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ORIGINAL PAPER

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Increased vasoconstrictor reactivity and decreased endothelial function in high grade varicocele; functional and morphological study

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Abstract The pathophysiology of human varicocele is not fully understood. We investigated vasoconstrictor reactivity, endothelial function and morphological changes in different grades of varicocele to clarify the pathophysiology. Contractile responses to phenylephrine, norepinephrine, serotonin and histamine were determined in isolated human varicose spermatic veins using the organ bath technique. Endothelial function was tested with acetylcholine-induced relaxation after phenylephrine-induced precontraction in the absence and presence of nitric oxide synthase inhibitor, L-NAME, and cyclooxygenase inhibitor, indomethacin. The cyclic guanosine monophosphate (cGMP) level was measured in the spermatic vein and peripheral plasma. Morphological changes were evaluated with light microscopy. Phenylephrine, norepinephrine, serotonin and histamine induced concentration-dependent contractions. The maximum contractions for all of these agents except norepinephrine were significantly higher in grade III than grade I and II ($P < 0.05$). The sensitivity to phenylephrine was significantly higher in grades II and III than in grade I ($P < 0.05$). In the presence of L-NAME and indomethacin, the difference from respective control phenylephrine-induced contractions was higher in grade I and II than grade III. Acetylcholine did not induce stable relaxation but the level of cGMP, which is responsible for the vasorelaxant effect of NO, in veins was lower in grades II and III than grade I ($P < 0.05$). Vessel wall thickness increased in grade II and dilatation developed in grade III when compared to grade I ($P < 0.05$). Our findings suggest that endothelium produces less vasorelaxant which results in the more enhanced effects of vasoconstrictor substances in grade III, indicating that endothelial dysfunction develops at high grades of varicocele.

Keywords Varicocele · Pathophysiology · Vasoconstriction · Endothelium · Nitric oxide

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Introduction

The left varicocele is one of the major causes of male infertility. It is responsible for progressive anatomical and functional testicular damage [19]. Constant monitoring of subjects suffering from varicocele is therefore necessary. The pathophysiology of varicocele still remains obscure and the efficacy of varicocelectomy is not clear because of inappropriate study design and information in the literature.

In vivo, vascular tonus is determined by the combined effects of both vasoconstrictor and vasodilator mediators. Imbalances between these vasoactive mediators may contribute to pathological events like vasospasm, hypertension and varicosis [5]. Endothelium plays a crucial role in the maintenance of vascular homeostasis. Endothelium-derived vasoactive mediators are important in controlling local vascular tone [5] including venous tone [14]. Among the various mediators released by the endothelium, nitric oxide (NO) is of major importance. It has vasodilator activity and its local level is controlled by biosynthesis from the inactive precursor L-arginine.

In the present study, we aimed to test the hypothesis that there is an imbalance between vasoconstrictor and

vasodilator mechanisms in human varicocele. We tested the vasoconstrictor reactivity and endothelial function by measuring the vasorelaxant effect of endothelium-released nitric oxide and cyclooxygenases products in different grades of varicocele. We also measured cGMP levels in varicocele tissue and in the plasma. Structural changes in the varicose spermatic vein were also evaluated.

Material and methods

Tissue

Human spermatic vein was obtained from patients undergoing varicocelectomy. All of the patients had symptomatic varicocele and were suffering from pain and discomfort from swelling. None of our patients had been operated for infertility. The study was approved by the local Ethics Committee. We obtained the written permission from all patients before the study. Varicoceles were graded in severity as follows: grade I (palpable only during the Valsalva maneuver), grade II (palpable without the Valsalva maneuver), or grade III (visible without the need for palpation). The age of the patients and duration of symptoms were 22 ± 1, 23 ± 2, 23 ± 1 years old; 6.0 ± 1.6, 8.5 ± 1.8, 8.8 ± 1.6 months for grade I, II and III, respectively.

Isometric contractions

Spermatic veins were placed in +4°C Kreb's solution and transferred immediately into the laboratory. Kreb's solution contained 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM glucose, and 25 mM $NaHCO_3$, at pH 7.4. The veins were cleaned and cut into 3–4 mm length rings. Care was taken not to damage the endothelium. The rings were mounted in an organ bath with a volume of 10 ml. Kreb's solution was gassed with %95 O_2 and %5 CO_2. Changes in venous tensions were recorded isometrically by a force-displacement transducer (FT03, Grass Instruments, Astro-Med, West Warwick, R.I.) and recorded on a multi-channel polygraph recorder (P122, Grass Instruments, Astro-Med). Resting tension was adjusted to 10 mN at the beginning of the experiments. The equilibration period was 60 min and the bathing medium was changed every 20 min during the equilibration period.

Experimental protocol

After equilibration, the rings were exposed to 80 mM KCl. After a 30 min equilibration period with repeated washing every 10 min, tissues were challenged with either phenylephrine, norepinephrine, histamine or serotonin (5-hydroxytryptamine, 5-HT). Agonists were added to the bath in a cumulative fashion in 0.5 $\log_{10}$-units, in a concentration range of 1 nM to 300 μM. Each preparation was used for only one experimental protocol.

After the completion of agonist experiments, the rings were washed three times with a 10 min interval between washings, and then exposed to 10^{-6} M phenylephrine. When the contractions reached a plateau, ACh (10^{-6} M) was added to test endothelium-induced relaxation. Whether the relaxation was obtained or not, the rings were taken from the organ bath 5 min later and immediately frozen at –70°C to measure the cGMP level. The rings were maintained at –70°C until the time of measurement.

cGMP level measurement

The cGMP content was determined following the instructions of an enzyme immunoassay kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Venous tissues were homogenized in a glass/glass homogenizer in ice-cold 6% trichloroacetic acid. The homogenates were centrifuged at 3,000 *g* for 15 min at 4°C and the supernatants extracted three times in five volumes of ether. The aqueous phase was lyophilized.

Effects of L-NAME and indomethacin on phenylephrine responses

In another set of experiments, the relaxant effects of endothelium-derived nitric oxide and cyclooxygenase products were examined. After the equilibration period, paired vessel rings were incubated with either NO synthase inhibitor, L-NAME (100 μM), or the cyclooxygenase inhibitor, indomethacin (10 μM), for 30 min, and then the phenylephrine concentration-response curve was obtained as described above.

Morphological studies

On completion of each concentration-response curve, the vessel rings were removed from the organ baths and fixed in formalin. These segments were subsequently cut into 5 μm thick sections and stained with hematoxylin-eosin. Sections were examined under a light microscope and analyzed with an image analyzer to measure outer (OD) and internal vessel (ID) diameter. The extraluminal area (EA) was calculated with the equation $EA = \pi(OD/2)^2$ and the intraluminal area (IA) with the equation $IA = \pi(ID/2)^2$. Vessel wall thickness, (WT; mm^2) was calculated according to the following equation: $WT = EA - IA$.

Drugs

Phenylephrine hydrochloride, norepinephrine hydrochloride, histamine dihydrochloride, 5-HT creatinine sulfate, acetylcholine (ACh), indomethacin and N(G)-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma (St Louis, Mo., USA). All chemicals were dissolved in distilled water in a single batch and stored at +4°C until use. Indomethacin was dissolved in 0.5% w/v sodium bicarbonate. Dilutions were made fresh daily by using Krebs solution as a diluent.

Data analysis

Data are expressed as mean ± SEM. Agonist-evoked contractile responses are expressed as arithmetic mean ± SEM of the % of 80 mM KCl. EC_{50} values (concentration of agonist required to produce 50% of the maximum response) were used to determine pEC_{50} values (negative $\log_{10}$ of the EC_{50} value). One-way analysis of variance (ANOVA) with post hoc Duncan's multiple range test was used for statistical comparisons. P values < 0.05 were considered significant.

Results

Isometric contractions

Phenylephrine, norepinephrine, serotonin and histamine induced concentration-dependent contractions in isolated human spermatic veins. Maximum contractions of all agonists except norepinephrine were significantly higher in grade III than grades I and II ($P < 0.05$). Phenylephrine-induced contractions were higher at nearly all concentrations in grade III than grade I ($P < 0.05$). Whereas phenylephrine-induced contractions

in grade II were apparently but not significantly different from grade I (Fig. 1a), norepinephrine, serotonin and histamine-induced contractions in grade II were similar to grade I (Fig. 1b–d). The concentration-response curve to serotonin and histamine showed that the contractions in response to these agonists were significantly higher in grade III than grades I and II at higher concentrations (Fig. 1c, d). Norepinephrine induced contractions were slightly, but not significantly, higher in grade III (Fig. 1b). In addition, the sensitivity to phenylephrine (as shown by the pEC_{50} value), but not to other vasonstrictors (data not shown), was significantly higher in grades III (6.56 ± 0.59) and II (6.25 ± 0.34) than grade I (5.67 ± 0.15) ($P < 0.05$, ANOVA and post hoc Duncan's multiple range test).

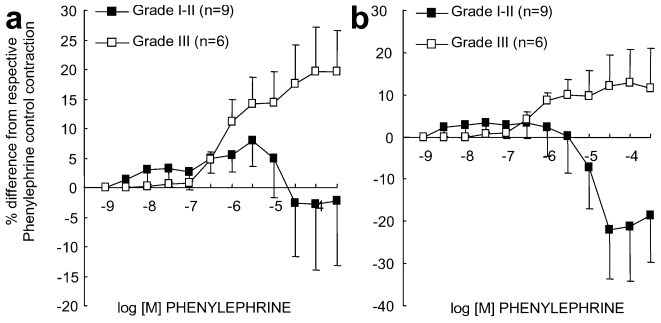

Fig. 2 Concentration-response curves for phenylephrine **a** in the presence of nitric oxide synthase inhibitor, L-NAME (100 μM), and **b** cyclooxygenase inhibitor, indomethacin (10 μM). The number of patients is indicated in *parentheses*. Data are expressed as differences from respective phenylephrine control contraction (% of the response to 80 mM KCl); *vertical lines* show the SEM

Effects of L-NAME and indomethacin on phenylephrine-induced contractions.

L-NAME (100 μM) caused more enhancement in phenylephrine-induced contractions in grades I and II than grade III. This enhancement was demonstrated as a difference from the respective control phenylephrine-induced contractions (Fig. 2). In the presence of indomethacin (10 μM), phenylephrine-induced contractions were increased at lower concentrations of phenylephrine (10^{-9}–10^{-6} M) in all grades. But at the higher concentrations of phenylephrine, indomethacin increased the contractions in grades I and II but attenuated them in grade III (Fig. 2).

ACh experiments and cGMP levels

ACh did not induce stable and evaluable relaxation in spermatic veins and we could not achieve a stable plateau with the precontractile substance phenylephrine in

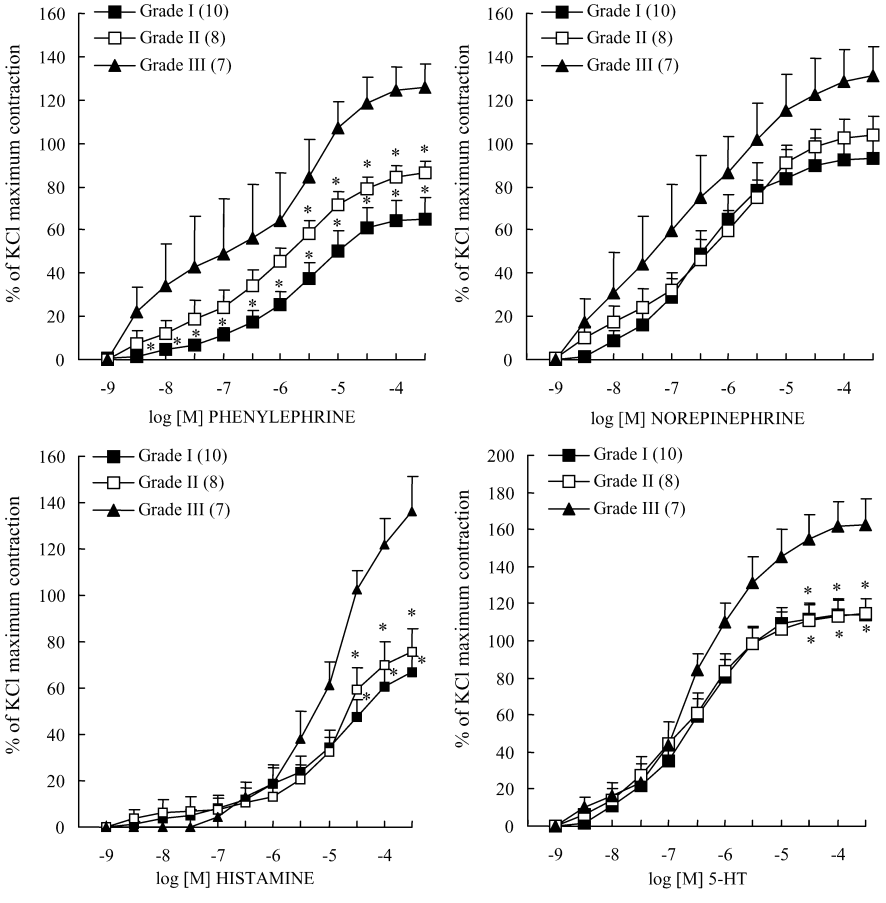

Fig. 1 Concentration-response curves (**a**–**d**) for phenylephrine, norepinephrine, histamine and 5-HT, respectively. Data are expressed as % of the response to 80 mM KCl; *vertical lines* show the SEM. The number of patients is indicated in *parentheses*. An *asterisk* indicates $P < 0.05$, grades I and II vs grade III, ANOVA with post hoc Duncan's multiple range test

most of the vessels. Therefore, we measured cGMP, a representative of endothelial NO production, in peripheral plasma and varicocele tissue to assess endothelial function. cGMP levels of varicocele tissue in grade I (81.47 ± 11.11 pmol/g, $n = 10$) was significantly higher than in grades II (28.78 ± 2.50, $n = 8$) and III (27.28 ± 5.81, $n = 7$) ($P < 0.05$). However, the plasma cGMP levels of these patients were similar (data not shown).

Histological study

The intraluminal area in grade III (94.9 ± 20.2 mm^2) was increased when compared to grades I (22.7 ± 3.5) and II (26.5 ± 6.3) ($P < 0.05$). The extraluminal area was also increased in grade III (377.3 ± 56.9 mm^2) when compared to other groups (80.4 ± 6.2, 228.2 ± 5.2 for grades I and II, respectively) ($P < 0.05$). In addition, the extraluminal area of grade II was significantly larger than that of grade I ($P < 0.05$). The vessel wall thickness in grades II (201.7 ± 54.8 mm^2) and III (282.5 ± 49.4) was higher than grade I (57.7 ± 4.1) ($P < 0.05$).

Discussion

The present study is the first demonstration of functional and morphological changes in the vascular smooth muscle and the endothelium of human spermatic varicose veins in different grades. Vasoconstrictor reactivity, especially α-adrenergic, was increased in parallel with the grade. In the presence of NOS inhibitor L-NAME and the cyclooxygenase inhibitor indomethacin, phenylephrine-induced contractions were more enhanced in grade III. In addition, vessel cGMP level, which is responsible for the vasorelaxant effect of NO, was lower in high grades of varicocele.

We found a generalized increase in maximal contractions to vasoconstrictors used and the sensitivity to α_1-adrenoceptor agonist phenylephrine was higher in high grades. Such an increase in sensitivity to α_1-adrenoceptor agonists was previously shown in different models of hypertension [4, 12]. Our findings support the hypothesis of increased venous pressure in the spermatic vein, which might precede to the development of varicocele.

There are several studies suggesting that intraluminal pressure is increased in varicocele [11, 21]. One of the reasons for increased intraluminal pressure might be aortomesenteric compression. Such compression of the left renal vein may lead to an increase in pressure in the spermatic vein. Recently, Graif et al. showed that the flow velocity in the proximal left renal vein is decreased in parallel with a decrease in the aortomesenteric distance and angle [7]. They also found that testicular vein diameters greater than 3 mm were statistically associated with a decreased superior mesenteric artery angle. Previously, Strakhov et al. diagnosed stenosis, aortomesenteric compression and left-side venous renotesticular hypertension of the left renal vein in 12, 342 and 158 of 356 patients, respectively [22]. They concluded that secondary genesis of the left varicocele had been demonstrated.

Increased vascular tone due to increased intraluminal pressure results in structural and functional changes in vessels [15]. Depending on the pressure and the structure of the veins, the degree of morphological change, hyperplasia and dilatation, may be different among the patients. We showed that hyperplasia was developed in grade II and dilatation in grade III. Our study is the first to show morphological changes related to the grade of varicocele.

Traditionally, a response to the administration of ACh in precontracted vessels is taken as an indication for endothelial NO production. We did not observe significant relaxation with ACh in spermatic veins but found that phenylephrine-induced contractions were enhanced in grade III, more than in grades I and II, in the presence of L-NAME and indomethacin. Endothelium-derived relaxing factor (EDRF) was discovered by Furchgott and Zawadzki [6], which was later identified as NO [9, 17]. NO is a non-stable radical which has a short half-life. Assessment of the direct role of NO in vascular wall tonus has been precluded by technical difficulties [8]. Therefore, an indirect method has been used to evaluate NO production in isolated organ bath experiments. L-NAME, a nitric oxide synthase (NOS) inhibitor, is commonly used for this purpose. Although the primary EDRF is NO, there are a number of additional endothelium-derived vasodilator substances, cyclooxygenase pathway products and inhibitors of this pathway, such as indomethacin, which are used to evaluate possible roles in relaxation. Our findings suggested that endothelium produced vasorelaxant factors less, indicating decreased endothelial function, which resulted in the enhanced effects of vasoconstrictor substances in grade III.

Some recent studies showed an excessive release of NO in the dilated spermatic vein [13, 16, 18]. Mitropoulos et al. showed that serum NOS activity was greater in the varicose spermatic vein when compared to the peripheral vein [13]. However, Romeo et al. reported that endothelial NOS was not overexpressed in the spermatic vein of varicocele patients [18]. Therefore, the dilated spermatic vein was not the major source for the increase in NO level [18]. Mitropoulos et al. also showed that xanthine oxidase activities and nitric oxide levels were greater in the spermatic vein than the peripheral vein [13]. Our results agree with these studies. We suggest that endothelial dysfunction develops in high grades of varicocele, which secrete less vasodilator mediators including NO. Therefore, the endothelium does not seem to be the source of increased NO levels in the dilated human spermatic vein. In our study, tissue cGMP level was decreased and the endothelium-dependent release of NO tended to decrease in high grades when compared to low grades. Moreover, eNOS activity

in serum [13], but not in spermatic venous tissue [18], was higher in patients with varicocele.

The principal target of NO is the soluble guanylate cyclase in adjacent smooth muscle. This interaction leads to the release of cGMP. cGMP is responsible for the vasorelaxant effect of NO. Although in our study, cGMP was higher in grade I than grades II and III, we did not observe significant relaxation with ACh in spermatic veins. Therefore, the cGMP level indirectly indicates the NO level produced by endothelium. Although cGMP may be produced, its level might be insufficient to induce relaxation in spermatic veins in our study. However, our findings in the human spermatic vein do not agree with the findings for primary human varicosis [20]. Schuller-Petrovic et al. reported that cultured endothelial cells derived from human varicosis saphenous veins had more accumulated cGMP levels than healthy veins [20]. Upregulation of the nitric oxide-cGMP system in diseased veins may shift the balance of vasoactive factors towards vasodilatation [20]. This imbalance might result in reduced active wall tension due to weak smooth muscle contraction and contribute to the development of primary varicosis which leads to chronic venous insufficiency [20]. The reason for this discrepancy between varicosis and varicocele might be due to differences in the structure of the vessels. Previous studies showed that intraluminal pressure was increased both in primary varicosis [2] and varicocele [11, 21]. In this case, why is the imbalance between vasodilator and vasoconstrictor factors different between varicosis and varicocele? The answer is because the factors preceding the development of pathological events are different. In human varicose saphenous and the hand veins of patients with varicosis, a decrease in α-adrenoceptor responsiveness has previously been reported [1, 10, 23]. Therefore, a primary defect in vasoconstrictor reactivity might precede the development of primary varicosis which leads to a subsequent increase in venous pressure. Varicocele probably develops secondarily to an increase in pressure. Increasing pressure might precede to the development of varicocele which in turn leads to functional and morphological changes.

We have shown that functional and morphological differences between grades I and III were present. Functional and morphological changes correlated well with each other in grades I and III. However, the degree of correlation between functional and morphological changes was not clear in grade II. Morphological evaluation showed that the intraluminal area of grade II was similiar to grade I, but the extraluminal area was higher in grade II than grade I, suggesting that vessel wall thickening, hyperplasia, occurred in grade II. This finding supports the diagnostic grading criteria we used. However, norepinephrine, histamine and serotonin-induced contractions were similar in grades I and II, but phenylephrine-induced contractions in grade II were, although apparent, not significantly different from grade I. On the other hand, sensitivity to phenylephrine was higher in grades II and III than grade I. The cGMP level of grade II, a representative marker of NO production, was similar to grade III. These findings suggest that grade II is a transition stage in which morphological and functional changes are continuous to varying degrees. Therefore, morphological and functional changes in grade II were not correlated with each other as in grades I and III.

In this study, the vessels we used were obtained from patients with symptomatic varicocele. We did not evaluate the fertility of these patients, whose major complaint was pain and discomfort from swelling. However, most patients with varicocele need to be treated because there is convincing evidence that a varicocele may have a progressive toxic effect on the testes [3]. We have proposed that endothelial dysfunction develops in high grades. Such dysfunction may be related to the development of infertility, but further investigation is needed to clarify this point. In addition we did not find a correlation between e grade and the duration of the symptoms for the vascular functions, but the number of patients was limited.

In conclusion, the present data provide evidence for the occurrence of endothelial dysfunction and increased vasoconstrictor reactivity in high grades of varicocele.

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