Protective effect of vascular endothelial growth factor on histologic changes in testicular ischemia–reperfusion injury

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Objective: To evaluate the efficacy of vascular endothelial growth factor (VEGF) on long-term histologic damage in testicular ischemia–reperfusion injury.
Design: Controlled experimental study using rats.
Setting: University of Mersin School of Medicine, Mersin, Turkey.
Animal(s): Sixteen adult male Wistar rats.
Intervention(s): Five rats underwent 2 hours of testicular torsion. Six rats received VEGF injection into the testis before detorsion. Five rats underwent sham operation.
Main Outcome Measure(s): Mean seminiferous tubular diameter (MSTD), germinal epithelial cell thickness (GECT), mean testicular biopsy score (MTBS), and apoptosis (caspase-3-positive cells).
Result(s): Testicular torsion–detorsion caused a significant decrease in MSTD, GECT, and MTBS and a significant increase in the mean value of caspase-3-positive cells in ipsilateral testes but not in the contralateral testes. The MSTD, GECT, and MTBS of the ipsilateral testes were significantly higher in the group treated with VEGF than in the torsion–detorsion group. The animals treated with VEGF had a significant decrease in the mean value of ipsilateral testicular caspase-3-positive cells compared with the torsion–detorsion group.
Conclusion(s): Vascular endothelial growth factor might have a role in testicular damage caused by ischemia–reperfusion. Administering VEGF before reperfusion might have the potential to decrease the long-term histologic damage after testicular torsion. (Fertil Steril® 2005;84:468–73. ©2005 by American Society for Reproductive Medicine.)

Key Words: Testis, torsion, VEGF, reperfusion injury

Testicular torsion is a surgical emergency. Misdiagnosis and inappropriate treatment of this condition can lead to male factor infertility (1). It seems that the main pathophysiology of testicular torsion is ischemia–reperfusion injury of the testis caused by the twisted spermatic cord and its release (2). Ischemia–reperfusion injury initiates a pathophysiologic cascade, including an activation of neutrophils, inflammatory cytokines, and adhesion molecules with increased thrombogenicity, release of massive intracellular Ca^{2+}, and generation of oxygen-derived free radicals (3). Reactive oxygen species, including superoxide anions, hydrogen peroxide or hydroxyl radicals, and nitric oxide or peroxynitrite, cause DNA damage, endothelial damage, and germinal cell necrosis (2, 4).

Vascular endothelial growth factor (VEGF), an angiogenic peptide, mediates angiogenesis and vasculogenesis and promotes endothelial cell survival (5). Over the past decade, extensive research has been done on VEGF, and the protective effect of VEGF has been shown in various forms of ischemia–reperfusion injury, including brain (6), liver (7), and heart (8). However, no study has investigated the role of VEGF in testicular ischemia–reperfusion injury. The aim of this study was to evaluate the effect of VEGF injection into the testis, especially on spermatogenesis and apoptosis, in a rat testicular ischemia–reperfusion injury model.

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MATERIALS AND METHODS

The study included 16 adult male Wistar rats weighing 280–310 g. The rats were maintained on a 12-hour light/dark cycle. All animal experiments followed a protocol approved by the ethics committee on animal research at our institution. We performed appropriate care and use of laboratory animals as recommended by Board of Registry publication guidelines.

Animal Preparation and Surgical Procedure

The rats were divided into three groups. Surgery was performed with a single dose of intraperitoneal ketamine (50 mg/kg) anesthesia. All surgical procedures were performed through standard ilioinguinal incisions.

In the torsion–detorsion group (n = 5), the gubernaculum was divided, and the testis was freed from its longitudinal

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and distal pole attachment to the epididymis. Torsion was created by rotating the left testis 720° clockwise and maintained by fixing the testis to the scrotum with a 4-zero silk suture placed through the tunica albuginea. After 2 hours of torsion, the testis was counter-rotated back to the natural position and reinserted into the scrotum. The wound was closed with 3-zero catgut suture.

In the VEGF group (n = 6), the same surgical procedure was done as in the torsion–detorsion group, but 4 μg of VEGF 165 (Calbiochem, San Diego, CA) was injected into the upper, lower, and mid-portions of the testis immediately before detorsion.

In the sham group (n = 5), similar to the torsion–detorsion group, the testis was rotated 720° clockwise, but it was then immediately detorsed. A 4-zero silk suture was then placed through the tunica albuginea.

Histologic Preparation

After 60 days, bilateral orchiectomy was performed, and the rats were euthanized by pentobarbital overdose (200 mg/kg) and bilateral thoracotomy. The testicles were fixed in Bouin's solution (7.5 mL of saturated picric acid, 2.65 mL of glacial acetic acid, and 2.5 mL of 7% formaldehyde), post-fixed in 70% alcohol, and embedded in paraffin blocks. Sections of 5 μm were obtained, deparaffinized, and stained with hematoxylin and eosin.

Histologic Evaluation

Testicular tissue was evaluated with standard light microscopy by a blinded observer in random order. Three slides prepared from the upper, lower, and mid-portions of the testes were evaluated completely for each testis. The mean seminiferous tubular diameter (MSTD), germinal epithelial cell thickness (GECT), and mean testicular biopsy score (MTBS) were used to evaluate 20 seminiferous tubules of each section. The MSTD was calculated with a microscope-adaptable micrometer. The MSTD of each testis was determined in micrometers. Germinal epithelial cell thickness was determined by counting the number of epithelial cells from the basement membrane to the lumen at 90°, 180°, 270°, and 360° and averaged. The MTBS was graded with Johnsen's score (9). A score of 0–10 was given to each tubule according to epithelial maturation.

Immunohistochemistry

Sections were dewaxed (xylene 3 × 5 minutes) and rehydrated by passing through graded alcohols and rinsed in water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 10 minutes. Non-specific binding sites were blocked with 2% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline for 30 minutes at room temperature. Afterward, 1/200 diluted polyclonal anti-caspase-3 primary rabbit antibody (Neo Markers, Los Angeles, CA) in phosphate-buffered saline containing solution was dropped on the sections, then the sections were incubated for one night in a refrigerator at 4°C.

The next day, biotin-bound secondary goat antirabbit antibody solution (Jackson Immunoresearch Laboratories, West Grove, PA) was dropped on the sections, then the sections were incubated for 1 hour. They were incubated for an additional 30 minutes with avidin-biotin-peroxidase enzyme reagent and washed. Finally, peroxidase substrate diaminobenzidine was dropped, and the sections were incubated for 10 minutes while staining intensity was checked under the microscope. The sections were washed in distilled water for 5 minutes. Buffer including 0.5% bovine serum albumin and no primary antibody was dropped on the sections separated for negative control. Positive control slides were prepared from human tonsil tissue block.

Evaluation of Apoptosis

Immunohistochemical labeling for caspase-3 was evaluated with standard light microscopy. At ×400 magnification, caspase-3-positive cells were counted in 20 fields per section.

Statistical Analysis

All data were expressed as mean ± SD. Analysis of variance was used for statistical analysis of the data among the groups. Multiple comparisons were made with Tukey's procedure, with $P<.05$ considered statistically significant.

RESULTS

The values of MSTD, GECT, MTBS, and caspase-3-positive cells of each group are shown in Table 1 and Figures 1 and 2. Compared with the sham group, the MSTD, GECT, and MTBS obtained from the torsion–detorsion group were significantly lower and the mean value of caspase-3-positive cells was significantly higher in the ipsilateral testes ($P<.001$). Ipsilateral testicular MSTD, GECT, and MTBS in the VEGF group were significantly higher than the values in the torsion–detorsion group ($P<.01$). The animals treated with VEGF had a significant decrease in the mean value of ipsilateral testicular caspase-3-positive cells compared with the torsion–detorsion group ($P<.01$). All the parameters of contralateral testes did not reveal any significant differences among these groups ($P>.05$).

DISCUSSION

Testicular torsion is a urologic emergency. Salvage rates are directly proportional to the duration of torsion. Early diagnosis with detorsion is the current management for the preservation of the testis and fertility (1). Although reperfusion is essential for the survival of ischemic tissue, the main pathophysiology of testicular torsion is ischemia–reperfusion injury of the testis caused by the twisted spermatic cord and its release (2).

TABLE 1

Mean values of MSTD, GECT, MTBS, and caspase-3-positive cells in ipsilateral and contralateral testes.

Group	MSTD ± SD (μm)		GECT (mean cell layers ± SD)		MTBS ± SD		Caspase-3-positive cells (mean ± SD)	
	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Torsion–detorsion[a]	127.3 ± 23.9	231.4 ± 12.1	1.2 ± 0.6	6.9 ± 0.3	2.9 ± 1.1	9.7 ± 0.2	0.7 ± 0.3	0.2 ± 0.1
VEGF[b]	160.9 ± 15.9	262.1 ± 14.8	3.8 ± 0.2	8.1 ± 0.5	5.1 ± 0.6	9.7 ± 0.1	0.3 ± 0.2	0.1 ± 0.4
Sham	211.2 ± 55.4	273.0 ± 36.8	8.4 ± 0.8	9.3 ± 0.9	9.8 ± 0.4	9.8 ± 0.7	0.2 ± 0.9	0.2 ± 0.1

Note: No statistically significant differences in the contralateral testes among the groups ($P>.05$).

[a] Versus sham in the ipsilateral testes, $P<.001$.

[b] Versus torsion–detorsion in the ipsilateral testes, $P<.01$.

Tunçkıran. Effect of VEGF on testicular ischemia–reperfusion injury. Fertil Steril 2005.

Ischemia–reperfusion injury initiates a pathophysiologic cascade, including an inflammatory response with liberation of cytokines and reactive oxygen species generation (3). Previous studies with a rat model of testicular torsion have demonstrated that a 2-hour, 720° rotation of the testis followed by reperfusion causes a significant increase in testicular lipid peroxidation products, nitric oxide content, and myeloperoxidase activity (an indicator of neutrophil accumulation) (10). The events result in the permanent loss of spermatogenesis (11, 12). The lesions in our study are characterized by a decrease in seminiferous tubule diameter, germinal epithelial cell thickness, and degree of spermatozoal maturation.

Apoptosis is another indicator of tissue injury. It has been documented that testicular ischemia–reperfusion resulted in apoptosis of germ cells, and it is induced by reactive oxygen species arising from neutrophils (4, 13). In the present study, we demonstrated that testicular torsion–detorsion caused a significant increase in caspase-3-positive cells in ipsilateral testes. Results of the current investigation indicate that testicular torsion–detorsion induces progressive histologic changes due to ischemia–reperfusion injury, and all these observations are in agreement with those of previous studies (4, 10–13).

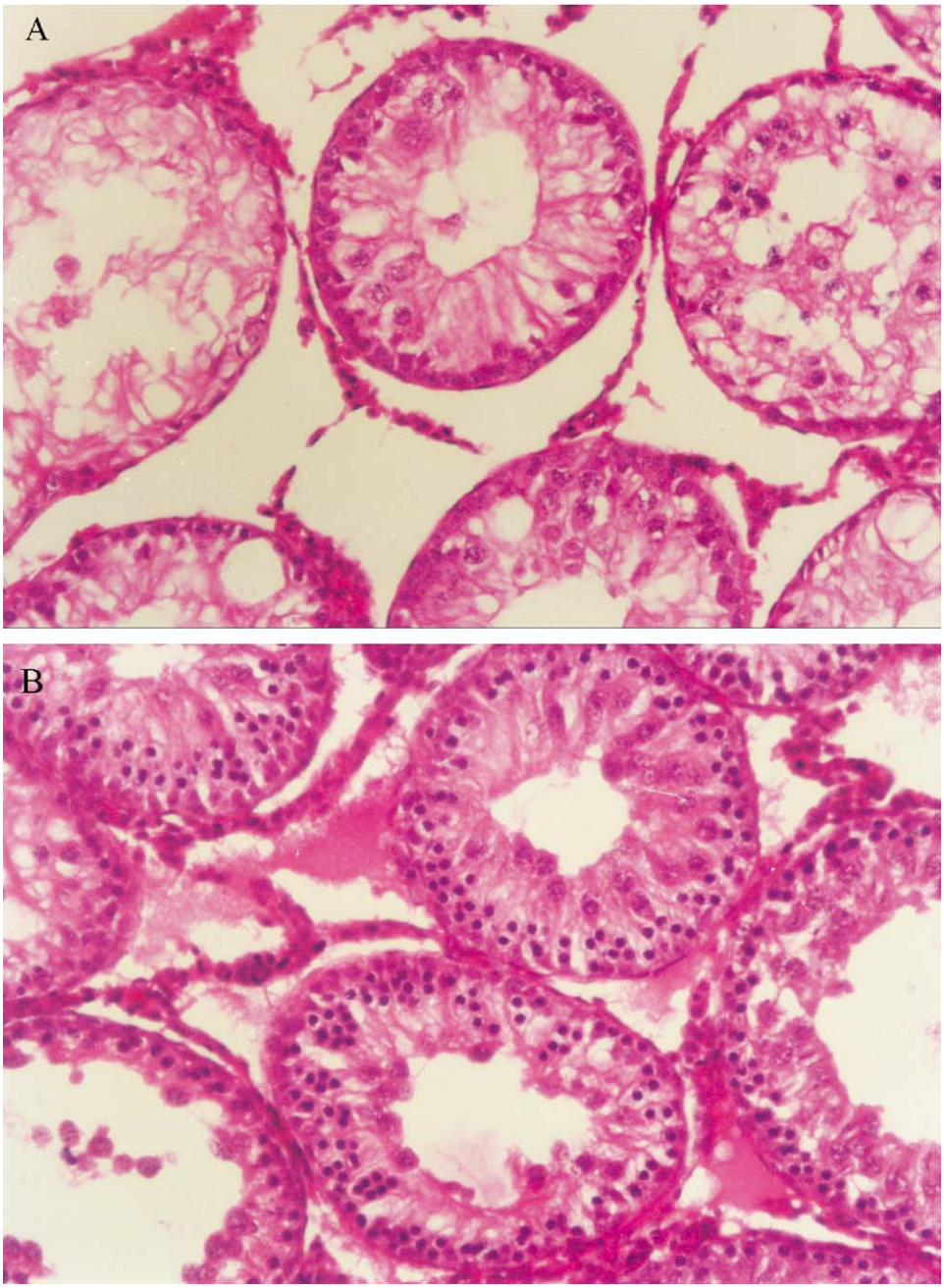
Vascular endothelial growth factor, also known as vascular permeability factor, is one of the most potent angiogenic and permeability-increasing factors known. There are six VEGF isoforms in human beings (VEGF 121, 145, 165, 183, 189, and 206) (5) and at least three isoforms in mice (VEGF 120, 164, and 188) (14). In recent years, an increased proportion of basic science research has been directed toward evaluating mechanisms and treatments involving cell injury, and use of VEGF after ischemia and reperfusion has been shown to improve the status of various organs after ischemia in animal models (5). Administration of VEGF leads to a reduction of ischemic brain damage (6), a protection of the sinusoidal endothelial cell damage of the liver (7), and the oxidative stress to the heart (8). This success has led us to attempt such treatment with models of testicular torsion.

In the present study, we have obtained data showing for the first time that VEGF is effective in reducing long-term histologic damage resulting from testicular ischemia-reperfusion, and preservation of the histologic parameters, including MSTD, GECT, and MTBS, are significantly maintained in animals treated with VEGF. The results of the present study support the hypothesis of improved seminiferous tubule diameter, the survival of the germinal epithelium, and spermatogenesis with intratesticular VEGF administration before detorsion of the testis. On the basis of the data presented in this study, we propose that administration of VEGF might be a novel approach for the therapy of ischemia–reperfusion injury of the testis.

One strategy to reduce oxidative stress involves the elimination of reactive oxygen species by reactive oxygen

FIGURE 1

Hematoxylin and eosin staining of seminiferous epithelium for evaluation of MSTD, GECT, and MTBS of an ipsilateral testis in the (**A**) torsion–detorsion group and the (**B**) VEGF group. Original magnification, ×100.



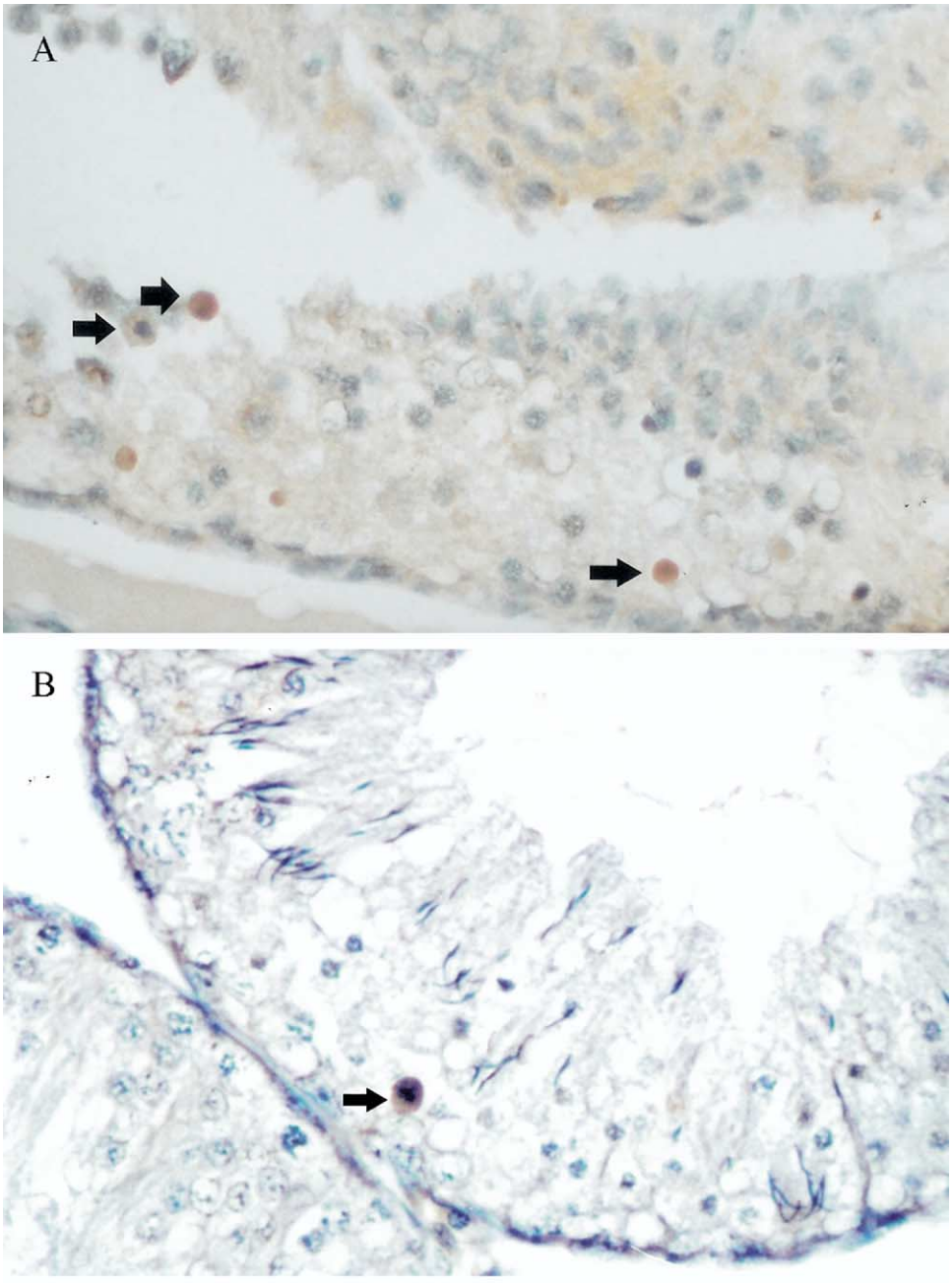
Tunçkiran. Effect of VEGF on testicular ischemia–reperfusion injury. Fertil Steril 2005.

species scavengers (3). Although the treatment with reactive oxygen species scavengers had a palliative effect on the histologic changes in testes that underwent 1 hour of experimental torsion, no significant rescue was seen after 2 hours of testicular ischemia (15). In contrast, a recent study from our institution using a rat model of 2 hours' testicular torsion has reported a significant rescue with the use of poly(adenosine diphosphate-ribose) polymerase inhibitors (12).

In the present study, we also found that administration of VEGF provides a significant decrease in histologic damage after 2 hours of testicular torsion followed by detorsion. The protective effect of VEGF on testicular

FIGURE 2

Caspase-3-positive labeled germ cells in seminiferous epithelium in the (**A**) torsion–detorsion group and the (**B**) VEGF group. *Arrows* show caspase-3-positive cells. Original magnification, ×400.



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ischemia–reperfusion injury might not be explained only by angiogenic activity to maintain the microvasculature. As stated previously, reperfusing leukocytes are potent generators of reactive oxygen species, and the recruitment of neutrophils to the testis after torsion is essential for the observed pathology (4, 10, 13).

Extravasated neutrophils become activated once in the inflammatory sites and secrete a variety of substances, such as growth factors, chemokines and cytokines, complement components, proteases, nitric oxide, reactive oxygen metabolites, and peroxynitrite, all important mediators of tissue injury (3). However, Kupatt et al. (8) demonstrated that

leukocyte recruitment in the ischemic myocardium was reduced when hearts were transfected with VEGF 165. Turner et al. (13) and Lysiak et al. (4) reported that loss of spermatogenesis after ischemia–reperfusion of the testis was due to germ cell–specific apoptosis, and an influx of neutrophils to the testis was essential for this pathology.

In the present study, we found that VEGF administration caused a significant decrease in testicular caspase-3-positive cells compared with the torsion–detorsion group. The exact mechanism of the antiapoptotic function of VEGF is still unclear. However, it has been demonstrated that the activation of the phosphatidyl inositol 3′ kinase/Akt signaling pathway and the increased expression of the anti-apoptotic proteins Bcl-2 and A1 are important elements in the VEGF protective actions (5, 16). There is strong evidence that VEGF prevented the apoptosis of human microvascular endothelial cells by activating the mitogen-activated protein kinase/extracellular signal regulated kinase and by inhibiting the stress-activated protein kinase/c-jun-NH_3-kinase (17).

On the other hand, recent studies have emphasized the anti-apoptotic effect of VEGF and the protective effect of VEGF against apoptotic changes in several tissues and cells (5, 7, 16, 17). Our study is the first report in the literature to show that the apoptotic changes seen in germ cells were reduced by VEGF in testicular torsion of the rat model. In light of these data, we propose that inhibition of apoptosis in testicular ischemia–reperfusion represents an important additional protective effect provided by VEGF.

The effect of unilateral torsion on contralateral testis is controversial. It has been demonstrated that ipsilateral torsion did not result in contralateral testicular damage in rats (18). Previous studies from our institute using a rat model of 2 hours' testicular torsion have demonstrated no significant effect of detorsion on biochemical (10) and long-term histologic changes (12) of the contralateral testis. Histologic parameters of contralateral testes did not reveal any significant differences, and results of the present study support our earlier findings.

CONCLUSIONS

Gene therapy with VEGF is a new potential treatment of ischemic disease. The previous use of VEGF in ischemic injury is limited in human clinical trials, and their results have been preliminary (19, 20). Although there has been relatively little research into the role of VEGF within the testis (21, 22), we have demonstrated for the first time the role of VEGF in the pathogenesis of testicular ischemia–reperfusion injury. The current study shows that VEGF can play a role in improving testicular function after torsion. We propose that VEGF might be a novel approach for the therapy of ischemia–reperfusion injury of the testis; however, further studies are required to elucidate the mechanism of ischemia–reperfusion injury of the testis that involves VEGF activation.

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