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Testicular tissue nitric oxide and thiobarbituric acid reactive substance levels: evaluation with respect to the pathogenesis of varicocele

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Abstract The aim of the present study is to evaluate tissue nitric oxide (NO) and thiobarbituric acid reactive substance (TBARS) levels in testicular tissue, and to determine their relationship with seminal parameters in order to explain possible effects on varicocele pathophysiology.Ten adult male Wistar rats at 8 weeks old underwent partial left renal vein ligation. A sham operation was performed on control rats in a second group of another ten rats. All animals were killed 4 weeks after surgery. The testes were removed and histological changes were observed by light microscopy with haematoxylin and eosin stain on half of each testis. The rest of testis was used for the evaluation of testicular tissue NO and TBARS levels. Epididymal aspirated seminal plasma was used for semen analysis and morphological analysis was carried out according to Kruger's criteria. Statistical analysis was performed by using Mann-Whitney U-tests and Spearman rank correlations between the two groups for NO and TBARS levels and for seminal parameters. Testicular tissue NO and TBARS levels (mean ± SEM) were 62.8 ± 10.1 μmol/g protein and 4.7 ± 0.3 nmol/g protein in group 1. These parameters were 16.9 ± 2.2 μmol/g protein and 3.1 ± 0.2 nmol/g protein in the group 2 controls. There were significant differences between these parameters ($P_{NO} = 0.000$, $P_{TBARS} = 0.001$). Although a positive and significant correlation between testicular tissue NO and TBARS levels was found ($r_s = 0.739$, $P = 0.014$), there was only a strong negative correlation between NO levels and sperm motility in group 1 ($r_s = -0.815$, $P = 0.004$). We found that this effect of NO on sperm motility was independent from TBARS levels after regression analysis ($r^2 = -0.687$, $\beta = 0.825$, $P = 0.034$). Although there were statistically significant differences in seminal parameters between the two groups, there was no difference between them in the histopathological examination. We found that sperm motility was significantly related to testicular tissue NO levels only. Thus, we suggest that NO is an important mediator in the pathogenesis of varicocele. TBARS and other substances have been effective via NO pathways.

Keywords Varicocele · Nitric oxide · TBARS · Infertility

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Introduction

Varicocele is an abnormal dilatation of the spermatic vein which is observed in around 30-50% of men in infertile couples [9, 22]. Although varicocele repair improves the quality of seminal parameters and subsequently increases pregnancy rates, the exact mechanism of varicocele pathogenesis has not been explained clearly [28]. Several mechanisms involving the pathophysiology of testicular dysfunction in the varicocele population have been defined [17, 26, 30]. Testicular hypoxia due to venous stasis, Leydig cell and germinal cell dysfunction due to small vessel occlusion, retrograde flow of adrenal and renal metabolites from the renal vein into the spermatic vein, elevation of scrotal and testicular temperature and decreases of gonadotropins and androgens secretion are the other pathophysiological mechanisms of varicocele.

Nitric oxide (NO) is a free radical molecule which is generated from the guanidine nitrogen of L-arginine. NO has an important role in several biological processes such as neurotransmission, tumour cell killing, immunity and inflammatory process [6, 10]. In addition, NO has been found to have a modulating role on sexual and reproductive function [16]. Recent studies show that NO

might be related to inhibited sperm motility, germ cell degeneration and stress-impaired testicular steroidogenesis [16, 19]. Moreover, the seminal plasma NO concentration was found to be higher in patients with varicocele than a healthy control group [1].

Human spermatozoa are rich in polyunsaturated fatty acids, and are therefore susceptible to reactive oxygen substances (ROS) attack and lipid peroxidation [21, 29]. Thiobarbituric acid reactive substances (TBARS) are implicated in lipid peroxidation. These substance are found in seminal plasma, and high levels of TBARS can increase sperm membrane permeability, cause teratozoospermia, and decrease fertility. In the literature, TBARS concentrations are found to be higher in more than 50% of infertile men than healthy controls [8, 29].

The aim of the present study is to evaluate NO and TBARS levels in testicular tissue and their relationship with seminal parameters in rats with experimentally created varicocele.

Materials and methods

Twenty adult male Wistar rats at 8 weeks old and weighing 170–210 g were used in the study. They were kept under a 12/12 h light/dark cycle, and were allowed free access to a standard rat laboratory diet and tap water. The study was started after obtaining permission from the local ethics authority. Unnecessary suffering was avoided during the study, and all procedures were performed under anaesthesia.

Rats were divided randomly into two groups. The first group underwent partial left renal vein ligation as described by Choi and Köksal and co-workers [5, 13]. This operation was performed under intramuscular 5% ketamine hydrochlorideanaesthesia. A midline incision was made to expose the left renal vein. A 2-0 silk suture was tied around the left renal vein 1 cm proximally to the renal vein and vena cava junction. The midline incision was closed with 3-0 silk sutures. A sham operation was performed on control rats.

All rats were killed 4 weeks after surgery and all showed left spermatic vein dilation. The testes were removed and half of each testis was fixed in Bouin's solution and embedded in paraffin. Histological sections were prepared and stained with haematoxylin and eosin. Histological changes were observed by light microscopy.

The rest of the testicular tissue was frozen in liquid nitrogen and stored at −80°C until investigation. After washing with 0.9% NaCl, tissue was homogenized (Labor Technique, Germany) in ice with 1 ml 0.9% NaCl solution. Homogenized tissue was centrifuged at 1,500 *g* for 10 min at 4°C. Supernatants were used for protein, NO and TBARS determination. Protein level was measured using Lowry's method [14]. TBARS levels, indicating lipid peroxidation, were measured by the method described by Armstrong and Al-Awadi, which was modified from the Yagi method [3]. The calibration curve was prepared with 1, 1, 3, 3-tetramethoxypropane (Sigma, USA) standards of 1–25 nM dilutions. The results were measured as nmol/g protein.

Nitrite and nitrate levels were measured as an indication of NO production by the method described by Miranda et al. [15]. Serial solutions of 0.5–250 μM of $NaNO_3$ (Merck, Germany) were used to determine standard curves and the results were measured as μmol/g protein.

Epididymal aspirated seminal plasma was used for semen analysis just before the death of the rats. Count and motility were evaluated in a Makler chamber with a light microscope under 40× magnification. Morphological analysis was performed after staining with SpermMac (Ferti Pro, Beernem, Belgium) according to the Kruger's criteria [12]. Since there in no report on normal rat semen parameters, our control group's results were accepted as normal.

Statistical analysis was performed by using the Mann-Whitney U-test and the Spearman rank correlation between the two groups and NO and TBARS levels and seminal parameters. Statistical significance was accepted at $P < 0.05$.

Results

Testicular tissue NO and TBARS levels (means ± SEM) and seminal plasma parameters are shown in Table 1. Although there were significant differences in sperm counts, sperm motility rate and sperm morphology, there was no difference in agglutinated sperm between the two groups. Leukocyte numbers were less than 1×10^6 in both groups.

Although a positive, significant correlation was detected between testicular tissue NO and TBARS levels ($r_s = 0.739$, $P = 0.014$), there was only a strong, negative correlation between NO levels and sperm motility in group 1 ($r_s = -0.815$, $P = 0.004$). Although there was no effect of TBARS on sperm motility ($P = 0.114$), we found that the effect of NO on sperm motility was independent from TBARS levels after regression analysis ($r^2 = 0.687$, $\beta = -0.825$, $P = 0.034$).

In the histopathological investigation, there was no difference between the two groups. Seminiferous tubules and germinal epithelium were normal in appearance. Every type of spermatogenic cell in the seminiferous tubules was detected using normal counts. Moreover, Leydig and Sertoli cells were normal in appearance; their nucleus and cytoplasmic organelles were normal. A slightly increased oedema and congestion were observed in group 1.

Discussion

In humans, NO is an important messenger which is found in several tissues, including the reproductive system. In males and females, different cell types such as endothelial cells, smooth muscle cells, fibroblasts and phagocytes are capable of NO release. Moreover, different isoforms of nitric oxide synthase (NOS) have been described in different structures [4, 11, 18].

The effects of NO in seminal plasma change with its concentration. At a physiological concentration, NO

Table 1 Testicular tissue NO and TBARS levels, and seminal plasma parameters in the study (group 1) and control (group 2) groups

	Group 1	Group 2	*P*
NO (μmol/g protein)	62.8 ± 10.1	16.9 ± 2.2	0.000
TBARS (nmol/g protein)	4.7 ± 0.3	3.1 ± 0.2	0.001
Sperm count (10^6/ml)	2.5 ± 0.4	3.4 ± 0.2	0.035
Sperm motility (%)	37.2 ± 2.9	67.1 ± 1.6	0.000
Sperm morphology (%)	3.9 ± 0.3	6.7 ± 0.4	0.000

acts as a mediator of the aforementioned functions [25, 32]. However, the harmful effects of NO are mediated by biologically activated molecules produced by the reaction of NO with oxidant molecules [24]. In previous studies, sperm motility and sperm concentration in patients with varicocele were found to be correlated with the seminal plasma NO concentration [19, 20]. A decreased semen concentration caused from increased NO was due to the direct inhibition of mitochondrial respiration and DNA synthesis. NO can reduce ATP levels in cells inhibiting ATP synthase [7, 31]. Therefore, a decreased ATP content or production might result in insufficient energy and poor sperm motility because approximately 90% of the energy is produced as ATP.

The relationship between infertility, varicocele and NO concentration has not been clearly identified. In patients with varicocele, seminal plasma NO levels were found to be 2–25-fold higher than that of control cases [19, 20]. Moreover, increased NOS activity has been observed in dilated spermatic veins, as well as in Leydig cells in varicocele patients [23, 24, 27]. Aksoy et al. [2] reported that increased NO levels were specifically related to the varicocele and not to infertility, since NO production in oligo and/or asthenozoospermic patients without varicocele is not increased. Because localization of NOS activity has been described in the literature, we aimed to determine the quantitative amount of nitrate/nitrite levels in testicular tissue.

In human ejaculate, TBARS and ROS are produced by both spermatozoa and leucocytes. These substances play a role in many physiological sperm functions such as hyperactivation, capacitation and acrosome reaction. Increased levels were found in infertile men. A significant increase was also demonstrated in some selected andrological conditions such as leucospermia and varicocele [11, 13, 29].

In the present study, we did not observe any histological differences between the two groups. This finding suggests that the molecular and functional effects of varicocele on spermatozoa occur earlier than the structural effects. Therefore, we consider that histopathological changes in varicocele will be observed in the long-term.

The relationship between ROS and varicocele has recently been documented by many authors, who reported that varicocele is associated with elevated sperm ROS production and diminished seminal plasma antioxidant capacity [7, 24]. Pasqualotto et al. [21] reported that the total antioxidant levels in patients with idiopathic infertility, varicocele, vasectomy and infection were significantly less than those in the control group. However, many findings support the hypothesis that infertility associated with varicocele is at least in part related to oxidative stress. Varicocele patients have been found to have decreased antioxidant defences both at the seminal and blood plasma level.

Finally, although varicocele is the most common cause of, and one of the curable diseases in, infertile patients, its pathophysiology has not been adequately described. After its identification, NO has been thought to play an important role in several tissues. It also has some detrimental effects on spermatogenesis in patients with varicocele. However, another controversial issue is whether NO has these harmful effects on spermatogenesis alone or in conjunction with other substances.

In conclusion, we found that sperm motility was significantly related to testicular tissue NO levels only. However, this relation was not detected in testicular tissue TBARS levels. Therefore, we consider that NO is an important mediator in the pathogenesis of varicocele. This study may support the concept that the post-varicocelectomy use of anti-oxidants in case of surgical failure can be useful.

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