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Essential Role of Neutrophils in Germ Cell-Specific Apoptosis Following Ischemia/Reperfusion Injury of the Mouse Testis[1]

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ABSTRACT

This study investigates the role of neutrophils in ischemia-induced aspermatogenesis in the mouse. Previous studies in the rat have demonstrated that ischemia-inducing testicular torsion followed by torsion repair and reperfusion resulted in germ cell-specific apoptosis. This was correlated with an increase in neutrophil adhesion to subtunical venules, an increase in reactive oxygen species, and increased expression of several apoptosis-associated molecules. In the present investigation, wild-type C57BL/6 mice were subjected to various degrees and duration of testicular torsion. A torsion of 720° for 2 h caused disruption of the seminiferous epithelium and significantly reduced testis weight and daily sperm production. An immunohistochemical method specific for apoptotic nuclei indicated that these effects were due to germ cell-specific apoptosis. An increase in myeloperoxidase (MPO) activity and an increase in the number of neutrophils adhering to testicular subtunical venules after torsion repair/reperfusion demonstrated an increase in neutrophil recruitment to the testis. In contrast, E-selectin knockout mice and wild-type mice rendered neutropenic showed a significant decrease in neutrophil recruitment as evidenced by MPO activity and microscopic examination of subtunical venules. Importantly, germ cell-specific apoptosis was also reduced. Thus, germ cell-specific apoptosis is observed after ischemia/reperfusion of the murine testis, and this apoptosis is directly linked to the recruitment of neutrophils to subtunical venules. Endothelial cell adhesion molecules, particularly E-selectin, play an important role in mediating this pathology.

apoptosis, testis

INTRODUCTION

Testicular torsion is a pathologic condition in humans that renders the testis ischemic, and surgical intervention is usually required to reestablish blood flow. Even with successful surgical repair, however, testicular atrophy is a common clinical outcome and is a significant urological issue [1]. In a rat model of testicular torsion, permanent loss of spermatogenesis is observed after torsion repair despite the return of blood flow [2] and the maintenance of Leydig [3] and Sertoli [4, 5] cell function. This loss of spermatogenesis has been shown to be due to germ cell-specific apoptosis [6, 7]. Previous work using the in situ TUNEL technique has demonstrated that spermatogonia are the primary cell types undergoing apoptosis after a 1-h, 720° testicular torsion followed by torsion repair [6]. This observation has recently been confirmed using the same rat model system but employing an immunohistochemical method of detecting apoptotic cells in situ [7]. Spermatogonia in stages II and III of the seminiferous tubule epithelial cycle were the primary cells targeted by this injury [7]. Germ cell-specific apoptosis coincided with an increase in polymorphonuclear (PMN) cells adhering to subtunical venules and a corresponding increase in interstitial reactive oxygen species (ROS) as assessed by thiobarbituric acid reactive substances (TBARS) assay [6].

Recent studies from this laboratory have focused on the molecular mechanisms of the induction of germ cell-specific apoptosis after torsion-associated testicular ischemia in the rat. These studies have demonstrated that induction of germ cell apoptosis in this model is most likely through an intrinsic pathway involving the mitochondria, as indicated by increases in Bax, Bcl-X_S, and cytoplasmic cytochrome c; however, stimulation of the Fas/FasL, or extrinsic pathway is also a potential contributor to the initiation of germ cell apoptosis [7]. The upstream events responsible for stimulating these two apoptotic pathways have not been addressed, but it has been hypothesized that PMN recruitment to subtunical venules of the testis is involved in the initial steps of the pathway to germ cell death [6, 7].

An increase in adhesion of PMNs to venules has been noted after ischemia/reperfusion in several organs [8] and is believed to be involved in tissue damage subsequent to reperfusion [9, 10]. This pathologic response is commonly referred to as ischemia/reperfusion injury and resembles an inflammatory response [11, 12]. Damage to endothelial cells following ischemia/reperfusion of other tissues results in the cell surface expression of cell adhesion molecules involved in PMN recruitment [13]. One class of endothelial cell adhesion molecules are the selectins [14]. The selectin family consists of three members; L, P, and E selectins. L-Selectin is found on leukocytes, P-selectin can be expressed on platelets and endothelial cells, and E-selectin is found exclusively on endothelial cells [15]. E-selectin is not present on the surface of unstimulated endothelial cells, however, upon stimulation with interleukin-1 (IL-1), tumor necrosis factor α (TNFα), or endotoxin it is transcriptionally up-regulated. Expression is maximal 3–8 h after stimulation and gradually decreases within 12–24 h [16].

The aim of the present study was to investigate the role of neutrophil recruitment in the pathway to germ cell-specific apoptosis after ischemia/reperfusion of the testis. Determining the involvement of neutrophils and factors required for their recruitment may lead to the design of new therapies to protect spermatogenesis as well as to new insights into ischemia/reperfusion injury in other organs.

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MATERIALS AND METHODS

Animals

This work was conducted in accordance with the *Guiding Principals of the Care and Use of Research Animals* promulgated by the Society for the Study of Reproduction. Male adult, C57BL/6 mice (wild-type) were obtained from the University of Virginia's vivarium sources. Adult male E-selectin knockout homozygous mice [17] are maintained in colonies at the Center for Comparative Medicine at the University of Virginia. E-selectin heterozygous mice were achieved by mating E-selectin homozygous knockout males with C57BL/6 females. Male E-selectin heterozygotes derived from these matings were housed until 5–6 wk of age, after which they were considered mature and were used in experiments. Wild-type C57BL/6 mice were rendered neutropenic by an i.p. injection of a 1:20 dilution of antineutrophil antiserum (Inter-Cell Technologies, Inc., Hopewell, NJ). Mice were bled prior to and 24 h after the injection, and blood neutrophil counts were performed to ensure neutropenia. Briefly, 5 μl of blood was collected from the tail vein, blood cells were diluted and stained in Kimura stain, and neutrophils were counted with the aid of a hemocytometer. Pilot experiments revealed that mice remained neutropenic for approximately 45 h after the administration of the antiserum. All mice were maintained on a 12L:12D cycle with food and water ad libitum.

Experimental Testicular Torsion

Adult male C57BL/6 mice were anesthetized with an i.p. injection of 0.01 mg/g sodium pentobarbital, and the testis was rotated as described by Turner et al. [6]. Briefly, the testis was exteriorized through a low midline laparotomy, the gubernaculum was divided, and the testis was freed from the epididymo-testicular membrane. To determine the appropriate degree and duration of torsion, mice were divided into 7 groups: 1) control (nonoperated), 2) 360° sham, 3) 720° sham, 4) 360°/1 h, 5) 720°/1 h, 6) 360°/2 h, and 7) 720°/2 h. During the sham or torsion period the testis remained in the abdomen with a closed incision. At the appropriate time the incision was reopened, the testis was counter-rotated to the natural position, the gubernaculum was rejoined, and the testis was reinserted into the scrotum via the inguinal canal. At the time of repair testes were examined and scored for apparent degree of ischemia and of reperfusion, respectively. Testes were collected 15 days after the repair of torsion, fixed in Bouin solution, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histological examination. Sham-operated animals were treated identically except that upon completion of the torsion maneuver, the testis was immediately counter-rotated.

After histological assessment, the degree and duration of torsion that demonstrated the most consistent disruption of spermatogenesis was then applied to another group of wild-type mice as well as a sham control group (n = 5 per group). Animals were killed 30 days after the repair of torsion and the testes were examined for atrophy and daily sperm production (DSP).

Measurement of Testicular Blood Flow

A laser-Doppler flow meter and flow probe (ALF-21, Transonic Systems Inc., Ithaca, NY) attached to a micromanipulator were used to monitor testicular microvascular flow in real time. The flow probe (1-mm diameter) is capable of monitoring microvascular perfusion in a tissue volume of approximately 1 mm^3. The principles of laser-Doppler flowmetry have been described elsewhere [18, 19]. Briefly, the fiber-optic flow probe directs monochromatic light to a microvascular bed where the light is subjected to a Doppler shift by moving red cells. Reflected light travels back through the fiber-optic cable to a photo detector, and the output signal is processed to report blood flow as relative perfusion units (PUs).

The laser-Doppler flow probe was carefully positioned to monitor flow over microvascular fields only. Measurements of blood flow were recorded over 1 min and averaged. A computer interface and FlowTrace software (Transonic Systems) were used to analyze the flowmeter output. Background noise was determined in each experiment by positioning the probe over the testis 10 min after cessation of a heartbeat. This value was subtracted from all blood flow data before any subsequent calculation. Blood flow was recorded prior to torsion, 110 min after torsion during the ischemic period, and 5 min after the repair of a 2-h torsion.

Histological Assessment of Testes after the Repair of Testicular Torsion

Histological sections of testes from the above-mentioned seven groups were qualitatively scored for disruption of spermatogenesis by two independent investigators. Mid-testis cross-sections were given a score from 0 to 3 on basis of the following criterion: 0 = normal seminiferous histology, no disruption of spermatogenesis; 1 = slight effect, few tubule cross-sections show disruption of seminiferous epithelium; 2 = moderate effect, many tubule cross-sections show disruption of seminiferous epithelium. Disruptions are largely limited to loss of spermatids and spermatozoa, but some tubules can exhibit complete loss of germinal elements. The majority of tubules remain apparently normal; and 3 = severely affected, the majority of tubule cross-sections show disruption of seminiferous epithelium and complete loss of germinal elements.

Evaluation of Sperm Production

Thirty days after the repair of testicular torsion or sham operation, testes were removed and immediately weighed to assess torsion-induced atrophy. DSP was also determined according to the method of Robb et al. [20] as reported previously from this laboratory [6]. Briefly, testes were weighed and then homogenized in 50 ml of 0.154 M NaCl, 0.5% Triton X-100 (v:v), 2% sodium azide, and eosin Y. The concentration of sperm nuclei was calculated using a hemocytometer and expressed as DSP (sperm $\times$ 10^6/g testis per day). Testicular sperm output (TSO; sperm $\times$ 10^6/testis per day) was also calculated as an indicator of the net effect on the individual testis.

Evaluation of Germ Cell Apoptosis

Germ cell apoptosis was examined immunohistochemically with the monoclonal antibody F7-26 (Apostain; Alexis Corporation, San Diego, CA) directed against single-stranded DNA (ssDNA). At the specified time points after torsion, repair testes were removed from the scrotum, rinsed in saline, immersed in Bouin fixative for 6 h, and paraffin-embedded. The Apostain technique was performed according to the manufacturer's protocol. Briefly, sections were deparaffinized, rehydrated, rinsed in 5 mM $MgCl_2$ in PBS, rinsed in distilled H_2O, and incubated for 15 min in ice-cold 0.1 N HCl. Subsequently, sections were rinsed in distilled H_2O and incubated for 5 min in 5 mM $MgCl_2$ and 0.2% Triton X-100 in PBS. The slides were then placed into 50-ml centrifuge tubes containing 30 ml of 50% formamide, and the tubes were immersed for 20 min in water preheated to 56°C. After heating the slides were immediately removed and transferred into ice-cold PBS for 10 min. Slides were then immersed in 3% H_2O_2 to block endogenous peroxidases, immersed in 0.1% BSA, 1% nonfat dry milk to block nonspecific binding of antibody, rinsed in PBS, and incubated overnight with a 1:100 dilution of F7-26. Slides were washed, incubated for 1 h with biotinylated rat anti-mouse antibody (Zymed, San Francisco, CA), and washed. The biotinylated secondary antibody was visualized with avidin-biotin-peroxidase complex (Elite ABC Kit; Vector Laboratories, Burlingame, CA) and diaminobenzidine (DAB; Sigma Chemical Co., St. Louis, MO) as the chromogen. Sections were counterstained with hematoxylin, dehydrated, and mounted.

The number of apoptotic cells was evaluated by counting the positively stained nuclei in 30 circular seminiferous tubule cross-sections per testis section. Data were averaged for each testis and expressed as apoptotic cells per tubule cross-section. Statistical evaluations were by analysis of variance followed by the Tukey range test ($P < 0.05$) after evaluation of each data set for homogeneity by Chauvenet's criterion [21].

Evaluation of Testicular Neutrophil Content

Testicular neutrophil content was determined by myeloperoxidase (MPO) assay. MPO is stored in primary granules of neutrophils and the enzyme activity is a common measure of neutrophil accumulation [22]. Following testicular torsion and repair, testes were removed and snap-frozen at −80°C. Tissues were homogenized in 2.0 ml of ice-cold 20 mM KPO_4 buffer pH 7.4, centrifuged at 17 000 $\times$ *g* at 4°C for 30 min, and the pellets were resuspended in ice-cold KPO_4 buffer pH 7.4. Suspensions were then spun two more times and 0.5% (w/v) hexacyltrimethylamonium bromide, 10 mM EDTA in 50 mM KPO_4 pH 6.0 was added to the remaining pellet. Suspensions were sonicated five times for 1 sec each on ice, freeze-thawed three times, incubated at 4°C for 20 min, and centrifuged at 17 000 $\times$ *g* for 15 min at 4°C. The resulting supernatant was assayed for protein concentration using bicinchoninic acid assay (BCA Protein Assay, Pierce, Rockford, IL) to ensure equal amounts of protein in the resulting supernatants were assayed. Duplicate samples of the supernatant were incubated with 0.2 mg/ml o-dianisidine, 158 μM H_2O_2 in 50 mM KPO_4 pH 6.0 at a ratio of 4:1, and changes in absorbance were

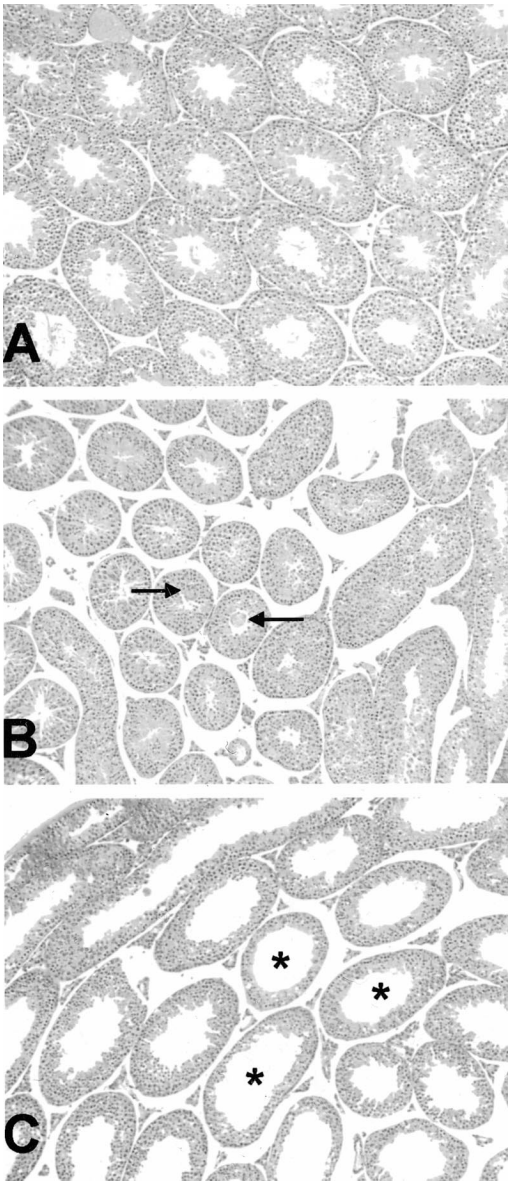

FIG. 1. Testis sections from wild-type C57BL/6 mice 15 days after **A**) sham operation, **B**) after 1-h 720° torsion, **C**) 2-h 720° torsion; stained with H&E. Arrows point to multinucleated cells and asterisk indicates seminiferous tubules with severe disruption of the seminiferous epithelium. Magnification ×60.

detected at 460 nm using a microtiter photometric plate reader (Titer-Tek, Huntsville, AL).

Evaluation of Testicular Subtunical Venule Neutrophil Margination

Subtunical venules were examined for the accumulation of neutrophils from various groups 4 h after the repair of torsion as well as from sham controls. Briefly, H&E-stained sections of testes were examined using a Nikon Microphot SA microscope with an attached CCD-camera and ISee software (Inovision Corporation, Raleigh, NC). Images were captured and subtunical venules were traced using ISee software. The intraluminal area of each vessel was determined. Adherent neutrophils were then counted and the numbers expressed as neutrophils per mm^2 of luminal area.

Statistical Analysis

All statistical evaluations were either by ANOVA followed by the Tukey range test or the Student *t*-test ($P < 0.05$) after evaluation of each data set by Chauvenet's criterion for homogeneity.

TABLE 1. Qualitative histological scores* (mean ± SEM) of mouse testes after various times (h) and degrees (°) of testicular torsion.

Group	Score	Group	Score	Group	Score
Control	0.0 ± 0.0	—	—	—	—
360°/sham	0.2 ± 0.2	360°/1h	0.7 ± 0.5	360°/2h	1.8 ± 0.5
720°/sham	0.5 ± 0.3	720°/1h	0.7 ± 0.2	720°/2h	2.5 ± 0.3

* Mid-testis cross sections scored independently by two investigators for overall disruption of spermatogenesis. 0 = Normal seminiferous histology, no disruption. 1 = Slight effect, few tubule cross-sections show disruption of seminiferous epithelium. 2 = Moderate effect, many tubule cross-sections show disruption of seminiferous epithelium; disruptions are largely limited to loss of spermatids and spermatozoa but some tubules can exhibit complete loss of germinal elements; the majority of tubules remain apparently normal. 3 = Severe effect, the majority of tubule cross-sections show disruption of seminiferous epithelium; tubule cross-sections often exhibit complete loss of germinal elements.

RESULTS

Evaluation of Testicular Blood Flow During and after Repair of Torsion

Testicular microvascular blood flow prior to testicular torsion was 16.3 ± 1.9 PU. One hundred ten minutes after testicular torsion, mean microvascular blood flow dropped to 0.2 ± 0.2 PU. Five minutes after the repair of torsion, mean microvascular blood flow was 7.1 ± 1.0 PU (n = 4, for each group).

Evaluation of Experimental Testicular Torsion

Sections of testes from control (nonoperated) wild-type C57BL/6 mice; or from the same strain mice 15 days after sham operation; or after torsion of 360° for 1 h, 720° for 1 h, 360° for 2 h, or 720° for 2 h were examined for disruption of spermatogenesis. Figure 1 illustrates sections from testes having received a sham operation, a 1-h, 720°; or a 2-h, 720° torsion. Sham-operated mice demonstrated normal seminiferous tubule morphology. Mice that received a 720° testicular torsion had moderate-to-severe disruptions of the seminiferous epithelium, depending on the duration of the torsion. Mice that underwent a 720° testicular torsion for 2 h displayed the most severe disruption of the seminiferous epithelium compared with the other torsion groups (Table 1).

A 2-h, 720° testicular torsion demonstrated a severe phenotype by histology; thus, this degree and duration of torsion was applied to other groups of wild-type mice that were killed 30 days later. A significant decrease in testis weight, DSP, and TSO was observed in this group compared with control and sham-operated mice (Fig. 2). Thus, a 2-h, 720° torsion causes a morphological disruption to the seminiferous epithelium, testicular atrophy, and a decrease in DSP. A 2-h, 720° testicular torsion was employed in all subsequent torsion experiments.

Evaluation of Germ Cell Apoptosis in Wild-Type C57BL/6 Mice

Tissue sections from testis from wild-type, sham-operated animals revealed very few nuclei detected by the Apostain technique (Fig. 3A). Sections from mice 4 h after the repair of torsion demonstrated a trend toward an increase in the number of stained germ cell nuclei (Fig. 3, B and D), which became significantly different 24 h after the repair of torsion (Fig. 3, C and D). Spermatogonial nuclei were the predominant cell type undergoing apoptosis following the repair of testicular torsion.

Neutrophil Recruitment Following Testicular Torsion in Wild-Type C57BL/6 Mice

Sections from sham-operated, wild-type C57BL/6 mice revealed that very few neutrophils adhered to subtunical venules (Fig. 4, A and C), whereas sections from wild-type mice 4 h after the repair of torsion displayed a significant increase in the presence of neutrophils in subtunical venules (Fig. 4, B and C). Results from MPO assays revealed a significant increase in MPO activity from wild-type testes 4 h after the repair of torsion (Fig. 4D). This increase in MPO activity indirectly indicates a recruitment of neutrophils to the testis after torsion repair.

Evaluation of Germ Cell Apoptosis in E-Selectin-Deficient and Neutropenic Mice Following Testicular Torsion

As in wild-type mice, tissue sections from sham-operated, E-selectin knockout mice displayed very few apoptotic nuclei (Fig. 5A). In contrast to wild-type testes, E-selectin knockout mice testes did not show histological evidence of increased apoptosis at either 4 (Fig. 5B) or 24 (not shown) h after repair of torsion. In fact, a significant decrease in apoptotic germ cells was noted in E-selectin knockout mice at both 4 and 24 h after the repair of torsion compared with wild-type mice (Fig. 6). E-Selectin heterozygous animals displayed values of germ apoptosis intermediate to E-selectin knockout mice and wild-type mice when assessed 4 h after the repair of torsion (Fig. 6).

Wild-type mice were rendered neutropenic by the administration of antineutrophil antiserum. The concentration of neutrophils in wild-type mice prior to antiserum administration was 690 ± 77.5 neutrophils/μl, and this value decreased to 290 ± 28.5 neutrophils/μl, 24 h after the administration of the antineutrophil antiserum. Neutropenic mice killed 4 h after the repair of torsion revealed a decrease in germ cell apoptosis compared with normal wild-type mice (Fig. 6).

Evaluation of Neutrophil Recruitment in E-Selectin-Deficient and Neutropenic Mice Following Testicular Torsion

In contrast to the data obtained from wild-type mice (Fig. 4), MPO assays performed on testes from E-selectin knockout mice revealed no significant increase in MPO activity 4 h after the repair of torsion (Fig. 7). MPO assays performed on testes from mice rendered neutropenic revealed an increase in MPO activity 4 h after the repair of torsion (Fig. 7); however, this response to ischemia/reperfusion is reduced compared with that observed in wild-type mice (Fig. 4). Consistent with the MPO data above, no recruitment of neutrophils to subtunical venules was observed in the E-selectin knockout animals, whereas few neutrophils could be observed in the subtunical venules of the neutropenic mice (Fig. 8).

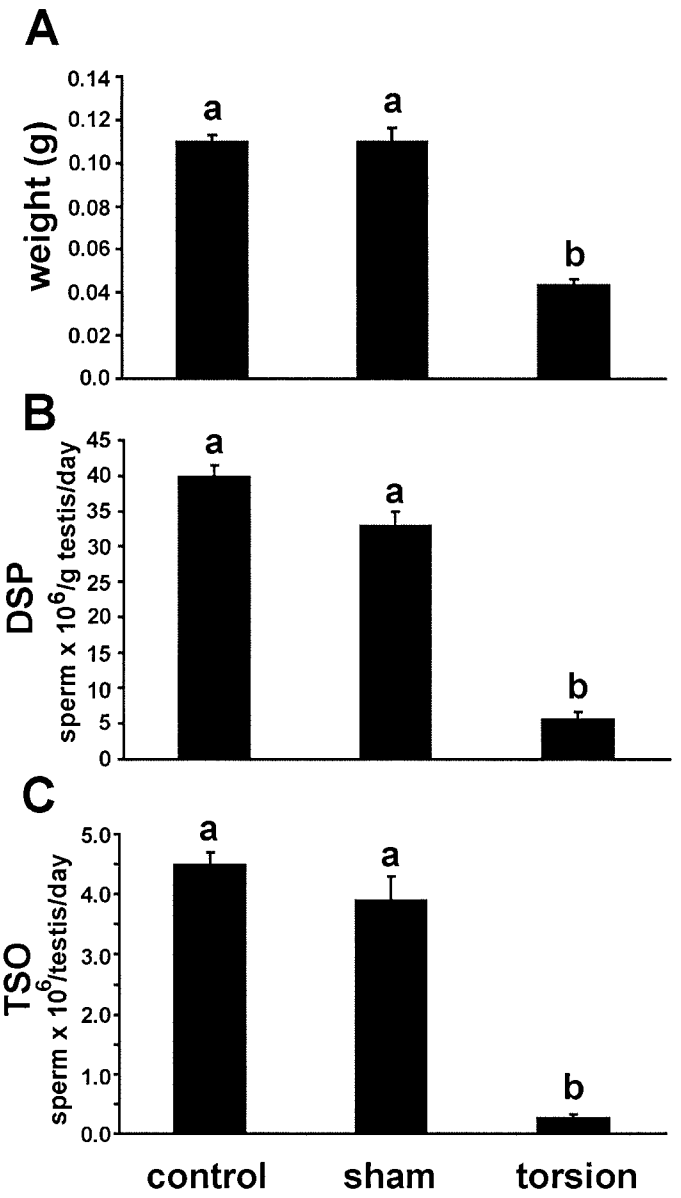

FIG. 2. Histograms of **A**) testis weight, **B**) daily sperm production (DSP), and **C**) testicular sperm output (TSO) from wild-type C57BL/6 mice 30 days after subjection to control (no procedure; n = 6), sham-operated (n = 7), and 2-h, 720° torsion followed by repair (n = 5). Bars represent the mean ± SEM. Bars sharing the same letter are not statistically different ($P < 0.05$).

DISCUSSION

In ischemia/reperfusion injury of the rat testis germ cell-specific apoptosis has been observed contemporaneously with an increase in leukocyte margination and diapedesis [6]. An increase in ROS as evident by an increase in testicular lipid peroxidation and by the ability of certain reactive oxygen scavengers to partially abrogate the torsion-induced apoptosis has also been observed [6]; thus, it has been hypothesized that the pathology seen after testicular torsion is a classical ischemia/reperfusion injury, and that neutrophils were the initiator cells for the molecular events leading to germ cell apoptosis [6]. However, the recruitment and involvement of neutrophils in torsion-induced germ cell apoptosis has not been directly linked. Results from the present study demonstrate an essential role of neutrophils in the germ cell death observed after testicular ischemia/reperfusion.

This is the first report of germ cell-specific apoptosis in a mouse model of testicular torsion. Previous studies in a rat model of testicular torsion found that a 720° torsion is sufficient to virtually eliminate testicular blood flow [2, 18], and that reperfusion begins immediately after repair of a 1-h or 2-h torsion with mean blood flow returning to control values within 1 h [18, 23]. In the rat model, a 1-h, 720° torsion is sufficient to cause germ cell apoptosis and render the animal permanently azoospermic [6]. In the present

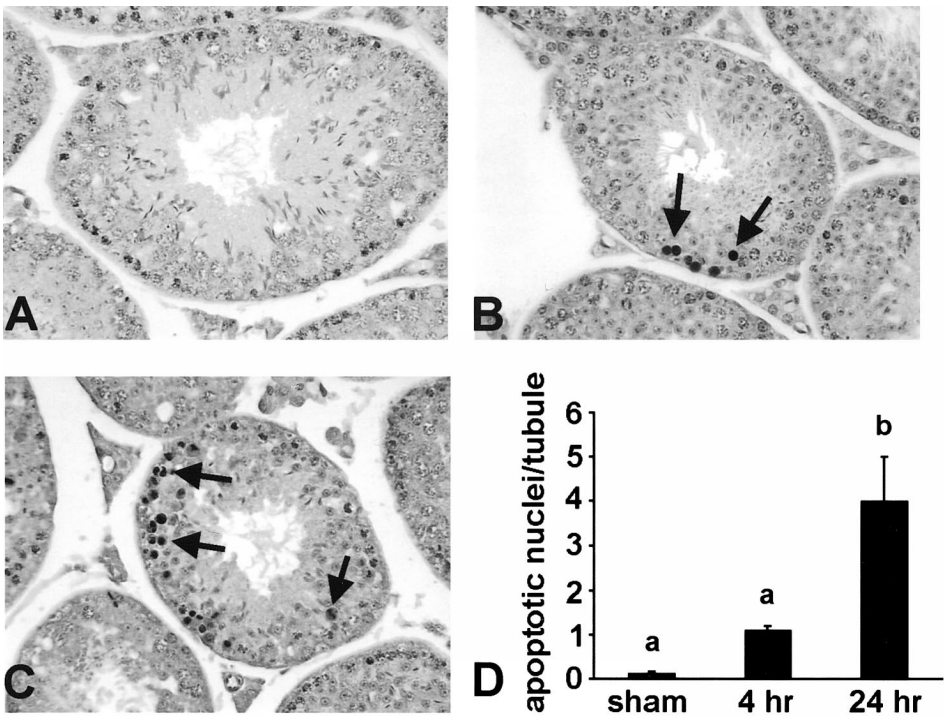

FIG. 3. Representative sections of wild-type C57BL/6 mice testes from **A**) sham-operated, **B**) 4 h after the repair of a 2-h, 720° torsion, and **C**) 24 h after the repair of a 2-h, 720° torsion stained with Apostain and counterstained with hematoxylin. Arrows point to Apostain-positive nuclei. Magnification ×219. **D**) Apostain-positive germ cell nuclei from C57BL/6 mice sham operated (n = 10), and those killed at 4 h (n = 5) and 24 h (n = 9) after the repair of a 2-h, 720° torsion. Bars represent the mean ± SEM. Bars sharing the same letter are not statistically different ($P < 0.05$).

study the model was shifted to the mouse, in which it was found that 720° torsion virtually eliminated testicular microvascular blood flow as well (~16 PU in controls vs. ~0.2 PU during torsion), and reperfusion was well underway 10 min after torsion repair (microvascular blood flow ~7 PU). This pattern of return flow also fits well with data from the rat [18, 23] and demonstrates in the mouse that experimental, 720° testicular torsion followed by repair induces ischemia followed by reperfusion. A 720° torsion applied for 2 h was necessary to cause disruption of the seminiferous epithelium, germ cell-specific apoptosis, and reduced DSP (Figs. 1–3; Table 1) in wild-type C57BL/6 mice. TSO was more markedly reduced after torsion repair than DSP, due to the added effect of loss of testis weight.

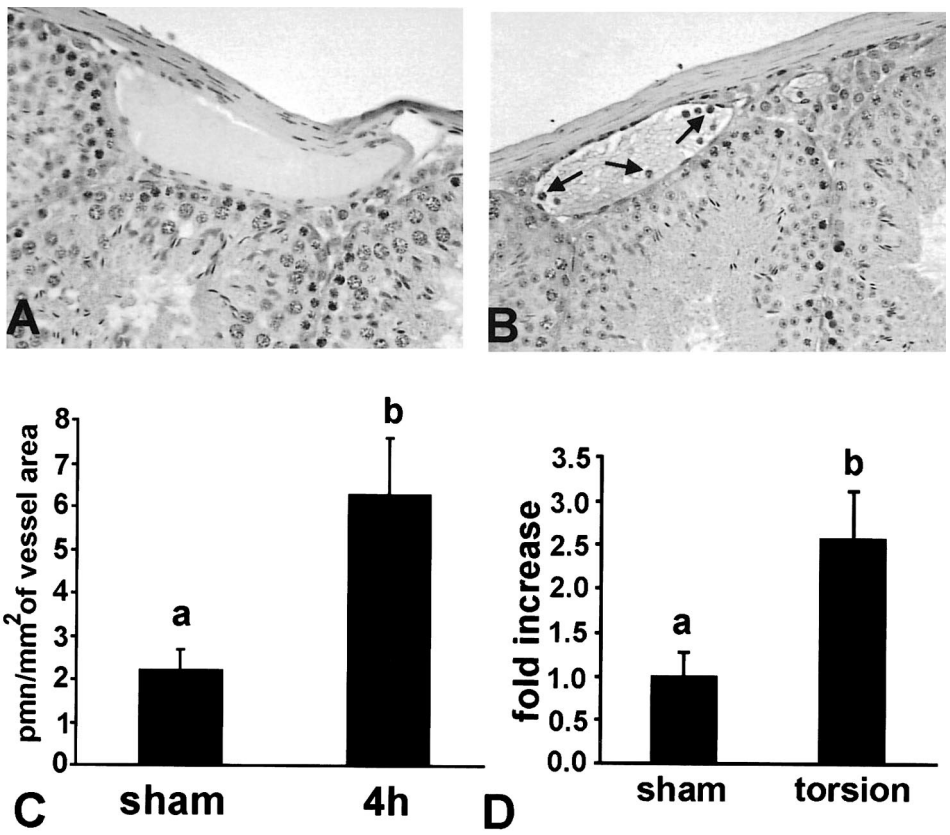

FIG. 4. Representative sections of testes from wild-type C57BL/6 mice killed 4 h, after receiving **A**) a sham operation or **B**) a 2-h, 720° torsion. Arrows indicate adherent polymorphonuclear (PMN) cells in subtunical venules. Magnification ×219. **C**) Adherent PMNs in subtunical venules from wild-type mice from the same groups as above (n = 5 for all groups). **D**) Histogram of myeloperoxidase activity from proteins from wild-type mice killed 4 h after receiving a sham operation (n = 5) or a 2-h, 720° torsion (n = 5). Bars represent the mean ± SEM. Bars sharing the same letter are not significantly different ($P < 0.05$).

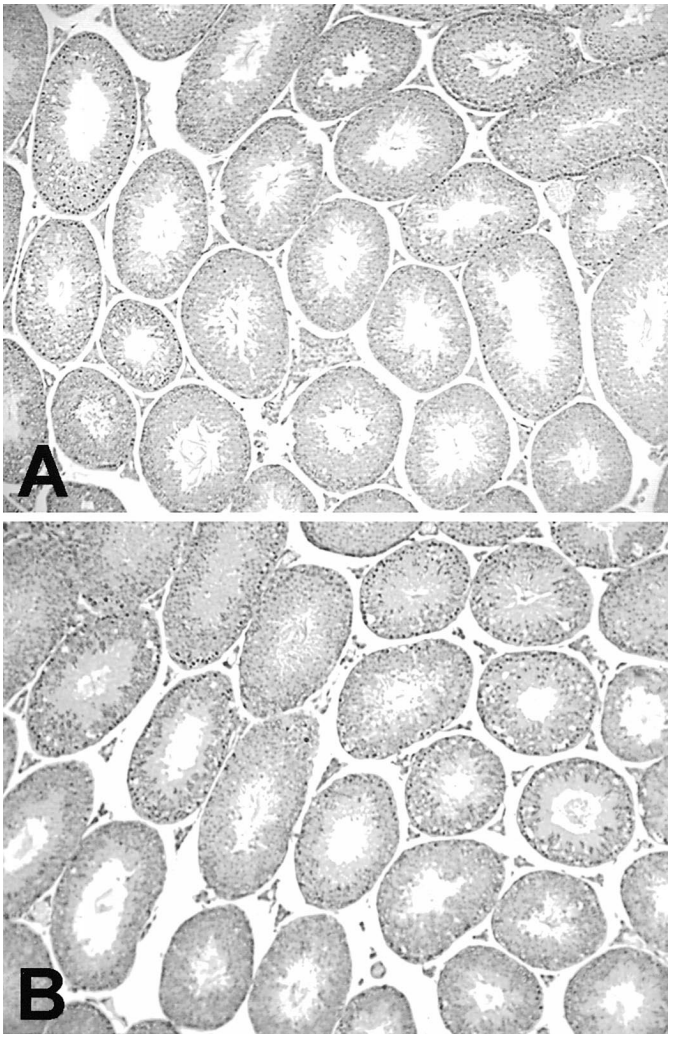

FIG. 5. Representative sections of E-selectin knockout mice testes from **A**) sham-operated and **B**) 4 h after the repair of a 2-h, 720° torsion stained with Apostain and counterstained with hematoxylin. Magnification ×60.

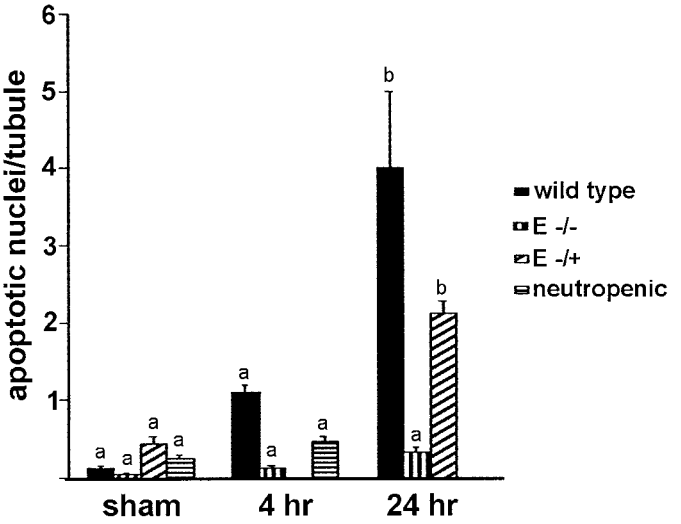

FIG. 6. Apostain-positive germ cell nuclei from wild-type C57BL/6 mice (from Fig. 3) and E-selectin knockout mice from sham-operated, and those killed at 4 and 24 h after the repair of a 2-h, 720° torsion. Apostain-positive germ cell nuclei were also examined in E-selectin heterozygous mice after sham-operated and 24 h after the repair of torsion as well as from neutropenic C57BL/6 mice after sham-operated and 4 h after the repair of torsion. Bars represent the mean ± SEM. Bars sharing the same letter are not significantly different ($P < 0.05$).

Spermatogonia were the primary cell type undergoing apoptosis after ischemia/reperfusion injury. In no experiments were Leydig or Sertoli cells observed to be undergoing apoptosis. This corresponds well with the previous rat data that demonstrated Leydig [3] and Sertoli [4, 5] cells remained functional following torsion. As in the rat model, an accumulation of neutrophils was also noted in the mouse testis after repair of torsion. This was shown by an increase in MPO activity after the repair of torsion and by histological examination of neutrophils in subtunical venules.

Neutrophil recruitment during an inflammatory response involves a series of regulated events involving at least four families of cell adhesion molecules [24]. Selectins aid in the capture and attachment of blood leukocytes to the vascular endothelium. This initial step results in slow, downstream rolling of the leukocyte on the vascular endothelium [25, 26]. Other cellular adhesion molecules, such as integrins, then aid in the firm adhesion and transmigration of leukocytes [24]. Similar leukocyte recruitment cascades have been shown to occur after ischemia/reperfusion in other tissues [12, 27]. The increase in neutrophil adhesion seen 4 h after the repair of torsion corresponds well with the timing of E-selectin expression seen in other models of ischemia/reperfusion injury [28] or after the administration of inflammatory cytokines [16, 29].

The failure of testicular torsion to stimulate apoptosis in mice rendered neutropenic indicates that neutrophils are essential for the observed pathology. Neutrophils recruited to the subtunical venules may secrete inflammatory cytokines such as TNFα, IL-1β, and IL-8 [30]. These aid in the recruitment of other leukocytes and enhance expression of the endothelial cell adhesion molecules, E-selectin and in-

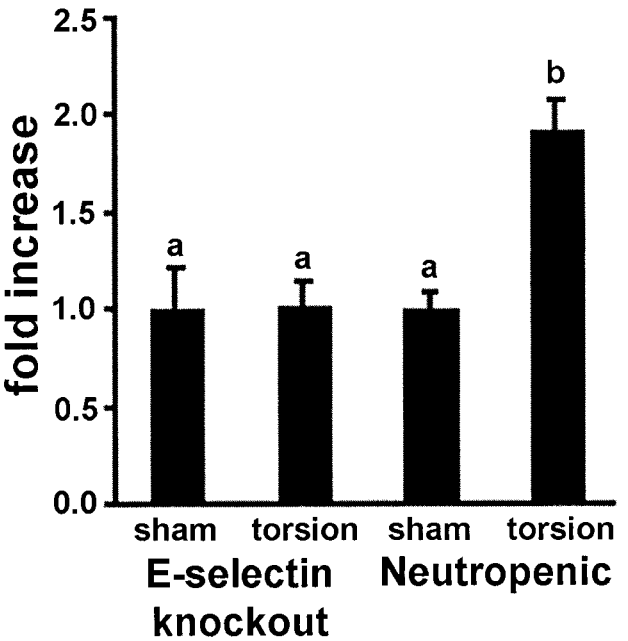

FIG. 7. Myeloperoxidase activity from proteins from E-selectin knockout mice and neutropenic C57BL/6 mice killed 4 h after receiving a sham operation or a 2-h, 720° torsion. Bars represent the mean ± SEM; n = 5 for all groups. Bars sharing the same letter are not statistically different ($P \leq 0.05$).

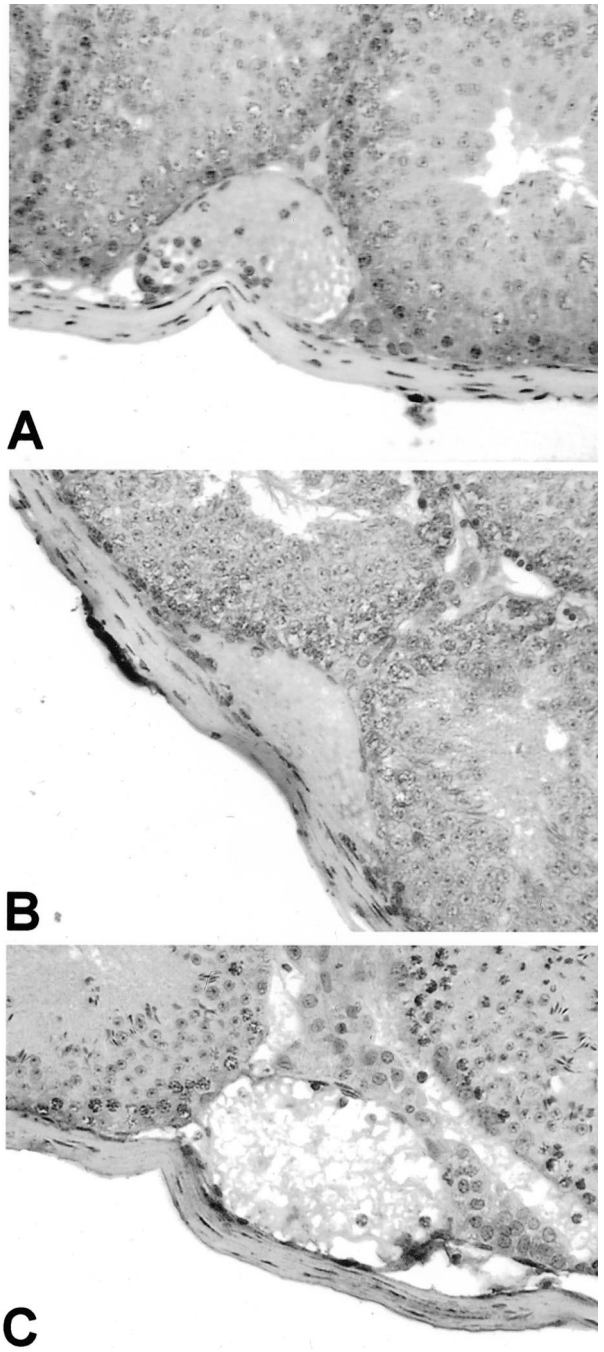

FIG. 8. Representative sections of testes from **A**) wild-type C57BL/6 mice, **B**) E-selectin knockout mice, and **C**) neutropenic C57BL/6 mice killed 4 h after receiving a 2-h, 720° torsion. Magnification ×219.

tercellular adhesion molecule (ICAM)-1 [31]. Once the neutrophils transmigrate through the endothelium into the interstitium of the testis they are poised to release factors such as ROS or FasL that may directly cause apoptosis in the germ cell. Alternatively, the invading neutrophils may secrete factors that influence Sertoli cells to secrete FasL, thus indirectly effecting germ cell death. Indeed, increases in ROS [6] and FasL [7] have been previously reported after the repair of testicular torsion in the rat.

It is also known that macrophages can secrete IL-8 [32] as well as 25-hydroxycholesterol [33], a compound believed to be involved in spermatogenesis, but which at high concentrations can induce apoptosis in a number of cell types [34, 35]. Thus, resident testicular macrophages may play a role both in the recruitment of neutrophils and in the observed germ cell apoptosis seen after ischemia/reperfusion of the testis. This is a possibility yet to be examined.

Many studies have focused on endothelial cell activation and leukocyte recruitment in several models of tissue injury. A recent study examining acute renal failure following ischemia/reperfusion injury of the kidney demonstrated that E-selectin expressed on endothelial cells plays a key role in that pathology, which is abrogated by administering a blocking E-selectin antibody [36]. Similar to the results reported in the present study, a protective effect was observed after ischemia/reperfusion of the kidney in mice rendered neutropenic or in E-selectin-deficient mice [36]. While the absence of E-selectin has not always been found to alter the pathological outcomes in models of inflammation [37], the results of Singbartl and Ley [36] and the present results indicate that strategies that block neutrophil recruitment may be useful in reducing ischemia/reperfusion injury in certain affected tissues. These insights suggest potential medical therapies to improve the rescue of the testis, in particular, after surgical repair of torsion, and future experiments will address these possibilities.

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