PATHOPHYSIOLOGICAL AND PATHOMORPHOLOGICAL ASPECTS OF RELAPSE OF VARICOSE VEINS AFTER ENDOVASCULAR LASER VEIN COAGULATION

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ABSTRACT
The aim: With the help of biochemical and morphological methods of investigation to identify the causes of a false postoperative recurrence of varicose veins after the EVLC. Materials and methods: In 173 patients with varicose veins of the lower extremities, the level of markers of endothelial dysfunction was determined: P-selectin, E-selectin, tissue plasminogen activator, endothelin-1, adhesion molecules of type 1 vascular endothelium (sVCAM-1-soluble vascularcellularmolecules), circulating endothelial cells (CEC) before surgery (EVLC), on the 10th and 60th day of the postoperative period. At the same time, a morphological and electron microscopic examination of the state of the deep venous system in 31 patients with varicose vein disease of the lower extremities who died from acute heart failure, was performed. Results: Increased values of markers of endothelial dysfunction in patients with varicose veins of the lower extremities before surgery of EVLC were established. We found that, despite the operation, the parameters of endothelial dysfunction decrease, but in the remote postoperative period do not come to the norm. Morphological and electron microscopic studies of the deep vein wall revealed pathomorphological changes in all of their layers, especially the endothelial layer. At the heart of the development of endothelial dysfunction in the postoperative period, the leading role belongs to changes in mitochondria. Conclusions: 1. Based on our research, we can state that there are significant pathomorphological and pathophysiological changes in the deep venous system of the lower extremities in conditions of varicose vein disease. 2. The initiator of postoperative relapse of varicose veins are structural changes in the wall of deep veins with a violation of the integrity of the endothelial lining, contributing to the absorption of plasma and leukocyte contents from the blood stream in the interstitium, with the following pathological changes in the layers of deep veins. Such changes are the basis for the manifestations of endothelial dysfunction in the postoperative period.

KEY WORDS: EVLC, varicose veins recurrence, endothelial dysfunction

INTRODUCTION
Endovascular laser coagulation (EVLC) of veins due to its low traumatic and cosmetic effect became a priority area in the treatment of varicose veins of the lower extremities (VVLE) [1, 2, 3]. Ultrasound diagnostics of the state of the superficial and deep venous system plays a significant role in improvement of functional results after EVLC, which makes it possible to assess the presence of blood flow in the veins, the diameters and their lumen, deformation and saccular transformation of the veins, wall thickness, homogeneity, elasticity of the valves, their function during loading hydrostatic samples, presence of blood reflux, the duration of the retrograde flow along the venous lines, as well as its distribution to the anatomical segments, the state of the sapheno-femoral and sapheno-popliteal junction, to determine the localization and perforator veins, their diameter and duration of venous blood reflux in them [4, 5, 6]. Thus, individual hemodynamic features of the venous system of the lower extremities in a patient are detected and, based on this, the EVLC is selected in combination with other minimally invasive operations.

Thus, individual hemodynamic features of the venous system of the lower extremities in the patient are detected and, based on this, the EVLC is selected in combination with other minimally invasive surgeries. It should be noted that the introduction of highly informative diagnostic technologies and innovative methods of surgical treatment of VVLE significantly reduced the number of postoperative recurrence of varicose veins of lower extremities. At the same time, in the vast majority of scientific publications, the main emphasis of causes of postoperative recurrence of varicose veins of lower extremities is on tactical and technical errors during saphenectomy [7]. Thus, according to the analysis of the results of surgeries performed in specialized clinics where the diagnostic, tactical and technical principles of VVLE treatment are observed, the percentage of postoperative recurrence of varicose veins of lower extremities is 5–12.5% [8, 9, 10, 11, 12, 13, 14]. In our opinion, a certain number of postoperative recurrence of varicose veins of lower extremities falls on a “false relapse”, which may be due to the progression of varicose veins, but already the system of deep veins. We did not find a definite answer to this problem in an accessible scientific literature.
THE AIM
With the help of biochemical and morphological methods of investigation, to identify the causes of a false postoperative recurrence of varicose veins after the EVLC.

MATERIALS AND METHODS
The results are based on an analysis of data from a study of 173 patients operated on varicose vein disease of the lower extremities. According to the international classification of CEAP, there were 84 patients with C2, 66 – with C3, and 23 patients – with C4. The age of the patients was (45±5.7) years. There were 98 women and 75 men.

The functional state of the deep and superficial venous system of the lower extremities was determined by ultrasound color duplex scanning of veins. To do this, the Vivid 3 (General Electric, USA) device was used with a 5–10 MHz frequency sensor and a corresponding standard software package of the mentioned company to test the venous system. During the ultrasound examination, the presence of blood flow in the veins, the diameters and forms of the lumen of the veins, their deformation and sacrificial transformation, wall thickness, homogeneity, elasticity of the valves, their function during loading hydrostatic tests, presence of blood reflux, duration of retrograde flow through the venous lines, and also its distribution on anatomical segments, a state of sapheno-femoral and sapheno-popliteal junctions. In all patients, the failure of the sapheno-femoral and sapheno-popliteal junctions was noted. The blood collection was performed intraoperatively by puncture of the ulnar vein of the forearm. The blood was taken in a 5 ml test glass S-Monovette (Germany). The method is based on the isolation of endothelial cells with platelets, followed by platelets sedimentation with adenosine diphosphate (ADP). To obtain plasma-rich platelets, immediately after the blood was centrifuged for 10 minutes at a speed of 1000 rpm, then 1 ml of plasma was mixed with 0.23 ml of sodium adenosine diphosphate salt at a concentration of 1 mg/ml. The obtained mixture was mechanically stirred on a shaker ELMI-S3 for 10 minutes, at a speed of 100 rpm. Free platelet supernatant was transferred to another container and centrifuged at 9000 rpm for 10 minutes for the sedimentation of endothelial cells. Then, the free plasma was carefully removed, and the obtained precipitate was suspended in 0.1 ml of 0.9% sodium chloride solution and mixed with a disposable tip. Hemocytometer was filled with a finished suspension. The number of cells in the endothelium was counted in 2 chambers by phase-contrast microscopy (Biomed 5, Russia) and divided by 2, to obtain the average result in two chambers. Taking into account the relationship between the number of cells in the grid and the volume of the hemocytometer, the volume of the obtained suspension and volume of plasma, when counting the number of endothelial cells, was multiplied by 10^4/l. With normal endothelial function, the number of CEC is in the range of 0–4 10^4/l.

The regions of the femoral vein were removed, up to 2 cm in length for determination of morphological state of the deep venous system of the lower extremities, in a sectional study, in 31 patients with varicose veins of the lower extremities. Histologic preparations were made according to generally accepted methods. Coloring was done by hematoxylin and eosin. Morphometric studies were performed using a histological analysis system. The image on the computer monitor was output from the LOMO Biolam microscope with a help of Vision CCD Camera and InterVideoWin DVR.

For electron microscopic studies, the venous wall region was pre-fixed in 2.5% glutaraldehyde solution with an active medium pH reaction of 7.2–7.4 prepared on Millonig’s phosphate buffer. Post-fixation of vein parts was carried out for enzyme immunoassay (EIA) according to the manufacturer’s instructions. Concentration of endothelin-1 was established using Biomedica (Canada) sets for EIA according to manufacturer’s instructions. The reaction was measured on a SUNRISE (Tecan, Austria) microplate semiautomatic photometer using a Hydroflex washing station (Tecan, Austria), which allowed standardizing these methods. The control was the study of 30 healthy people. Endothelial dysfunction markers were also detected in the early (10 days after surgery) and the late postoperative period (60 days after surgery). In these cases, the blood for the study was taken from the femur of the limb, operated on the VVLE, by puncture.

To determine the CEC, we used the method of J. Hladovec and N.N. Petryshchev and co-authors (2001). Blood collection was carried out in the morning, on an empty stomach, by the puncture of the ulnar vein of the forearm. The blood was taken in a 5 ml test glass S-Monovette (Germany). The method is based on the isolation of endothelial cells with platelets, followed by platelets sedimentation with adenosine diphosphate (ADP). To obtain plasma-rich platelets, immediately after the blood was centrifuged for 10 minutes at a speed of 1000 rpm, then 1 ml of plasma was mixed with 0.23 ml of sodium adenosine diphosphate salt at a concentration of 1 mg/ml. The obtained mixture was mechanically stirred on a shaker ELMI-S3 for 10 minutes, at a speed of 100 rpm. Free platelet supernatant was transferred to another container and centrifuged at 9000 rpm for 10 minutes for the sedimentation of endothelial cells. Then, the free plasma was carefully removed, and the obtained precipitate was suspended in 0.1 ml of 0.9% sodium chloride solution and mixed with a disposable tip. Hemocytometer was filled with a finished suspension. The number of cells in the endothelium was counted in 2 chambers by phase-contrast microscopy (Biomed 5, Russia) and divided by 2, to obtain the average result in two chambers. Taking into account the relationship between the number of cells in the grid and the volume of the hemocytometer, the volume of the obtained suspension and volume of plasma, when counting the number of endothelial cells, was multiplied by 10^4/l. With normal endothelial function, the number of CEC is in the range of 0–4 10^4/l.

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RESULTS AND DISCUSSION

The analysis of biochemical markers (Table 1) indicates the presence of obvious signs of endothelial dysfunction (ED) in patients with VVLE.

Thus, the number of circulating endothelial cells in the venous blood affected by varicose veins of lower limbs was significantly higher compared with venous blood taken from the ulnar vein. This indicates the location of pathological processes when VVLE and proves the significant significance of this marker of endothelial dysfunction in this pathology.

Also, the difference in sVCAM-1 measured in venous blood taken from LSV and ulnar veins was found out. This indicates a violation of the interaction of endothelial cells with peripheral blood cells. Thus, the increased expression of the adhesion molecule sVCAM-1 in the vein wall is a sign of migration of leukocytes through the endothelium and maintaining the inflammatory process in it. The foregoing proves that in the development of varicose disease of the lower extremities, one of the key roles is inflammation against the background of a functionally altered and activated endothelium.

In a repeated study of ED markers in the early (10 days) and late (60 days) postoperative period, we stated that the main indicators of ED were still increased, although at their level they were less than preoperative. Thus, it can be argued, that after saphenectomy, ED is not completely eliminated.

To establish the causes of this phenomenon, we conducted an additional morphological and electron microscopic examination of the state of the deep venous system of the lower extremities in 31 patients with varicose veins of the lower extremities. Material for research (femoral vein regions, up to 2 cm in length) was collected during sectional study.

As a result of investigations of the morphological state of the deep venous system in patients with varicose disease, the following types of morphological changes in their walls were distinguished: a) hypertrophy of structural elements of the wall; b) initial phenomena of venous wall sclerosis on the background of hypertrophy; c) initial phenomena of atrophy of the vein wall on the background of evident sclerosis.

In the first category of patients, changes in the inner membrane were associated with the endothelial layer and the development of arterialization of the venous wall, which was manifested in the appearance of spindle-shaped regions protruding in the lumen of the vessel by endothelial cells. This indicates the location of patholog...
Fig. 1. Hypertrophy of the vein wall. Hematoxylin-eosin. Amplification x 100.

Fig. 2. Hypertrophy of the vein wall. The vessel in the vessel. Ultra-thin slice. Amplification × 400

Fig. 3. Endothelial hypertrophy, desquamation, development of collagen fibers, collagenosis, fragmentation of individual collagen fibers. Hematoxylin-eosin. Amplification x 100.

Fig. 4. Initial phenomena of atrophy against the background of severe sclerosis. Endothelial hypertrophy, desquamation, development of collagen fibers, collagenosis, fragmentation of individual collagen fibers. Hematoxylin-eosin. Amplification × 100.

Fig. 5. Initial atrophy of the venous system against the background of severe sclerosis. Hematoxylin-eosin. Amplification x 100.

Fig. 6. Significant atrophy of the venous system against the background of severe sclerosis. Hematoxylin-eosin. Amplification × 100.
of multi-nucleated cells of the symplastic structure and the area of hypertrophy of the subendothelial layer. In this case, the subendothelial layer is moderately developed, whereas the muscular layer is constructed of two well-differentiated layers of muscle fibers forming the inner circular and outer longitudinal layer, the fibers are hypertrophied, interfascicular sclerosis is noted among them. The adventitious membrane is thin, sometimes not visualized. The outer and inner elastic membranes are visualized separately. In the upper third of the large subcutaneous vein, in the abdominal region, these elements were more pronounced, and there is also an additional middle layer of the muscular membrane with skewed direction of fibers (Fig. 1).

In the subendothelial layer there is a slight increase in the content of glycosaminoglycans. The valves of the veins were characterized by the presence of a well-developed elastic component of the connective tissue in them. In some cases, the areas of two-sided epithelization were visualized in the valves.

In the second group of patients, we established the changes that characterize the intensive development of collagen fibers, the appearance of a network of collagen fibers on the border between the internal endothelial and middle – muscular layer, in addition, the direction of collagen fibers was parallel to the endothelium, and in some cases at an angle to him. Elastic fibers in the muscle layer were largely unchanged, occasionally their fragmentation occurred. The content of sour glycosaminoglycans was within the normal range. They were also in the muscle of the vessels (Fig. 2), which had a typical structure, in some cases, we noted hyalnosis. In adventitia, elliptical bundles of smooth myocytes, well-developed elastic membranes in the arteries of the vessel were visualized, as well as the usual thickness and number of collagen fibers in the absence of sclerotic changes and, within the norm, the content of acidic glycosaminoglycans. Collagen fibers are thick enough, mostly intact.

In this case, the construction of the endothelial layer is practically no different from the first group. There is a thickening of the subendothelial layer, in some cases, uniform around the vessel, a cluster in the form of a pillow of smooth myocytes with spindle-shaped nuclei, located along the axis of the vessel. Areas of desquamation of the endothelium in places of its hypertrophy (Fig 3–4). Accumulation of acidic glycosaminoglycans, initial sclerosis due to the development of collagen fibers was noted in the subendothelial layer. Elastic fibers on the border with the muscular membrane are thickened, in some cases, even with the phenomena of initial hyalnosis. The muscular membrane contains smooth myocytes with areas of their folding, most pronounced in the outer layers of the membrane. Sclerotic changes in the muscle and their degree were expressed in different parts of the venous wall. On some regions, there was a folding of the muscular membrane with separation of their connecting tissue. The elastic fibers were hypertrophied. Sclerosis with thickening and homogenization of elastic fibers was visualized in the adventitious membrane.

The third group of patients was characterized by the predominance of processes of atrophy of elements of the vascular wall against the background of multiple sclerosis (Fig. 5).

The thickness of the vascular wall was different and characterized by the presence of regions in the form of spindle-shaped knots, there were sites of desquamation of the endothelium. In some regions the spindle-shaped knots of the subendothelial layer, to which smooth myocytes with rounded nuclei are adjacent, penetrated the endothelium into the lumen of the vessels. The subendothelial layer contains a large amount of sour glycosaminoglycans. Fibrinoid

Fig.7. Destruction and homogenization of mitochondria, fragmentation of the endoplasmic complex membranes, increase of the number of ribosomes and polysomes. Amplification × 18000.

Fig.8. (A, B, C). Ultrastructure of endothelial cells of the lower extremities veins with varicose disease. Diluted nuclear membrane, condensed chromatin, expanded perinuclear space, A × 5000, B × 8000, C × 8000.
and mucoid edema of the connective tissue of the inner membrane of the vein with the phenomena of metachromasia and homogenization of collagen fibers, swelling and folding of elastic fibers of the inner elastic membrane were observed. The muscular membrane was characterized by pronounced atrophic changes of loss of myocytes, elastosis and folding. In some cases, the muscular membrane was represented by separate islets of smooth myocytes lying between thick and coarse collagen fibers, in a raw – absent completely, in a raw – thinned to 2-3 layers of myocytes. Myocytes contained hyperbazophilic, often spiral-twisted, very thin nuclei. There were areas without myocytes in areas of extremely thinned wall in the muscular layer. The adventitious membrane was characterized by pronounced sclerotic changes, elastosis, hyalinosis. In some cases, atrophic changes in the venous wall were characteristic in the same group of patients (Fig. 6).

In the intima there was a complete destruction of the elastic elements with the accumulation of a large number of acidic glycosaminoglycans in the subendothelial layer. In the large part of the vascular wall there was a significant thinning of the muscular membrane with the effects of elastosis and an increase in the number of collagen fibers. In some cases, myocytes were absent, and those that survived acquired structures with a sharp thinning, some elongation, spiral twisting or deformation of nuclei; in the adventitia – a certain thickening of elastic and collagen fibers.

In the electron microscopy study, endothelial cells did not differ significantly from the known cellular indexes of this type and had a standard set of organelles. The nuclei are in most cases with an uneven surface, condensed on the marginal type of chromatin. Micropinocytotic vesicles, which are mainly located near the basal cell surface, testify to normal transendothelial transport in vessels. Endothelial cell mitochondria had an average electron density and a homogeneous matrix.

Cristas of a large part of mitochondria were not reduced, individual mitochondria contained totalized cristas and a bright matrix. The outer membranes were with the islets of destruction (Fig. 7). Ductuli of granular of endoplasmic reticulum were significantly expanded, and some were electron-transparent vacuoles. There are practically no ribosomes on their membranes, and in the cytoplasm a large number of ribosomes and polysomes were detected. The gialolasma of the endothelial cells was moderately enlightened, the centers of lysis of membranes of the granuloid endoplasmic reticulum. Also, in the endothelial layer there were some enlarged cells with an enlightened cytoplasm, which we regarded as edema of endothelial cells. In some endothelial cells, vacuolation of the cytoplasm was determined. In the cytoplasm of the processes there was a large number of micropinocytosis vesicles filled with an electron-transparent substance (Fig. 8).

The cytoplasm of smooth myocytes was filled along fibers with actin and myosin microfilaments, and their cytoplasmic membrane was clear with small lysis foci. Organelles were not always localized in the perinuclear region of smooth myocytes, occasionally placed in the form of aggregates in other parts of the cytoplasm and were surrounded by bundles of filaments. The mitochondria and membranes of the Golgi cytoplasmic complex were destructively altered.

In the wall of the veins in the places of the damaged endothelial layer, there was a contact of erythrocytes directly with myocytes. In these places, red blood cells closed with each other and underwent focal lysis.

In the places of endothelial layer damage, intracellular processes in fibroblasts intensified. The nuclei of fibroblasts acquired a festonic look with multiple deep and superficial invaginations of the nuclear membrane. The nuclear chromatin was partially condensed and concentrated near the nuclear membrane. In the central part of the karyoplasm, ribosome clusters are present. Perinuclear spaces were not expanded.

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In the cytoplasm of fibroblasts, there were well-developed tubules of the granular endoplasmic complex, their tanks.
were thinned. On the membranes there are numerous ribosomes. A small number of mitochondria contained cristae, the number of which corresponded to this type of cells, the basal membrane was thinner, had a diverse density.

Numerous vacuoles are concentrated around nuclei, or occupy a considerable area of their cytoplasm. Expressive vacuolation of myeloid cells, as a rule, was accompanied by damage to the myofibrils of these cells, which is an evidence of partial violation of their contractile function. In the vascular system, there are places where red blood cells accumulate, which completely block the lumen of arterioles, venules and capillaries. Some of them were in a state of hemolysis, which, under ultra microscopy, was shown to be heterogeneous in content density. The tight contact of erythrocytes with endothelial cells testified to the slowing of blood flow. The conducted ultrastructural studies of the organization of endothelial cells in varicose veins revealed a violation of the metabolic activity of organelles. Mitochondria were subject to significant destructive changes, indicating a violation of bioenergy supply of synthetic processes. As a result of the violation of bioenergetic processes, the synthesis of substances decreases as well, manifested by the sharp expansion of the tanks of the granular endoplasmic complex, the disappearance of ribosomes, and the reduction of the Golgi cytoplasmic complex. There were also catabolic processes, as indicated by the increase in the number of lysosomes located near the Golgi complex.

The data of electron microscopic studies made it possible to reveal at the ultrastructural level pathological changes that occur in the layers of the venous wall and draw some conclusions about the pathogenesis of relapse of varicose disease. So, in our opinion, the basis of the development of endothelial dysfunction and development of varicose disease, the leading role belongs to changes in mitochondria. Mitochondrial insufficiency, manifested by lysis of cristas and external membranes, disrupts bioenergetic processes in cells, while the reparative possibilities of intracellular membrane structures and the metabolism of endothelial cells in general are also reduced. The absence in the cytoplasm of the processes of endothelial cells, micropinocytosis follicles indicates a decrease in the activity of transcellular transport of substances and electrolytes through the endothelium.

Further destructive changes of leiomyocytes indicate a violation of contractile properties of the venous wall and a decrease in their tone. Condensation of nuclear chromatin indicates a decrease in metabolic activity, and destruction of external membranes of mitochondria, disorganization and lysis of cristas and sealing of their matrix is a structural confirmation of the low level of contractile capacity of leiomyocytes under conditions of bioenergy deficiency. And the state of ultrastructural organization of smooth muscle cells in the case of varicose veins of the lower extremities does not allow maintaining a normal vascular tone. Activation of the fibroblast of the venous wall producing collagen and elastic fibers testifies to the compensatory reaction aimed at maintaining the normal configuration of the vessel wall in the places of damage of the endothelial layer.

**CONCLUSIONS**

Based on our research, we can state that there are significant pathomorphological and pathophysiological changes in the deep venous system of the lower extremities at VVLE. The initiator of postoperative recurrence of varicose disease is the structural changes in the wall of deep veins with violation of the integrity of the endothelial insulin, which contributes to the contents of plasma and leukocytes from the bloodstream in the interstitium, with the following pathological changes in the layers of deep veins. Such changes are the basis for the manifestation of endothelial dysfunction in the postoperative period. There is a need to develop criteria for predicting postoperative recurrence of varicose veins, as well as means to prevent its development.

**REFERENCES**


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