and epithelium (Supplemental Fig. 1C), suggesting a significant decrease in germ cell numbers. The number of apoptotic germ cells was assessed further by TUNEL (Table 2 and Supplemental Fig. 1D). We observed a significant dose-dependent increase in the number of TUNEL-positive cells per tubule (Table 2).

Despite the decreased number of germ cells, mature spermatozoa were produced and germ cell separations could be done. After the 0.6× BEP treatment, the purity of the pachytene spermatocyte fractions was decreased significantly (Supplemental Fig. 2A). However, the purity of the round spermatid fractions was about 89% (Supplemental Fig. 2B). Although the treatment did not affect the purity of round spermatids, a significantly lower number of cells was recovered due to the lower rate of spermatogenesis.

Effects of BEP Treatment on Gene Expression in Round Spermatids

Using whole rat genome Affymetrix 230 2.0 microarrays, we assessed the impact of the BEP treatment on gene

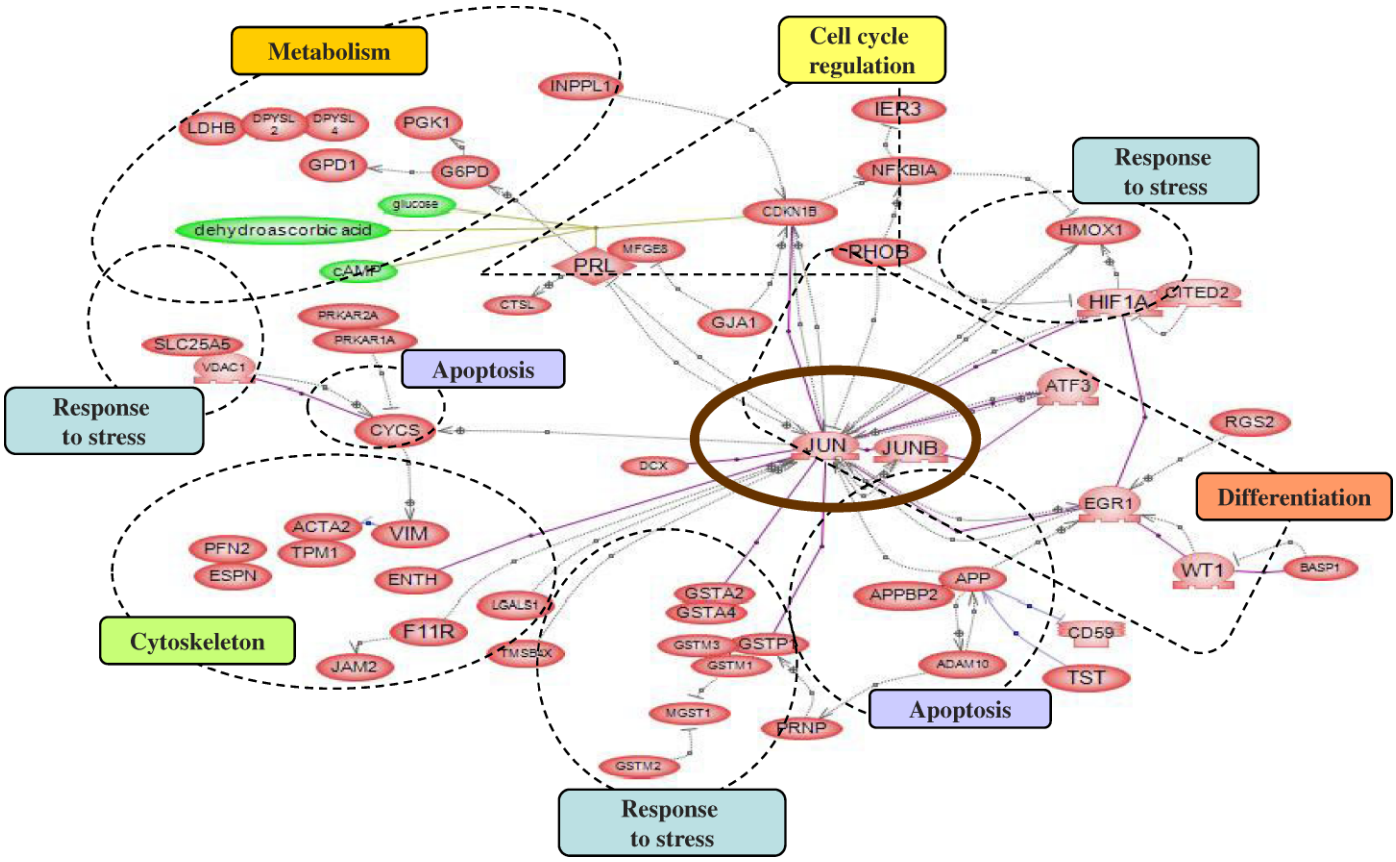

FIG. 2. Probable direct linkages among genes significantly upregulated by BEP treatment. The genes significantly upregulated by BEP were analyzed using Pathways Assist 3.0 software. Only direct relationships among genes in the published literature to date are shown. Clusters of genes were circled according to their biological function. Gray arrows indicate positive regulation, gray T junctions indicate regulation, and purple lines indicate binding.

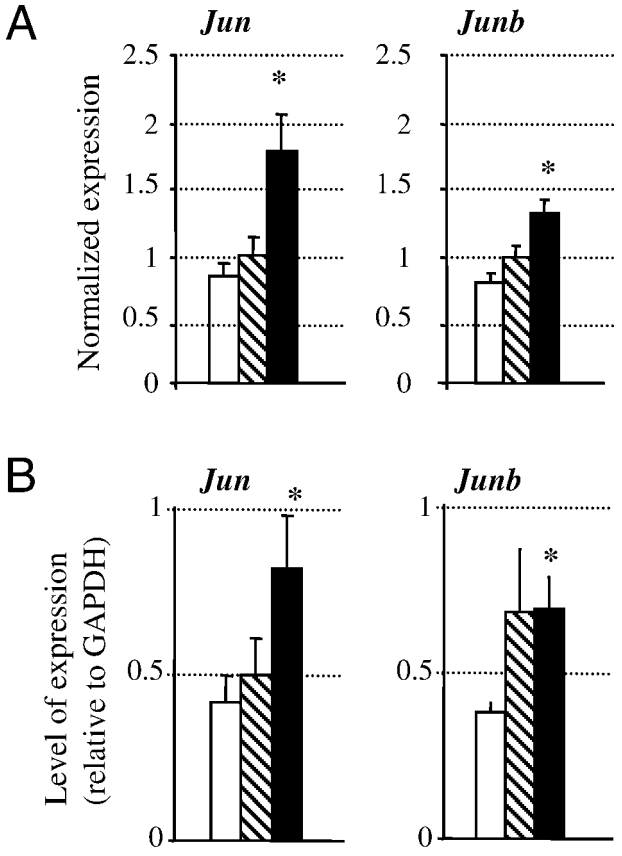

FIG. 3. *Jun* and *Junb* protooncogene RNA relative expression in the round spermatids after BEP treatment. After 9 wk of treatment with 0× (open bars), 0.3× (striped bars), or 0.6× (black bars) doses of BEP, round spermatids were isolated, and RNA was extracted and used to measure *Jun* and *Junb* expression level using microarrays (**A**; n = 3–4) and quantitative qRT-PCR (**B**; n = 6). Values represent the means ± SEM. $^*P < 0.05$ using a one-way ANOVA test.

expression in the round spermatid fractions. Of the 31 099 probe sets present on the array, 18 484 (59.4%) were considered expressed in the control round spermatids (Fig. 1A). Interestingly, an additional 685 probe sets were considered expressed after BEP treatment: 203 after 0.3× BEP treatment and 216 after 0.6× treatment (Fig. 1A). The treatment significantly affected 221 probe sets, with at least a 1.5-fold difference in expression from the control cells (1.5- to 3.8-fold change; Fig. 1B). BEP treatment induced the novel expression of 22 probe sets, eight of which were sensitive only to the high dose (Fig. 1B). Among the treatment-affected 221 probe sets, five were downregulated and 178 were upregulated after treatment, whereas 38 showed a transient decrease after the 0.3× treatment but levels comparable to controls in the 0.6× dose group (Supplemental Fig. 3). Using k-means cluster analysis (Supplemental Fig. 3 and Supplemental Tables 1–5), we determined that upregulated probe sets could be subdivided into three groups: those showing a dose-dependent increase (49 probe sets; Supplemental Table 2), those showing an increase only in the high-dose treatment (124 probe sets; Supplemental Table 3), and those showing a maximum increase in the 0.3× treatment group (five probe sets; Supplemental Table 4). Downregulated probe sets (Supplemental Table 1) could be subdivided into two groups: one showing a dose-dependent decrease (four probe sets), and the other in which the maximum decrease was observed with the lower dose (one probe set).

TABLE 3. Number of probe sets significantly decreased or increased by the BEP treatment according to their biological function.

Function	Decreased	Increased
Unclassified	2	34
Response to stress/hypoxia		27
Metabolism	1	23
Cell cycle regulation		21
Transcribed sequences		16
Transcription regulation		11
Cytoskeleton		11
Cell adhesion		7
Protein modification		7
Reproduction related	2	4
Cell/cell interaction		3
Vesicle transport		3
Response to DNA damage		1

Among the 183 probe sets showing an upregulation or downregulation after the 0.6× dose, 16 were transcribed sequences and 34 had unclassified biological functions (Table 3). Although many of these probe sets (32.7%) were of unknown biological function, 14.7% were involved in the stress response, 12.5% in metabolism, and 11.4% in cell cycle regulation (Table 3). Possible interactions among these putative genes were analyzed further using Pathway Studio. The software recognized 79.7% of the 178 upregulated genes imported. Interactions among 28% of the original genes on this list could be found and are represented in Figure 2. The most affected biological functions were all represented by a cluster of genes having potential interactions among each other. Interestingly, many pathways were related to the Jun protooncogene (*Jun*) family, known to play a decisive role in determining cell fate.

Effects of BEP Treatment on Jun *and* Junb *Expression in Round Spermatids*

Given the potentially central role of *Jun* and *Junb* in the pathways obtained (Fig. 2), we analyzed the impact of BEP treatment on this protooncogene family. The mRNA expression of both *Jun* and *Junb* was increased significantly in the 0.6× dose group (Fig. 3A). These results were further confirmed by qRT-PCR (Fig. 3B). Expression of these proteins in the maturing germ cells was characterized by immunofluorescence of isolated cells (Fig. 4). JUN is expressed in the nucleus of the pachytene spermatocytes and spermatids at all stages of elongation, from round to elongated spermatids (Fig. 4A). The expression pattern of JUNB is very similar before spermiogenesis, where JUNB is present in the nuclei of pachytene spermatocytes and round spermatids (Fig. 4B). Interestingly, JUNB could not be observed in the nuclei of elongating spermatids from stages 10 to 17, but JUNB was observed in elongated spermatids at about stages 18 to 19 (Fig. 4B). High levels of JUN and JUNB were seen in the cytoplasm of spermatids during elongation, with an accumulation in the resulting residual body (Fig. 4). We analyzed the protein levels of JUN and JUNB after treatment in the round (Fig. 5, A–C) and elongating (Fig. 5, B–D) spermatids using Western blots. JUN protein levels were not affected by BEP treatment in the round spermatids (Fig. 5A) but were significantly increased by 0.6× BEP treatment in the elongating spermatids (Fig. 5B). We did not observe any change in the protein content of JUNB in either round spermatids (Fig. 5C) or elongating spermatids (Fig. 5D).

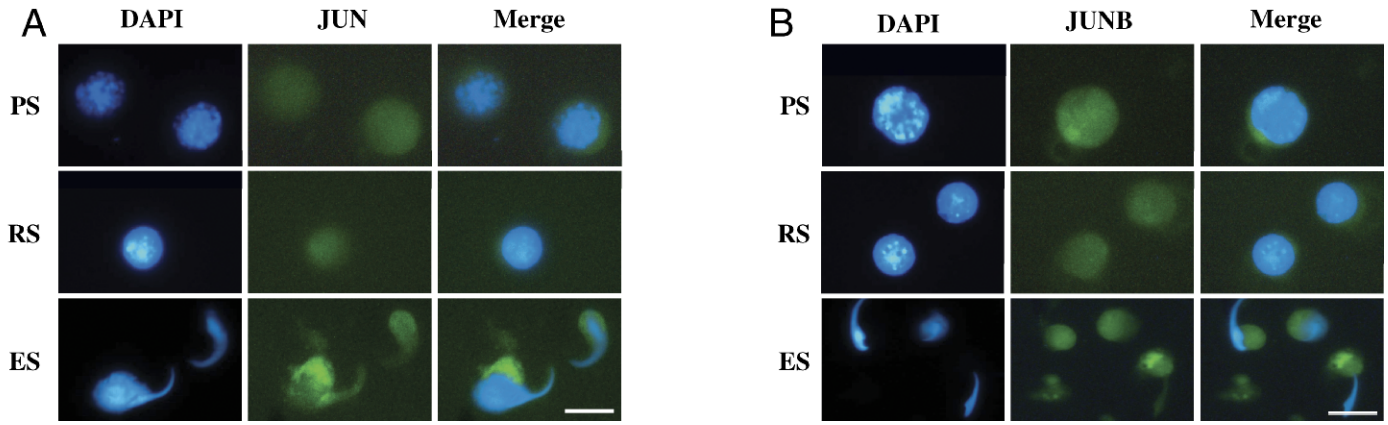

FIG. 4. Immunofluorescent staining for JUN (**A**) and JUNB (**B**) in pachytene spermatocytes (PS), round spermatids (RS), and elongating spermatids (ES). Rat male germ cells were purified by the STA-PUT method, collected, and spun on slides. Cells were fixed in methanol, stained for *Jun* or *Junb* as revealed by the fluorescein probes, and counterstained with DAPI. Bars = 5 μm.

DISCUSSION

This study shows that the BEP chemotherapeutic cocktail affects gene expression in round spermatids. Using the Brown-Norway rat, we first described the negative impact of a 9-wk treatment on reproductive organ weights, spermatogenesis, and sperm production. The Brown-Norway is more sensitive to BEP than the Sprague-Dawley rat, because effects were observed with a lower concentration of these drugs (0.6× in the present study vs. 1× in the Sprague-Dawley rat [15]). This is consistent with rat strain-specific differences in sensitivity to drugs [25]. We describe here for the first time the endocrine status of these animals. We observed a significant increase in FSH that is likely the result of disruption of spermatogenesis [26]. We also describe a trend toward a decrease in serum testosterone concentration and a significant decrease in serum LH. A decrease in serum testosterone would be consistent with the decreased weights of androgen-dependent organs (seminal vesicles and prostate); the lower serum LH concentration may be responsible for the decreased testosterone concentration. The impact of BEP on LH secretion may be the result of a direct impact of the drugs on the hypothalamus and/or the pituitary.

BEP treatment affected spermatogenesis, resulting in a reduction in the production of spermatozoa. The number of DNA strand breaks in those cells, as measured by the COMET assay, was increased after treatment. Using the cell separation technique [21], we obtained a relatively low purity of pachytene spermatocytes in our controls (75%–80%); following treatment, we were not able to collect these cells with an acceptable purity (41%–69%) for expression studies. After treatment with 0.6× BEP, the percentage of Sertoli cells in the pachytene spermatocyte fractions was increased significantly, suggesting that pachytene spermatocyte density or size was affected by the treatment. Although no abnormalities in Sertoli cells were observed, this also suggests that Sertoli cell size and density were affected, or that the junctions between these cells were altered in such a way that the dissociation procedure did not separate these cells effectively. In addition, although BEP treatment did not affect the purity of round spermatids, a significantly lower number of cells were recovered.

To our knowledge, this is the first study to examine the effects of toxicants on the whole-genome expression profile in a pure population of germ cells, as opposed to whole-testis extracts [27, 28] or specific arrays [29, 30]. We have shown that the expression of 221 probe sets was significantly affected after treatment, with at least a 1.5-fold change compared with controls. Although it has been shown that treatment with a single chemotherapeutic agent, cyclophosphamide, induces a decrease in the expression of stress response genes [30], it is interesting to note that the expression of most probe sets (80%) was increased after BEP treatment. This apparent discrepancy suggests that there are distinct effects of different drugs, and/or

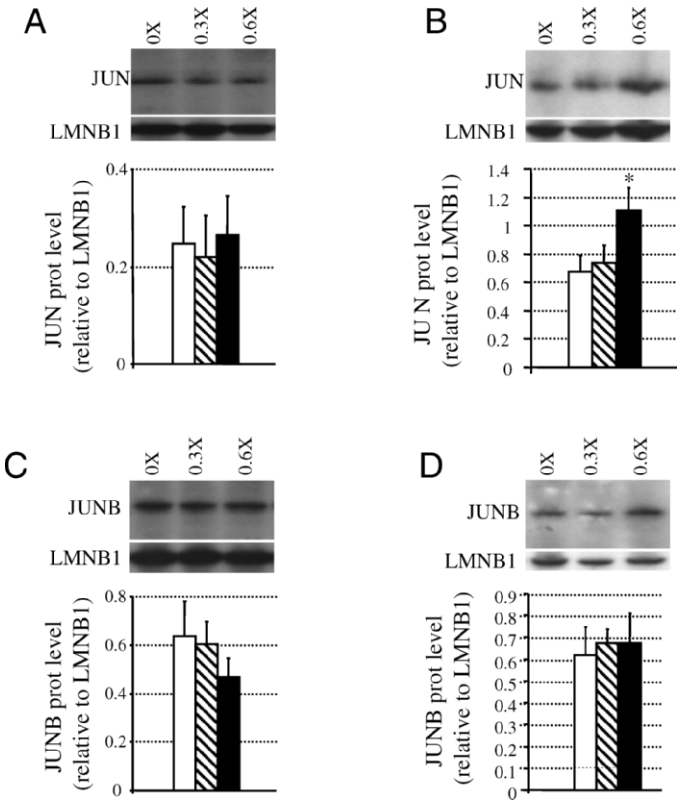

FIG. 5. Effects of BEP treatment on JUN and JUNB protein (prot) levels in round (**A**, **C**) and elongating (**B**, **D**) spermatids. After 9 wk of treatment with 0× (open bars), 0.3× (striped bars), or 0.6× (black bars) BEP, round and elongating spermatids were isolated, and proteins were extracted and used to assess JUN (**A**, **B**) and JUNB (**C**, **D**) expression levels by Western blot analysis. For each protein, a representative blot is shown, and its quantification by densitometry is expressed relative to the loading control, LMNB1. Values represent the means ± SEM (n = 4–7) $*P < 0.05$ compared with controls.

that differences in the length of treatment (5–6 wk vs. 9 wk in the present study) are an important factor. According to the main biological functions of the affected genes, the round spermatids show a stress response after treatment, because the expression of 27 genes involved in the response to stress was activated. Alternatively, because alkylating agents cause DNA breaks [18], the cells that can repair their DNA may be the ones that survive the insult. Interestingly, the expression of only one gene (Non-POU-domain-containing, octamer-binding protein: *Nono*) known to be involved in DNA repair processes [31] was affected. Although we did not measure protein activity, these results suggest that DNA repair in round spermatids is not a primary target of BEP treatment. We can hypothesize that DNA repair occurred earlier in spermatogenesis [32], with the result that DNA damage may have already been repaired in round spermatids.

Because chemotherapeutic compounds affect DNA, potentially creating DNA strand breaks and mutations [18], a possible mechanism by which these drugs deregulate transcription would be by affecting the integrity of regulatory sequences in the genome. Anticancer drugs, such as bleomycin and cisplatin, may also induce a DNA damage response, triggering the expression of genes involved in cell fate determination. Alternatively, BEP may indirectly affect the gene expression profile in male germ cells through hormonal changes. Indeed, spermatogenesis is an endocrine-regulated function, and testosterone, LH, and FSH regulate gene expression in germ cells [33]. Other indirect mechanisms are also possible. Indeed, because germ cells are transcriptionally active during spermatogenesis from spermatogonia to early spermatids [34], the RNA population present in the round spermatids may result from transcription that occurred in any preceding cell type. Considering the 9-wk treatment length, any change in gene expression in the round spermatids could be due to an effect on spermatogonia or spermatocytes because these cells would have had time to mature into round spermatids. Some mechanisms of transcriptional regulation have been shown to be testis specific [34], all of which could potentially be affected by BEP treatment; these include DNA methylation status [35, 36]; levels and activity of transcription factors, such as TATA-binding protein; polymerase II (*Tbp*); and/or cAMP-responsive element-binding protein and modulator family (*Creb* and *Crem*; reviewed in Kimmins et al. [37]). Interestingly, BEP activated the expression of 11 genes having potential transcription regulation functions, including CREB-binding protein/p300-interaction transactivators 1 and 2 (*Cited1* and *Cited2*). Because CREM is activated in the testis by FHL5 (also known as ACT) [38], a LIM domain protein, it is also interesting to note that BEP increased the expression of the LIM domain-only 4 (*Lmo4*). LMO4 protein is highly expressed in the testis [39] and forms a complex with CREB in cortical neurons [40].

Further analysis of the potential interactions between the genes affected in the round spermatids after BEP treatment was done to elucidate the pathways that may lead to the survival of damaged cells and production of abnormal sperm. BEP induced the expression of many converging pathways with multiple proteins interrelated with JUN and JUNB. The activation of these immediate early genes is probably the result of the DNA damage response induced by the drugs. The *Jun* protooncogene family is involved in cell proliferation and differentiation (reviewed in Angel and Karin [41]). Thus, an increase in the expression of these genes might result in a deregulation of the cell cycle checkpoints in turn, leading to the survival of abnormal cells. The expression patterns of *Jun* and *Junb* have been described in germ cells during spermatogenesis as stage-specific, with higher levels of these mRNAs in premeiotic cells [42]. Here, we describe their protein expression patterns: JUN was present in the nuclei of all male germ cells, from pachytene spermatocytes to elongated spermatids, whereas JUNB was present in pachytene spermatocytes, round spermatids up to steps 8–9 of elongation, and mature spermatozoa. Schultz et al. [43] described a stage-specific expression pattern of JUN, with expression restricted to the primary pachytene spermatocytes. The discrepancy with our results could be due to a number of reasons, including a difference in the techniques used (testicular sections vs. isolated germ cells on slides) and the very low level of expression.

We have assessed the impact of BEP treatment on the expression of *Jun* and *Junb* compared with controls that were handled using the exact same procedures. Thus, our treatment-related induction of *Jun* and *Junb* expression is not due to any effect of the cell separation technique used [44]. The increase in *Jun* mRNA observed in round spermatids did not lead to an increase in the Jun protein product in these cells, but did so at a later stage, during elongation. The accumulation of mRNA in the cytoplasm of spermatids as translationally inactive messenger ribonucleoprotein (mRNP) particles has been described for other genes [45]; the translation of these mRNP particles is activated later during spermiogenesis. JUNB was present only in elongated spermatids; no increase in JUNB protein was detectable by Western blot analysis in these germ cells. BEP treatment-induced expression of *Jun* and *Junb* may alter the program of germ cell development and cell fate determination, because a role for these protooncogenes in the regulation of spermatogenesis [42, 44] and in regulating gene expression guiding developmental transition has been proposed.

Our study identified genes involved in the response of male germ cells to BEP treatment, providing new insight into cellular pathways driving germ cell differentiation and identifying potential new biomarkers of abnormal male germ cells that could lead to adverse progeny outcome.

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