Angiogenesis inhibition impairs testicular morphology in experimental left varicocele rat model

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Received 10 November 2013; accepted 1 December 2013
Available online 12 June 2014

KEYWORDS
Angiogenesis; Experimental left varicocele; Rat; Spironolactone; Varicocele; Vascular endothelial growth factor

Abstract
Introduction: It has been reported that varicocele might promote angiogenesis. However, it is not clearly identified how angiogenesis affect testicular morphology or spermatogenic activity. The objective of the study is to investigate the effect of spironolactone, as an angiogenesis inhibitor, on the ipsilateral testis morphology in left varicocele-induced rats.

Materials and methods: Twenty-four adult (12–14 mo), male Wistar albino rats were randomly assigned to four groups (n = 6, for each): 1. Control group, 2. Sham-operated group, 3. Experimental left varicocele group and, 4. Spironolactone (20 mg/kg/day)-treated experimental left varicocele group. Histopathological findings in rat testis were investigated.

Results: Microvessel density increased in varicocele group and spironolactone inhibited angiogenesis neither by antimineralocorticoid, nor by antiandrogenic effect. However, spermatogenesis impaired in spironolactone treated varicocele group.

Conclusion: Angiogenesis seems to be a protective process in varicocele. Spironolactone treatment, probably by inhibiting angiogenesis, impairs testicular morphology.

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La inhibición de la angiogénesis deteriora la morfología testicular en modelo de rata con varicocele izquierdo experimental

Resumen

Introducción: Se ha señalado que el varicocele podría favorecer la angiogénesis. Sin embargo, no se ha identificado claramente cómo la angiogénesis afecta a la morfología testicular o a la actividad espermogénica. El objetivo de este estudio es investigar los efectos de la espironolactona, como inhibidor de la angiogénesis, en la morfología del testículo ipsilateral en ratas con varicocele inducido en el lado izquierdo.

Materiales y métodos: Veinticuatro ratas albinas Wistar, adultas (12-14 meses) y de sexo masculino, fueron asignadas aleatoriamente a 4 grupos (n = 6, para cada uno): 1) grupo de control; 2) grupo con operación simulada; 3) grupo experimental con varicocele izquierdo; y 4) grupo experimental con varicocele izquierdo tratado con espironolactona (20 mg/kg/día). Se investigaron los resultados histopatológicos en testículos de rata.

Resultados: La densidad microvascular aumentó en el grupo con varicocele y la espironolactona no inhibió la angiogénesis ni por efecto antimineralcorticoide ni por efecto antiandrográfico. No obstante, la espermogénesis se vio afectada en el grupo con varicocele tratado con espironolactona.

Conclusión: La angiogénesis parece ser un proceso protector en el varicocele. El tratamiento con espironolactona, probablemente al inhibir la angiogénesis, afecta a la morfología testicular.

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Introduction

Varicocele, one of the most common factors in male infertility, is defined as the dilatation and tortuousity of plexus pampiniformis leading to a retrograde reflux caused by various etiological factors. According to the results of epidemiological trials, varicocele is estimated in 19–41% of fertile couples as a cause of primary infertility. However, the incidence of varicocele may be as high as 81% in patients with secondary infertility.1,2

The most accepted theories regarding the etiology of varicocele are anatomical differences between left and right testicular veins, the lack of venous valves and nutcracker phenomenon.3–5 The pathogenesis of varicocele-related infertility is not completely identified. Although there have been some theories such as hyperthermia, alterations in the testicular blood flow, renal and/or adrenal reflux, hormonal disorders, autoimmunity, apoptosis, and oxidative stress, none of them can enlighten the process.6–12

It has been reported that varicocele promotes angiogenesis; however, it is not exactly shown how spermatogenesis is affected by angiogenesis.13 To our hypothesis, if angiogenesis increases the distribution of the toxic metabolites through the testis, inhibition of angiogenesis may improve spermatogenesis. However, if angiogenesis occurs as a protective process, inhibiting angiogenesis may impair spermatogenesis more.

Spironolactone (SPL), a competitive antagonist of aldosterone, has been widely used as a potassium-sparing diuretic drug. Recently, the antiangiogenic effect of SPL has been described, which is unrelated to the antimineralocorticoid effect.14

The objective of the present study is to investigate the effect of SPL on a rat model of experimental left varicocele. We hypothesize that SPL improves the detrimental effect of varicocele on spermatogenesis in experimentally-induced varicocele in rats by inhibiting the angiogenic process.

Material and methods

After being approved by the Baskent University Local Ethical Committee (DA 07/12), the study was performed in 24 adult (12–14 months) male Wistar albino rats (282, 75 ± 20.47 g). The animals were fed by standard rat chow and tap water ad libitum and maintained in the animal facility with constant environmental conditions (room temperature: 20 ± 2 °C, relative humidity: 50 ± 10%, light-dark cycle: 12:12-h). The rats were randomized into 4 groups (n = 6, for each); 1. Control, 2. Sham operated 3. ELV 4. ELV + Spironolactone (20 mg/kg/d, i.p., 45 days)-treated (V+S).

Each animal was anesthetized by 10% ketamine hydrochloride (60 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). After an abdominal midline incision; the left renal vein, inferior vena cava, and left spermatic vein were identified. A 4/0 silk ligature was loosely placed at the site of the left renal vein and left spermatic vein insertion over a rigid hydrophilic guide wire of 0.64 mm that was placed on the left renal vein. The ligature was loosely tied and the guide wire was removed leading to an immediate dilation of the left renal and left spermatic vein. The incision was sutured with 4/0 silk ligature. All of the surgical procedures in the sham-operated group were identical to that of the ELV group, except for the vein ligature step.

Animals in the V+S and ELV were weighed on the first day postoperatively, to adjust the dose of spironolactone or the volume of saline to be given, respectively. The rats in the V+S received spironolactone at the dose of 20 mg/kg/day through an orogastric catheter, for 45 days, beginning the first day postoperatively. Spironolactone was dissolved in saline (5 mg/ml). The ELV group received only the corresponding volume of saline for 45 days.
Angiogenesis inhibition in experimental left varicocele

All rats were sacrificed by ketamin overdose (150 mg/kg, i.p.) on the postoperative 45th day and then underwent bilateral orchidectomy. The testis tissue was fixed in Bouine’s solution and embedded in parafin, then stained with hematoxylin and eosin for histopathological examination.

The degree of angiogenesis was assessed by counting stained microvessels. Only vessels with a clearly defined lumen or well-defined linear vessel shape were counted as microvessels. Newly forming vessels that consisted of only one layer of endothelial cells were excluded. In each specimen, the 3 areas with the highest degree of vascularization were identified by ×100 magnification. Then, the number of vessels in the selected field was counted at high power (×400). For each testis the highest of these 3 vessel counts were recorded as microvessel density (MVD). Mean MVD values ± SEM in each group were compared.

To determine the biochemical effects of spironolactone, peripheral venous blood samples were achieved preoperatively and postoperatively from each animal. Serum sodium and potassium levels were determined by ion selective electrode method and total testosterone level was determined by radioactive immune assay (Testo-RIA-CT KIP 1709, Biosource, Belgium).

**Statistical analysis**

The data were taken as the mean and standard error of mean (SEM). For biochemical data, one way ANOVA was used as parametric test and post hoc Bonferroni test was used to determine the difference among groups. For intra-group comparisons (such as basal vs. post administration), paired t test was used. For statistical evaluation of the histopathological data, Kruskal–Wallis analysis of variance was used. If any difference was detected between the groups, post hoc Dunn’s test was used to compare the groups. P < 0.05 was accepted as statistically significant.

**Results**

Histopathological changes in testes were reported as atrophied germ cells at any stage of spermatogenesis, hyperplasia in Leydig cells, and decline in Sertoli cell numbers (mixed atrophy) in ELV group (H&E staining ×200 HPF). (D) Germinal cells are disturbed and calsification in Sertoli cells in V+S group [indicated by *] (H&E staining ×200 HPF).

![Figure 1](http://www.elsevier.es) **Figure 1** Histopathological findings. (A) Normal histopathological findings in control group (H&E staining ×200 HPF). (B) Normal histopathological findings in sham group (H&E staining ×200 HPF). (C) Atrophied germ cells in any stage of spermatogenesis, hyperplasia in Leydig cells, and decline in Sertoli cell numbers (mixed atrophy) in ELV group (H&E staining ×200 HPF). (D) Germinal cells are disturbed and calsification in Sertoli cells in V+S group [indicated by *] (H&E staining ×200 HPF).
Basal and post-sacrification serum sodium ($Na^+$) levels were significant in neither intra-group nor inter-group comparison. Basal potassium ($K^+$) levels were higher in ELV and V+S groups when compared with the control group ($p < 0.05$). Although the last $K^+$ values were not significantly different between the groups, $K^+$ level tended to increase in the control group ($p < 0.05$) and to decrease in the V+S group ($p < 0.01$). Basal and the last TT values were not different between the groups.

**Discussion**

Angiogenesis is the formation of new vessels from an existing vasculature. Varicocele may be inducing angiogenesis; to trigger an inflammatory process in the testes such a cascade that angiogenesis may stimulate new collateral formation, and eventually increases in testicular blood flow, pressure, and temperature may disrupt spermatogenesis. Early repair of varicocele returns testicular temperature and blood flow to normal, possibly by preventing angiogenesis in the testes, earlier. On the other hand, an ongoing and long lasting angiogenic process in the testes may explain why testicular atrophy occurs or blood flow returns to normal after a late varicocele repairment.

Turner suggested that mitogenic factors or vasoactive agents might be responsible for the increase in blood flow and total ligation of spermatic vein could return the blood flow to the normal ranges. Improvement in testicular blood flow and spermatogenesis after varicocele repair in ELV models may be described by this opinion.

Vascular endothelial growth factor (VEGF), one of the most potent angiogenic factors, promotes a tyrosine kinase cascade, stimulates the production of the agents, such as nitric oxide (NO), which leads to a stimulation of vessel permeability, proliferation, migration and new vessel formation. Isoyama and Sofikitis have suggested that factors inducing vascularization improve the spermatogenic factors by regulating the counter-current heat system and the distribution of the nutrients in varicoceleized testes, in line with our findings. This might explain the more marked disruption in spermatogenesis in the V+S than that in ELV, in the present study.

In our study, MVD was more prominent in the ELV compared with the V+S and control groups suggesting that spironolactone inhibited the varicocele-induced angiogenesis. However, spermatogenesis was disturbed in the ELV and V+S groups being more marked in the latter. A more comprehensive study may be performed in two more groups, control + spironolactone and sham + spironolactone, to truly test the effects of medication or to reduce the false positive results about medication. We did not determine significant micro vessel density in sham or control groups. Therefore, we suggest that, if this was not caused by antimineralocorticoid and/or antiandrogenic activity of SPL, angiogenesis plays a protective role in varicocele.

Few authors investigated the relationship between varicocele and vascular factors such as angiogenesis and vasculogenic factors. The expression of VEGF has been detected in male genital organs such as testis, epididymis, prostate and seminal vesicles, and VEGF receptor-2 (VEGFR-2) expression in the blood vessels supplying the above-mentioned organs. Shiraiishi and Naito examined the expression and the role of VEGF in human testes with varicocele and showed the increased expression of VEGF which was inversely correlated with total motile sperm count and testicular volume. The authors concluded that excessive VEGF expression impaired spermatogenesis in testes with varicocele. However, Tek et al. investigated the effect of VEGF injection into the testes on spermatogenesis and apoptosis in an ELV model and stated that VEGF might improve testicular damage and play a significant role in decreasing apoptosis. In an experimental left varicocele rat model, the effect of angiogenesis inhibition on vas deferens was investigated. The authors showed that angiogenesis negatively affected functional pathways and morphometric results in rats vas deferens and SPL pretreatment changed biometric responses such as 5-HT sensitivity.

Recently, it has been shown that spironolactone inhibits angiogenesis and decreases vascular damage and/or fibrosis...
regardless of its antiandrogenic and antimineralocorticoid effects. Miternique-Grosse et al. determined that spironolactone inhibited angiogenesis in human umbilical vein endothelial cells and in fibrin gel chambers implanted to rats. Since the inhibitory effect of spironolactone could not be prevented by VEGF and aldosterone had no effect, the authors concluded that spironolactone inhibited angiogenesis in vivo and in vitro regardless of its antimineralocorticoid activity.

In the present study, MVD was found to be higher in the ELV than that in the V+S and control groups in the left testes. The basal and final TT values did not differ among the groups. Since spironolactone administration did not affect testosterone levels in the present study, we suggest that the antiangiogenic effect of spironolactone is independent of its antiandrogenic effect. This finding is in accordance with Miternique-Grosse et al. However, in contrast to Ge et al., who have shown that SPL alters TT values, SPL pre-treatment failed to cause such an effect in this study.

Spermatogenesis in the left testis was found to be worse in V+S than that of the ELV, while it did not show such a differential effect in the right testes. Thus, we suggest that the antiangiogenic effect of spironolactone is independent of its antiandrogenic activity. MVD was not different between the control and V+S groups. Spironolactone inhibited angiogenesis in both testes, while spermatogenesis was not affected in the right testis. TT values were not different among the groups. We may not know exactly how Leydig functions are affected in 45 days. However, testicular morphology was normal in right testes for all groups and TT levels did not change. Therefore, we suggest that spironolactone administration impairs spermatogenesis through a mechanism involving its antiangiogenic but not antiandrogenic effect, yet further studies are required.

The diversity in the basal K+ values is difficult to explain, since the rats were randomized at the beginning of the study. The higher serum potassium concentration detected in some of the rats might have been due to the hemolytic process resulting in the leakage of intracellular K+ to serum.

Conclusion

We showed that varicocele increased MVD and angiogenesis in both testes, to result in an impairment in testicular morphology. Spironolactone inhibited angiogenesis in the varicocele group, while it significantly impaired spermatogenesis as well. Therefore, we conclude that angiogenesis may play a protective role in the varicocele-related process. The antiangiogenic effect of spironolactone seemed to be associated with neither its antimineralocorticoid nor antiandrogenic effect.

Conflict of interest

The authors declare that they have no conflict of interest.

In vitro data of the study have been published in Urology, (Elsevier) 2012; 80: 816–821.

References


