Proliferation and apoptosis of smooth muscle and endothelial cells with immune inflammation in cerebrovascular diseases on atherosclerosis background

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ABSTRACT

The aim – to determine the role of vascular wall cells proliferation and apoptosis with the participation of immune inflammation, and their impact on the development of cerebrovascular disease (CVD) development on the atherosclerosis (AS) background.

Materials and methods. We studied 50 cases of death with ischemic stroke and 50 cases of death with hemorrhagic stroke on the cerebral vessels AS background.

Outcomes. Lymphocytes are one of the atheroma components and are mainly localised at the sites of plaque rupture in close contact with macrophages and smooth muscle cells. Smooth muscle cells are able to synthesize important collagen and elastin for the vascular wall, but potentiated apoptosis of smooth muscle cells may contribute to destabilisation and plaque rupture. Smooth muscle cells apoptosis was triggered by proinflammatory factors and took place with the participation of cytotoxic T-lymphocytes (T-killers) therefore in the atherosclerotic lesions focus we registered an accumulation of multitude cytotoxic T-lymphocytes.

Conclusions. The macrophages and smooth muscle cells susceptibility to apoptosis was significantly higher directly in the atheroma, but macrophage apoptosis is a useful process for the atherosclerotic plaque stability. Desquamation and endothelial cell apoptosis are interrelated processes that play an important role in atheromatous plaque formation.

Keywords: atherosclerosis, lymphocytes, macrophages, smooth muscle cells, apoptosis

INTRODUCTION

Cerebrovascular diseases (CVD) should be considered as a complex of various neurological, vegetovascular and metabolic disorders, the dynamics of which is mainly stipulated by the degree of vascular insufficiency, cerebral atherosclerosis, blood pressure and risk factors influence [9,14,15,24]. Significant prevalence, high mortality and disability of the population due to CVD developed against the background of atherosclerosis (AS), and their most severe manifestation – cerebrovascular strokes – make prevention and treatment of these diseases one of the most pressing medical and social issues [6,12,14].

The development of acute and chronic cerebrovascular disorders is often preceded by AS. These conditions are accompanied by structural and functional changes in the vascular wall, mainly in its endothelial lining [6,12,14,19]. Vascular tone (total vascular resistance, blood pressure), vascular wall atrombogenicity, platelet and coagulation system activity, inflammatory and oxidative process, as well as vascular wall layers structural preservation and atherogenesis depend on the endothelial cells adequate functioning [12,16]..

In physiological conditions endothelium has low proliferative activity, mainly because of its own elements, due to amitotic division of endothelial cells

[7,11]. It is able to maintain cell layer continuity through intact cells migration and division. Deendothelialization caused by a pathological condition triggers reparative regeneration process by active proliferation of endothelial cells. Violation of this process, most often upon atherogenesis, leads to pathological regeneration, when the proliferation of subordinate smooth muscle cells with the formation of myointimal thickenings – atheroma precursors – takes place in deendothelialized areas of the vascular wall [12,17,22].

Immunocompetent cells play a key role in the atheromatous plaque formation and atherosclerosis progression. Foam cells are able to express surface receptors and produce inflammatory mediators that engage other monocytes, activate endothelial and smooth muscle cells. Lymphocytes are also present in all stages of atherogenesis [2,3].

If trigger agents are not neutralized by immunocompetent cells, and the inflammatory response progresses, it changes from protective to damaging. Involvement of monocytes and lymphocytes occurs as a result of adhesion molecules activation on both endothelium and immunocompetent cells. Activation of monocytes and lymphocytes causes activation and possible plaque rupture. Lymphocytes are one of atheroma components and are mainly localised at the plaque rupture points in close contact with macrophages and smooth muscle cells. Activated lymphocytes produce pro-inflammatory cytokines that can enhance the inflammatory response, and activated macrophages, in turn, release proteolytic enzymes that contribute to fibrous capsule thinning and plaque destabilisation. Both lymphocytes and macrophages produce cytotoxic factors that potentiate apoptosis [2,5,8].

Smooth muscle cells proliferation is positive for atherogenesis, despite the fact that derived from smooth muscle cells macrophage-like cells promote an inflammatory response [1,4]. A number of studies have shown that smooth muscle cells that actively express the main histocompatibility complex of class II antigens were identified in atherosclerotic plaques of blood vessels. These activation proteins are specific for T-lymphocytes and macrophages and are involved in the receptor transmission of immune information that indicates the ability of SMC to participate in immune responses in AS [2,10,23].

Smooth muscle cells (SMC) are an important component of atherosclerotic formations that ensure the plaque stability. However, smooth muscle cells apoptosis is sufficient for the negative effects of AS, such as plaque rupture, inflammation and calcification [14,19,23].

The aim of our study was to determine the role of vascular wall cells proliferation and apoptosis, and their impact on the CVD development on the AS background.

MATERIALS AND METHODS

We studied 50 cases of death with ischemic stroke on the cerebral vessels AS background, 50 cases of death with hemorrhagic stroke on cerebral vessels AS background and 50 cases of death not related to CVD and AS (comparison group). We studied arteries of two structural and functional levels: main – carotid arteries and extracerebral – cerebral floor arteries, where we took 2-3 segments with lipid and fibrous plaques, and in the comparison group we studied unchanged areas. We performed immunohistochemical (IHC) study using the following markers CD4 (CD4 Ab-8), CD8 (SP-16), CD68 (CD68/Macrophage Marker Ab-4), p53 (Clone Y5), CD31/PECAM-1 (Endothelial Cell Marker, Ab -1), Vimentin Ab-2 (Clone V9).

We fixed the material in a 10% solution of neutral buffered formalin according to established procedure. To perform IHC reactions, sections 4-5 μm thick were mounted on Super Frost Plus adhesive slides (Menzel), dewaxed, hydrated, and treated with 3% hydrogen peroxide solution to block endogenous peroxidase. The Ultra Detection System kit (Thermo Scientific) was used as the second antibody. To separate non-specific structures, sections were additionally stained with Mayer's haematoxylin.

The results of IHC reactions of the CD4 (CD4 Ab-8) markers – T-helpers marker, CB8 (SP 16) – T-suppressors marker and CD68 (CD68/Macrophage Marker Ab-4) were evaluated by counting cells with positive staining in 10 randomly selected microscope fields of view at 400 magnification. In addition, staining intensity degree was evaluated: 0 – no staining, 1 (+) – weak staining of light brown colour, 2 (++) – moderate staining of brown colour, 3 (+++) – pronounced staining of dark brown colour.

Vimentin Ab-2 (Clone V9) was used to determine mesenchymal cells and their derivatives – endotheliocytes, smooth myocytes, fibroblasts, pericytes, which were estimated as the specific volume of immunopositive cells per unit area. The results of the IHC reaction were appraised by the semi-quantitative method by points from 0 to 6 according to the conventional method, taking into account the stained cells [66]. 0 points were rated in the absence of staining, 1 point – up to 10%, 2 points – up to 20%, 3 points – up to 30%, 4 points – up to 40%, 5 points – up to 50%, 6 points – more than 50% cell staining. In addition, the degree of staining intensity was evaluated: 0 – no staining, 1 (+) – weak staining of light brown colour, 2 (++) – moderate staining of brown colour, 3 (+++) – pronounced staining of dark brown colour.

Immunopositivity of the CD31 marker is observed in the cytoplasm and cell membrane. The results of the IHC reaction CD31/PECAM-1 (Endothelial Cell Marker) Ab-1 were apprised by a semi-quantita-

tive method by points from 0 to 6 according to the conventional method, taking into account the stained cells [1]. 0 points were rated in the absence of staining, 1 point – up to 10%, 2 points – up to 20%, 3 points – up to 30%, 4 points – up to 40%, 5 points – up to 50%, 6 points – more than 50% stained cells. In addition, the staining intensity degree was evaluated: 0 – no staining, 1 (+) – weak staining of light brown colour, 2 (++) – moderate staining of brown colour, 3 (+++) – pronounced staining of dark brown colour.

The p53 (Clone Y5) marker was used to determine cerebral arterial walls cells apoptosis. The IHC reaction results were appraised by a semi-quantitative method. The cell nuclei staining intensity degree was determined: 0 – no staining, 1 (+) – weak staining, 2 (++) – moderate staining, 3 (+++) – pronounced staining of nuclei. We determined the area occupied by immunopositive structures. The relative area of expression (S,%) was calculated by the formula: S=S immunopositive nuclei/S nuclei x100 [332].

Histological examination and micropreparations photographing were performed on an AxioScop 40 microscope (Zeiss). Data from morphometric studies were subject to statistical processing on a personal computer using the standard Microsoft Excel program; the results were processed by the variation statistics method and considered reliable at p< 0,05.

OUTCOMES

In recent years, the AS origin and development are considered from the standpoint of monoclonal proliferation of smooth muscle cells (SMC), immune inflammation and apoptosis, where great importance is attached to immunocompetent, smooth muscle cells and endothelium role. Upon atherogenesis we observed apoptosis of endothelial cells, mononuclear cells and smooth muscle cells at all stages of atherosclerotic lesions formation.

We noted SMC proliferation and connective tissue fibers hyper production, which explains the cause of the intima thickening with the subsequent fibrous plaque formation.

Immunomorphological analysis of the arterial walls showed that, with the exception of the endothelium, all intima and media cells react with antibodies to Vimentin (Figure 1). In the study of Vimentin in the arterial wall in the group of patients with ischemic stroke on the background of AS, its expression constituted 59.6 ± 4.8% (p >0.05) against the total area; in the group with hemorrhagic stroke on the AS background – 52.8 ± 3.7% (p >0.05), so there is no significant difference in the quantitative content of Vimentin in the study groups. In the comparison group the expression of Vimentin was 24.2 ± 6.4 (p >0.05).

At IHC reaction with Vimentin we noted the presence of connective tissue components, and also the expressed artery wall fibrosis.
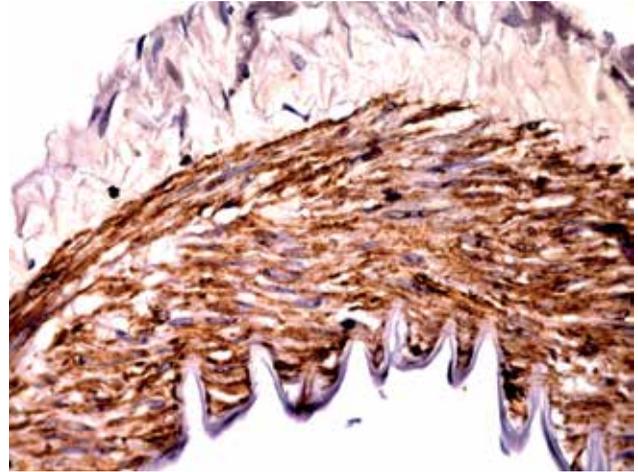

FIGURE 1. Intensive expression of the Vimentin Ab-2 marker in the artery wall of a patient with ischemic stroke (↑). Staining: IHC procedure with primary antibodies of Vimentin Ab-2 positive cells. Magnification: ocular lens 10, field lens 20

Vimentin expression showed that vascular wall fibrosis increases as the underlying disease progresses. We observed the activation of SMC proliferation in blood vessels intima, the myocytes migration from the middle membrane and an increase in their number, which depends on fibroblast and endothelial factors.

We also detected the synthesis of the connective tissue matrix components, i.e. collagen and elastin, which subsequently led to intima thickening and fibrous plaque formation.

In the process of atherogenesis in the developed atheroma, along with the smooth muscle cells proliferation we also noted their apoptosis (Figure 2). The expression of p53 in the arterial wall in the group of deceased of ischemic stroke averaged 25.7% (p <0.05), of hemorrhagic stroke – 17.9% (p <0.05).

SMC can synthesize collagen and elastin important for the vascular wall, but potentiated SMC apoptosis may contribute to the plaque destabilisation and rupture [19,21,23].

SMC apoptosis was triggered by proinflammatory factors and took place with the participation of cytotoxic T-lymphocytes (T-killers) therefore in the atherosclerotic lesions focus we registered an accumulation of multitude cytotoxic T-lymphocytes (Figure 3). T-lymphocytes (CD8, membrane expression) in atherosclerotic lesions constituted 8.56 ± 1.16 (p >0.05) in the group with ischemic stroke and, respectively, 9.12 ± 1.64 (p >0.05) in the group with hemorrhagic stroke.
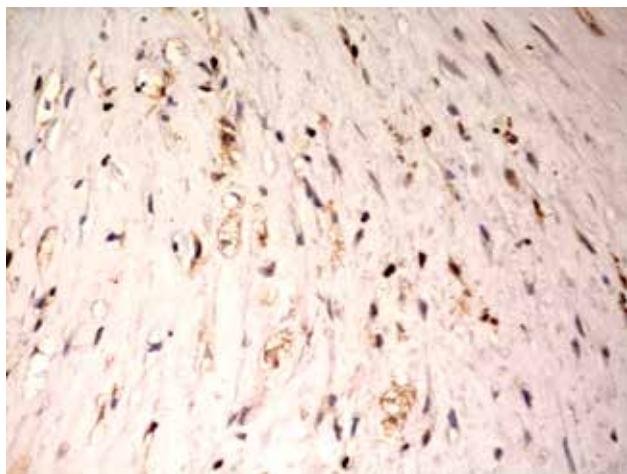
FIGURE 2. Moderate expression of the p53 marker in artery wall cells of a patient with hemorrhagic stroke (↑). Staining: IHC procedure with primary antibodies p53 of positive cells. Magnification: ocular lens 10, field lens 40.
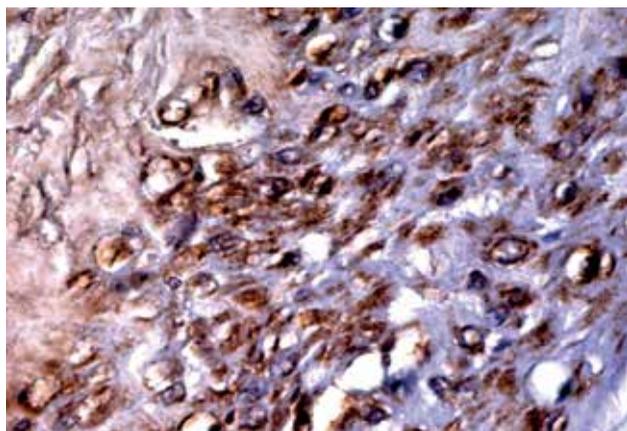

FIGURE 3. Intense expression of the CD8 marker in the artery wall of a patient with ischemic stroke (↑). Staining: IHC procedure with primary antibodies of CD8 positive cells. Magnification: ocular lens 10, field lens 40

Multitude immunocompetent cells, i.e. macrophages and T-lymphocytes, were observed subendothelially and in the fibrous capsule, with apoptosis being common, possibly to suppress the inflammatory response.

We observed monocytes adhesion on the luminal surface of the arteries, presence of multitude monocytes under the endothelium, and more mature macrophages in the intima depths, that indicate these cells entry into the arterial wall from the blood.

In atherosclerotic lesions formation, we noted a significant number of CD68-positive cells with marker expression in the cytoplasm (Figure 4), which constituted 16.68 ± 1.82 (p >0.05) in the group with ischemic stroke and 14.56 ± 1.28 (p >0.05) in the group with hemorrhagic stroke. In the comparison group, there was no expression of markers in the artery wall, in some cases it was observed in the form of single cells.
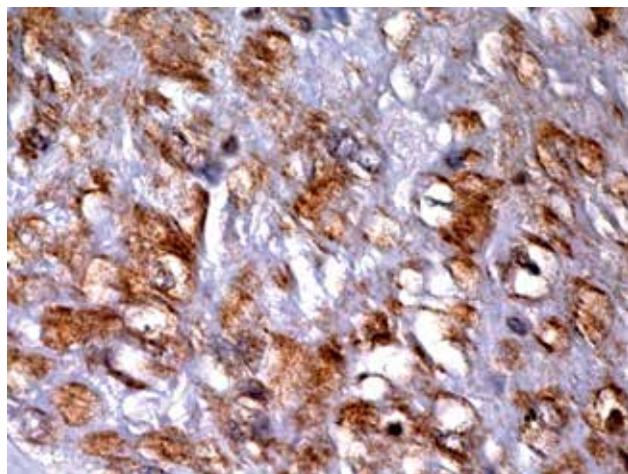

FIGURE 4. Intensive expression of the CD68 marker in the artery wall of a patient with ischemic stroke (↑). Staining: IHC procedure with primary antibodies of CD 68 positive cells. Magnification: ocular lens 10, field lense 40

Multitude macrophages contain extensive apoptotic material, which indicates the apoptotic cells phagocytosis. However, the apoptosis development in such macrophages and the presence of multitude apoptotic material extracellularly may indicate insufficient mechanisms of apoptotic cells neutralization in atherosclerotic vascular lesions.

However, at present, it is impossible to reliably determine whether this process is due to a pronounced inflammatory response and active cell proliferation, with potentiated apoptosis, lack of mechanisms of apoptotic material neutralization, or possibly due to a combination of all these factors.

The expression of p53 in the arterial wall in the group of deceased of ischemic stroke averaged 25.7% (p< 0.05), of hemorrhagic stroke – 17.9% (p< 0.05). In the arterial wall in the group of deceased of hemorrhagic stroke, the level of p53 expression was
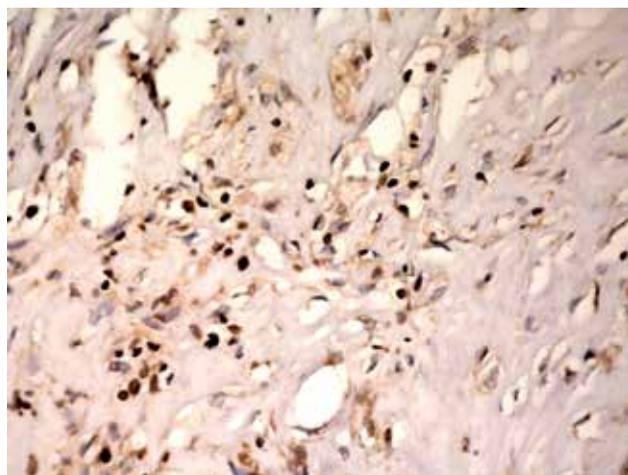

FIGURE 5. Intense expression of the p53 marker in mononuclear cells of the artery wall around the atherosclerotic plaque of a patient with ischemic stroke (↑). Staining: IHC procedure with primary antibodies of p53 positive cells. Magnification: ocular lens 10, field lens 40

probably lower as compared to the group of deceased of ischemic stroke; immunopositive cells usually allocated diffusely (Figure 5).

In lipid atherosclerotic plaques, apoptotic cells were found both around the atheroma and among the foam cells. Individual apoptotic cells were also localised below the atheroma at the edge of the middle membrane. More pronounced expression of apoptosis p53 marker was observed in the marginal parts and around atherosclerotic plaques.

The endothelium of the cerebral vessels in ischemic and hemorrhagic strokes on the AS background assumes structural changes in the form of rupture, desquamation and exfoliation (Figure 6). In many cases we noted on sections that the endothelial cells are distinctly elongated, the cell thickness is very small and the nuclei are elongated. An accumulation of desquamated apoptotic endotheliocytes is observed in the vascular lumen, as indicated by their intense expression.

Iimmunopositivity of the CD31 marker is observed in the cytoplasm and cell membrane. In the study of the CD31 marker in the arterial wall in the group of patients with ischemic stroke we revealed uneven antigen deposition in the endothelium – 3.2 ± 0.3 (p >0.05) points.

Uneven expression was also observed in the group with hemorrhagic stroke, at that, high-focal expression approached to its absence, averaging 3.8 ± 0.2 points (p >0.05).

IHC studies have shown that there is a close relationship between structural changes in endothelial cells and intima damage. The endothelium functions are aimed at permeability regulating and adapting to external factors, and at the same time at maintaining its integrity.

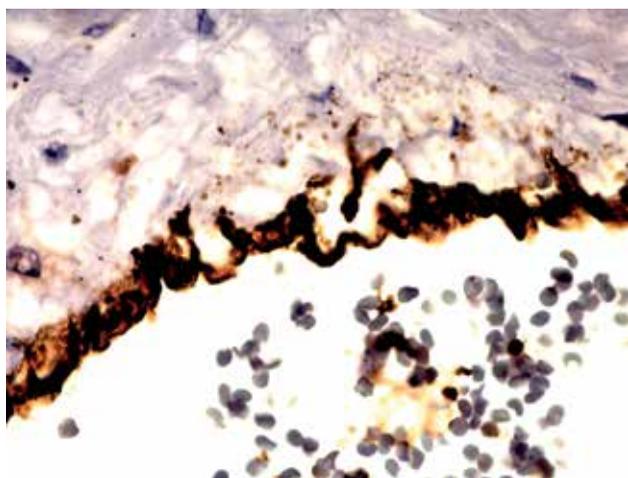We observed different degrees of CD31 marker expression in the arterial walls, ranging from mod-
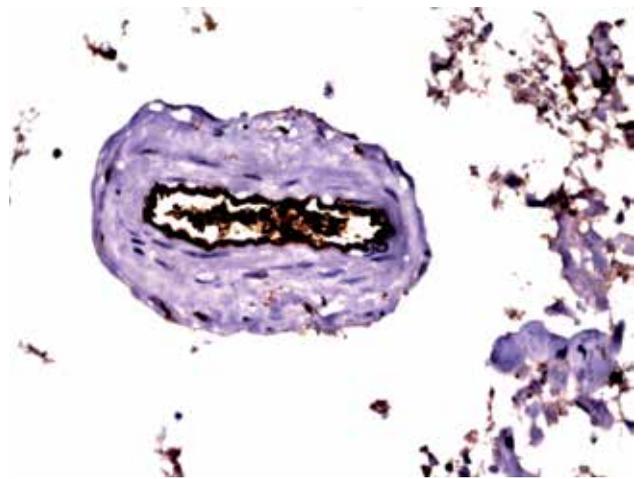
FIGURE 7. Intensive expression of the CD31 marker in the arterial endothelium of a patient with hemorrhagic stroke – the presence of endothelial cells in the vessel lumen (↑). Staining: IHC procedure with primary antibodies of CD31 positive cells. Magnification: ocular lens 10, field lens 40

erate with uneven antigen deposition to high-focal, combined with its complete absence in large areas.

Deendothelial areas showed no antigen expression or single endothelial cells expression. Desquamated endothelial cells clusters were noted in the vessels lumen, as indicated by their intense expression (Figure 7).

Morphologically, the results obtained in the study did not differ from the data presented in other modern studies. The data obtained indicate and coincide with the foreign authors' data that the endothelial barrier plays an active role in the development of atherosclerotic plaque by regulating the endothelial cover permeability and vasoactive mediators local secretion [12,16,22].

At present, it is necessary to develop methods that will accurately quantify apoptosis in the vascular wall. A clear understanding of the inducing mechanisms will help to find ways to suppress cell death in the vessel wall, and specify directions for further medicinal treatment development.

It is obvious that disorders in the vascular wall upon AS will lead to changes in organs and systems that are the pathogenetic basis for many pathological processes, such as cerebrovascular and cardiovascular pathology. That is, the development of an individual program for AS modification should still be based on an objective quantitative assessment of the risk factors spectrum and concentration, which shape the AS prognosis.

FIGURE 6. Intense expression of the CD31 marker in the arterial endothelium of a patient with ischemic stroke. Staining: IHC procedure with primary antibodies to CD31 positive cells. Magnification: ocular lens 10, field lens 100

CONCLUSIONS

1. The number of apoptotic cells increased as the underlying disease progressed. And the intima cells

apoptosis is actively involved in atherosclerotic changes development.

2. The macrophages and SMC susceptibility to apoptosis was significantly higher directly in the atheroma as compared to other atherosclerotically changed vascular wall areas.

3. Increase in the immunocompetent cells number and their apoptosis in the fibrous capsule, reduced number of the fibrous capsule cells, increased proliferation and intense SMC apoptosis in atheroma contribute to atheromatous plaque destabilisation and rupture.

4. Apoptosis of macrophages is a useful process for the atherosclerotic plaque stability, provided that there are mechanisms of apoptotic material neutralisation.

5. Desquamation and endothelial cell apoptosis are interrelated processes. Reduction in the number of endothelial precursor cells that are actively involved in endothelial regeneration plays an important role in the formation of atheromatous plaque. The endothelial dysfunction severity has a prognostic value for the CVD development.

Conflict of interest: none declared
Financial support: none declared

REFERENCES

1. Aguado A, Fischer T, Rodríguez C, Manea A, Martínez-González J, Touyz RM, Hernanz R, Alonso MJ, Dixon DA, Briones AM, Salaices M. Hu antigen R is required for NOX-1 but not NOX-4 regulation by inflammatory stimuli in vascular smooth muscle cells. *J Hypertens.* 2016 Feb;34(2):253-65. doi: 10.1097/HJH.0000000000000801. PMID: 26682942; PMCID: PMC4947528.

2. Allahverdian S, Chehroudi AC, McManus BM, Abraham T, Francis GA. Contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis. *Circulation.* 2014 Apr 15;129(15):1551-9. doi: 10.1161/CIRCULATIONAHA.113.005015. Epub 2014 Jan 30. PMID: 24481950.

3. Ammirati E, Moroni F, Magnoni M, Camici PG. The role of T and B cells in human atherosclerosis and atherothrombosis. *Clin Exp Immunol.* 2015 Feb;179(2):173-87. doi: 10.1111/cei.12477. PMID: 25352024; PMCID: PMC4298395.

4. Bennett MR, Sinha S, Owens GK. Vascular Smooth Muscle Cells in Atherosclerosis. Circ Res. 2016 Feb 19;118(4):692-702. doi: 10.1161/CIRCRESAHA.115.306361. PMID: 26892967; PMCID: PMC4762053.

5. Bobryshev YuV, Karagodin VP, Kovalevskaya ZhI. Kletochnye mekhanizmy ateroskleroza: vrozhdennyy immunitet i vospalenie [Cellular mechanisms of atherosclerosis: innate immunity and inflammation]. *Fundamental'nye nauki i praktika.* 2010;1(4):140-48. (in Russian).

6. Bornfeldt KE, Tabas I. Insulin resistance, hyperglycemia, and atherosclerosis. Cell Metab. 2011 Nov 2;14(5):575-85. doi: 10.1016/j.cmet.2011.07.015. PMID: 22055501; PMCID: PMC3217209.

7. Buie JJN, Oates JC. Role of interferon alpha in endothelial dysfunction: insights into endothelial nitric oxide synthase-related mechanisms. *Am J Med Sci.* 2014 Aug;348(2):168-75. doi: 10.1097/MAJ.0000000000000284. PMID: 24796291; PMCID: PMC4526236.

8. Chistiakov DA, Melnichenko AA, Myasoedova VA, Grechko AV, Orekhov AN. Mechanisms of foam cell formation in atherosclerosis. *J Mol Med* (Berl). 2017 Nov;95(11):1153-1165. doi: 10.1007/s00109-017-1575-8. Epub 2017 Aug 7. PMID: 28785870.

9. Fartushna OYe, Vinychuk SM. Detection and removal of vascular risk factors as important area of primary prevention of transient ischemic attack. Ukrainian Medical Journal. 2015;1(105):23-27. (in Ukrainian) http://nbuv.gov.ua/UJRN/UMCh_2015_1_8.

10. Feil S, Fehrenbacher B, Lukowski R, Essmann F, Schulze-Osthoff K, Schaller M, Feil R. Transdifferentiation of vascular smooth muscle cells to macrophage-like cells during atherogenesis. *Circ Res.* 2014 Sep 12;115(7):662-7. doi: 10.1161/CIRCRESAHA.115.304634. Epub 2014 Jul 28. PMID: 25070003.

11. Gimbrone MA Jr, García-Cardeña G. Vascular endothelium, hemodynamics, and the pathobiology of atherosclerosis. Cardiovasc Pathol. 2013 Jan-Feb;22(1):9-15. doi: 10.1016/j.carpath.2012.06.006. Epub 2012 Jul 18. PMID: 22818581; PMCID: PMC4564111.

12. Gimbrone MA Jr, García-Cardeña G. Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. *Circ Res.* 2016 Feb 19;118(4):620-36. doi: 10.1161/CIRCRESAHA.115.306301. PMID: 26892962; PMCID: PMC4762052.

13. Karaflou M, Lambrinoudaki I, Christodoulakos G. Apoptosis in atherosclerosis: a mini-review. *Mini Rev Med Chem.* 2008 Aug;8(9):912-8. doi: 10.2174/138955708785132765. PMID: 18691148.

14. Mishchenko TS. Epidemiology of cerebrovascular diseases and organization of medical care for patients with stroke in Ukraine. *Ukrains'kyi Visnyk Psykhonevrolohii.* 2017;1(90):22-24. (in Russian)

15. Moshenska O.P. Fatal ischemic stroke: peculiarities of the most acute period. *Ukrainian Medical Journal.* 2011; 1, (81), 29–35.

16. Perticone F, Ceravolo R, Pujia A, Ventura G, Iacopino S, Scozzafava A, Ferraro A, Chello M, Mastroroberto P, Verdecchia P, Schillaci G. Prognostic significance of endothelial dysfunction in hypertensive patients. *Circulation.* 2001 Jul 10;104(2):191-6. doi: 10.1161/01.cir.104.2.191. PMID: 11447085.

17. Pircher A, Treps L, Bodrug N, Carmeliet P. Endothelial cell metabolism: A novel player in atherosclerosis? Basic principles and therapeutic opportunities. Atherosclerosis. 2016 Oct;253:247-257. doi: 10.1016/j.atherosclerosis.2016.08.011. Epub 2016 Aug 20. PMID: 27594537.

18. Regina C, Panatta E, Candi E, Melino G, Amelio I, Balistreri CR, Annicchiarico-petruzzelli M., Di Daniele N., Ruvolo G.Vascular ageing and endothelial cell senescence: Molecular mechanisms of physiology and diseases. *Mechanisms of ageing and development.* 2016;159:14–21.doi: http://doi.org/10.1016 / j.mad.2016.05.003

19. Rudijanto A. The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis. *Acta Med Indones.* 2007 Apr-Jun;39(2):86-93. PMID: 17933075.

20. Ryter SW, Kim HP, Hoetzel A, Park JW, Nakahira K, Wang X, Choi AM. Mechanisms of cell death in oxidative stress. *Antioxid Redox Signal.* 2007 Jan;9(1):49-89. doi: 10.1089/ars.2007.9.49. PMID: 17115887.

21. Slee EA., Adrain C, Martin SJ. Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *Journal of Biological Chemistry.* 2001; 276(10):7320–7326. doi: 10.1074/jbc.m008363200.

22. Vanhoutte PM. Endothelial dysfunction: the first step toward coronary arteriosclerosis. *Circ J.* 2009 Apr;73(4):595-601. doi: 10.1253/circj.cj-08-1169. Epub 2009 Feb 18. PMID: 19225203.

23. Yan H., Peng X., Xu H., Zhu J., Deng C. Inhibition of aortic intimal hyperplasia and vascular smooth muscle proliferation and extracellular matrix protein expressions by astragalus-angelica combination. Evidence-Based Complementary and Alternative Medicine, vol. 2018, Article ID 1508637, 15 pages, 2018. https://doi.org/10.1155/2018/1508637

24. Zinchenko OM., Mishchenko TS. The state of the neurological service in Ukraine in 2015; 2016; 23.