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ORIGINAL ARTICLE

CHANGES IN THE SENSORIMOTOR CORTEX OF THE RAT BRAIN UNDER THE MODELING OF HEMORRHAGIC STROKE

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Tetiana M. Oliinyk[1], Liudmyla M. Sokurenko[2], Rostyslav F. Kaminsky[2], Viktoria E. Lavrynenko[3], Olena V. Kancer[3], Svetlana N. Chuhray[2], Sergii T. Omelchuk[2], Anna V. Blagaia[2]

[1] NATIONAL UNIVERSITY OF UKRAINE ON PHYSICAL EDUCATION AND SPORT, KYIV, UKRAINE

[2] BOGOMOLETS NATIONAL MEDICAL UNIVERSITY, KYIV,UKRAINE

[3] TARAS SHEVCHENKO NATIONAL UNIVERSITY, KYIV, UKRAINE

ABSTRACT

The aim: To assess the structural and metabolic changes in the sensorimotor cortex of the rat brain under conditions of hemorrhagic stroke.

Materials and methods: The experiment was carried out on rats of the control and experimental groups with a model of hemorrhagic stroke. We used histological, electron microscopic, biochemical methods and biological markers.

Results: In the sensorimotor cortex of the ipsilateral cerebral hemisphere of rats under conditions of hemorrhagic stroke, cerebral edema and progression of neurodegenerative changes were observed; an increase in the size of mitochondria, which is caused by edema of their matrix; activation of lipid peroxidation processes and a decrease in the activity of enzymes of the antioxidant system, a decrease in the level of apoptosis markers and inhibition of ERK1/2 expression. The study of DNA fragmentation in the cerebral cortex revealed a significant number of manifestations of necrosis and an insignificant number of cells in a state of apoptosis.

Conclusions: after modelling a hemorrhagic stroke in the right hemisphere of the brain, perivascular and pericellular edema of the energy apparatus, cell death by necrosis and apoptosis, and activation of lipid peroxidation processes were established as well as a decrease in the activity of enzymes of the antioxidant system.

KEY WORDS: hemorrhagic stroke, ischemia, electron microscopy, biochemical changes, hypertension

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INTRODUCTION

Hemorrhagic stroke occurs due to hemorrhage in brain tissue after rupture of blood vessels and comprises about 15-20% of all strokes [1]. The common sites of hemorrhagic stroke are the basal ganglia (50%), cerebral lobes (10% to 20%), the thalamus (15%), pons and the brain stem (10% to 20%), as well as the cerebellum (10 %) [2]. In modern conditions, under the threat of Coronavirus disease to which the patients with major chronic diseases such as hypertension and diabetes are most vulnerable, these are also key risk factors for hemorrhagic stroke [3]. Thus, the study of morphological and biochemical changes, as well as biomarkers of apoptosis and necrosis will allow a deeper understanding of the mechanisms of hemorrhagic stroke and minimizing mortality and morbidity of patients in the future.

THE AIM

The aim of the study was to evaluate structural and metabolic changes in the sensorimotor cortex of the rat brain under conditions of hemorrhagic stroke.

MATERIALS AND METHODS

The experiment was performed on 30 rats, which were divided into 3 groups, the control was intact and pseudooperated rats, the differences between which were not detected, the experimental group - with a model of hemorrhagic stroke. Experiments with animals (weighing, measuring blood pressure, injections, operations) were carried out in accordance with the Law of Ukraine "On protection of animals from cruelty" (2006), "General ethical principles of animal experiments", adopted by the First National Congress on Bioethics (Kiev, 2001) and in accordance with the requirements of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1985).

Simulation of local hemorrhagic stroke in rats [4] was achieved by mechanical destruction of the inner capsule (SI dextra, L = 3.5-4.0; H = 6.0; AP = 0.6-1.0) by 4 -6 rotational movements of the bent mandrel-knife, followed by the introduction into the inner capsule of 0.15-0.2 ml of autologous blood. Ten days after stroke

simulation, intracardiac brain perfusion was performed with 4% paraformaldehyde solution on 0.1 N phosphate buffer (pH 7.4) and frontal sections of the motor area of the neocortex of the ipsilateral hemisphere [5] 2-3 mm thick were obtained.

In order to study histological changes, paraffin frontal sections of the brain [6] with a thickness of 6-8 μm were made on a Thermo Microm HM 360 microtome, stained with hematoxylin and eosin [7] and toluidine blue according to Nissl's method [8]. Morphometric and morphological analyzes were performed using Carl Zeiss software (AxioVision SE64 Rel.4.9.1) and Olympus BX 51 microscope (Japan).

In order to study the ultrastructural changes, fragments of the motor site of the neocortex of the ipsilateral hemisphere were removed and fixed in 2.5% solution of glutaraldehyde on phosphate buffer with fixation in 1% buffered saline of osmium tetroxide (OsO_4) and embedded into a mixture of epoxy resins epon-araldite (Epon 812, Araldite 502) [9]. Epoxy blocks were used to make semi-thin and ultra-thin sections on a Reihart ultratome. Ultrathin sections after contrast with 2% uranyl acetate solution were examined on an electron microscope Tescan Mira 3 LMU (Czech Republic).

THE STUDY OF DNA FRAGMENTATION OF CEREBRAL CORTEX FRAGMENTS

DNA was separated in 1.7% agarose gel (Agarose Serva Premium, "Serva", Germany) into fragments depending on molecular weight using the program "Gel Pro Analyzer". In agarose gel, apoptosis is defined as "DNA stairs" [10]. In necrosis, there is a rapid nonspecific cleavage of DNA into shorter fragments, which are registered as a "smear". In order to assess the activity of enzymes, determination of the amount of protein in the homogenate was performed by the method of Bradford [11]. The following monoclonal antibodies were used in Western bloting [12]: Bax, caspase-3 and ERK1/2 ("Cell Signalling", USA).

Catalase activity (CAT) was determined by the method of Aebi H. [13]. Superoxide dismutase (SOD) activity was determined by the Mirsa H. method [14]. NAD(P)H-quinone oxido-reductase activity was established in liver homogenates in a mixture of NADPH-generating glucose-6-phosphate dehydrogenase system, menadione (2-methyl-1,4-naphthoquinone) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [15]. LPOP products were detected by the method of determination of malonic dialdehyde (MDA) in the reaction with thiobarbituric acid (TBA) by the method of Uchiyama M [16]. The content of diene conjugates (DC) in the sample was calculated based on the molar coefficient of extinction at 233 nm for conjugated dienes of polyunsaturated higher fatty acids [17].

Statistical processing of the obtained data was performed using Student's t-test and Mann's U-test using Origin Lab (version 8.0) and Statistica 6.0 depending on the normality of the distribution.

RESULTS

Histological changes of the sensorimotor cortex of the cerebrum show (Fig. 1) neurodegenerative changes, namely edema of the cytoplasm and cell nuclei. Hyperchromic staining of neurons, deformation of nuclei and perikaryons, single intact gliocytes were determined. Altered lumen and necrosis of endothelial cells, concomitant perivascular edema with signs of infiltration of cells of monocytic-macrophage series are observed in capillaries.

The results of morphometric study confirm the progressive neurodegenerative changes in the sensorimotor cortex of the cerebrum of rats (Table I).

Gliocytes show signs of karyopicrosis as one of the morphological forms of apoptosis. The area of gliocyte nuclei in the experimental comparison groups decreased statistically significantly by 37.3% ($p < 0.05$).

Based on the results of electron microscopic examination, in the experimental group of rats with simulated hemorrhagic stroke significant dystrophic changes were found: severe edema of the neuropile and processes of neurons and gliocytes; a sharp decrease in the number of synapses. Only single interneuronal contacts were detected, and in some areas, they were completely destructed. The nuclei of neurons were swollen, the perinuclear space was increased, and the electron density of chromatin was reduced, which is a manifestation of fragmentation of nucleoprotein complexes. Nerve fibers were characterized by stratification of the lamellae of myelin sheaths (Fig. 2). Due to pronounced ultrastructural changes in cells, reduction of organelles and disruption of cell membrane integrity, we were unable to differentiate damaged small neurons from gliocytes, indicating the development of total neurodegenerative changes in the cerebral cortex during stroke, without significant differences between neurons and gliocytes.

Investigation of the energy apparatus of neurons - mitochondria**:** increase in the diameter of mitochondria (matrix and crystae edema of the organelles) was observed, which is a sign of edema of the cytoplasm of neurons. A statistically significant increase in the average diameter of mitochondria in experimental rats was determined – 342.5 ± 21.9 nm (control – 215 ± 41.2 nm). The increase in the size of mitochondria was due to edema of their matrix.

Analysis of biochemical changes in the integrated marker parameters of free radical oxidation of biomolecules showed a significant intensification of this process under the conditions of experimental stroke. In particular, this applies to the content of TBA-active products (MDA) and

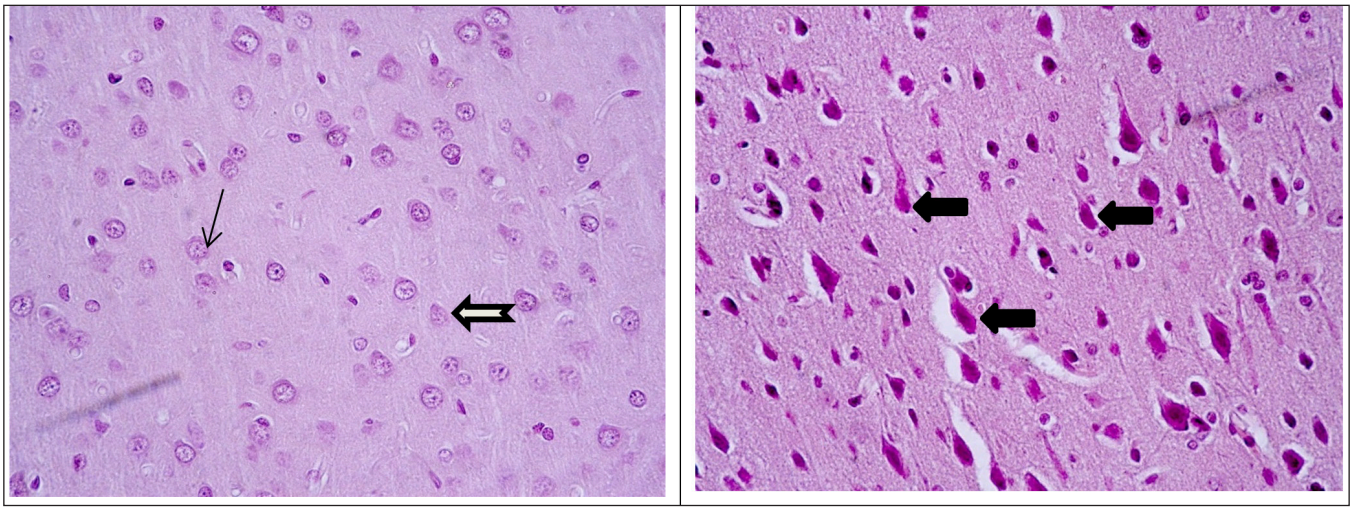

Fig. 1. Sensorimotor cortex of the cerebral hemisphere of rats with hemorrhagic stroke.
Note: ⬅ apoptotic neurons; ⇦ neurons in a state of hydropic dystrophy; neurons without changes; ← gliocytes. Hematoxylin-eosin. Obj. 40, oc. 10.

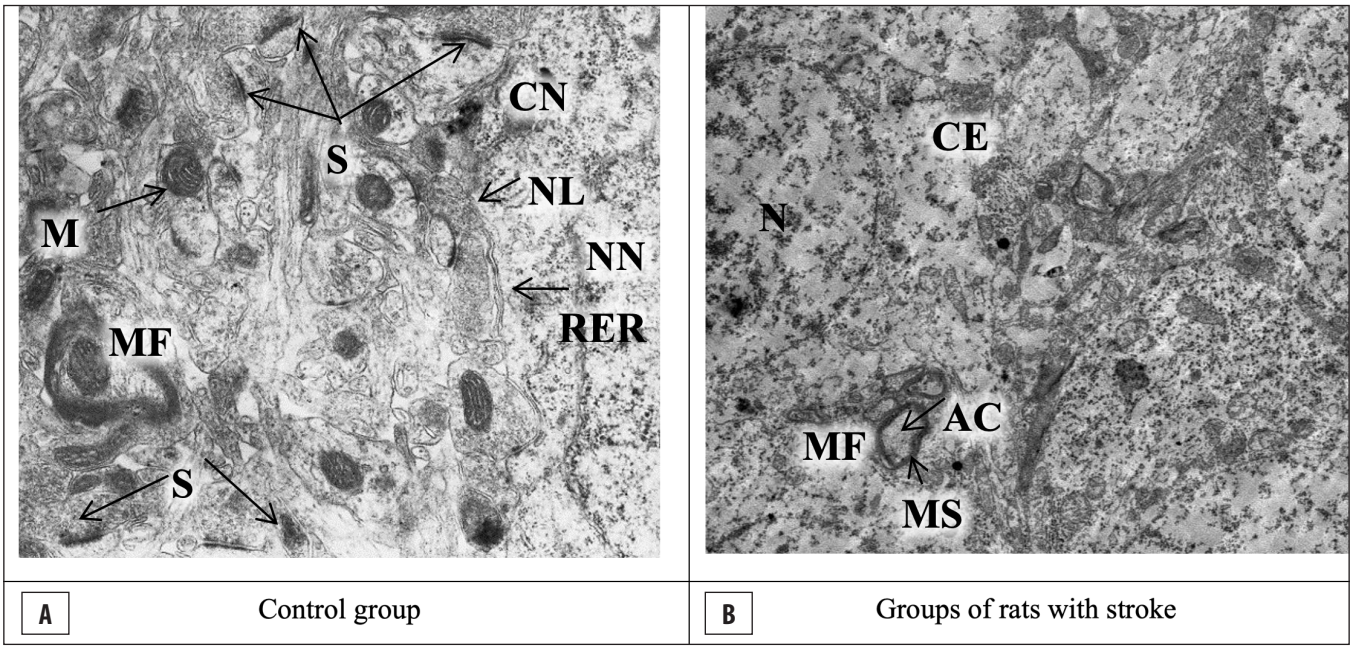

Fig. 2. Ultrastructural disorders in the cerebral cortex. A. Neuropil and neuron. Nerve cell processes are located densely. Synapses and single myelin fibers are registered. Note: NN is the nucleus of a neuron; NI - neurolemma; CN - cytoplasm of the neuron; MF - myelin fiber; S - synapse; M - mitochondria; RER - rough endoplasmic reticulum. Electronogram: × 25000.
B. Dystrophic changes of neuropil and neuron. Acute edema of the cytoplasm of the neuron with loss of integrity of membrane structures. Development of necrotic processes. Most myelin fibers have a detachment of the myelin sheath from the axial cylinder. Note: N – the nucleus of a nerve cell in a state of necrosis; CE - cytoplasmic edema; MF - myelin fiber; AC - axial cylinder; MS - myelin sheath. Electronogram: × 11400.

DC. It was found that the level of MDA in the homogenate of the cerebral cortex of normotensive rats was 2.2 times higher ($p < 0.01$) compared with the control group of rats, while DC was 1.5 times higher ($p < 0.01$) (Table II).

Our results showed that during stroke the activity of SOD and CAT decreased by 10.9% and 16.2% ($p < 0.05$) in rats with hemorrhagic stroke (Table II). According to the results of biochemical studies, it was found that in rats with hemorrhagic stroke, the activity of NAD (P) H-quinone oxidoreductase DT-diaphorase increased by 25.9% ($p < 0.05$) (Table II). Thus, under the conditions of hemorrhagic stroke, the activation of LPOP processes takes place, as evidenced by the increase in the content of LPOPs - MDA and DC products and the decrease in the activity of AES - SOD and CAT enzymes.

In the cells of the ipsilateral hemisphere of the cerebrum of rats on day 10 after simulation of hemorrhagic stroke, the dominance of necrosis over apoptosis was observed. This is accompanied by structural abnormalities in neurons that are associated with hyperproduction of endogenous cytotoxic molecules. The latter are formed as a result of

Table I. Morphometric parameters of sensorimotor cortex of rats on the background of modeled hemorrhagic stroke

Indicator / Group	Control	Experiment
Total density of neurons	104,7±3,9	99,4±5,1
Density of damaged neurons	5,0±0,9	39,9±4,3*
The area of the perikaryon of pyramidal neurons of the sensorimotor cortex, μm2	366,7±9,1	439,9±20,5*
The area of the nuclei of pyramidal neurons of the sensorimotor cortex, μm2	192,5±5,3	256,8±12,0*
The area of nuclei of gliocytes of the sensorimotor cortex, μm2	36,1±0,4	22,6±1,2*

Note: * - reliable to the control group (p <0,05).

Table II. The content of lipid peroxidation products in the cerebral cortex of control rats and rats with hemorrhagic stroke

Group of animals	Lipid peroxidation products(LPOPs)		Antioxidant Enzyme Systems (AES)		
	MDA, nmol/mg prot.	SOD U/mg/min prot	CAT mgmol/min prot	DT-diaphorase, nmol/mg/min	DC, nmol/mg prot.
Control	7,16±0,37	14,43±0,21	1,54±0,02	4,16±0,14	4,46±0,39
HS	15,54±1,05*	12,86±0,28*	1,29±0,05*	5,24±0,24*	6,93±0,87*

Note: HS - hemorrhagic stroke; * - changes are significant in relation to control (p <0,05).

oxidative stress in damaged mitochondria and cause the death of nerve cells. However, the decrease in the activity of the antioxidant system can be considered as a consequence of peroxidation of protein molecules of enzymes that perform the function of cell protection.

STUDY OF DNA FRAGMENTATION IN THE CEREBRAL CORTEX

DNA fragmentation was almost absent in control rats. DNA degradation was observed in the cerebral cortex tissue during hemorrhagic stroke, in the form of short DNA fragments (100-200 base pairs (bp)), which can be assessed as a manifestation of necrosis. However, on the electrophoregram we additionally found clear areas of DNA fragments divisible by 400 bp, which can be explained by the presence of a small number of cells in a state of apoptosis.

STUDY OF THE EXPRESSION LEVEL OF CASPASE-3 AND BAX IN THE CEREBRAL CORTEX

Quantitative analysis of the level of expression of caspase-3 indicates a decrease in its level compared to the level of its expression in control rats by 19.4% (p <0,05). In rats with hemorrhagic stroke, a similar pattern of reduction in the level of expression of Bax by 40.5% compared with controls (p <0.05) was found.

STUDY OF THE EXPRESSION LEVEL OF ERK1/2 IN THE CEREBRAL CORTEX

The expression level of ERK1/2 is lower in experimental rats compared to control rats. Inhibition of ERK1/2 expression can be considered as one of the mechanisms of cytoprotection.

DISCUSSION

Thus, the results of studies in the development of hemorrhagic stroke have shown cerebral edema and progression of degenerative changes neurons and capillaries, which confirms the morphological picture described by other researchers [4].

Electron microscopy revealed significant dystrophic changes in neurons and gliocytes, a sharp decrease in the synapses number, the absorption of which involves microglia/macrophages and astrocytes [18]. The observed damage of gliocytes is confirmed by new experimental data that even small changes in the structure of astrocytic processes in synapses can radically change the strength of synaptic transmission in the brain [19].

Neurons nuclei had manifestations of nucleoprotein complexes fragmentation, and nerve fibers were characterized by delamination of myelin sheaths lamellae, which was confirmed by own research results and data of other authors [20].

The study of mitochondria determined an increase in the diameter of mitochondria due to the swelling of their matrix. These data may explain the patterns of development of structural disorders on the background of stroke, namely the dominance of necrosis with underlying energy deficiency in cells and the development of cytopathological peroxidation of macromolecules (proteins, lipids, nucleic acids) and their subsequent decay. Mitochondrial dysfunction can lead to mitochondrial membrane potential collapse, overproduction of reactive oxygen species, disorders of

mitochondrial dynamics, and activation of mitochondria-related inflammation [21].

It was established that the level of MDA and DC in rats exceeded the indicators of the control group of rats. SOD and CAT decreased. A decrease in catalase activity leads to the formation of a hydroxyl radical, which causes lipid peroxidation. The activity of NAD(P)H-quinone oxidoreductase DT-diaphorase increased, which can be considered as a compensatory reaction of nerve cells to oxidative stress.

In the cortex of the ipsilateral hemisphere of rats with hemorrhagic stroke, cell death processes are observed, which are based on pronounced nonspecific fragmentation of deoxynucleoproteins, i.e. elimination of cells by necrosis. This assumption is confirmed by the data of other authors, who showed that in the condition of a stroke during the first hours the cells die by necrosis, and then the mechanisms of apoptosis join [22].

Signal pathways of apoptosis and necrosis initiation are often common, but the implementation of a certain mechanism of cell death depends on the characteristics of cell metabolism. Thus, it has been shown that ATP, reactive oxygen species (ROS), and nitrogen metabolites (NO, NO_2, $ONOO^-$) play an important role in switching between apoptosis and necrosis [23]. In addition, caspases and a number of other enzymes have been shown to play a key role in necrotic cell death. Thus, the decrease in the level of markers of apoptosis (caspase-3, Bax) and the dominance of total DNA fragmentation, i.e. nucleolysis indicate the progression of necrosis mechanisms over apoptosis. Simultaneous DNA fragmentation and activation of caspase-3 expression in the first 3 days of the experiment and activation of cytoprotective mechanisms on day 7 are known [24], which confirms the violation of apoptotic processes in the cerebral cortex. In our own studies, the tendency to decrease the synthesis of ERK1/2 indicates a violation of the mechanism of apoptosis activation, which may be interrelated with the development of necrosis and inhibition of regenerative processes in the cells of the cortex of large hemispheres in hemorrhagic stroke. This results in oligaemia, neuro-transmitter release, mitochondrial dysfunction, and cellular swelling [2].

CONCLUSIONS

After simulation of hemorrhagic stroke in the right hemisphere of the brain, perivascular and pericellular edema, dystrophic changes of neurons of III-V layers of the neocortex and cytoarchitectonics disorders as well as energy disorders, cell death by necrosis and apoptosis, activation of processes of peroxidation of lipids and reduction of activity of antioxidant system enzymes were observed.

REFERENCES

1. Montaño A, Hanley DF, Hemphill JC 3rd. Hemorrhagic stroke. Handb Clin Neurol. 2021; 176:229-248. DOI: 10.1016/B978-0-444-64034-5.00019-5.
2. Unnithan AKA, Mehta P. Hemorrhagic Stroke. 2023. https://pubmed.ncbi.nlm.nih.gov/32644599/ [date access 21.01.2023]
3. Wang H, Tang X, Fan H et al. Potential mechanisms of hemorrhagic stroke in elderly COVID-19 patients. Aging (Albany NY). 2020; 12(11): 10022-10034. DOI: 10.18632/aging.103335.
4. Wadowski PP, Schörgenhofer C, Rieder T et al. Microvascular rarefaction in patients with cerebrovascular events. Microvasc Res. 2022; 140: 104300. doi: 10.1016/j.mvr.2021.104300.
5. Wang D, Liu K, Wake H et al. Anti-high mobility group box-1 (HMGB1) antibody inhibits hemorrhage-induced brain injury and improved neurological deficits in rats. Scientific Reports. 2017; 7: 46243. doi: 10.1038/srep46243.
6. Savosko SI, Chaikovsky JB, Pogorela NKh, Makarenko AN. Features of histostructural changes in rat cerebral cortex in hemorrhagic stroke modeling. International Journal of Physiology and Pathophysiology. 2013; 4(2): 113-121. doi: 10.1615/IntJPhysPathophys.v4.i2.40.
7. Liu S, Grigoryan MM, Vasilevko V et al. Comparative analysis of H&E and Prussian blue staining in a mouse model of cerebral microbleeds. J Histochem Cytochem. 2014; 62(11): 767-73. doi: 10.1369/0022155414546692.
8. Benner S, Kakeyama M, Endo T et al. Application of NeuroTrace staining in the fresh frozen brain samples to laser microdissection combined with quantitative RT-PCR analysis. BMC Research Notes. 2015; 8: 252. doi: 10.1186/s13104-015-1222-9.
9. Cherkasov VG, Vasilenko DA, Parakhin AA. Ultrastructural modifications in neurons of the rat frontal cortex induced by intoxication by tert-butyl methyl ether. Neurophysiology. 2010; 42(6): 471-481. doi: 10.1007/s11062-011-9174-8.
10. Matassov D, Kagan T, Leblanc J et al. Measurement of apoptosis by DNA fragmentation. Methods Mol Biol. 2004; 282: 1-17. doi: 10.1385/1-59259-812-9:001.
11. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72(1-2): 248-254. doi: 10.1006/abio.1976.9999.
12. Mahmood T, Yang P-C. Western Blot: Technique, Theory, and Trouble Shooting. North American Journal of Medical Sciences, 2012; 4(9): 429-434. doi: 10.4103/1947-2714.100998.
13. Aebi H. Catalase in vitro. Meth. Enzymol. 1984; 105: 121-126. doi: 10.1016/s0076-6879(84)05016-3.

14. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972; 247(10): 3170-5.
15. Petrova GV, Donchenko GV. Cytotoxicity of troglitazone, a structural analogue of α-tocopherol is mediated by inhibition of NAD(P)H: Quinone oxidoreductase. Ukr. Biokhim. Zhurn. 2009; 81(4): 82-88. doi: 10.1016/0003-2697(78)90342-1.
16. Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem, 1978; 86(1): 271-278. doi: 10.1016/0003-2697(78)90342-1.
17. Gavrilov VB, Gavrilova AR, Khmara NF. Measurement of diene conjugates in blood plasma by UV absorption of heptane and isopropanol extracts. Laboratory business. 1988; 88(2): 60-63.
18. Shi X, Luo L, Wang J et al. Stroke subtype-dependent synapse elimination by reactive gliosis in mice. Nat Commun. 2021;12(1):6943. doi: 10.1038/s41467-021-27248-x.
19. Scimemi A. Astrocytes and the warning signs of intracerebral hemorrhagic stroke. Neural Plast. 2018; 2018:7301623. doi: 10.1155/2018/7301623.
20. Shen D, Wu W, Liu J et al. Ferroptosis in oligodendrocyte progenitor cells mediates white matter injury after hemorrhagic stroke. Cell Death Dis. 2022;13(3):259. doi: 10.1038/s41419-022-04712-0.
21. Zhang Z, Zhang A, Liu Y et al. New Mechanisms and Targets of Subarachnoid Hemorrhage: A Focus on Mitochondria. Curr Neuropharmacol. 2022; 20(7): 1278-1296. doi: 10.2174/1570159X19666211101103646.
22. Yavorska VO, Flomin YuV. Specific treatment of ischemic stroke: neuroprotection. International neurological journal. 2010; 6(36): 147-159.
23. Fricker M, Tolkovsky AM, Borutaite V et al. Neuronal Cell Death. Physiol Rev. 2018; 98(2):813-880. doi: 10.3390/ijms19103082.
24. Roh JH, Ko I-G, Kim S-E et al. Treadmill exercise ameliorates intracerebral hemorrhage-induced depression in rats. Journal of Exercise Rehabilitation. 2016; 12(4): 299-307. doi: 10.12965/jer.1632692.346.

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ORCID and contributionship:
Tetiana M. Oliinyk: 0000-0003-4685-1479[A-C]
Liudmyla M. Sokurenko: 0000-0002-6870-2290 [D,E]
Rostyslav F. Kaminsky: 0000-0001-5744-7581[C]
Viktoria E. Lavrynenko: 0000-0002-2570-1271[F]
Olena V. Kancer: 0000-0002-9567-8046[E]
Svetlana N. Chuhray: 0000-0001-7431-7375 [E]
Sergii T. Omelchuk: 0000-0003-3678-4241[F]
Anna V. Blagaia: 0000-0002-2451-9689 [E]

Conflict of interest:
The Authors declare no conflict of interest.

CORRESPONDING AUTHOR

Anna V. Blagaia
Bogomolets National Medical University
13 T. Shevchenko blvd., 01601 Kyiv, Ukraine
e-mail: profilactika@hotmail.com

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