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CLINICAL STUDY

Nitric oxide synthases in varicose vein wall

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Abstract: *Introduction:* Venous wall weakness is supposed to be the most probable reason of primary varicosis. There are conflicting findings in literature about its structural changes. NO is potent vasodilatator due to the smooth muscle relaxation. It is synthesized by nitric oxide synthase (NOS). There are 3 known isoforms of NOS: nNOS (neuronal NOS), iNOS (inducible NOS), eNOS (endothelial NOS).
Material and methods: 10 varicose vein and 10 control vein samples were processed by standard light microscopy method. Sections were then processed by standard immuno-histochemic technique using rabbit polyclonal antibodies against all 3 NOS isoforms: iNOS, eNOS (SantaCruz, USA), nNOS (BioScience, USA). Antibodies expression was evaluated semiquantitatively and then proved morphometricaly by 2D image analysis (ImageJ 1.34n, National Institute of Health, USA). Total area of NOS isoforms expressions was determined by color analysis and color digital substraction.
Results: Histomorphological and semi quantitative evaluation of NOS isoforms showed discontinuous and significantly lower expression of all 3 NOS isoforms in tunica media of varicose veins compared with control group, where the expression of all 3 NOS isoforms was continuous. For the statistical analysis unpaired t-test was used.
Discussion and conclusion: Our results suppose lower NO levels in varicose vein wall, deducing that varicose vasodilatation is due to other mechanism, although the stage of chronic venous disease of varicose vein samples was undetermined. Our results are in contradiction with previously published results of Howlader et al., who observed raised total NO levels in patients with severe stages of chronic venous disease*(Tab. 2, Fig. 13, Ref. 18)*.
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Key words: varicose veins, nitric oxide (NO), nitric oxide synthase (NOS).

Varicose veins are dilated, tortuous and elongated veins affecting especially the superficial veins of the lower limbs. Beside the posthrombotic syndrome and crural ulcer, they represent only one of the symptoms of the chronic venous disease- a relatively frequent vascular disease affecting the lower limbs veins of the persons in productive age (1). In the developed countries of Europe and USA the incidence of the chronic venous disease accounts for about 40 – 60 % in females and 15 – 30 % in males (2). According to its etiology we distinguish its primary form (cause unknown) and secondary form (occurring usually as a consequence of the survived deep lower limb phlebothrombosis). As the exact cause of the primary form has not been yet revealed, the therapy of this form still resides mainly in reducing the symptoms. The cause of the primary form of varicosis remains to be the subject of interest of several investigators in the world. There is etiopathogenetic association between:

a) venous wall weakness associated with alterations in connective tissue and smooth muscle cells (3, 4),
b) altered function of the venous endothelium (5),
c) venous valve damage (6, 7),
d) alterations in microcirculation and venous wall nourishment (8, 9).

Nitric oxide (NO) is an important cellular signaling molecule, a potent vasodilatator due to the smooth muscle relaxation. It also inhibits platelet adherence and aggregation, reduces adherence of leukocytes to the endothelium and suppresses proliferation of vascular smooth muscle cells.

Nitric oxide synthases (NOSs), from the biochemical point of view, are a family of complex enzymes that catalyze the oxidation of L-arginine to form NO and L-citrulline. Three human NOS isoforms have been identified to date: eNOS (endothelial constitutive NOS), nNOS (neuronal constitutive NOS), and iNOS (inducible NOS). Their genes are found on human chromosomes 7, 12, and 17, respectively, and so were named for the tissue in which they were first cloned and characterized (10). Vasculoprotective effect of individual NOSs isoforms in human organism is not sufficiently clarified yet (11). The knowledge of nitric oxide synthases (NOSs) is of extreme scientific importance, not

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only for understanding new pathophysiological mechanisms but, also as a target for therapeutic intervention.

Endothelial constitutive nitric oxide synthase (eNOS)

The role of nitric oxide in regulating vascular tone and mediating platelet function is attributable to the ongoing activity of the endothelial constitutive form of NOS. Endothelial NOS is pharmacologicaly identical with previously isolated EDRF (endothelium-derived releasing factor), exprimed by the intact endothelium (12). Inactivation of the eNOS pathway limits the contribution of NO to vessel homeostasis and results in increased vascular tone and platelet adhesion and aggregation. The activity of eNOS is regulated by the intracellular free calcium concentration and calcium- calmodulin complexes. Endothelial NOS is a constitutively expressed protein predominantly associated with the particulate subcellular fraction, suggesting that the native enzyme is a membrane-bound protein. A detailed analysis of the membrane association of eNOS showed that this enzyme is localized to the Golgi apparatus as well as to specific structures in the plasmalemmal membrane called caveolae. The association of eNOS with a region of the plasma membrane in which several key signal transducing complexes are concentrated (such as G-proteins) is likely to have profound repercussions on enzyme activity as well as on its accessibility to intracellular mechanisms of the pathway release, including mechanisms independent of intracellular calcium release (10).

Neuronal constitutive nitric oxide synthase (nNOS)

This isoform is present in central and peripheral neuronal cells and certain epithelial cells. Its activity is also regulated by Ca^{2+} and calmodulin. Its functions include long-term regulation of synaptic transmission in the central nervous system, central regulation of blood pressure, smooth muscle relaxation, and vasodilation via peripheral nitrergic nerves. It has also been implicated in neuronal death in cerebrovascular stroke (10).NO plays also important role in pathophysiology of some neurodegenerative diseases. The presence of NO and NOS should be proved indirectly through the histochemic positivity of nicotinamid dinucleotid phosphate diaphorase (NADPHd) (13). It was proposed that nerve stimulation directly activated the release of NO from nitrergic nerves and, in fact, NO appears to be the dominant neurotransmitter responsible for the nerve-mediated, endothelium-independent vasodilation (12). Esteban et al (1998) documented the nNOS presence in the livers of cat and rat; deducing from this that nitrergic fibres should be involved in the blood flow regulation and further metabolic functions of this organ (14).

Inducible nitric oxide synthase (iNOS)

The expression in this enzyme is induced in a multitude of different cells, including macrophages, endothelial cells, vascular smooth muscle cells and cardiac myocytes after stimulation with lipopolysaccharide (LPS), cytokines (such as IL-1b, TFN-a, IFN-g, IL-6), and others; thus it has an important role in antimicrobial, antiparasitic and antineoplastic activity. This isoform is not regulated by Ca^{2+}. It produces large amounts of NO that have cytostatic effects on parasitic target cells by inhibiting iron-containing enzymes and causing DNA fragmentation. The induction of iNOS is involved in the pathophysiology of autoimmune diseases and septic shock (10).

Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed mainly in endothelial cells and nitrergic nerves, respectively, synthesizing a small amount of NO under basal conditions and on stimulation by various agonists. By contrast, inducible NOS (iNOS) is expressed when stimulated by inflammatory stimuli, synthesizing a large amount of NO in a transient manner. In an experiment on mice, NO derived from iNOS suppressed the development of constrictive remodeling of the left common carotid artery (11).

Abd-El-Aleem et al (2000) reported increased levels of eNOS and iNOS in the chronic venous ulcers compared with normal skin (15). Howlader et al (2002) found an increased levels of total NO in the blood of patients with severe forms of chronic venous disease (healed venous ulcers and lipodermatosclerosis) (16). Our study was aimed to find a linkage between the NOSs and the venous dilatation of primary varicose veins. The changes in NOSs isoforms expression should help to elucidate the cause of the vessel wall structural changes of the primary varicosis.

Material and methods

10 varicose vein samples of great saphenous veins (17) (5 males and 5 females) (Tab. 1) for the age structure of varicose vein group of samples) taken by the stripping surgery in years 1997–2005 were compared with 10 control samples of the lower limb superficial veins (7 males and 3 females (Tab. 2) for the

Tab. 1. Varicose vein group of samples.

	Males	Females	Together
Number	5	5	10
Average age (in the time of pickup, in yrs)	29.8	32.8	31.3
Age range (years)	22–37	24–42	22–42
Median (in years)	30	30	30

Tab. 2. Control group of healthy (non-dilated) samples.

	Males	Females	Together
Number	7	3	10
Average age (in the time of pickup, in yrs)	26.143	30.333	27.4
Age range (years)	19–40	20–39	19–40
Median (in years)	26	32	26.5

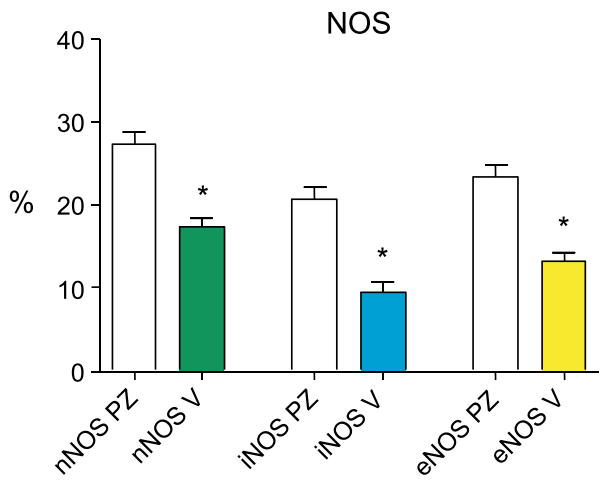

Fig. 1. The graph of morphometric analysis results of the expression of all 3 NOSs isoforms in the varicose and control groups of samples.

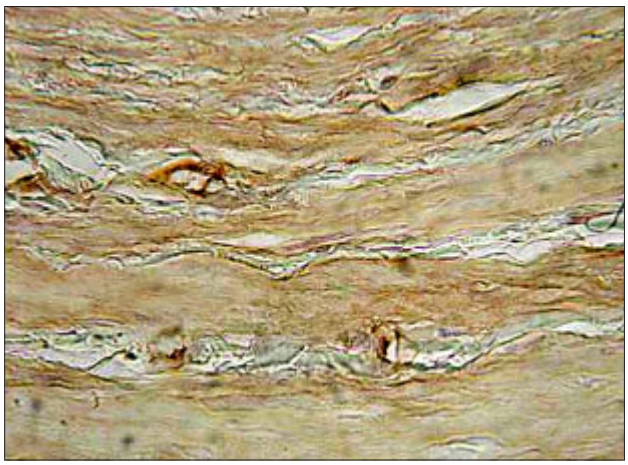

Fig. 2. Expression of eNOS in the tunica media of control (healthy) vein.

Fig. 3. Expression of eNOS in the tunica media of varicose vein.

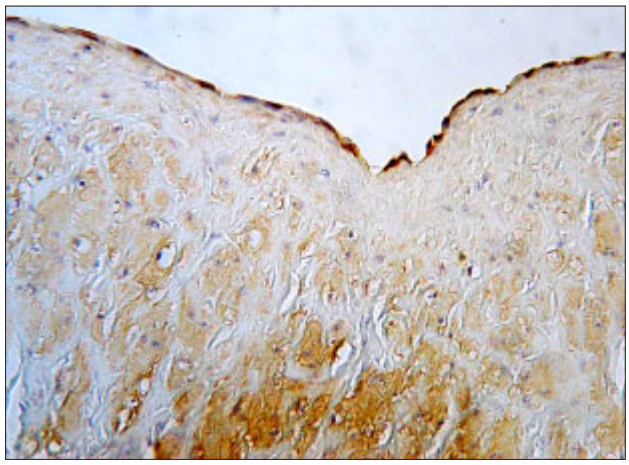

Fig. 4. Continuous eNOS cytoplasmatic expression in the endothelium of tunica intima of the control (healthy) vein sample (DAB, x40).

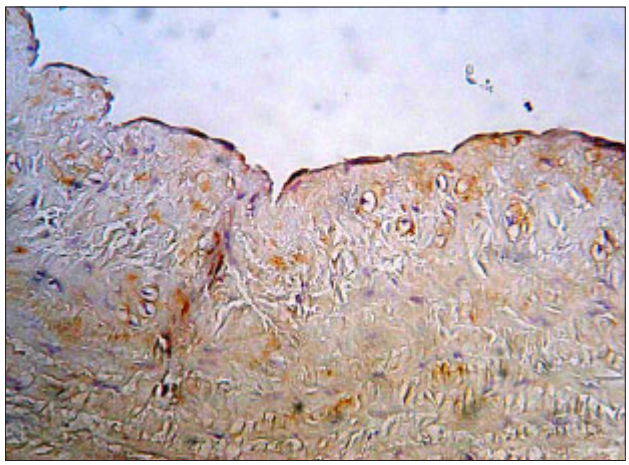

Fig. 5. Discontinuous eNOS cytoplasmatic expression in the endothelium of tunica intima of the varicose vein sample (DAB, x40).

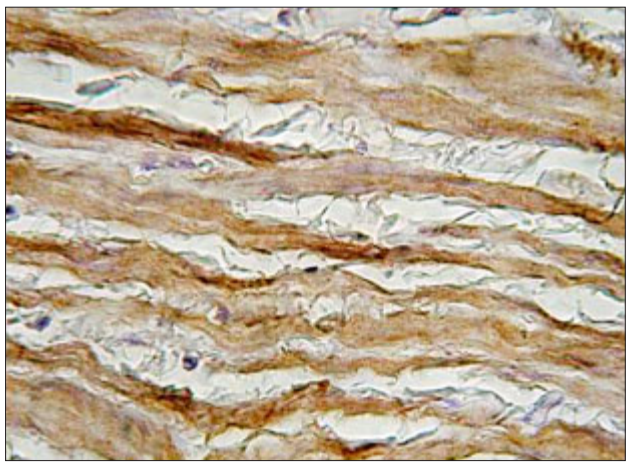

Fig. 6. Expression of nNOS in tunica media of control (healthy) vein sample.

age structure of the control group of samples) taken from the necroptic material of the Institute of Forensic Medicine (Medical Faculty, Comenius University) in years 1999–2004 with no previous history of any vessel disease. The samples were immediately fixed in the buffered formaline, processed by the standard light microscopy method into the paraffin blocks. The sections were then processed by the standard immunohistochemic technique using the rabbit polyclonal antibodies against all 3 NOS isoforms (iNOS and eNOS from SantaCruz, USA; nNOS from BioScience, USA). The expression of the antibodies was evaluated semi quantitatively and also proved morphometricaly. By the semi quantitative evaluation we concentrated on the histologic characteristics of each section; the localization and intensity of marker positivity in the vein wall layers (– negative, ± irregular positivity, + weak positivity, ++ medium positivity, +++ strong positivity). The semi quantitative analysis was proved morphometricaly by the 2D image analysis (ImageJ 1.34n, National Institute of Health, USA). The total area of NOS isoforms expressions in tunica media was then determined by the color analysis (the brown color of NOSs expression) and by the color digital substraction was determined its portion of the total area of tunica media.

Results

The histomorphological and semi quantitative evaluation of NOS isoforms showed discontinuous and significantly lower expression of all 3 NOSs isoforms in the tunica media of the varicose veins compared with control group, where the expression of all 3 NOSs isoforms was continuous (Fig. 1). For the statistical analysis was unpaired t-test used.

For the endothelial isoform of NOS (eNOS) we found continuous and diffuse expression of the followed marker in tunica intima and media of the control samples, whereas in the varicose samples its expression was discontinuous. Enhanced eNOS positivity was observed especially in the endothelial cells of tunica intima; in the healthy control samples its expression was continuous and in majority of the varicose samples its positivity showed discontinuity and interruptions in its expression (Figs 2, 3, 4, 5).

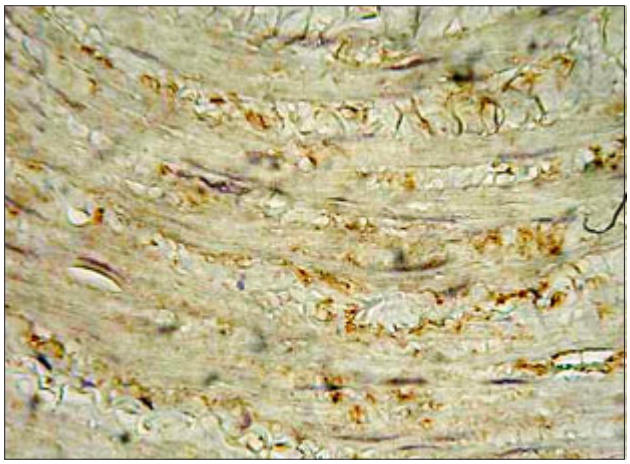

Fig. 7. Expression of nNOS in tunica media of varicose vein sample.

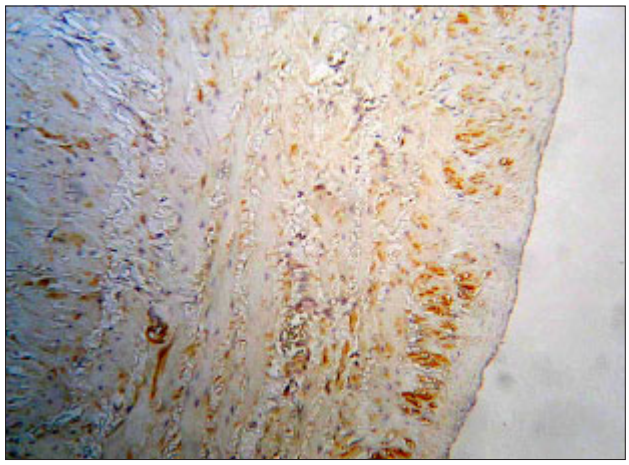

Fig. 8. Enhanced nNOS positivity in tunica intima (compared with tunica media) of varicose vein of 30yrs old man (V54).

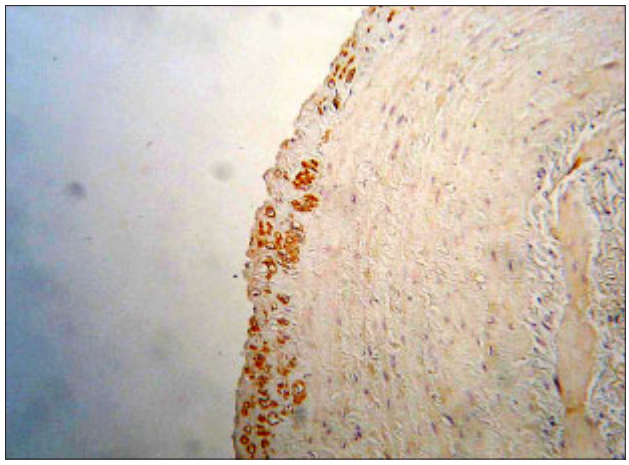

Fig. 9. Enhanced nNOS positivity in tunica intima (compared with tunica media) of varicose vein of 32yrs old man (V55).

*For the neuronal and inducible N*OSs the histomorphological and semi quantitative evaluation showed lower and discontinuous expression of both followed NOSs isoforms in tunica intima and tunica media of the varicose vein samples in comparison with the control samples (Figs 6–7, 10–11). Only exceptions were two varicose samples (V54, V55); in these varicose samples the expression of nNOS and iNOS was higher in tunica intima than in tunica media (Figs 8–9, 12–13). Both samples represent the varicose great saphenous vein samples of relatively young men (30 and 32 years respectively), taken by the stripping surgery in 2004 and 2005 years.

Discussion and conclusion

Abd-El-Aleem et al. found increased levels of total NOS, eNOS, iNOS and arginase in the skin of chronic venous ulcer by

Fig. 10. Expression of iNOS in tunica media of control (healthy) vein.

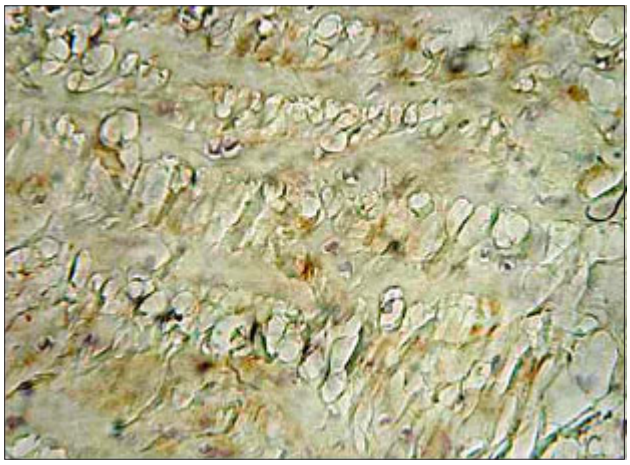

Fig. 11. Expression of iNOS in tunica media of varicose vein.

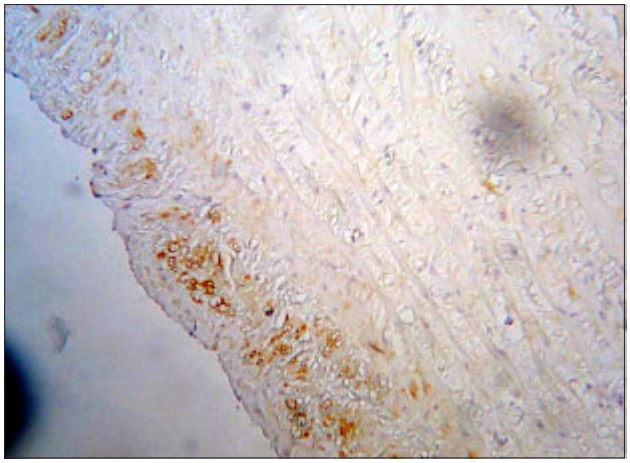

Fig. 12. Enhanced iNOS positivity in tunica intima of varicose vein of 30yrs old man (V54).

using immunocytochemistry, western blotting, and enzyme assays (15). Howlader et al (2002) by the colorimetric method observed raised levels of total NO in the blood plasma of the patients with severe chronic venous disease- healed venous ulcer-

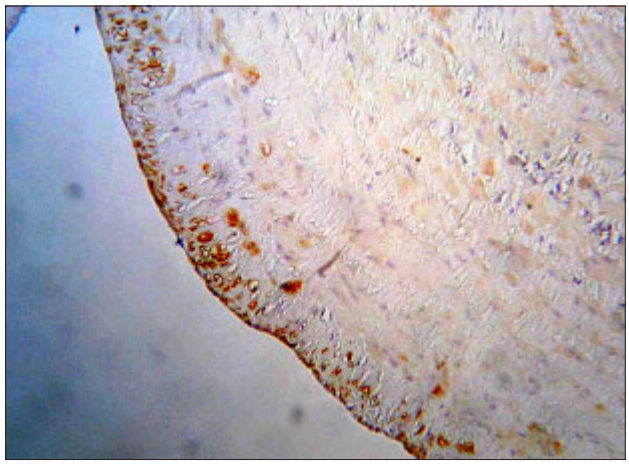

Fig. 13. Enhanced iNOS positivity in tunica intima of varicose vein of 32yrs old man (V55).

ation and lipodermatoclerosis (corresponding to C4 and C5 of CEAP classification). In the less severe CEAP stages of the chronic venous disease (C2 and C3) the difference of total NO levels between diseased and control subject were not statistically significant (16).

In our study the stage of CEAP of the varicose (dilated) venous samples was not associated (not marked). Only retrospectively, deducing from the fact that these varicose great saphenous veins were decided for extraction (performing the stripping surgery); we should suppose that they should be probably C2 stage according to CEAP classification (more detailed C2-EP-AS-PR).

For the endothelial NOS (eNOS) we confirmed the enhanced eNOS expression in the endothelium of tunica intima in both followed groups: in the healthy (control) samples the eNOS expression was diffuse and continuous; whereas in the varicose samples the eNOS expression was discontinuous (Figs 4–5). This fact confirms that eNOS is exprimed by the intact endothelium and that varicose veins are characterized by impaired endothelial function.

The expressions of nNOS and iNOS were also significantly lower and discontinuous in varicose compared with control (healthy) vein samples; deducing that varicose vasodilatation is due to other mechanisms than potentiated by peripheral nitrergic fibres or by inflammatory stimuli in the varicose vein wall. This is in correlation with one of our previous works; in which we observed lower presence of mast cells in varicose vein samples (18). Enhanced positivity of nNOS and iNOS in the tunica intima of two varicose samples (V54 and V55) (Figs 10–13) are probably due to other unrecognized coinciding disease; by other varicose samples the expression of these two markers in tunica intima copied its expression in tunica media. As was mentioned above; both samples were varicose great saphenous veins of 30 and 32 yrs old males (respectively) and there was no difference between the fixation and processing of these two samples compared with the rest of group.

Our results of significantly lowered expressions of all 3 NOS isoforms suppose lower nitric oxide (NO) levels in varicose vein

wall, deducing that the varicose vasodilatation is due to other mechanism, although the stage of chronic venous disease of varicose vein samples was undetermined (only retrospectively supposing the C2 stage of CEAP); but we confirmed the impaired function of the varicose vein endothelium. These results also suggest that NO level in varicose vein wall may undergo changes depending on the severity of chronic venous disease.

Several possibilities exist for proving the results of our work: we suggest to repeat the method in more numerous groups of samples (with marked CEAP stage of chronic venous disease); to compare Ca^{2+} presence in vein walls; to check the NOS and NO presence by the indirect histochemic evidence of NADPHd positivity; the comparison of the expression of selected inflammatory stimuli known for their potentiation of iNOS expression (IL- 1â, IL-6, TNF-á, etc.)

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