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The effect of aminoguanidine on sperm motility and mitochondrial membrane potential in varicocelized rats

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ABSTRACT

Objective(s): Increased levels of nitric oxide (NO) in the testicular veins of people suffering from varicocele have already been reported. However, the role of NO-synthase (NOS) isozymes and their inhibitors have not been extensively studied. We aimed to evaluate the inhibitory effects of aminoguanidine (AG), on sperm motility, vitality, and mitochondrial membrane potential (MMP) in varicocelized rats.

Materials and Methods: Twenty fore male Wister rats were divided into control, sham, varicocele, and treatment groups. Varicocele and treatment groups underwent partial ligation of left renal vein. Rats in the sham group underwent the same procedures as the varicocele group with the exception of vein ligation. 10 weeks after varicocele induction, sperm parameters were evaluated in all groups. The treatment group received 50 mg/kg AG injection daily for 10 weeks after which they were sacrificed prior to assessment of the parameters. Sperm viability and MMP were assessed by flow cytometry using propidium iodide (PI) and rhodamine 123 (Rh123), respectively.

Results: The results of this study show a decrease in sperm viability, motility and MMP in the varicocele group compared with the other groups. After AG injection, we observed that all the parameters were significantly enhanced in the treatment group compared with the other groups. Rh123 staining revealed a positive relation between MMP and sperm motility, whereas PI staining showed a positive relation between sperm motility and viability.

Conclusion: The findings of our study show that AG improves sperm motility and MMP, and thus, might be useful in the management of varicocele-related infertility.

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Introduction

Varicocele is an abnormal enlargement of the testicular vein (pampiniform venous plexus) above and around the testes (1). It usually happens in 19% to 41% of men with primary infertility and in around 80% of men with secondary infertility. Thus, this abnormality is probably one of the most frequent reasons for low sperm production and decreased semen quality (2).

Varicocele causes excess generation of reactive oxygen species (ROS), in both the testes and semen (3). Oxidative stress (OS) is one of the main factors involved in the etiology of impaired sperm function in infertile patients (4). Men with varicocele have a low number of potentially functional spermatozoa per ejaculate, coupled with a decreased progressive motility and sperm vitality (5). However, little is known about the cellular mechanisms underlying varicocele-related infertility (6). Over the past decade, mitochondrial involvement in infertility has been reported, among infertile men, by some studies (7). Mitochondria are the main source of intracellular ROS production (8), and mitochondrial membrane potential (MMP) is usually used to evaluate mitochondrial function (9). Increased ROS production (10) and mitochondrial membrane dysfunction have been reported in infertile men (11).

Nitric oxide (NO) and peroxynitrite ($ONOO^-$), powerful ROS oxidants, have been found to be generated in numerous concentrations in enlarged testicular veins. In view of this, it is suspected that NO and $ONOO^-$ may play important roles in the high OS level in varicocele (12). Inducible NO-synthase (iNOS) isoform is associated with the generation of

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high amounts of NO synthase (13). The fertility potential of varicocele patients is not predictable with available markers (14). A number of tests, performed by flow cytometry, have been recently proposed which are of potential benefits largely because of their capacity to increase the precision, their distinguishing power, and the velocity of semen analysis (15-17).

According to the results of a study, antioxidant supplementation resulted in a large number of sperms with high mitochondrial activity in MMP after cryopreservation (18), therefore, antioxidant supplementation may be beneficial in increasing MMP and motility in varicocelized rats. Aminoguanidine (AG) has relatively greater interaction with iNOS isoforms that are responsible for the massive NO production in varicocele (19-21). Moreover, AG demonstrates antioxidant and free radical scavenging properties which are especially observed in $ONOO^-$ generation (22).

The vitality and MMP of spermatozoa, assessed by flow cytometry, appear to be beneficial markers related to fertility potential. In this study, the vitality and MMP of sperm cells were assessed by PI and Rh123 staining, respectively. Rh123 is the most commonly used dye for the evaluation of mitochondrial function in mammalian cells. However, the influence of AG on sperm MMP in varicocele has not been shown yet.

The aim of this study was to assess the influence of induced varicocele on rat sperm function such as motility, viability, and MMP. The study also aimed to determine the effect of AG on these sperm parameters.

Materials and Methods

Animals

24 adult male Wister rats (300±55 g) were selected for this study. During the study, the rats were housed three per cage under controlled lighting (12:12 hr light/dark schedule) and temperature (22± 2 ˚C) and had free access to standard rat laboratory diet and tap water. The rats were randomly divided into 4 groups. Two groups (varicocele and treatment) of rats underwent partial ligation of the left renal vein (23, 24). The third group underwent a sham procedure, and the fourth was the control group. The ethical committee of Tehran University of Medical Sciences approved all experiments and animal handling processes.

Surgical procedure

The experimental left varicocele was induced using the same procedure employed by Köksal *et al* (25). General anesthesia was induced using an intraperitoneal (IP) injection of ketamine (100 mg/kg) and xylazine (1 mg/kg) (26-29). A midline laparotomy incision was made to show the left renal vein which was then partially ligated by 4-0 silk sutures tied around the vein. Then, the left renal vein was dissected with a midline incision. The ligature was located around the renal vein at a point medial to the insertion of the spermatic vein by using a 20-gauge needle and a 4-0 silk suture. After placing the ligature, the needle was carefully picked up and the midline incision was sutured using 3-0 silk sutures (25, 30). The sham-operated rats underwent a similar operation in which the left renal vein was dissected freely without ligation. The animals in varicocele, sham, and treatment groups were killed 10 weeks after surgery, and dilatation of the testicular veins was assessed.

Treatment

Ten weeks after varicocele induction, rats in the treatment group received 50 mg/kg AG (sigma/aldrichchemical Cat No.109266) injection. The drug was dissolved in distilled water just before IP administration, and the injection was done daily for 10 weeks.

Caudal epididymal sperm parameters

Sperm collection and motility

With laparotomy, the left and right caudal epididymides were carefully separated from the testis and minced into small pieces in 5 ml of Hank's solution at 37 ˚C. The sperm suspension was observed on a glass slide at 37 ˚C for motility evaluation. The percentage of motile spermatozoa was evaluated by counting more than 200 sperms in 10 randomly selected fields under a light microscope (Olympus BX51, Germany); any cell exhibiting flagellar movement was considered motile (27, 28, 31).

MMP assessment by flow cytometry

Sperms were separated by centrifugation (500 g, 3 min, and 37 ˚C) to eliminate pellet serum and then evaluated by flow cytometry. The cells were re-suspended in phosphate-buffered saline (PBS; pH= 7.4, 37 ˚C) to a final concentration of 1×10^6 cells/ml (32). Aliquots of 1×10^6 sperms were placed in PBS containing 26.2 M Rh123 (Sigma-Aldrich, Cat NO. R8004) for 10 min in a dark room at room temperature. The samples were then immediately analyzed for MMP.

Before analysis, all aliquots were counterstained with PI (Sigma-Aldrich, Cat NO. 11348639001) to evaluate cell vitality. This fluorochrome enters dead sperms through their damaged plasma membrane and thus can be used to assess vitality. The samples stained with Rh123 were then incubated in PI with 23.9 M for 6 min in a dark room at 37 ˚C and then analyzed immediately.

All samples were analyzed using a Partec PAS FacScan Flow cytometer (DAKO Cytomation, Denmark) with a 488-nm excitation laser and Flowmax software. A forward and side scatter gate was used to detect

single spermatozoa from among clumps and debris. Approximately, 10,000 gated events were analyzed per sample. Fluorescence from the Rh123-stained spermatozoa was collected in a fluorescence detector 1 with a 585-nm bandpass filter, and fluorescence from the PI-stained spermatozoa was collected in a fluorescence detector 3 with a 620-nm bandpass filter. Fluorescent measurements were compensated to minimize spillover fluorescence between red and green spectrum (28).

Statistical analysis

All data were shown as mean±standard error of mean (SEM). Using the SPSS/PC program (version 13.0 SS), one-way analysis of variance (ANOVA) and *post hoc* Duncan test were performed to identify any significant differences between the groups in terms of the different parameters. $P<0.05$ was considered statistically significant.

Results

The effect of varicocele on sperm MMP, vitality and motility are shown in Figures 1 and 2. The percentage of viable sperms with normal MMP, assessed using Rh123-positive and PI-negative

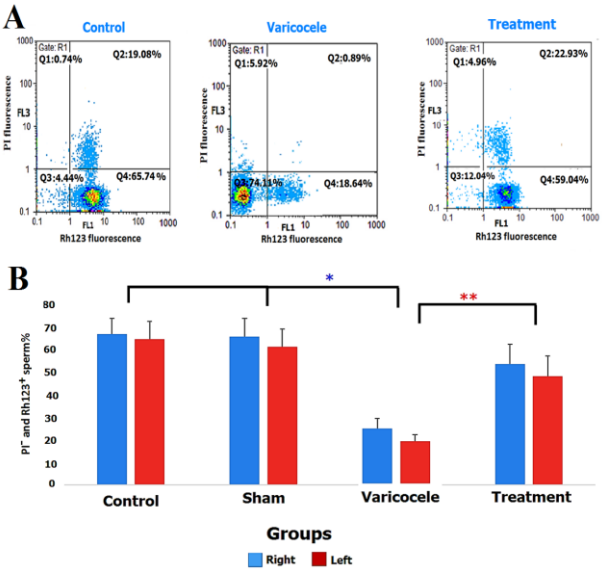

Figure 1. (A) Flow cytometric two-dimensional dot-plots of fluorescence intensities of 10,000 individual rat sperm stained with Rh123 and PI. Data in quadrants 2 and 4 represent sperms with normal MMP, and Rh123 fluorescence and data in quadrants 3 and 4 represent viable sperm with no PI fluorescence. Only 18.64% of the varicocele sperm population (quadrant 4) contain viable cells with normal MMP, compared with 65.74% of the control sperm population and 59.04% of the treatment sperm population. The fluorescence of Rh123 and PI were collected in fluorescence detectors 1 (FL1) and 3 (FL3), respectively. (B). Comparison of MMPs in different groups. Experiments were independently repeated three times. Each column represents mean± SEM
*Significant difference observed when varicocele group was compared with the control and sham groups ($P<.05$)
**Significant difference observed when treatment group was compared with control and sham groups ($P<.05$)

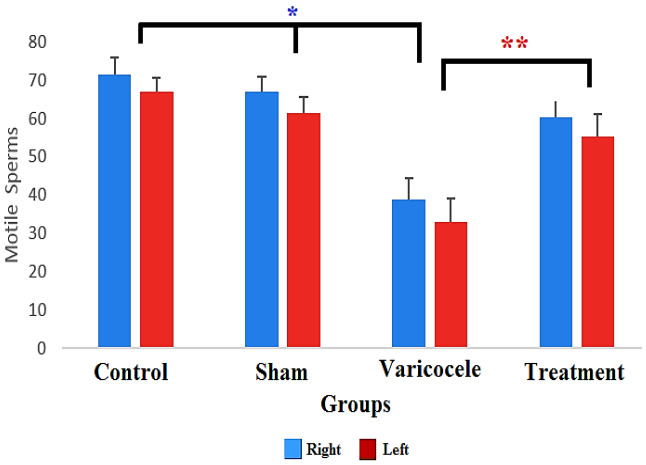

Figure 2. Evaluation of sperm motility in different groups Experiments were independently repeated three times. Each column represents mean±SEM
*Significant difference observed when varicocele group was compared with control and sham groups ($P<.05$)
**Significant difference observed when treatment group was compared with control and sham groups ($P<.05$)

Percentages was significantly lower in the varicocele group compared with the control and sham groups ($P<0.05$), (Figure 1). The percentage of dead (*PI-positive*) spermatozoa was significantly higher in the varicocele group as compared with the control and sham groups ($P<0.05$).

Sperm motility decreased significantly in the varicocele group compared with that in the control and sham groups ($P<0.05$) (Figure 2). There was no significant difference between control and sham groups with regard to all the evaluated data.

The treatment group was injected with AG for 10 prior to the evaluation of the parameters. In this group, Rh-positive cells, and the percentages of vital cells and motile sperm were significantly higher when compared with the varicocele group ($P<0.05$), (Figures 1 and 2).

Discussion

Many studies have indicated OS in varicocele-related infertility is caused by increased ROS production. This has been associated with increased NO level and the release of NOS in the enlarged testicular veins of varicocele patients (33, 34). Another factor that may also result in increased ROS production is decreased antioxidant defense which normally exists in seminal and blood plasma (35).

In this study, we evaluated the effects of induced varicocele on some sperm parameters. We showed that varicocelized rats had significantly low levels of sperm vitality and motility compared with the control group, which is consistent with the result obtained in previous studies (35, 36). In our study, differences between viable and dead sperms were assessed using PI staining. We observed a reduction

in sperm vitality was observed in varicocele rats which are also consistent with the results obtained by Jafari *et al* (37). Increased NO generation seems to be the cause of reduction in the number of viable sperms. Motility is one of the most important parameters that have been found to be greatly influenced by ROS. It is as well a powerful predictor of the ability of a given sample to cause fertilization *in vitro*. Despite its importance, the mechanism by which motility is decreased has not been clarified. Motility of spermatozoa is partially dependent on mitochondrial function (38). Researchers reported that sperm motility in varicocele patients is significantly decreased compared to fertile donors (36). Some researchers reported that ROS in human spermatozoa is related to a decline in sperm motility and vitality. These researchers suggested that the inhibition of sperm motility after incubation with ROS was caused by a defect in ATP (39, 42).

It has also been suggested that mitochondrial function is similarly affected by ROS in varicocele patients (38). In a previous report, NO was found to inhibit ATP synthase in cells resulting in decreased ATP levels, which subsequently caused poor sperm motility (40). In this study, we observed low percentage of sperms with normal MMP in the varicocele group. Sperms with high MMP show a subpopulation of sperms with a high fertilizing capacity because of their normal morphology and high motility values (9). Decreased MMP observed in the varicocelized rats may have been caused by high NO levels. This indicates that decrease in sperm motility may be caused by changes in mitochondrial activity (38).

Evaluation of mitochondrial function in the sperms of varicocelized rats showed that the percentage of viable sperms stained by PI staining was positively correlated with the percentage of Rh123 fluorescent sperms in the total population. However, the incidence of Rh123 fluorescence was much less in the sperms of varicocelized rats. These results are in agreement with a previous study (37).

Analysis of sperm cells after Rh123 staining showed that there is a significant relation between MMP and sperm cell motility. Our study suggests that mitochondrial function is essential for the maintenance of sperm motility. Nonetheless, it may also be that NO-injury to mitochondria sets an apoptosis-like mechanism in motion. Previous study has also illustrated a correlation between R123 fluorescence and sperm motility (37, 38, 41). More likely decreased MMP reduced ATP generation to the point at which the amount of ATP produced is not adequate to retain motility (42).

It is suggested that high levels of NO mediate MMP in the spermatozoa of varicocelized rats. Thus, it may be concluded that reduced MMP in spermatozoa of varicocelized rat is associated with the role of seminal NO in mediating such damage. It is speculated that mitochondrial damage in spermatozoa of varicocele patients is related, at least in part, to NO increase. Some researchers demonstrated that iNOS activity is up-regulated in varicocele patients and it is a possible reason for the high NO levels (43). In view of this, iNOS inhibitors may be useful in decreasing NO amounts in infertile men with varicocele. On the other hand, AG has been reported to have $ONOO^-$ scavenger property.

The results of this study indicate a significant increase in sperm motility, viability, and MMP in varicocelized rats, following AG administration, compared with the varicocele group. The enhancement of sperm motility, viability and MMP observed in the treatment group was caused by the antioxidant effect of AG. In our previous study, we reported improving the effects of AG in sperm count and morphology in varicocelized rats (19-21). Pena *et al* also evaluated the effect of antioxidant supplementation on mitochondrial function (18). It is possible that the interference of AG with free radical production may be one of the factors in varicocele repair. The action of AG is likened to l-arginine which suppresses iNOS in a competitive method leading to a reduction in NO generation (44). It seems likely that this mechanism contributed to the declined levels of NO in AG-injected rats. Therefore, we can conclude that increased levels of iNOS and NO in the varicocelized rat can trigger a decrease in MMP, which is subsequently followed by decreased ATP generation and sperm motility.

Conclusion

AG appears to have promising positive effects on improving sperm motility, vitality and MMP in the varicocelized rat, and might thus be helpful in the management of varicocele-related infertility.

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Conflict of interest

Authors declare that there is no conflict of interest in this study.

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