The effects of nitric oxide synthase inhibitor (L-NAME) on epididymal sperm count, motility, and morphology in varicocelized rat

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ABSTRACT

Introduction: Increase in the nitric oxide in the spermatic veins of men by varicocele has been reported. Although Several studies have considered the relationship between varicocele and semen NO concentrations, no study on the effects of nitric oxide synthase inhibitor (L-NAME) on epididymal sperm count, motility and morphology which are important in fertility of the individual has been reported. The aim of study was to evaluate the effects of nitric oxide synthase inhibitor (L-NAME) on epididymal sperm count, motility, and morphology in varicocelized rat.

Methods: Twenty four Wistar male rats divided into four groups. The group A and B underwent a left experimental varicocele (by 20-gauge needle). Group C, underwent a procedure similar to groups A and B without any change on spermatic vein (as sham group). Group D referred to as control. Animals in group A were killed 10 weeks after the operation and both left and right epididymal sperm were counted and their morphology and motility were analyzed. Animals in group B received 10mg/kg L-NAME intraperitoneally daily for ten weeks.

Results: In group A, Sperm count decreased and the morphology changed significantly in comparison with the groups C and D. The sperm morphology in groups A and B showed statistically significant differences ($P<0.0001$). Sperm motility decreased significantly in the group A in comparison with the groups C and D. Although motility in group A of animals were different in comparison with group B , it was not statistically significant.

Conclusion: These findings suggest that nitric oxide synthase inhibitor (L-NAME) improved sperm count and morphology.

Keywords: Nitric Oxide Synthase Inhibitor, Sperm parameters, Varicocele, Rat

INTRODUCTION

Varicocele is an abnormal dilatation and stasis of veins of the pampiniform plexus that drain the testis. It occurs within the spermatic cord (1, 2), it occurs in 15-20% of the male population and causes infertility in 30-50% of married men (2).

Various theories have been proposed to explain that 90% of all varicocele are left-sided (10, 17). Although many infertile people have varicocele, its relationship with male infertility still remains unexplained. Many factors seem to be involved in sperm alterations: backflow of noxious substances from the kidney or adrenal glands, increase in the testicular temperature, tissue hypoxia induced by venous stasis, hypothalamic or pituitary dysfunctions (1,3-7). Recently it has been proposed that varicocele results from oxidative stress and testicular backflow is a cause of gonadal injury which is mainly caused by reactive oxygen species (ROS) (1, 7- 13).

Varicocele repairs improve intratesticular temperature, but fertility will reverse only in about one half of the patients. Thus the mechanism of varicocele induced infertility is not clear and requires further investigations (7, 9, 14).

Nitric oxide (NO) is a free radical gaseous molecule synthesized by three isoforms of NO synthase (NOS), namely the neuronal, endothelial and inducible isoforms. NOS isoforms have been shown to regulate a number of functions, e.g. sperm motility and maturation and germ cell apoptosis in the testes. (15). In adult patients with varicocele the amount of NO levels in the varicose veins are 25 times higher than in serum of peripheral veins. In these

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patients high oxidative stress is postulated and NO is considered as a causative factor for bad sperm function in subfertile men with varicocele (1, 5, 7, 16). Some authors have believed that a partial obstruction of the spermatic vein is the only procedure which is able to induce a varicocele similar to that happen in human beings (9, 17).

The harmful effects of varicocele on spermatogenesis are still not clear. High levels of NO could determine sperm toxicity and reduce sperm motility through formation of peroxy-nitrite, a highly toxic anion of peroxidation (12).

NG-Nitro-L-arginine methyl ester is extensively used as NOS inhibitor in pharmacological tests. On the basis of reports (15, 18) it inhibits all three NOS isoforms and lower NO content in tissues (15, 18). Although several authors have considered the relationship between varicocele and semen NO concentrations, no study about the effects of nitric oxide synthase inhibitor (L-NAME) on epididymal sperm parameters has been reported. The aim of study was to evaluate the effects of nitric oxide synthase inhibitor (L-NAME) on epididymal sperm count, motility, and morphology in varicocelized rat.

MATERIALS AND METHODS

Twenty four Wistar male rats (mean weight 250 g) were maintained under standard laboratory conditions. For fertility test, after 1-week acclimatization, under a 12 h: 12 h light: dark cycle at room temperature of 22 ± 2ºC all male animals which were fertile mated with adult female rats and pregnancy was evaluated.

Animals were divided randomly into four groups. The group A and B underwent a left experimental varicocele (partial spermatic vein ligation). By this method the lumen of the spermatic vein was reduced to 20-gauge effectively. The group C (sham group), underwent a similar procedure but without legation of spermatic vein. The group D served as control.

Operation

General anesthesia was induced by intraperitoneal (I. P) injection of 5% ketamine hydrochloride (100 mg/kg) and xylazine (1mg/kg)(19). By means of a Zeiss OPM1 operative microscope (200X magnification), the left spermatic vein was identified and isolated from testicular emergence as far as its outlet in the left common iliac vein. Groups A and B were subjected to ligation in left selective spermatic vein by a ‘20-gauge needle. The needle was positioned parallel to the spermatic vein and a 4-0 silk suture was tied around the spermatic vein and needle. Then the needle was removed to induce an evident dilation with blood engorgement inside the spermatic vein without complete obstruction of the lumen. By this method the lumen of the vein was reduced to 20-gauge effectively. The wound was closed with a 4-0 silk suture (9). In group C (sham group) rats underwent a similar procedure without ligation of the spermatic vein. Animals in group A were killed 10 weeks after the operation.

Treatment

Ten weeks after operation, animals in group B received 10mg /kg of L-NAME [NG-Nitro-L-arginine methyl ester] (Sigma Chemicals Co). The drug was dissolved in distilled water immediately before intraperitoneal administration. Animals received drugs for ten weeks.

Epididymal Sperm Counting, Motility and Morphology

Sperm collection and count

The laparotomy was conducted and the reproductive tract was exposed. The left and right epididyms were carefully separated from the testis and placed in a Petri dish containing Ham's F10. Epididymal caudal was minced with scissors to release sperm and then was placed in the incubator for 15min.

Approximately 10µL of the diluted sperm suspension was transferred to each counting chamber of the hemocytometer and allowed to stand for 5 min. The cells which settled during this time were counted by a light microscope at 200X magnification. (The sperm heads were counted and expressed as million/ml of suspension) (22).

Sperm morphology

The sperm morphology was also determined using Eosin/Nigrosin stain as earlier described (23, 24). To a test tube containing 40µl of sperm suspension was added 10 µl of 1% eosin Y and Nigrosin and were mixed by gentle agitation. Sperm were incubated at room temperature for 45-60 minutes for staining and then re-suspended with a Pasteur pipette. It was found that it is better to examine 200 sperms per animal morphologically at 400-1000X magnification.

Morphological abnormalities were classified as; Headless sperm, flattened head: reduced hook or banana head pin or nail, bent neck, bent tail, kinked tail, multiple abnormalities (23,24).

Sperm motility

The spermatozoa were classified as motile or immotile. Aliquots of the sperm suspension

prepared for analysis were placed on a slide. The slides were evaluated with phase contrast microscopy in 10 microscopic fields and 200 sperms per animal were analyzed at a final magnification of × 1 000. The assessment of the motile sperm fraction was defined as the mean number of motile sperm × 100 /total number of sperm (22)

Statistical Analyses

All values were presented as mean ± SEM. One-way analyses of variance (ANOVA) and post hoc Duncan test were performed to determine the differences among all groups for the whole parameters using the SPSS/PC computer program (version 13.0SS).

RESULTS

Macroscopic findings

All the rats in group A and B showed a conspicuous dilatation of the left spermatic vein with blood engorgement. In group C no dilatation of the left spermatic vein was observed.

Microscopic findings

Sperm count, motility and morphology decreased significantly in the group A (rats were killed 10 weeks after operation) and the group B (rats were killed 20 weeks after operation and treated for ten weeks) of animals that received L-NAME injection in comparison with the control group (Table 1).

The sperm count and normal morphology increased in the group B of animals that received L-NAME in comparison with the corresponding group A (Table 1).

Sperm motility decreased significantly in the group A in comparison with the control group (Table 1). But it did not show any significant differences in in comparison with treated group B. In general, group C (sham) did not show any significant alteration in sperm count, motility or normal morphology in comparison with the control group (Table 1).

DISCUSSION

Varicocele is characterized by the stasis of the internal spermatic vein, leading to elevated scrotal temperature, testicular hypoxia and retrograde blood flow of adrenal and renal metabolites especially in left side (1, 5, 9, 19). Several studies have confirmed the role of NO in modulation of sexual and reproductive function and, it has also been suggested that NO might be involved in different testicular abnormalities, i.e. in inhibiting human sperm motility, in germ cell degeneration and in stress-impaired testicular steroidogenesis (1,7, 9,16). Effects of a varicocele on semen parameters in adults have been extensively studied and consistent findings have been; decreased sperm motility, lower total sperm counts, and increased number of abnormal sperm forms (1-3).

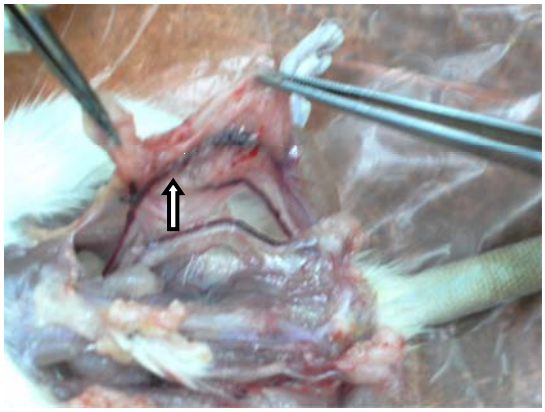

Figure 1. Spermatic vein that was distended after varicocele (arrow).

In this study, Sperm count, motile sperm and normal morphology were decreased significantly in the group A (varicocelized rat). There were statistically significant differences between the groups A and D. These effects of varicocele may be mainly due to the reversal of direct effects of NO on these parameters. These findings support the previous conclusion that in experimental studies of varicocele, NO at supra physiological levels can be harmful for both testicular and sperm function (9,12) and increased NO production could reduce sperm motility and adversely influence sperm function and infertility. There were significant differences in sperm counts, motility and morphology between the two groups (varicocelized rat and control group).

Since sperm motility was significantly related to the testicular tissue NO levels it has been suggested that NO is an important mediator in the pathogenesis of varicocele (12).

Reduction in number of mobile spermatozoids and increase in the number of abnormal ones are the result of the effects of varicocele on spermatogenesis.

An abnormal spermogram is more frequent in men with varicocele than in those without varicocele. The qualitative and quantitative improvement of the spermogram, lead to a greater number of pregnancies (26). Sperm motility and concentration in patients with varicocele have been correlated with the seminal plasma NO concentration (1, 6, 27). A decreased in semen concentration resulting from increase in NO is due to the direct inhibition of mitochondrial respiration and DNA synthesis. NO may reduces ATP levels in cells due to inhibition of ATP synthase. Therefore, a decrease in ATP content or production might results to insufficient energy and poor sperm motility since

Table 1. Effects of nitric oxide synthase inhibitor (L-NAME) on epididymal sperm count, motility, and morphology in varicocelized rat

parameters	Groups							
	Control (D)		Sham (C)		Varicocele (A)		Varicocele (B)	
	Right	Left	Right	Left	Right	Left	Right	Left
Sperm count ($\times 10^6$ /mL)	144 ±5.1	146 ±7.1	136 ±16.9	143 ±11	123 ±17.8	68 ±19.3*	130 ±9.1	116 ±11.9**
Motile sperm (%)	86 ±4.1	87 ±2.6	91 ±2	88 ±5.5	84 ±5	58 ±6.9*	91 ±1	65 ±2.5
Normal morphology sperm (%)	90 ±1.5	89 ±2.2	86 ±1.7	88 ±1.6	74 ±5.9	58 ± 9*	86 ±2.2	80 ±4.5**

Note. Results are given as means ± SD., * p <.0001 compared with groups C&D, ** p <.0001 compared with group A

approximately 90% of the energy is produced by ATP (12, 28). Some investigations in animal and human have shown that NO concentration increases in seminal plasma, spermatic veins, and leydig cells of patients with varicocele (5, 7,16). Experimental studies have shown that NO at supra physiological levels can be harmful for both testicular and sperm function. Several authors have considered the relationship between varicocele and semen NO concentrations, concluding that increased NO production may influence sperm production, motility and morphology in these patients (1, 4-7). Moreover, increase in NOS activity has been observed in dilated spermatic veins, as well as in Leydig cells in varicocele patients (7, 16). Oxidative status, which reflects a relative balance between generated ROS and its free radicals scavenging, may not be responsible for the testicular dysfunction associated with experimentally induced varicocele during adolescence in rats (9). NOS isoforms have been shown to regulate a number of functions, e.g. sperm motility and maturation and germ cell apoptosis in the testes (18). A common pharmacological approach to study the role of a biological mediator is to investigate the effects of specific inhibitor on the processes related to the mediator. Thus, Guanidine such as L-NAME and Amino-guanidine are extensively used as inhibitors in pharmacological tests, since it is believed that their pharmacological effects are exerted by inhibition of NOS isoforms and presumably by lowering NO content in tissues. (15). An L-arginine analog, NG-nitro-L-arginine methyl ester (L-NAME) inhibits all three NOS isoforms and is a potent inhibitor of NO synthesis in all tested tissues. To our knowledge, this is the first investigation for a relation between the effects of nitric oxide synthase inhibitor (L-NAME) on epididymal sperm count, motility and morphology in varicocelized rat (1).

The results of this investigation is in accordance with other studies in which increase in NOS activity were found in dilated spermatic veins, as well as in Leydig cells in varicocele patients. (7,16)

This study has demonstrated that there were few differences in sperm motility between groups A (varicocelized rat) and B (treated) and suggests that sperm motility is significantly related to testicular tissue NO levels. There is a negative correlation between seminal plasma NO concentration and sperm motility, and it is concluded that increase in NO concentration may be one of the reasons for damages to sperm in patients with varicocele (4, 28). These negative effects of NO on sperm can be abrogated by inhibiting NO synthesis by L-NAME (25, 29). NO regulates sperm motility, in a way that low concentration of NO enhances and medium and/or high concentrations of NO decrease sperm motility. It may be speculated that under physiological conditions, small amounts of NO are generated to neutralize free radicals which inhibit sperm motility. Thereby low concentrations of NO may protect O2-mediated reduction of sperm motility. In contrast, excessive generation of NO under pathological conditions such as varicocele reduce sperm motility by contribution in formation of peroxynitrite, a highly toxic anion of peroxidation (11, 25).

CONCLUSION

The findings of this study show that nitric oxide synthase inhibitor (L-NAME) may improve sperm count and morphology that are associated with infertility in varicocelized rat. Therefore, it might be considered that NO is an important mediator in the pathogenesis of varicocele. This study may support the concept that the use of nitric oxide synthase inhibitor in infertile men with varicocele may be useful.

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