ORIGINAL ARTICLE
PRACA ORYGINALNA

DISCRETE LABORATORY AND MORPHOMETRIC MARKERS OF ATHEROSCLEROTIC LESIONS OF LOWER EXTREMITY VESSELS

DOI: 10.36740/WLek202003130

Olesya M. Horlenko, Viktoriya M. Studenyak, Fedir V. Horlenko, Lyubomyra B. Prylypko
SHEI «UZHHOROD NATIONAL UNIVERSITY», UZHHOROD, UKRAINE

ABSTRACT

The aim: To investigate discrete laboratory and morphometric features of atherosclerotic lesions in patients with chronic ischemia of the lower extremities (CILE).

Materials and methods: The examined contingent consisted of 47 patients (age 56.32 ± 1.09 years) diagnosed with obliterating atherosclerosis of the lower extremities. The study included determination of levels of Homocysteine, Folic acid, C protein, quantitative determination of circulating Endothelial cells (DEC) in blood plasma, and morphometric study of DEC.

Results: Protein C levels are within the reference values (0.97 ± 0.12 mg / l. In 37 (78.7%) patients Hypoacidofoliemia (<3.0 mmol / l) was observed. Homocysteine levels were clearly elevated in all patients. In the vast majority Hyperhomocysteinemia mild form (91.5%) was observed. The number of DEC in patients was -3.22 ± 0.39x10^5/l and after compression –6.12 ± 0.21x10^5/l

Conclusions: Protein C levels were within the reference values (0.97 ± 0.12 mg / l); Folic acid levels in the vast majority (37 patients, 78.7%) were <3.0 mmol /l. Blood plasma Homocysteine levels were clearly elevated in all patients. The mild form of Hyperhomocysteinemia (91.5%). was observed in the vast majority The number of DEC in patients was -3.22 ± 0.39x10^5 and after compression –6.12 ± 0.21x10^5/l, which confirmed the presence of the endothelial dysfunction.

KEY WORDS: patients, obliterating atherosclerosis of lower extremity vessels, endothelium, hyperhomocysteinemia, morphometry

Wiad Lek. 2020;73(3):561-564

INTRODUCTION

The attention of scientists is focused on the direction of endothelium-targeted research, as the earliest target organ and the key link in disease pathogenesis, according to literary sources, in our time [1]. The endothelium is an active endocrine organ, one of the largest in the body, diffusely located intravascularly across all tissues. The endothelium, which is distributed throughout the body, is essentially an organ with a huge surface.It is a differentiated complex structure which has specific functions in the body. It is a part and a control object of one of the organism's systems – of the circulatory system. By histologists' definition, the endothelium is a single-layer of specialized cells lining the entire cardiovascular tree from the inside, weighing about 1.8 kg. [2]. The blood capillaries, which are the ultimate executive link in the implementation of the transport functions described, are sometimes referred to as blood vessels. They complete one of the main functions of the circulatory system – the bilateral metabolism between the blood and the interstitial fluid of body tissues. From the microcirculatory vascular system of blood into the interstitial fluid comes the substances necessary for tissue metabolism, and from the interstitial fluid into the blood are transported the final products of tissue metabolism. The efficiency of such two-way transport of substances is ensured by the considerable duration, time of contact of blood with a huge surface of exchange of microcirculatory channel. This surface is formed by endothelial cells, whose total surface area exceeds ≈ 1000 m2[1,2].

An important pathogenetic link in the development of atherosclerotic vascular damage is the homocysteine theory. The development of atherosclerosis explains the possible connection of hyperhomocysteinemia (HHC) with the formation of vascular pathology on the basis of this theory, the fasting homocysteine concentration of more than 12.1 μmol / l is associated with a 2-times increase in the risk of atherosclerosis, including obliterating atherosclerosis of the lower extremities (OALE), coronary heart disease (coronary artery disease), acute coronary artery disease regardless of other risk factors, according to European studies. A study of the role of Homocysteine (HC) in the development of atherosclerosis revealed that the correlation coefficient between Coronary artery disease and cerebrovascular accident is 0.5-1.0 for every 5 μmol / l increase in Homocysteine, according to a meta-analysis. Approximately 30-40% of patients with OALE have elevated levels of Homocysteine. HHC is thought to increase the risk of progression of OALE, but the etiological role of HHCs remains unknown, as no studies have been reported to study the reduction of HCC levels in OASNCs. Much of the HC is remethylated with the formation of methionine. Folic acid and its derivatives are the main source of tetrahydrofolate, in which deficiency can develop HHC. At this point, other potential mechanisms for the emergence of endothelial dysfunction and its defeat at HHC have become known. Circulating desquamated endothelial cells are cells that separate from the vessel wall in the course of its damage

Table I. Laboratory markers of vascular atherosclerosis development (n=47)

Parameters	Number of leukocytes (x10⁹/л)	Protein C screening (0,8-1,15мg/l)	Folic acid (3,0-17,0 mmol/l)	Homocysteine (5,0-12,0 mmol/l)
M±m	8,2±0,3 (5-14)	0,97±0,12 (1,26-0,63)	2,22±0,37 (6,71-1,22)	18,29±1,18 (40,20-11,50)

and are a direct cellular marker of endothelial dysfunction [1, 2]. The concentration of desquamated endothelial cells in the peripheral blood is very low in the in the physiological state, since the endothelial self-renewal process is slow and the desquamated cells are intensively absorbed in the bloodstream by the macrophage system [3]. At the same time, there were increase in the number of these cells in various pathological conditions associated with the defeat of the vascular system (vasculitis, cardiovascular disease etc.) [1, 3]. It can be argued that the level of desquamated endothelial cells in the peripheral blood is a reflection of systemic damage of the endothelium based on this fact [4,5].

THE AIM

To investigate discrete laboratory and morphometric features of atherosclerotic lesions in patients with chronic ischemia of the lower extremities (CILE).

MATERIALS AND METHODS

The study was conducted on the basis of the ZRKH, named after Andrew Novak, Uzhhorod. The examined contingent consisted of 47 patients (age 56.32 ± 1.09 years) diagnosed with obliterating atherosclerosis of the lower extremities. The study included determination of levels of Homocysteine, Folic acid, C protein, quantitative determination of circulating Endothelial cells in blood plasma, and morphometric study of Endothelial cells. The study of HC levels in the blood plasma of patients was performed by enzyme-linked immunosorbent assay (ELISA) with using the test of Axis-Shield (UK) kit. The concentration of Folic acid in the serum of patients was investigated using an immunochemical method with electrochemiluminescent detection on ACL TOP 700 coagulometer, manufactured by Instrumentation Laboratory, USA. For the study of protein C the test systems of reagents manufacturer by "Roche Diagnostics" (Switzerland) was used. "A set of reagents for screening assessment of disorders in the system of protein C (Protein C-screening test) according to TU 9398-276-05595541-2009"was used. All patients underwent venous blood sampling, followed by determination of the number of desquamated endothelial cells (taking into account morphometric characteristics), which determined the degree of endothelial dysfunction. For the diagnosis of endothelial dysfunction in patients in the shoulder region create a positive pressure in excess of systolic blood pressure by 40-50 mm Hg, after 4 minutes carry out decompression, followed by sampling of venous blood with subsequent determination of the number of desquamated endothelial cells by Hladovec J. in terms of 1 liter of plasma [6]. Endothelial damage was assessed in the presence of desquamated endothelial cells (DEK) in the blood plasma of patients at a value greater than 2.77×10^5 /l of plasma. The method has a high sensitivity and allows to diagnose latent endothelial dysfunction (patent RU (11) 2234094 (13) C2). MicrosMCX-100 Daffodil microscope was used for the work[1]

RESULTS

The first stage of the study was the laboratory search for disorders that contribute to the development of vascular atherosclerosis and clinical presentation in patients with CILE. Disorders of Homocysteine amino acid metabolism and interdependent markers of folic acid and protein C were investigated (Table 1)

Protein C levels are within the reference values (0.97 ± 0.12 mg / l) according to table I. In 5 patients, from the experimental group, the values (<0.8 mg / l) were decreased, in 4 patients -> 1,5 mg / l. Protein C is an indicator of the blood coagulation system, one of the major inhibitors of the coagulation process and is one of the most important components of the anticoagulant blood system. The data obtained indicate the controlled state of the hemostasis in patients. The following picture was observed in the study of folic acid levels. In 37 patients, hypoacidofoliemia (<3.0 mmol / l) was observed, accounting for 78.7% of the total number of patients. Increasing Folic acid was not fixed.

When assessing the degree of homocysteine, blood plasma has the following gradation of abnormalities: mild HHC (10–30 μmol / l), moderate HHC (30–100 mmol / l) [7] Homocysteine levels were clearly elevated in all patients. In 4 patients a mild form of hyperhomocysteinemia (8.5%) was observed, in the vast majority was a mild form of HHC (91.5%). Studies over the past 15 years have found that homocysteine is a ranked independent risk factor for cardiovascular disease (CVD) – myocardial infarction, stroke, and venous thromboembolism, atherosclerosis [7,8]. HHC is a more informative indicator of the development of diseases of the cardiovascular system than cholesterol according to scientists data.

Homocysteine significantly reduces protein C activation by competitively inhibiting of the thrombo-modulin thrombin interaction required for thrombin S. protein activation. Homocysteine is also capable of interfering with the regulation of fibrinolysis. Vascular endothelium regulates local processes of hemostasis, proliferation, cell migration into the vascular wall and vascular tone. The idea of endothelial dysfunction was formed, which means the imbalance between the factors that provide all these processes. Fixation of endothelial cells on the basement membrane is carried out with the help of vitronectin, fibronectin, cadherins and more effective in young cells. The process of desquamation reflects the renewal of the endothelium, which has lost its ability to perform its inherent function as a result of aging or the effects of harmful factors. The endothelium desquamation is based on the activation of proteinases, necrosis and / or apoptosis of endothelial cells.

Table II. Counting of DEC in patients (n = 30) with obliterating atherosclerosis of the lower extremities

Parameters	Age (years)	Number DEC before probe	Number DEC after probe	P
	56,32±1,09	3,22±0,39x105	6,12±0,21x105	<0,001

In the case of injury and apoptosis, there is a disruption of the functioning of proteins that provide the connection of endothelial cells with the basement membrane, which leads to desquamation of endothelial cells. The duration of finding of circulated endothelial cells in the blood is about 24-42 hours, during which their capture and destruction by macrophages of the liver, lungs and spleen occurs. Apoptosis and necrosis of endothelial cells, increased production of proteinases, breaking the endothelial cell junction with underlying intimacy, promote the release of cytokines, free radicals and reactive oxygen species. The source of these biologically active substances may be leukocytes, especially those endogenous to the endothelial cells, which confirm the body's inflammatory response. Risk factors of atherosclerosis along with cardiovascular factors are infectious agents: Chlamydophilia pneumonia, symptoms of viral herpes, Helicobacter pylori and cytomegalovirus, which enhance the procoagulant properties of the endothelium. During the chronic inflammatory reaction, migration and proliferation of smooth muscle cells occur, which contributes to the further progression of atherosclerosis [9]. The endothelium is characterized by high stability, which is confirmed by the rare detection of apoptotic endothelial cells in the intima of vessels in normal state, in some other pathological processes, reflects the degree of damage of the vessels and allows to judge the severity of the disease, the effectiveness of the therapy. Adhesion of circulating leukocytes to the wall of blood vessels, as well as migration into the subendothelial space is carried out by adhesion molecules. During proteolytic cleavage, soluble forms of adhesion molecules appear, a sensitive indicator of the extent of atherosclerotic lesions of the arteries [10]. The table presents the quantitative characteristics of the level of desquamated plasma endothelial cells in patients before and after the test with compression of the vessels of the shoulder (Table II).

At the start of the study, the number of desquamated endothelial cells in patients exceeded the reference values (3.22 ± 0.39x105) and after of compression the DEC amount increased in 2-times (6.12 ± 0.21x105/1in of plasma). The high level of DEC in patients confirmed the presence of the endothelial dysfunction in the patients. A significant increasing in more than one percent of the DEC level in the blood after vessels compression of the shoulder vessels indicates their tendency to desquamation with minor mechanical effects (external – compression; internal – shear stress after decompression) and testifies to endothelial dysfunction. Based on the studies, it should be considered that the increase in the number of circulating desquamated endothelial cells above 2.77x105/l of plasma after a sample with short-term compression of the blood vessels is a diagnostic criterion of endothelial dysfunction, which was confirmed in our investigation. Based on a set of general clinical and special research methods, patients in the study group confirmed the presence of endothelial dysfunction. We also conducted a morphometric study of endothelial cells in the blood of patients with chronic lower extremity ischemia (Figure 1)

In the case of injury and apoptosis, there is a malfunction of proteins that provide endothelial cell connections with the basement membrane, which leads to endothelial cell desquamation. The source of these biologically active substances may be leukocytes.

DISCUSSION

Hyperhomocysteinemia damages the walls of the vessels, making their surface loose. Cholesterol and calcium precipitate, forming atherosclerotic plaques on the damaged surface. Increased levels of Homocysteineemia increase thrombosis. Inhibiting the work of the anti-coagulation system, Hyperhomocysteinemia is one of the links in the pathogenesis of early thrombovascular disease, as it increases the risk of vascular thrombosis. Homocysteine is also capable of interfering with the regulation of fibrinolysis. The Homocysteine detrimental effect on vascular endothelium is realized through a direct damaging effect on the blood vessels by auto-oxidation products, enhanced low-density lipoprotein peroxidation, and inhibition of endothelial cells DNA synthesis . The multifactorial genesis of atherosclerosis and thrombosis is undeniable. The authors, at the same time, who deny the significant role of HHC in the pathogenesis of cardiovascular disease, do not take into account the presence of other proven or actively investigated proatrogenic or prothrombotic determinants. One of these factors is HHC, which is an important determinant in the development of thromboembolic complications and endothelial dysfunction in various diseases and conditions. Adhesion of circulating leukocytes to the wall of blood vessels is carried out by adhesion molecules, as well as migration into the subendothelial space Examination for the detection of HHC is advisable to perform in patients with vascular pathology. Significant increase in the greater percentage of cases of the amount of DEC in the blood of patients after shoulder vessels compression indicates their tendency to desquamation with minor mechanical effects (external – compression; internal – shear stress after decompression) and indicates endothelial dysfunction. Based on the studies, it should be considered that the increase in the number of DEC circulating ($6.12 \pm 0{,}21x10^5$/l) in plasma after short-term compression of the shoulder vessels is a diagnostic endothelial dysfunction criterion, which was confirmed in ours. The presence of endothelial dysfunction, Hyperhomocysteinemia as determinants of the development of the lower extremities vessels atherosclerotic lesions was confirmed in the patients of the study group, on the basis of the complex of laboratory and morphometric methods of investigation.

CONCLUSIONS

1. The level of Protein C is within the reference values (0.97 ± 0.12 mg / l)

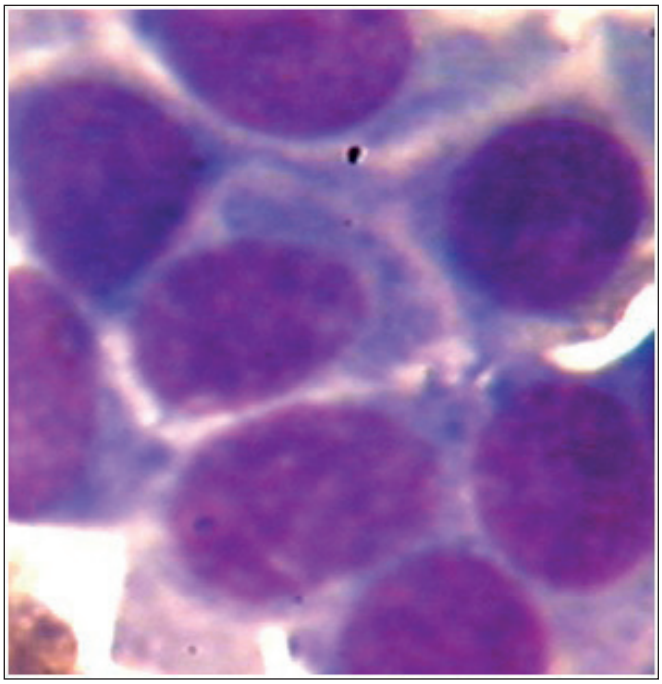

Figure 1. Endothelial cell layer: pronounced polymorphism and proliferation, nucleoli are visualized, coarse-grained chromatin, the nucleus occupies almost all the cytoplasm.

2. Hypoacidofoliaemia (<3.0 mmol / l) was observed in 37 patients in the study of folic acid levels, representing 78.7% of the total patients number. An increasing in folic acid levels has not been recorded.
3. Plasma Homocysteine levels were clearly increased in all patients. In 4 patients a mild form of Hyperhomocysteinemia (8.5%) was observed, in the vast majority was a Hyperhomocysteinemia mild form (91.5%).
4. The number of desquamated blood plasma endothelial cells in patients exceeded the reference values (3.22 ± 0.39x105) and the amount of DEC increased in 2-times(6.12 ± 0.21x105/l) of plasma after compression, which confirmed the presence of the investigated endothelial dysfunction.
5. At morphometric research of the endothelial cells layer expressed polymorphism and proliferation is observed; nucleoli, coarse-grained chromatin are visualized, the nucleus occupies almost all cytoplasm.

REFERENCES

1. Martynov AI, Avetyak NG, Akatova YeV i dr. Endotelial'naya disfunktsiya i metody yeye opredeleniya[Endothelial dysfunction and methods for its determination]. Rossiyskiy kardiologicheskiy zhurnal. 2005; 4 (54): 94–8. (In Russian)
2. Yushchuk YeN, Vasyuk YuA, Khadzegova AB i dr. Endotelial'naya disfunktsiya pri zabolevaniyakh serdechno-sosudistoy sistemy i metody yeye korrektsii. [Endothelial dysfunction in cardiovascular system diseases and methods for its correction]. Klinicheskaya farmakologiya i terapiya. 2005; 14 (3): 85–8. (In Russian)
3. Titov VN. Anatomicheskiye i funktsional'nyye osnovy endoteliy-zavisimoy vazodilatatsii, oksid azota i endotelin [Anatomical and functional basics of endothelium-dependent vasodilation, nitric oxide and endothelin]. Ros. kardiol. zhurnal. 2008; 1: 71–85. (In Russian)
4. Tabarov MS, Toshtemirova ZM, Saidmuradova RA i in. Uchast' v zdíysnennί angíogenezu yak v yembríonal'nomu rozvitku, tak í pri regeneratsíí" tkanin píslya poshkodzhennya. Fiziologiya i patologiya endoteliya [Participation in the implementation of angiogenesis both in embryonic development and in tissue regeneration after injury. Endothelial physiology and pathology]. Vestn. Avitsenny. 2012; 2: 196–202. (In Ukrainian)
5. Fadini GP, Avogaro A. Cell-based methods for ex vivo evaluation of human endothelial biology. Cardiovascular research. 2010; 87(1): 12-21.
6. Feoktistova V.S., Vavilova T.V., Sirotkina O.V i dr. Novyy podkhod k otsenke disfunktsii endoteliya: opredeleniye kolichestva tsirkuliruyushchikh endotelial'nykh kletok metodom protochnoy tsitometrii [A new approach to assessing endothelial dysfunction: determining the number of circulating endothelial cells by flow cytometry]. Klinicheskaya laboratornaya diagnostika. 2015; 60(4): 23-7. (In Russian)
7. Potter K. Homocysteine and cardiovascular disease: should we treat? Clin. Biochem. Rev. 2008 ; 29: 27–30.
8. Cho YI, Cho DJ, Rosenson RS. Endothelial shear stress and blood viscosity in peripheral arterial disease . Current Atherosclerosis Reports. 2014; 16 (4): 404–410.
9. Lagutchev VV, Shchupakova AN. Vzaimosvyaz' i dr. SVCAM-1 antitela k Chlamydophilia pneumoniae, Helicobacter pylori, Virus herpess implex i stepeni ateroskleroza arteriy nizhnikh konechnostey [The relationship of SVCAM-1 antibodies to Chlamydophilia pneumoniae, Helicobacter pylori, Virus herpes simplex and the atherosclerosis degree of the lower limb arteries]. Immunopatologiya, allergologiya, infektologiya. 2010; 4: 12–8. (In Russian)
10. Kade AKp, Çanin SA, Gubareva YeA i dr. Fiziologicheskiye funktsii sosudistogo endoteliya. [Physiological functions of the vascular endothelium]. Fundamental'nyye issledovaniya. 2011; (11)3: 611–7. (In Russian)

ORCID and contributionship:

Olesya M. Horlenko – 0000-0002-2210-5503 [A,D,F]
Viktoriya M. Studenyak – 0000-0003-3935-0537 [A,B]
Fedir V. Horlenko – 0000-0002-0496-2069 [A,D,F]
Lyubomyra B. Prylypko – 0000 0002 4131 5450 [B,E,F]

Conflict of interest:

The Authors declare no conflict of interest.

CORRESPONDING AUTHOR

Olesya M. Horlenko
Squ. Narodna 2, 88000, Uzhhorod, Ukraine
tel: +380505269658
e-mail: kaf-dithvorob@uzhnu.edu.ua

Received: 17.01.2020
Accepted: 05.03.2020

A – Work concept and design, **B** – Data collection and analysis, **C** – Responsibility for statistical analysis, **D** – Writing the article, **E** – Critical review, **F** – Final approval of the article