The effect of vascular endothelial growth factor on spermatogenesis and apoptosis in experimentally varicocele-induced adolescent rats

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Objective: To investigate the effect of vascular endothelial growth factor (VEGF) injection into the testes on spermatogenesis and apoptosis in a varicocele-induced adolescent rat model.
Setting: University hospital urology research laboratory.
Animal(s): Six-week-old male Wistar rats (n = 32).
Intervention: The rats were divided into six groups: control group (n = 6), sham operated group (n = 6), left varicocele-induced group (n = 6), varicocele + varicocelectomy group (n = 6), varicocele + VEGF-injected group (n = 4), and varicocele + varicocelectomy + VEGF-injected group (n = 4).
Main Outcome Measure(s): Johnsen's score and apoptotic cells.
Result(s): The mean Johnsen's score was lower in the varicocele group compared with in the control and sham groups. The mean apoptotic index was significantly higher in the varicocele group compared with in the control and sham groups. Compared with the varicocele group, the mean apoptotic index was significantly lower in the varicocele + varicocelectomy, varicocele + VEGF, and varicocele + varicocelectomy + VEGF groups.
Conclusion(s): Varicocele may cause a decrease in spermatogenesis and an increase in the apoptotic index. VEGF may play a positive role in improving testicular damage and may also play a significant role in decreasing apoptosis in a varicocele-induced adolescent rat model. (Fertil Steril® 2009;91:2247–52. ©2009 by American Society for Reproductive Medicine.)

Key Words: Varicocele, adolescent, rat, vascular endothelial growth factor, spermatogenesis, apoptosis

Varicocele is the most commonly seen and correctable pathology in men presenting with infertility (1–3). However, the pathogenesis of testicular damage or the mechanism by which varicocele produces sperm dysfunction has not yet been clearly identified. Many mechanisms have been proposed in the physiopathology of varicocele, such as hypoxia, hyperthermia, renal-adrenal reflux, hormonal dysfunction, autoimmunity, oxidative stress, and apoptosis (4).

Over the past decade, many studies have focused on apoptosis and hypoxia to clarify the pathophysiology of varicocele. Kilinc et al. reported that varicocele can lead to tissue hypoxia and hypoxia-related pathophysiological events, such as angiogenesis (5). In another study, Lee et al. showed that hypoxia-related pathophysiological changes such as reactive oxygen species (ROS) may occur in the testicular tissue of patients with varicocele (6). Whatever the mechanism, the common histopathological features of severe and prolonged varicocele are degenerative changes in the germinal epithelium and an increase in apoptosis. ROS cause DNA damage, endothelial damage, and germinal cell necrosis (7).

Vascular endothelial growth factor (VEGF) is an angiogenic peptide and mediates angiogenesis and vasculogenesis. There are six isoforms of VEGF in human beings ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$) (8) and at least three isoforms in rats ($VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$) (9). VEGF also promotes endothelial cell survival and decreases apoptosis. In recent years, an increased proportion of basic science research has been directed toward evaluating mechanisms and treatments involving cell injury and usage of VEGF after ischemia reperfusion has been shown to improve the status of various organs after ischemia in animal models (10–13). Intramyocardial injection of $VEGF_{165}$ significantly improved cardiac performance, stimulated angiogenesis, and reduced cardiomyocyte apoptosis in a rat model of acute myocardial infarction (13).

The success of such studies has led us to attempt such treatment with models of varicocele-induced rat testes because no study has investigated the effects of VEGF in experimentally varicocele-induced adolescent rat testes. Therefore, the aim of this study was to investigate the effect of VEGF injection into the testes on histologic changes including spermatogenesis and apoptosis in a varicocele-induced adolescent rat model.

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MATERIALS AND METHODS

The study included 32 adolescent (6-week-old) male Wistar rats weighing between 180 and 210 g. The rats were maintained on a 12 hour daylight and 12 hour dark cycle at 20 ± 1°C and 55% humid condition. During the study, all experiments were approved by the ethics committee on animal research at our institution. We performed appropriate care and use of laboratory animals as recommended by the Board of Registry Publication Guidelines. $VEGF_{165}$ (Calbiochem, San Diego) was used in the treatment groups in the same technique and concentration as described in our previous study (10). Each flacon contains 10 μg of $VEGF_{165}$ in 1 mL of solution.

Animal Preparation and Surgical Procedure

The 32 rats were divided into six groups. Surgery was performed with the subject under a single dose of IP ketamine (50 mg/kg) anesthesia. A standard midline incision was performed in all surgical procedures.

Control group (n = 6) No surgical procedure was performed in this group, which served as a control group.

Sham group (n = 6) As a sham operation, a midline incision was performed and the left renal vein was exposed. The renal vein was cleared of adhering tissue, and a 4/0 silk suture was put in place but not tied.

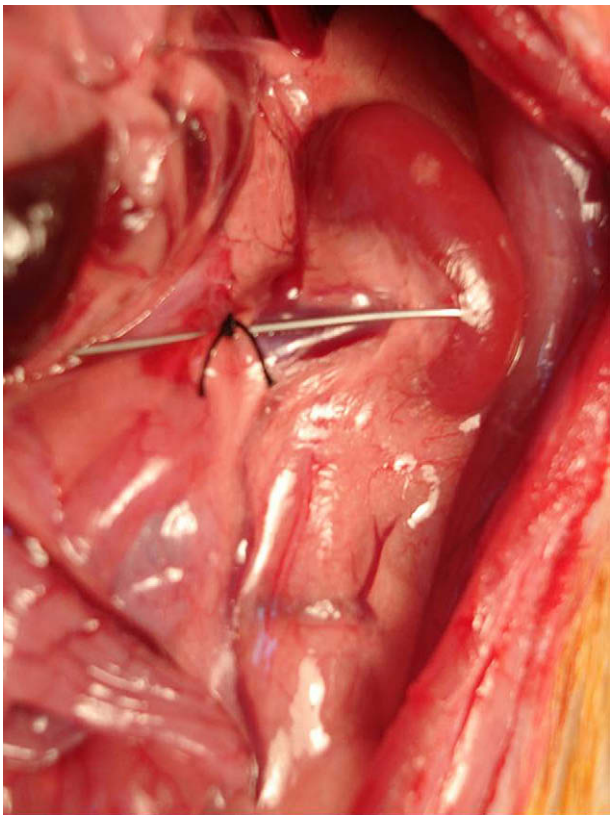
Varicocele group (n = 6) After a midline incision, the left renal vein was exposed. With a careful blunt dissection, a tunnel was made in the fat, and the renal vein was cleared of adhering tissue in a position medial to the insertion of the left spermatic vein and left adrenal vein. A metal probe with a 0.85-mm diameter was placed parallel to the left renal vein. Varicocele was created by partial ligation of the left renal vein at the point medial to the insertion of the adrenal and spermatic vein into the renal vein with a 4/0 silk suture (Fig. 1). After the procedure, the metal probe was carefully removed, and the vein was allowed to expand against the loop of the suture. The suture was positioned at the junction of the left renal vein and vena cava. Immediate dilatation of the renal vein was observed in all rats by which the renal vein diameter was reduced by approximately one-half.

Varicocele + Varicocelectomy group (n = 6) Varicocele was performed by the partial ligation of the left renal vein, and 4 weeks after the procedure all rats underwent varicocelectomy. Varicocelectomy was performed 4 weeks after the creation of varicocele because maximum apoptotic activity in rat testes may initiate 28 days after the creation of varicocele (7).

Varicocele + VEGF group (n = 4) First, left varicocele was performed by the same surgical procedure that was used in the varicocele group, and then 0.4 mL (4 μg) of $VEGF_{165}$ (Calbiochem) was injected into the upper, lower, and mid portions of the left testis, but no varicocelectomy was performed.

FIGURE 1

Partial ligation of left renal vein.



Tek. VEGF and varicocele. Fertil Steril 2009.

Varicocele + VEGF + Varicocelectomy group (n = 4) First, left varicocele was performed by the same surgical procedure that was used in the varicocele group, and then 0.4 mL (4 μg) of $VEGF_{165}$ was injected into the upper, lower, and mid portions of the left testes. Four weeks after the operation, varicocelectomy was performed.

Animals were sacrificied 4 weeks after the varicocelectomy operation, and modified Johnsen's score and caspase-3 staining apoptotic cells were examined in all groups.

Histological Preparation

One-half of the left testis was fixed in Bouin's solution (75% picric acid + 25% formaldehyde + 5% glacial acetic acid) for 4–6 hours and washed with 70% alcohol. Five-micrometer sections were obtained, deparaffinized, and stained with hematoxylin and eosin.

Histological Evaluation

Testicular tissue was evaluated with standard light microscopy by one blinded specialist only and in random order. In each section, 20 seminiferous tubules were assessed according to the presence of spermatogenic cells, and modified

TABLE 1

Mean Johnsen's scores and apoptotic indexes of the groups.

Groups	Mean Johnsen's score (range)	Mean apoptotic index (range)
Control group	11.88 ± 0.08 (10–12)	0.206 ± 0.058 (0.124–0.282)
Sham group	11.88 ± 0.02 (10–12)	0.146 ± 0.145 (0.108–0.226)
Varicocele group	9.50 ± 0.43 (2–10)	0.693 ± 0.148 (0.243–0.795)
Varicocele + varicocelectomy group	11.75 ± 0.12 (9–12)	0.196 ± 0.058 (0.121–0.274)
Varicocele + VEGF group	9.90 ± 0.58 (5–12)	0.256 ± 0.051 (0.152–0.344)
Varicocele + VEGF + varicocelectomy group	9.95 ± 0.31 (8–12)	0.086 ± 0.421 (0.068–0.312)

Tek. VEGF and varicocele. Fertil Steril 2009.

Johnsen's score (a score of 1–12 was given to each tubule according to epithelial maturation) was evaluated.

Rabbit anti-active caspase-3 polyclonal antibody (at 1:10 primer antibody dilution) was used for immunohistochemical staining. Caspase-3-positive cells were evaluated by the indirect immune peroxidase method. Caspase-3-positive cells were evaluated in 20 fields per section at a ×400 magnification with an Olympus BX 50 standard light microscope (10).

Statistical Analysis

Histopathological findings (Johnsen's score) were assessed by nonparametric Kruskal-Wallis test, and mean Johnsen's score was used for the comparison of the groups. Multiple comparisons were made using Tukey's procedure. $P<.05$ was considered statistically significant. Analysis of variance was used for statistical analysis of the apoptotic index among the groups.

RESULTS

The mean Johnsen's scores and apoptotic indexes of all groups are shown in Table 1. The mean Johnsen's score was lower in the varicocele group compared with the control and sham groups ($P=.05$ and $P=.003$, respectively; Figs. 2A and 2B). When compared with the sham group, the mean Johnsen's score was approximately similar in the varicocele + varicolectomy group. In the varicocele + VEGF group and varicocele + VEGF + varicocelectomy group, the mean Johnsen's score was lower compared with the sham group, but the difference was not statistically significant ($P=.126$ and $P=.057$, respectively).

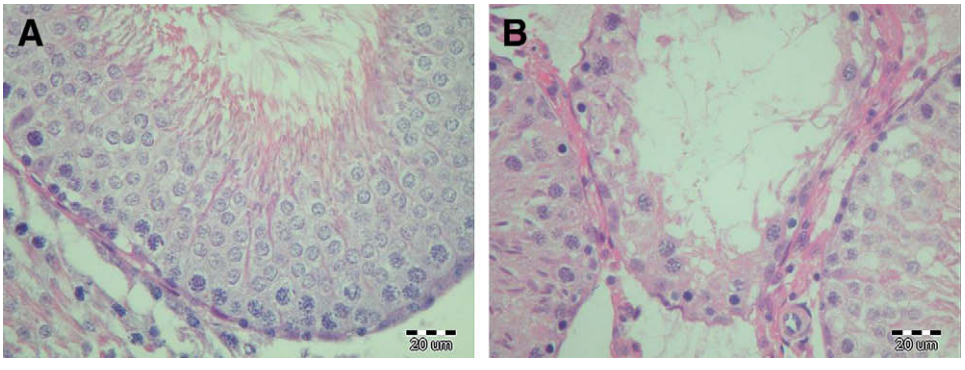
The mean apoptotic indexes were significantly higher in the varicocele group compared with the control and sham groups ($P=.001$ and $P=.0001$, respectively; Figs. 3A and 3B). Compared with the varicocele group, there was a significant decrease in apoptotic indexes in the varicocelectomy, VEGF-injected, and varicocelectomy + VEGF-injected groups ($P=.001$, $P=.018$, and .001, respectively; Figs. 3C and 3D).

DISCUSSION

Varicocele is the most common cause of male factor infertility and is present in 19%–41% of men presenting for

FIGURE 2

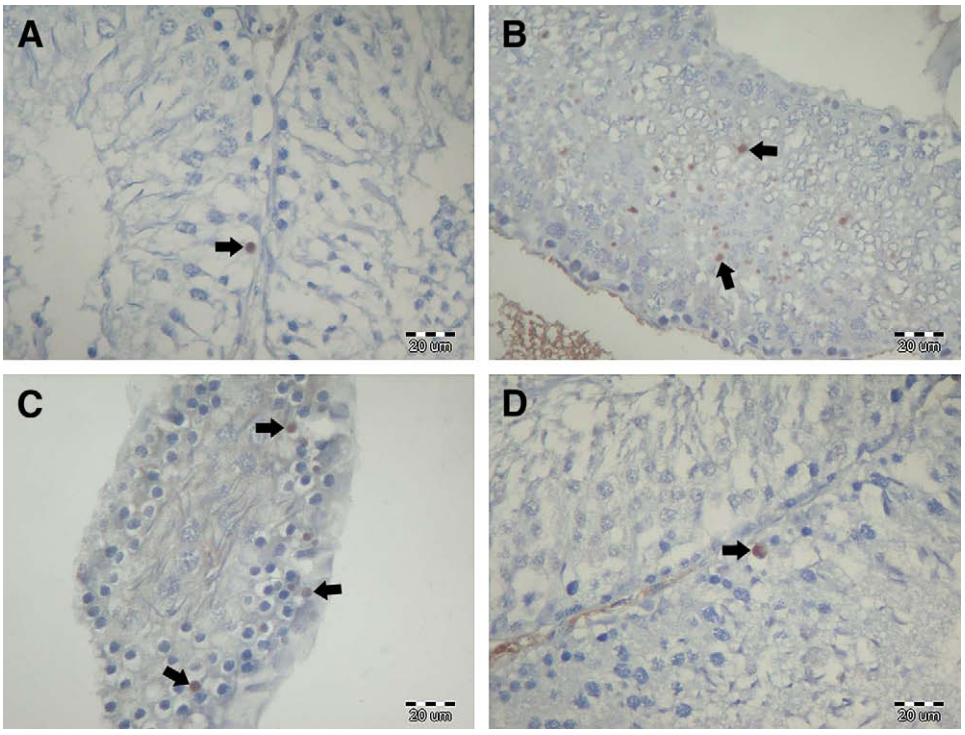
(**A**) Hematoxylin and eosin staining of seminiferous epithelium for evaluation of spermatogenesis in the control group, ×400. (**B**) Hematoxylin and eosin staining of seminiferous epithelium for evaluation of spermatogenesis in the varicocele group, ×400.



Tek. VEGF and varicocele. Fertil Steril 2009.

FIGURE 3

(**A**) Caspase-3-positive labeled germ cells in seminiferous epithelium in the control group, ×400. Note that the arrow shows the caspase-3-positive cell. (**B**) Caspase-3-positive labeled germ cells in seminiferous epithelium in the varicocele group, ×400. Note that arrows show caspase-3-positive cells. (**C**) Caspase-3-positive labeled germ cells in seminiferous epithelium in the varicocele group, ×400. (**D**) Caspase-3-positive labeled germ cells in seminiferous epithelium in the varicocele + varicocelectomy +VEGF group, ×400.



Tek. VEGF and varicocele. Fertil Steril 2009.

infertility evaluation (14). Since Tulloch in 1952 reported pregnancy and an increase in sperm count after varicocelectomy in men with nonobstructive azoospermia, varicocelectomy has become the most common surgical operation in male factor infertility (15). However, although sometimes varicocelectomy results in an improvement in semen parameters and fertility, sometimes the results may not be satisfactory, so it is still uncertain which mechanism in varicocele causes subfertility or infertility (16).

In our study, we demonstrated a regression in spermatogenesis in the varicocele group compared with control and sham groups, which is consistent with the literature. We created varicocele by partial ligation of left renal vein and then assessed Johnsen's score by hematoxylin and eosin staining. In the varicocele group, Johnsen's score was lower than in the control group, but the difference was not statistically significant. Compared with the sham group, Johnsen's score was significantly lower in the varicocele group. Johnsen's score increased in the varicolectomy group compared with the varicocele group, but the difference was not statistically significant.

There are many theories on the physiopathology of varicocele such as hyperthermia, renal-adrenal reflux, hormonal dysfunction, oxidative stress, and apoptosis (4). Over the last decade, many studies show that hyperthermia in varicocele causes an increase in germ cell apoptosis (17, 18). Thus many studies have focused on the relation between varicocele and apoptosis to clarify the physiopathology of varicocele.

Apoptosis is a normal physiological phenomenon in most tissues and has a critical regulatory role. Apoptosis is also an important aspect of normal spermatogenesis. Therefore, alterations in the apoptosis of germ cell may be crucial in varicocele-related human infertility. Kerr reported spontaneous apoptosis in testes, and germ cell apoptosis during normal spermatogenesis is estimated to result in the loss of 25%–75% of potential mature sperm cells in the adult testes. Apoptosis appears to affect all three classes of germ cells, that is, the spermatogonia, spermatocytes, and spermatids (18). Germ cell apoptosis also increased in varicocele-related infertility, and it was also reported that men with varicocele have 100 times more apoptotic germ cells in their ejaculate specimen than men without varicocele (19). In an another study, Fazlioglu et al. reported that in an experimental varicocele model, 14 days after creating varicocele

germ cell apoptosis was twice as high as in the control group and that 28 days after varicocele creation germ cell apoptosis reached a maximum rate (7). Also in that study, after varicocelectomy was performed, germ cell apoptosis decreased and apoptotic indexes become similar to those of the control group.

In our study, in the varicocele group, germ cell apoptosis increased as in other studies in the literature. We also created varicocele by partial ligation of the left renal vein and then assessed apoptosis by caspase-3 staining. The apoptotic index was lower in the control group and increased in the varicocele group. On the other hand, in the sham group, the apoptotic index was similar to that of the control group. When compared with the varicocele group, the apoptotic index decreased significantly in the varicocelectomy group. After varicocelectomy a regression was seen, and the apoptotic index became similar to that of the control group. The only study suggesting a decreased apoptosis in varicocele was reported by Fujisawa et al. In that study, testicular biopsy was performed on subfertile men with varicocele and without varicocele. As compared with the varicocele group, germ cell apoptosis was higher in men with no varicocele (20). The investigators reported that fixation of testes in formaldehyde instead of in Bouin's solution may have caused the different results in their study.

These data suggest that germ cell apoptosis increased in the varicocele group and that after varicolectomy a regression was seen. However, the changes in Johnsen's score were not statistically significant.

VEGF is an angiogenic peptide and mediates angiogenesis and vasculogenesis. VEGF also promotes endothelial cell survival and decreases apoptosis. VEGF has been used successfully to decrease ischemia reperfusion injury in several organ systems (8, 10–13). VEGF shows its effects on two receptors, VEGFR1 and VEGFR2. Martin and Risau reported VEGFR2 receptors in Sertoli cells in testes tissue (12). In an experimental study, Tunckiran et al. reported that intratesticular $VEGF_{165}$ injection has positive effects on ischemia reperfusion injury created by testicular torsion (10). Also, Ruixing et al. reported that after cardiac ischemia, apoptosis in cardiomyocyte decreased and cardiac performance increased by intracardiac VEGF injection (13). The success of such studies has led us to attempt such treatment with models of varicocele-induced adolescent rat testes.

In the present study, we aimed to evaluate whether VEGF injection into the testes has a decreasing effect on varicocele-related germ cell apoptosis or not. First, experimental varicocele was created; then we injected VEGF into the upper, lower, and mid portions of the left testes and evaluated spermatogenesis and germ cell apoptosis. The mean apoptotic index in the VEGF-injected group was lower when compared with the varicocele group. In addition, compared with the varicocele group, mean apoptotic indexes were lower in the varicocelectomy + VEGF-injected group. These data suggest that VEGF injection decreased apoptosis significantly. When compared with the varicocele group, Johnsen's score was higher in the VEGF-injected group, but the difference was not statistically significant. Also compared with the varicocele group, Johnsen's score was higher in the varicocelectomy + VEGF-injected group, but the difference was not statistically significant.

The present study was limited in that it did not reveal statistical significance according to the mean Johnsen's score between the varicocele group and the VEGF-injected + varicocele and the VEGF-injected + varicocelectomy groups. This outcome may be due to underpowering of the study. Currently, many factors are implicated in the pathophysiology of varicocele. Recently, varicocele-related apoptosis has become a popular topic. By which mechanism varicocele causes infertility is still uncertain, but these data suggest that VEGF may decrease germ cell apoptosis, and these findings may be an advance in the study of varicocele-related infertility.

In conclusion, these data suggest that varicocele may cause a decrease in spermatogenesis and an increase in apoptotic index. VEGF may play a positive role in improving the testicular damage that occurs after varicocele in ipsilateral testes. VEGF may also play a significant role in decreasing apoptosis in a varicocele-induced adolescent rat model; however, further studies are needed to elucidate the mechanism of VEGF in testis.

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