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ORIGINAL ARTICLE

Effects of melatonin on testicular tissue nitric oxide level and antioxidant enzyme activities in experimentally induced left varicocele

Atilla Semercioz, Rahmi Onur, Sezai Ogras & Irfan Orhan

Department of Urology, Faculty of Medicine, University of Firat, Elazig - TURKEY.

Correspondence to: Dr. Rahmi ONUR
Firat Universitesi, Firat Tip Merkezi
Uroloji Klinigi
TR-23200, Elazig - TURKEY
EMAIL: rahmionur@yahoo.com
PHONE: + 90 424 233 35 55 / 2522
FAX: + 90 424 238 76 88

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Abstract

OBJECTIVES: The pathophysiology of the testicular damage in varicocele have not been completely understood. There are several studies concerning the effects of increased seminal reactive oxygen species (ROS) and the role of nitric oxide (NO) in infertile patients with varicocele and antioxidants have been used successfully to decrease oxidative stress in testis. In this study, we determined the effects of melatonin on testicular NO and antioxidant enzyme levels and investigated whether melatonin can prevent or diminish testicular damage in adult rats with experimentally induced unilateral varicocele.

METHODS: Thirty adult male Wistar rats were randomly divided into three groups. In group I, using midline incision left renal vein was exposed but not tied. In group II, left renal vein was partially ligated to create varicocele. In group III, after creation of varicocele, daily and fresh-prepared melatonin was administered intraperitoneally for 4 weeks. Histopathological examination was performed and tissue malondialdehyde (MDA), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and tissue NO levels were determined.

RESULTS: In group II, there were severe degenerative changes of the germinal epithelium and atrophy of the seminiferous tubules in left testis. In group III, in which rats received melatonin intraperitoneally, there were focal areas showing decrease in spermatogenesis and some degenerative changes. Similarly, the levels of ROS and NO were significantly increased in testicular tissue of rats in group II, whereas group III rat testes in which melatonin was administered, showed increased antioxidant enzyme activity and decreased NO levels.

CONCLUSION: Infertility related to varicocele has multiple pathophysiological mechanisms and the effect of ROS, NO and antioxidant defense system are contributing factors in the disease state. Melatonin, a potent free radical scavenger, administration might be helpful in the prevention or at least, in the delay of the severe effects of varicocele on testicular tissue.

Abbreviations & units

ROS	Reactive oxygen species
NO	Nitric oxide
mg	milligram
kg	kilo gram
g	gram
°C	centigrade degrees
mm	millimeter
NaCl	Sodium chloride
MDA	Malondialdehyde
SOD	Superoxide dismutase
GSH-Px	Glutathione Peroxidase
nm	nanometer
µmol/g w t	mikromol / gram wet tissue
SD	Standard deviation

Introduction

Varicocele is the most common cause of male infertility and present in 19 to 41% of men presenting for infertility evaluation [1,2]. However, pathogenesis of testicular damage or the mechanism by which varicocele produces sperm dysfunction has not been clearly identified yet [2]. The proposed mechanisms include testicular hypoxia by venous stasis and small vessel occlusion, leading to Leydig cell and germinal cell dysfunction, retrograde blood flow of adrenal and renal metabolites, increased testicular and scrotal temperature and decrease in gonadotrophin and androgen secretion [3,4]. Whatever the mechanism, the common histopathologic features of severe and prolonged varicocele, on testicular examination, are severe degenerative changes of the germinal epithelium and atrophy of the seminiferous tubules [5].

Reactive oxygen species (ROS) were shown to be constantly produced by spermatozoa and the spermatozoal membrane, which is rich in polyunsaturated fatty acids, is susceptible to peroxidation in the presence of elevated seminal ROS levels [1]. Thus, sperm dysfunction may be a consequence of elevated seminal ROS and several studies demonstrated that in the presence of increased antioxidant enzyme levels testicular dysfunction can be prevented in varicocelized subjects [1,6,7]. Recently, nitric oxide (NO) which is an intracellular and intercellular messenger in different tissues, has been shown to be excessively released within the dilated spermatic vein that could lead to impaired sperm function [2,8]. It is a lipophilic molecule that may exert direct cytotoxic effects in the neighbouring sperm cells and was also shown that NO and superoxide released by monocytes within the seminal plasma could also react together to form peroxynitrite and this may further damage spermatozoa [8].

Treatments involving antioxidants have been used successfully to decrease oxidative stress related injuries in many organ systems, as well as in testis [6,9]. Melatonin, an endogenous hormone secreted in circadian rhythm from the pineal gland can enhance the antioxidant defense systems and acts as a potent free oxygen radical scavenger [10]. In the present study, we aimed to document the effects of experimental varicocele on rat testicular tissue and to determine the role of NO and antioxidant defense system in varicocelized rat testicular tissue. We also determined the effects of melatonin on testicular NO and antioxidant enzyme levels and investigated whether melatonin at 10 mg/kg dose can prevent or diminish testicular damage in adult rats with experimentally induced unilateral varicocele.

Materials and methods

This study was approved by the Local Ethics Committee of Firat University. Thirty adult male Wistar rats (aged 12–14 weeks) weighing 250–350 g were used in this study. All rats were kept in individual cages being exposed to 12 hours of daylight at 20 ± 1°C temperature, 55 ± 15% humid conditions and fed with standard rat food and tap water until experimentation.

Experimental Design and Creation of Varicocele

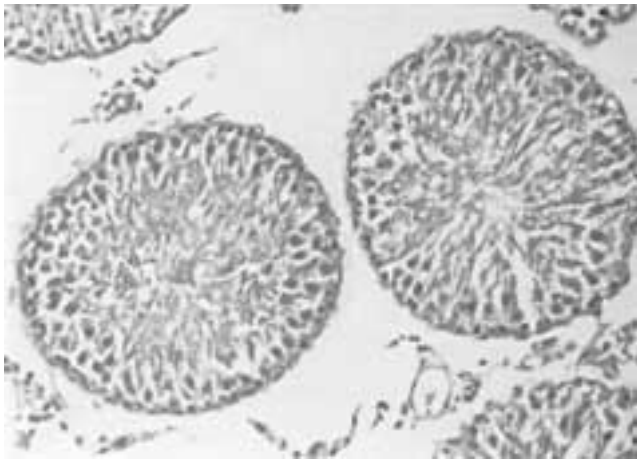
Thirty adult male rats were divided into 3 equal groups. In group I, rats underwent sham operation. Partial left renal vein ligation was performed to rats in groups II and III under intramuscular ketamine hydrochloride (Ketalar, Eczacibasi, Istanbul, Turkey), 44mg/kg and xylazine-hydrochloride (Rompun, Bayer, Istanbul, Turkey), 5mg/kg, anesthesia. Testes were examined 4 weeks after creation of varicocele in all groups. With a midline incision the left renal vein was exposed and after fine dissection of proximal left renal vein, a 4–0 silk suture was tied around the left renal vein. A metal probe with various diameters ranging from 0.4 to 0.85 mm diameter selected according to the size of the renal vein was placed on the left renal vein at the point medial to the insertion of the adrenal and spermatic vein into the renal vein. The ligature was tied around the vein to contain the metal probe and following ligature the probe was removed gently and the vein allowed to expand within the boundary of ligature. Immediate dilation of the renal vein was observed in all rats by which the renal vein diameter was reduced by approximately one half. Sham operated animals underwent the same procedure except that the ligatures were not secured. The midline incisions were closed with interrupted 3–0 silk sutures in all animals.

Preparation and Administration of Drug

Under sterile conditions and avoiding direct sunlight exposure, melatonin (Sigma Chemical, St. Louis, MO) was dissolved freshly in pure ethanol and mixed with 0.9 % NaCl amounting a final concentration of 1:10 in a freshly prepared solution form. Throughout the experiment, 10 mg/kg melatonin was administered intraperitoneally to the rats in groups III. Sham operated and varicocele-induced rats in groups I and II, respectively, received equal amount of physiological saline intraperitoneally, everyday.

Assessment of effects of experimental varicocele

Animals were sacrificed 4 weeks after creation of varicocele. Both, ipsilateral and contralateral testes were removed and half of each testes were washed with saline. One-half of each testes fixed in formaldehyde and stained with hematoxylin and eosin. Degenerative changes of the germinal epithelium and atrophy of the seminiferous tubules was assessed in histopathological specimens blindly by only one specialist and the severity was graded as mild, moderate and severe.



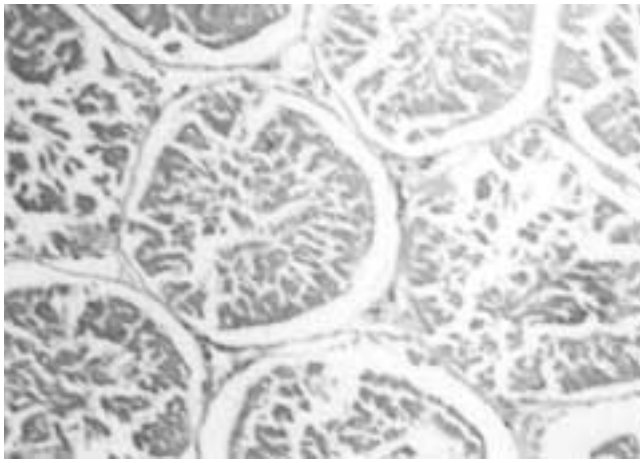


Figure 1. Normal testicular histopathologic appearance in sham-operated rats (H&E x 200).

Figure 2. A section of left testis from group II showing prominent decrease in spermatogenesis, maturation arrest and total loss of spermatogenetic cells and interstitital edema with vascular congestion (H&E x 200).

Figure 3. Focal areas showing decrease in spermatogenesis, basal layer thickening with slightly decreased seminiferous tubule diameter and some loss of germ cells (H&E x 200).

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The other samples obtained from both testes of all animals were immediately frozen and stored for few days at –20° C until tissue MDA and antioxidant enzyme activities was analyzed.

Determination of Tissue Malondialdehyde and Antioxidant Enzyme Activities

For the biochemical evaluation of oxidant-antioxidant system, the testicular tissue washed 3 times with 0.9% NaCl solution and 1.15% KCl was liquidified to amount 9 mL for each tissue. Homogenate was prepared with the teflon end on homogenizator (Elvenjem Potter, Du Pont Instruments, Newton,CT) and were centrifuged at 4000 rpm.

The determination of MDA in the homogenates was performed spectrophotometrically with the method described by Satoh and Yagi [11,12]. We assessed the tissue levels of tissue superoxide dismutase (SOD), and glutathione Peroxidase (GSH-Px) activities, similarly. The level of tissue SOD activity was measured using the commercially available measurement kit of RANSOD (Randox lab., Crumlin, BT 29, UK).

Determination of Tissue Nitric Oxide

Endogenously produced NO within the body is a highly unstable product that has readily oxidized to nitrite initially and then to nitrate. Thus, the concentration of NO in many biological systems is usually termed as nitrite and nitrate [13]. In our study, we also preferred to determine the level of NO by measuring spectrophotometrically its stable decomposition products. This method, as described previously, requires decomposition of NO and determination of the stable products by Griess reaction [13]. To avoid any nonspecific interactions we first deproteinized the tissue and then performed the measurement of nitrite/nitrate concentrations and represented it as tissue NO levels. Total nitrite concentration was assessed using modified Cadmium reaction method. The colour produced by nitrite was measured spectrophotometrically at 545 nm.

Data Analysis

The data were expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA and Student's *t-test* for the significance between the groups, whereas histopathologic examinations were graded as mild, moderate or severe destruction and compared using the non-parametric chi-square test. $p<0.05$ was considered statistically significant.

Result

Histologically, the testes of sham operated rats showed seminiferous tubules comprising a complex stratified epithelium containing spermatogenic cells and supporting cells (Sertoli cells) which was considered as normal (Figure 1). Contralateral testes in group I showed no significant difference with respect to left testes. In group II, testicular tissue of all rats were dramatically affected and severe degenerative changes of the germinal epithelium and atrophy of the seminiferous tubules were observed. Contralateral testes in the same group showed also degenerative changes but moderately affected. The testes in this group showed

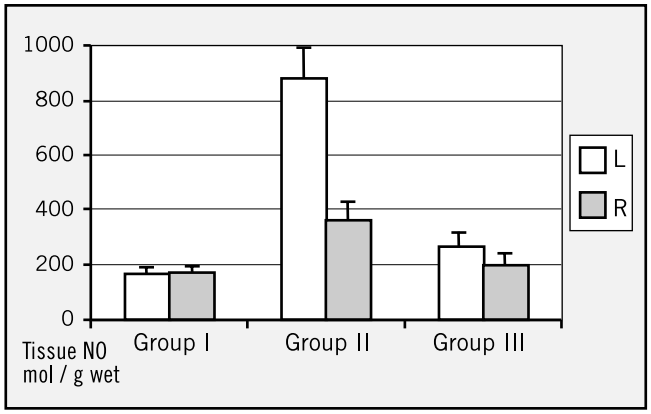

Figure 4. Comparison of left and right testicular tissue Nitric oxide levels in each group.

prominent changes and there was significant damage when compared to group I testes. However, the histology of right testes was better preserved than the varicocelized one ($p<0.05$). There was a prominent decrease in spermatogenesis, maturation arrest and total loss of spermatogenetic cells and interstitital edema with vascular congestion in many cases (Figure 2). In group III, in which rats received 10 mg/kg melatonin intraperitoneally, there were focal areas showing decrease in spermatogenesis, basal layer thickening with slightly decreased seminiferous tubule diameter and some loss of germ cells (Figure 3). However, there was still spermatogenetic activity with normal germ cells and partial preservation of normal histopathology when compared to testes of rats in group I.

Tissue levels of MDA and activities of antioxidant enzymes, GSH-Px and SOD in all groups are shown in Table 1. Melatonin administration in group III raised the level of antioxidant enzyme activities to the level of sham operated animals and no statistically significant difference was observed between these two groups ($p>0.05$).

Figure 4 demonstrates the amount of NO produced within ipsilateral and contralateral testes of sham operated, varicocelized and varicocele + melatonin administered rats. Nitric oxide levels in left testis of sham operated rats were excessively higher than the testicular tissue NO level in groups I and III. Contralateral testes NO measurements showed no statistically significant difference except in group II. There was a two-fold increase in NO level in left testis of varicocelized rats when compared to right testis (Figure 4). Tissue NO levels of left testes in group III were significantly higher than group I, however were found to be significantly lower when compared to group II. This was also shown histopathologically since testicular tissue was severely affected in group II, whereas it was moderately affected in group III.

Discussion

Nitric oxide is a highly reactive free radical participating in many physiological processes in a variety of organs [14]. In testis, it was shown to be important in the regulation of male reproductive function and fertility, as low concentrations of NO can influence male fertilization, sperm motility and has been postulated to have a possible paracrine/autocrine role in the regulation of testicular steroidogenesis [15,16,17]. Since NO is a free radical and has its biological roles at low concentrations, its production in supraphysiological levels can compromise both testicular and sperm function [2,15]. Moreover, high levels of NO reacting together with superoxide could contribute to the formation of peroxynitrite, a highly toxic anion [8,14].

Varicocele, which is the leading cause of male infertility, is associated with both increased production of NO and spermatozoal reactive oxygen species. Irrespective of the fertility status, it was identified that there's a strong relationship between a potent end effect of sperm dysfunction and varicocele [1]. Recently, many researches implicated the role of ROS in the testicular dysfunction and showed poor sperm motility in infertile men to be strongly related to lower antioxidant levels [1,18]. Hendin et al reported a 4-fold increase in the frequency of elevated ROS generation in the incidental varicocele group compared to their control patients [1]. In our study, we observed significantly increased levels of MDA and a statistically significant decrease in the level of antioxidant enzyme activities ($p<0.05$). Similarly NO, which is another free radical with more diverse actions in many biological systems, was also reported to be dramatically increased in spermatic veins of patients with varicocele compared to peripheral veins of same subjects. Mitropoulos et al reported a 25-fold increase in the rate of NO production in the varicocele vein compared to the peripheral vein [8]. In the present study, we determined more than 8-fold increase in the level of NO in varicocelized testis. It was also demonstrated that ROS significantly increased in varicocelized testis and contralateral testis with subsequent suppression of the antioxidant enzyme activities. Nitric oxide, with its direct cytotoxic effects react with superoxide to form peroxynitrite, an unstable species, that is protonated to peroxynitrous acid. This product spontaneously decomposes to nitrite and hydroxyl radi-

Table 1. The level of tissue Malondialdehyde and activities of antioxidant enzymes, Glutathion peroxidase and Superoxide dismutase.

	Group I		Group II		Group III	
	Left	Right	Left	Right	Left	Right
Malondialdehyde	6.67±1.01	6.74±1.14	13.7±1.77*	10.20±0.90*	6.81±0.72	6.90±1.03
(nmol / g Protein)	(6.0–9.30)	(6.1–9,40)	(8.10–18.50)	(7.0–16.0)	(6.10–8.50)	(5.80–9.20)
Superoxide Dismutase	133.65±30.69*	142.20±28.70*	43.27±6.57	61.38±8.90	127.232±15.01*	138.20±17.15*
(U / mgProtein)	(110–210)	(108–244)	(33–53)	(40 –799)	(92–115)	(88–167)
Glutathion Peroxidase	61.4±4.32*	58.7±7.40*	28.7±5.33	32.4±7.14	59.8±6.26*	60.2±7.01 *
(U / g Protein)	(55–67)	(51–65)	(21–38)	(23–44)	(56–75)	(53 –71)

* : $p<0.05$.

cals and the remaining peroxynitrite is directly isomerized to nitrate [19]. Peroxynitrite is an important mediator of free radical toxicity with strong oxidizing properties in many biological molecules, such as protein and nonprotein sulfhydrates, deoxyribonucleic acid and membrane phospholipids. As a result peroxynitrite appears as one of the responsible pathogenic agents in the testicular dysfunction in patients with varicocele [8]. Considering several other pathophysiologies in varicocele testis, the testicular damage as end result appears to be a complex interrelationship of different factors. In our study, we mainly focused on the effects of two different systems, nitric oxide and antioxidant defense system, and subsequently evaluated the protective role of melatonin. Our findings suggest that, experimentally induced left varicocele stimulates production of lipid peroxydation products and diminishes the level of antioxidant enzyme activities. At the end of experiment, testicular NO levels was observed to be increased excessively and together with increased level of MDA and decreased SOD and GSH-Px, a severely damaged testicular architecture was detected. However, melatonin administered intraperitoneally to varicocelized rats in group III successfully reduced the tissue MDA and NO levels while led to an increase in SOD and GSH-Px enzyme activities. As a result, both ipsilateral and contralateral testes in group III showed a moderately protected testicular histology from the destructive effects of left varicocele.

Although, exact mechanism related to varicocele has not been clearly identified yet, our data provide evidence for participation of ROS and NO as the mediators of testicular damage. Severe and prolonged varicocele, as performed in our study was found to be associated with prominent histopathological changes, increased NO, MDA production and decreased antioxidant levels. Since ROS is diffusely distributed in testicular tissue and NO, as a lipophilic molecule, may also diffuse out of the varicocele veins and exert cytotoxic effects in the neighbouring sperm cells, resulting in their damage. Nitric oxide and superoxide in the presence of varicocele are released excessively and could also form peroxynitrite and may further damage spermatozoa [8]. Here, melatonin plays a dual role and directly acts in the protection of normal histology by detoxifying superoxide ion and by inhibiting nitric oxide production [20]. As it was reported in previous studies, melatonin shows its beneficial effects with increasing doses and in our study had protective effects on testicular tissue by inhibiting lipid peroxidation and decreasing NO production [10].

The use of melatonin as an antioxidant is widely accepted and proven to be effective in many biological systems [10,21]. Our results contributed to its protective effects on oxidative damage in testicular tissue and suggest a novel action involving decreased production of NO in both ipsilateral and contralateral testicular tissue, in case of experimentally induced left varicocele. To our knowledge, there was no previous report regarding the relationship between NO and melatonin in animal testicular tissue but further studies are needed to show the complex relationship between NO, antioxidant defense system and the testes. Nonetheless, the use of melatonin in experimental varicocele may delay the detrimental effects until surgery, the gold standard treatment modality of varicocele, performed.

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