Original Article

Histological and Immunohistochemical Changes in the Adult Rat Testes after Left Experimental Varicocele and Possible Protective Effects of Resveratrol

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ABSTRACT

Introduction: Varicoceles are present in about 30-50% of infertile men. However, the histological changes in the testes are not well-defined. Also, Resveratrol (RES) is a powerful antioxidant used recently in the treatment of infertility with other causes.

Aim of the Study: So, this study aimed to elucidate the histological and immunohistochemical changes of both testes after left experimental varicocele and to evaluate the possible protective role of RES.

Materials and Methods: This study included forty adult male albino rats divided into four equal groups: Control group (I); sham-operated group (II); left varicocele group (III) and left varicocele+RES group (IV). Varicocele was induced by partial ligation of the left renal vein. RES was orally administered to group IV in a dose of 20 mg/kg body weight every day for five weeks. At the end, all animals were sacrificed and testes were excised. Paraffin sections were prepared and stained with H&E, PAS reaction and immunohistochemical staining for proliferating cell nuclear antigen (PCNA). PCNA-labeling index was calculated to assess spermatogenesis.

Results: Left experimental varicocele was shown to affect both ipsi- and contra-lateral testes. The changes in the ipsilateral testes were in the form of irregular degenerated seminiferous tubules with numerous blood vessels and thickened basement membranes. These changes were present in some tubules in the contralateral testes. PCNA-LI values were significantly lower than the control and sham-operated groups. Treatment with RES proved to improve these changes.

Conclusion: Left experimental varicocele has deleterious effects on the structure of both testes and supplementation with RES in cases of infertility with varicocele may have a protective effect.

Key Words: Testis, varicocele, resveratrol, PCNA.

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INTRODUCTION

Varicocele can be defined as varicose veins of the testicle. In the general population of healthy males, the overall incidence of varicocele is between 10 and 15 percent. In males presenting with primary infertility, somewhere between 30 and 50 percent will have a varicocele. Varicoceles present almost always on the left side and they usually appear early in puberty.

Most investigators believe that varicoceles occur on the left side because the venous drainage from the left testis and spermatic cord is directly into the left renal vein. On the other hand, the right side drains into the inferior vena cava. This direct drainage is a straight south-to-north conduit that can be adversely affected by increased hydrostatic pressure and valvular abnormalities of the veins. An ipsilateral varicocele carries a risk of bilateral testicular malfunction.

Oxidative stress has been an area of active research for many physiologic processes. Elevated levels of oxygen free radicals, or reactive oxygen species (ROS), have been implicated in a variety of conditions, including myocardial reperfusion injury and central nervous system damage following stroke. Recently, it has been hypothesized that infertility attributed to varicocele is, at least in part, associated with increased oxidative stress, in terms of both ROS generation and diminished antioxidative capacity. It appears that men with varicoceles have increased oxidative stress in their semen. This oxidative stress manifests as elevated levels of oxygen free radical production, as well as decreased levels of protective antioxidants in the seminal fluid.

Resveratrol (RES) is a natural strong antioxidant, polyphenolic compound that is present in peanuts.
grapes, blackberries and other plants. It exhibits a wide range of biological activities including antioxidant, anti-inflammatory and antitumour effects\(^9,10\). Some recent researches demonstrated that RES could increase sperm output and protect sperm from apoptosis caused by physical damage such as testicular torsion\(^11\). Whether RES could inhibit adverse effects of varicocele was not tested.

The exact effects of left varicocele on the structure of the testes remain uncertain. Therefore, this experimental study was designed to demonstrate histological and immunohistochemical changes in both testes of adult rat after induction of left unilateral varicocele and to evaluate the possible protective effect of the natural antioxidant Resveratrol.

**MATERIALS AND METHODS**

**Animals:**

Forty adult male albino rats of 200 – 220 gm body weight were used in this study; they were obtained and housed in the animal house of Kasr-El-Aini Faculty of Medicine, Cairo University. The animals received a standard diet for rodents and allowed free access to water. The animals were divided randomly into four groups of ten animals each:

- **Group I:** Control group.
- **Group II:** Sham operation.
- **Group III:** Left experimental varicocele.
- **Group IV:** Left experimental varicocele + RES.

**Surgical procedure:**

The rats of groups II, III & IV were anesthetized by intraperitoneal injection of pentobarbital (15mg/kg body weight) and a midline laparotomy was done. After displacing the viscera, the left kidney and its adjacent vasculature were exposed. In groups III and IV, a varicocele was created. The left renal vein, inferior vena cava and the left spermatic vein were identified and a clamp passed behind the left renal vein just distal to the spermatic vein insertion. A peripheral venous cannula (24 F) was placed over the left renal vein and using this as a guide, a 4/0 silk suture was tied around the left renal vein medial to the adrenal and spermatic veins. This occlusion caused increased intravenous pressure lateral to the obstruction and pressure is transmitted to the left spermatic vein. This causes a varicocele to develop. In group II, the 4/0 silk suture was passed behind the renal vein and not tied. The cannula was then carefully removed and the vein was allowed to expand against the suture. The abdominal incision was closed\(^12\).

**Drug and Treatment Protocols:**

Trans-Resveratrol (Sigma-Aldrich Saint Louis, Missouri, USA; Prod. # R5010): The drug was in the form of white powder with yellow cast. Because of its low solubility in water, RES was suspended in carboxymethylcellulose (Sigma-Aldrich Saint Louis, Missouri, USA; Prod. # 419273). It was orally administered to group IV, by gavage, 20 mg/kg body weight in a volume of 10 mL/kg body weight every day for five weeks. The doses were freshly prepared immediately before administration\(^13\). The other groups were administered the vehicle only.

**Evaluation Methods:**

**Histological Study:**

At day thirty-six, all the animals were sacrificed by decapitation and their testes were excised. They were fixed in Bouin’s solution and processed for paraffin sections of 5µm thickness. Sections were stained with H & E and PAS reaction with hematoxylin counterstaining.

**Immunohistochemical Study:**

Proliferating cell nuclear antigen (PCNA) is an intranuclear polypeptide that is involved in DNA replication, excision and repair. Its synthesis and expression is linked to cell proliferation\(^14\). Since spermatogenesis is a complex cell cycle of rapidly proliferating cells ending with liberation of sperms, PCNA was used in this study to quantitatively analyze spermatogenesis. Immunohistochemical staining was carried out using primary antiserum to PCNA (Clone PC 10, DAKO A/S Denmark). The primary antibody was diluted in Tris-buffered saline with a dilution of 1:50, as determined by the data sheet. The sections were incubated with the primary antibody overnight at + 4°C. The binding of the primary antibody was observed using a commercial avidin-biotin-peroxidase detection system recommended by the manufacturer (DAKO, Carpenteria, USA). A mouse monoclonal antibody was applied in place of the primary antibody to act as a negative control. Sections from the small intestine were used as a positive control. Then the slides were stained with diaminobenzene (DAB) as the chromogen and counterstained with hematoxylin\(^15\).

**PCNA-Labeling Index (PCNA-LI)**

Slides were examined under the light microscope with a magnification X 200. Then sections were evaluated for PCNA immunostaining. Microscopic fields were chosen at random. Five fields per slide and five slides per animal were evaluated. Only the basal germ cells of these tubules were counted, because they are the cells where active DNA synthesis took place\(^17\). The PCNA-LI for each seminiferous tubule was estimated as a percentage of immuno-labeled cells to all basal cells. For each
specimen, the mean ± SD was calculated. Then, the total PCNA-LI for all groups was estimated accordingly.

Statistical Analysis:

Statistical analysis of the obtained data was performed using ANOVA test. P values <0.05 were considered significant.

RESULTS

Histological Results:

Histological examination of the testicular sections from the control group and sham-operated group revealed similar appearance of the seminiferous tubules and interstitial tissue in-between. The basement membranes of the tubules appeared thin in both groups (Figs. 1&2).

Sections from the ipsilateral testes from group III (varicocele) revealed irregular variable sized seminiferous tubules. Spermatogenic cells were vacuolated and form only 1 to 3 layers. Numerous blood vessels were noted in the interstitial tissue. The tubules were separated from each other with interstitial acidophilic material (Fig. 3). The basement membranes of the tubules appeared thickened and the interstitial material was PAS positive (Fig. 4).

Conalateral testes from group III revealed alterations of some tubules. The alterations were in the form of irregular tubules with reduced thickness of the germinal epithelium. There were many blood vessels and interstitial acidoophilic material (Fig. 5). The basement membranes of the tubules appeared thickened (Fig. 6).

As regards group IV (varicocele+RES), the ipsilateral testes revealed increased spermatogenic cell layers in the tubules. However, the tubules were variable in size. Interstitial acidoophilic material and many blood vessels were still present (Fig. 7). The basement membranes appeared to be thickened (Fig. 8). For the contralateral testes, there were no differences in the histological findings when compared to the control and sham-operated groups except for the presence of acidoophilic material in the interstitium (Fig. 9). The basement membranes appeared thin (Fig. 10).

Immunohistological Results:

Testicular tissues demonstrated spermatogenic cells labeled in the sections. The labeled cells included mainly spermatogonia and primary spermatocytes. The staining appeared in the nuclei of the labeled cells. The staining was quite evident in the control, sham-operated and contralateral testes of group IV (varicocele+RES). It was less evident in the ipsilateral testes of group IV (varicocele+RES) and contralateral testes of group III (varicocele) and least in the ipsilateral testes of group III (Figs. 11,12,13,14&15).

PCNA-LI Results:

Comparing total PCNA-LI values of the different experimental subgroups to that of the control group, they were similar in both group I and group II. The values were very highly significantly lower in the testes of group III (ipsi- and contralateral) than those of the control group. There was significant improvement of the PCNA-LI values of group IV when compared to the corresponding testes of group III. However, they were still significantly different from the control group (Table 1 and Histogram 1).

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* Significant difference (p<0.05).
Histological and Immunohistochemical Changes in the Adult Rat Testes after Left Experimental Varicocele

**Histogram 1:** Mean PCNA labeling index in the various studied groups. Groups sharing the same letter are not significantly different from each other at p ≥ 0.05.

**Fig. 1:** A photomicrograph of a section in the testis of control rats (Group I) showing the structure of the seminiferous tubules with the interstitial tissue. Note the few interstitial blood vessels (arrows).

H & E X 200.

**Fig. 2:** A photomicrograph of a section in the testis of control rats (Group I) showing a thin basement membrane surrounding a seminiferous tubule (arrows).

PAS X 400.

**Fig. 3:** A photomicrograph of a section from an ipsilateral testis from group III (varicocele) showing irregular variable sized seminiferous tubules. Germ cells are vacuolated and form only 1 to 3 layers. Note the numerous blood vessels (arrows) and homogenous acidophilic material (asterisks).

H & E X 200.

**Fig. 4:** A photomicrograph of a section from an ipsilateral testis form group III (varicocele) showing thickened basement membranes of the tubules (arrows) and PAS positive material (asterisks).

PAS X 400.
Fig. 5: A photomicrograph of a section from a contralateral testis from group III showing alterations of some tubules (T) in the form of reduction the number of layers of the germinal epithelium with vacuolated spermatogenic cells. Note the many blood vessels (arrows) and deposition of acidophilic material (asterisks). H & E X 200.

Fig. 6: A photomicrograph of a section from a contralateral testis from group III (varicocele) showing thickened basement membranes of the tubules (arrows). PAS X 400.

Fig. 7: A photomicrograph of a section from an ipsilateral testis from group IV (varicocele+RES) showing increased spermatogenic cell layers in the tubules. The seminiferous tubules are variable in size. H & E X 200.

Fig. 8: A photomicrograph of a section from an ipsilateral testis form group IV (varicocele+RES). The basement membranes appear to be thickened (arrows). PAS X 400.

Fig. 9: A photomicrograph of a section from a contralateral testis form group IV (varicocele+RES) showing the seminiferous tubules with structure similar to the control group. However, acidophilic material is still present (asterisk). H & E X 200.

Fig. 10: A photomicrograph of a section from a contralateral testis form group IV (varicocele+RES) showing thin basement membranes (arrows). PAS X 400.
Histological and Immunohistochemical Changes in the Adult Rat Testes after Left Experimental Varicocele

Fig. 11: A photomicrograph of a section in the testis of control rats (Group I) showing positive immunostaining (brown nuclear reaction) in most of the basal germ cells in the seminiferous tubules (arrows).
PCNA immunohistochemical staining X 200.

Fig. 12: A photomicrograph of a section from an ipsilateral testis form group III (varicocele) showing few immunopositive germ cells (arrows).
PCNA immunohistochemical staining X 200.

Fig. 13: A photomicrograph of a section from a contralateral testis form group III (varicocele) showing some immunostained germ cells (arrows).
PCNA immunohistochemical staining X 200.

Fig. 14: A photomicrograph of a section from an ipsilateral testis form group IV (varicocele+RES) many immunostained germ cells (arrows).
PCNA immunohistochemical staining X 200.

Fig. 15: A photomicrograph of a section from a contralateral testis form group IV (varicocele+RES) showing many immuno-positive cells in the seminiferous tubules (arrows).
PCNA immunohistochemical staining X 200.

DISCUSSION

Varicocele is one of the most common clinical diseases associated with male infertility and occurs in human beings. Studies were obviously limited in understanding the effect of varicocele on the testicular structure since study designs on humans must be noninvasive. Because of this, animal models of varicocele have become valuable tools.

In the present study, varicocele was created by means of partial ligation of the left renal artery. This method mimics the nut-cracker phenomenon believed by some to be a major cause of varicocele in humans. This phenomenon occurs in humans when the left renal vein becomes compressed between the aorta and the superior mesenteric artery and it suffers a potential occlusion.
The present study showed that the experimental varicocele in rats induced testicular lesions. The changes affected both ipsi- and contralateral testes. Ipsilateral testes demonstrated marked degenerative changes in the form of irregular seminiferous tubules, with degeneration of the germinal epithelium, thickening of the basement membranes of the seminiferous tubules and deposition of PAS positive material in the interstitial tissue. Contralateral testes demonstrated patchy tubular changes. Also, the degree of spermatogenesis was evaluated using PCNA-LI. A significant decline in both testes after experimental varicocele was found.

Similar changes were demonstrated in previous work in the ipsilateral rat testes. Chakraborty et al. documented blood stagnation within the microcirculatory vessels of the testes in patients with varicocele with consequent tissue hypoxia. High hydrostatic pressure is exerted on the pampiniform plexus. When venous pressures exceed the arteriolar pressures in the testicular microcirculatory system, the same hypoxic effect on the testicular tissue occurs on both sides. This adverse effect is due to hydrostatic pressure and lack of proper drainage. The blood becomes stagnated and as a consequence, oxygenated arteriolar blood cannot flow normally into the testis. It results in impaired sperm quality and reduction of the quantity, eventually leading to mild, moderate, severe and extremely severe oligospermia and azospermia.

The present results demonstrated blood vessel proliferation in the testes of left experimental varicocele group. This could be explained by tissue hypoxia since it is known to be the key factor for growth and proliferation of new blood vessels. It initiates a cascade of molecular pathways including those involved in angiogenesis, which is the development of new blood vessels from existing vasculature. Angiogenesis occurs through complex interactions among endothelial cells, basement membrane and extracellular matrix. Vascular endothelial growth factor (VEGF) appears to be universal when hypoxia is present. VEGF activates endothelial cells. This causes the cells to secrete and activate matrix metalloproteinases and plasminogen activators that can result in degradation of the basement membranes. This allows the endothelial cells to migrate into the surrounding matrix. As these cells move, they proliferate and differentiate to form a new vessel with a clear lumen. Finally, endothelial cells deposit basement membrane and also secrete growth factors that attack supporting cells to stabilize the new vessel.

Also, it is presumed by some that retrograde blood flow down the spermatic vein is a part of the condition of varicocele. The idea of retrograde blood flow has been used to explain how renal and adrenal metabolites (e.g., catecholamines) arrive at the testis and this may lead initially to chronic testicular vasoconstriction and ultimately be toxic to testicular function.

Based on previous observations, it appeared that men with varicoceles had increased oxidative stress in their semen. This oxidative stress manifested as elevated levels of oxygen free radical production, as well as decreased levels of protective antioxidants in the seminal fluid. ROS had been implicated in reduced fertility in patients with varicoceles. ROS concentrations are higher in semen samples from both fertile and infertile men with varicoceles compared with controls. Patients with varicocele represent an interesting model because they exhibit an augmented ROS generation and high levels of nitric oxide, which is related to ROS generation. In fact, testicular varicocele results in defective spermatogenesis and spermiogenesis, with the subsequent decrease in sperm production and the release of immature ROS-producing spermatozoa into the seminiferous tubules. The spermatozoon has a high content of polyunsaturated fatty acids within the plasma membrane and a low concentration of scavenging enzymes within the cytoplasm. Therefore, it is susceptible to the peroxidation in the presence of elevated ROS seminal levels and oxidative stress induces DNA damage in both the mitochondrial and nuclear genomes.

Increased evidence has pointed hypoxia can cause increased cellular oxidative stress with consequent damage to lipids, proteins and DNA. Mitochondria reductive stress has been suggested as one possible source of free radicals involved in this apparently paradoxical phenomenon of hypoxia-induced oxidative stress.

Based on previous studies, hypoxia may result in the accumulation of reducing equivalents in the mitochondrial electron transport chain, which can lead to enhanced production of reactive oxygen species (ROS). Mitochondria themselves may become oxidative targets, leading to peroxidation of membrane lipids, protein oxidation, DNA cleavage and consequently impaired ATP production.

The present study demonstrated that the contralateral testes were affected to a lesser degree. Although there are opposing previous views, unilateral varicocele has usually been considered to affect the contralateral testis and results in diminished fertility. The pathophysiology of this condition remains unclear. Immunological mechanisms, changes in contralateral blood flow and reflexive systems induced autonomic nervous system have been proposed to be involved. Recently, it was shown that chemical sympathectomy could improve spermatogenesis in
the contralateral testes of rats with left experimental varicocele. Moreover, previous work suggested that changes in the spermatogenesis in the contralateral testis consequent to a unilateral varicocele are probably caused by local (contralateral) tissue hypoxia. As in unilateral testicular torsion, which decreases blood flow and increases biochemical indicators of tissue hypoxia in the contralateral testis, there may be similar changes in unilateral varicocele.

In the present study, RES was shown to have protective effects against the changes induced by varicocele. Previous studies demonstrated that RES is used for the treatment of male infertility and/or subfertility having different causes, such as idiopathic infertility, high levels of reactive oxygen species or inflammatory alterations.

The mechanism of RES could be mediated by the concentration of constitutive oxidative stress within the seminiferous tubules. In cases of overproduction of ROS, RES was found to be effective scavenger of hydroxyl, superoxide as well as having antioxidant abilities in cells producing ROS, RES exhibits a protective effect against lipid peroxidation in cell membranes and DNA damage caused by ROS.

Resveratrol also induces an increase in the serum concentration of follicle-stimulating hormone (FSH), luteinizing hormone and testosterone, which involves hypophyseal stimulation in the hypothalamic-hypophyseal-testicular axis.

In conclusion, left experimental varicocele caused structural changes with affection of spermatogenesis in both testes and Resveratrol could partially reverse these changes. Our findings support a possible rationale for antioxidant supplementation in cases of infertility with varicocele.

REFERENCES

التغيرات الهيستومناعية والهستوكيميائية المناعية لخروج الفئران البالغة بعد دوالى الخصية اليسرى التجريبى

و الدور الوقائي المحتمل لمادة الرسفيراترول

منة محمد عبدالدايم
قسم الحفاظ، كلية الطب، جامعة القاهرة

ملخص البحث

توجد دوالى الخصية بنسبة 30-40% تقريبا عند الرجال الذين يعانون من العقم. بالرغم من ذلك فإن التغيرات الهيستومناعية للخصية غير محددة. بالإضافة إلى ذلك فإن مادة الرسفيراترول من مضادات الأكسدة القوية التي تستخدم حديثا في علاج العقم لأسباب أخرى.

لذلك كان الهدف من هذه الدراسة تحديد التغيرات الهيستومناعية والهستوكيميائية المناعية للخصيتين بعد دوالى الخصية اليسرى التجريبى وتقييم الدور الوقائي المحتمل لمادة الرسفيراترول.

و قد شملت هذه الدراسة أربعين فأراً ذكوراً بالغاً وتم تقسيمهم إلى أربع مجموعات متساوية: المجموعة الأولى (المجموعة الضابطة)، المجموعة الثانية (مجموعة شام)، المجموعة الثالثة (مجموعة دوالي الخصية اليسرى) والمجموعة الرابعة (مجموعة دوالي الخصية اليسرى + الرسفيراترول). وقد تم إعداد دوالى الخصية اليسرى عن طريق ربط الوريد الكلوي الأيسررين جزئيا. وتم إعطاء المجموعة الرابعة مادة الرسفيراترول بجرعة 20 مجم/كم من وزن الجسم يوميا لمدة خمسة أسابيع. بعد انتهاء المدة تم التضحية بكل الفئران واستخلاص الخصى. تم تحضير قطعات شعاعية برايفينية وصبغها بالهيماتوكسيلين وإيوسين وتفاعل شحوم البيروبريديك وذكراً صبغها صبغة هستوكيميائية مناعية لمستقبلات أنيونية (الخلايا المتكترة) وتم حساب مؤشر PCNA (NPNA) لتقييم عملية تكون الحيوانات المنوية.

و كانت النتائج أن دوالى الخصية اليسرى التجريبى قد أدى إلى تأثر كل من الخصيتيين. و كانت التغييرات في الحالة اليسرى في صورة عدم التماثل و تحلل في الأنابيب المنوية وزيادة في سمك الغشاء القاعدي للأنابيب المنوية. أما الخصية اليمنى فقد كانت التغيرات موجودة في بعض الأنابيب المنوية. و أظهرت النتائج أيضاً انخفاض مؤشر PCNA إحصائياً. وقد أثبتت الدراسة أن تناول مادة الرسفيراترول قد أدى إلى تحسن هذه التغييرات.

و يمكن استنتاج أن دوالى الخصية اليسرى التجريبى له آثار ضارة على كل من الخصيتيين. و أن هذه الدراسة أن التكملية بمواد الرسفيراترول لحالات العقم نتيجة لوجود دوالى الخصية قد يكون له تأثير مفيد.