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OPTIMIZATION OF PREDICTION OF MORPHOLOGICAL DISORDERS OF SKELETAL MUSCLES IN EXPERIMENTAL ACUTE ISCHEMIA-REPERFUSION ON THE BASIS OF COMBINED CHANGES IN LIPID PEROXIDATION AND ANTIOXIDANT PROTECTION BY NEURAL NETWORK CLUSTERING

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Abstract

The paper proposes a method for optimizing the prediction of the development of morphological disorders of skeletal muscle in experimental acute ischemia-reperfusion based on combined changes in lipid peroxidation and antioxidant protection. The approach is based on the use of neural network clustering.

The purpose of the study – to propose a method for optimizing the prediction of the severity of morphological disorders in experimental acute ischemia-reperfusion on the basis of combined changes in lipid peroxidation and antioxidant protection by neural network clustering.

The experimental model of ischemic-reperfusion lesion was represented by five groups of rats with reperfusion terms of 1 and 2 hours, 1 day, 7 and 14 days (18 animals each). Acute limb ischemia-reperfusion was simulated by applying SWAT rubber tourniquets on the hind right limb of animals for two hours under thiopental-sodium anesthesia. Histological examination was performed at the Department of Pathological Anatomy with a sectional course and forensic medicine of I. Horbachevsky Ternopil National Medical University of Ministry of Health of Ukraine according to generally accepted methods. Determination of lipid peroxidation and antioxidant protection in the blood serum of each experimental group of rats was performed by the Central Research Laboratory of I. Horbachevsky Ternopil National Medical University of Ministry of Health of Ukraine. For in-depth analysis and clustering of the indicators of the studied groups in order to optimize the prediction of the course of ischemic-reperfusion lesion, a neural network approach was used using the NeuroXL Classifier add-in for Microsoft Excel. The greatest prognostic value in relation to the severity of morphological disorders in the early reperfusion period according to the data of neural network clustering have combined changes in the level of TBA-active products and catalase.

Key words: acute ischemia-reperfusion; morphological changes; lipid peroxidation; neural network clustering.

Introduction. Effective use of information methods helps to significantly improve the quality of scientific medical research. This is especially important in the field of morphology due to the need to analyze a large amount of digital information. Numerous scientific studies are aimed at solving the problems of introducing innovative information technologies [1-3], but the problem of their use in experimental medicine to optimize the prediction of pathological processes for effective application in practice remains incomplete. Especially relevant is the prediction of morphological tissue disorders in acute ischemia-reperfusion, which is one of the most common types of injuries in both military and civilian medicine [4-10].

The purpose of the study. To propose a method for optimizing the prediction of the severity of morphological disorders in experimental acute ischemia-reperfusion on the basis of combined changes in lipid peroxidation and antioxidant protection by neural network clustering.

Materials and methods

The work was performed on material obtained from adult white male rats weighing 200-240 g. The animals were kept on a standard diet and in standard identical vivarium conditions with free access to water. Animal care, nutrition, research and euthanasia met national and international conditions for the humane treatment of animals. According to the purpose and objectives of the study, the experimental animals were divided into six experimental groups. The experimental model of the early ischemic-reperfusion period is presented by groups of animals with reperfusion terms of 1 hour, 2 hours and 1 day, and the model of the late ischemic-reperfusion period – groups of experimental animals with reperfusion periods of 7 and 14 days (18 animals in each group). For comparative analysis, an intact group was selected, which included 15 animals.

The experiment was conducted in the morning (from 9-00 to 12-00) in a specially designated room of the Central Research Laboratory of I. Horbachevsky Ternopil National Medical University of Ministry of Health of Ukraine at a temperature of 18-20˚C, relative humidity of 40-60% and illumination of 250 lux and consisted of modeling limb ischemia by applying for two hours rubber harnesses SWAT (Stretch – Wrap – And – Tuck) 5 mm wide on the hind right limb of animals, at the level of the inguinal fold under thiopental-sodium anesthesia (40 mg x kg^{-1} body weight). The criteria for the correct application of the tourniquet were: no arterial pulse on the rheovasogram, no swelling of the limb, decreased temperature and pale color of the skin of the limb, which indicated compressive occlusion of the femoral artery. Reperfusion was modeled by restoring blood circulation in previously ischemic limbs due to removal of the hemostatic tourniquet two hours after its application. Animals were observed throughout the experiment (up to 14 days for the latter group). These terms allowed us to analyze the dynamics of vascular and skeletal muscle remodeling, the development of biochemical disorders in the blood and damaged skeletal muscles, submicroscopic changes in the hemomicrocirculatory bed and muscle tissue in acute ischemia and subsequent reperfusion.

Euthanasia of animals was performed by decapitation under thiopental-sodium anesthesia. The drug was administered intraperitoneally at a rate of 500 mg x kg^{-1} body weight, followed by sampling of biological material.

Histological examination was conducted at the Department of Pathological Anatomy with Sectional Course and Forensic Medicine of I. Horbachevsky Ternopil National Medical University of Ministry of Health of Ukraine according to generally accepted methods [11]. Staining of histological specimens was also performed using special histological techniques:

resorcinol-fuchsin solution according to Weigert, van Gieson and azan according to the Heidenhain's method [11].

Morphometric study. The specimens were studied using a Bresser Trino Researcher 40x-100x microscope and a MICROmedSEO SCAN laboratory microscope with a camera and a polarizing nozzle. The most demonstrative histological specimens were photographed using a Digital Camera for Microscope Science Lab DCM 820 (Resolution 8.0 Mp). Morphometric changes were studied with magnification of the microscope lens – 4, 10, 20 and 40, and the eyepiece – 10.

The morphometric examination of the muscles determined the following average indicators: the diameter of the muscle fiber, the area of the nuclei of the muscle fiber, the cross-sectional area of the muscle fiber, which was calculated according to the formula:

$$S = \frac{3,14 \times d^2}{4},$$

$$(1)$$

Also determined the ratio of these indicators in the experimental groups with ischemic-reperfusion lesions to the corresponding indicators of the control group. These parameters were calculated using the software for image processing and analysis "SEO Image Lab" company "Sumy Electron Optics".

The state of lipid peroxidation (LPO) in the body can be objectively assessed by determining diene (DC) and triene (TC) conjugates and TBA-active products (TBA-AP). And the state of antioxidant protection (AOP) can be judged from the activity of superoxide dismutase (SOD) and catalase. The study was conducted in the Central Research Laboratory of I. Horbachevsky Ternopil National Medical University of Ministry of Health of Ukraine. Determination of LPO and AOP was performed in blood serum of experimental rats.

The content of DC and TC was determined by the method of V. B. Gavrilov and M. I. Myshkorudna, the essence of which is that extracted with heptane-isopropyl mixture hydroperoxides give maximum light wave absorption at a length of 232 nm [12]. The result was calculated in conventional units per 1 ml of serum.

Determination of the concentration of TBA-AP was carried out according to the following method: at high temperature in an acidic environment malonic dialdehyde reacts with thiobarbituric acid, forming a colored complex with a maximum absorption at 532 nm. The concentration of TBA-AP is proportional to the intensity of the resulting color and is determined using a photometer [13].

Determination of SOD activity was performed using the method proposed by S. Chevari. [14]. The principle of the method is based on the ability of the enzyme to inhibit the reduction of nitrotetrazolium blue.

Determination of catalase activity is based on the ability of hydrogen peroxide to form a stable colored complex with ammonium molybdate, the intensity of which is inversely proportional to the activity of catalase in the studied substrate [15].

Statistical processing of the material was performed using the software package "Microsoft Excel" (2010). Verification of indicators for normal distribution was carried out by the Kolmogorov-Smirnov test. The statistical significance of the difference between the arithmetic means was assessed by the nonparametric criterion (U-test) Mann-Whitney.

For a more in-depth analysis and clustering of the indicators of the studied groups in order to optimize the prediction of the course of ischemic-reperfusion lesion, a neural network approach was used using the NeuroXL Classifier add-in for Microsoft Excel. NeuroXL Classifier (developed by AnalyzerXL) implements self-organizing neural networks that perform categorization by studying trends and relationships within data. The key advantages of using NeuroXL Classifier are ease of use; the optional need for in-depth knowledge in the field of neural networks; integration with Microsoft Excel; providing valid neural network technology for high-precision classification; identification of relationships and trends that cannot be determined by traditional methods [16, 17].

Results

Histological, morphometric, and electron microscopic examination of skeletal muscle micropreparations of ischemic rat limbs in all study groups was performed.

Histological examinations revealed significant structural changes in the system of hemomicrocirculatory bed in the early reperfusion period, which were manifested by spasm of the afferent and hyperemia of the capacitive microvessels, intravascular stasis, changes of microarchitectonics of microvessels, significant depletion of vascular pattern, edema and violation of the integrity of the basal membrane of microvessels, diapedetic hemorrhages (Fig. 1).
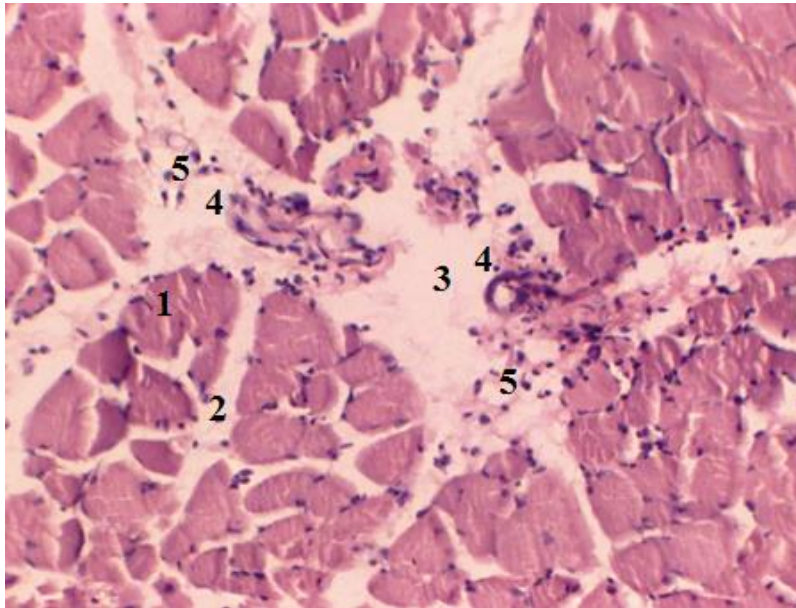
Figure 1. Cross section of the quadriceps muscle of the rat. Reperfusion after 1 day. Hematoxylin and eosin staining, magnification x100. Symbols: 1 – edema of muscle fiber; 2 – edema of endomysium with the release of nuclei; 3 – edema of paravasal space; 4 – vessels of haemomicrocirculatory bed; 5 – areas of leukocyte infiltration

These changes reached the greatest severity after 1 day of reperfusion and gradually were decreasing during the late reperfusion period.

In the hemomicrocirculatory bed submicroscopically were revealed edema of endothelial cells with their prolapse into the lumen of hemocapillaries, ultrastructural changes in the intracellular structures of endotheliocytes and their nuclei, uneven thickening of the basal membrane and changes in its the electron-optical density, protein and erythrocyte extravasates, as well as platelets in the lumen of microvessels on the surface of damaged endothelial cells, indicating the development of microthrombosis in the hemocapillaries.

Ultrastructural abnormalities in the vessels of the hemomicrocirculatory bed and muscle fibers of the ischemic skeletal muscles of the hind limbs of rats were observed as early as 1 hour after removal of the tourniquet, and reached maximum severity in rats after 1 day of reperfusion, which confirms the development of ischemic reperfusion syndrome.

In the late reperfusion period after 7 and 14 days there was a reverse development of pathological changes. During this period, the ultrastructure of the vessels of the hemomicrocirculatory bed and skeletal muscles was largely restored (Fig. 2).
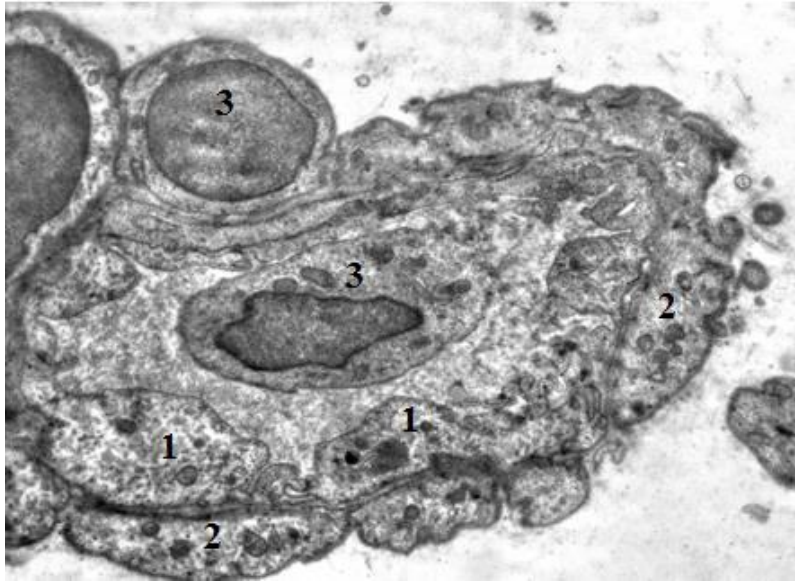
Figure 2. Ultrastructure of the skeletal muscle arterioles of the thigh. Reperfusion after 7 days, magnification x 12000. Symbols: 1 – swollen endotheliocytes with a slight prolapse into the lumen of the arterioles; 2 – slight edema of the leiomyocytes of arterioles; 3 – leukocyte and lymphocyte

At the end of the experiment submicroscopically hemocapillaries were the ordinary shape, their walls were without visible damages, there was a partially altered shape of the endothelial cells nuclei with intussusception of karyolemma, the basal membrane of microvessels was of the uniform thickness and slightly increased electron density, in the perivasal space often single lymphocytes were found. The ultrastructure of the myosymplast was also largely restored, but there was a slight edema of the sarcoplasm of muscle fibers with preserving the structure of intracellular organelles, and a moderate increase in the number of pinocytic vesicles and caveolae, indicating that after 14 days of reperfusion complete recovery of the ultrastructure of the vessels of the hemomicrocirculatory bed and myosimplast still does not occur.

Pathological changes in the muscle tissue of the extremities occurred in the early period of post-ischemic lesions of skeletal muscles and increased by the end of the first day. Histological examination of skeletal muscles revealed disorganization of the structural components of myosymplasts, reduction of striation, edema, defibering and rupture of muscle fibers (Fig. 3).
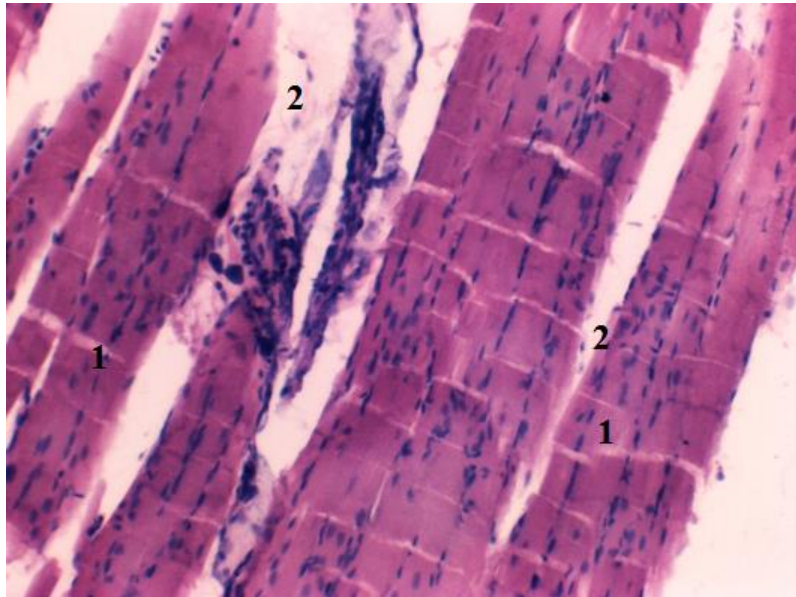
Figure 3. Longitudinal section of the quadriceps muscle of the rat. Reperfusion after 1 day. Staining with hematoxylin and eosin, magnification ×300. Symbols: 1 – fragmentation and rupture of muscle fibers; 2 – edema of endomysium and the release of myosymplast nuclei into endomysias

With polarization microscopy in this period, damage of myosymplasts with the convergence of anisotropic disks, the contracture of myofibrils of third and fourth stages, ruptures and the myocytolysis of muscular fibres were observed (Fig. 4).

Electron microscopy has shown significant edema of muscular fibers and endomysium, hyperthrophy ans edema of mitochondria, their uneven placement with accumulation under the sarcolemma, partial defragmentation and destruction of mitochondrial cristae, autolysis of some mitochondria, phagosomal activation, myofibrils fragmentation, changes in the shape and electron density of the myosymplast nuclei, signs of dilation of the tubules of the sarcoplasmic reticulum, violation of visualization and parallel orientation of Z-disks, which indicates violation of the spatial organization of sarcomere proteins (Fig. 5).

The experiment has determined the dynamics of changes in such morphometric parameters as the average diameter and area of muscle fibers, the area of their nuclei, as well as the nuclear-cytoplasmic ratio in the skeletal muscles of middle third of the thigh of rats in different periods after removal of the tourniquet. Also counting the average number of nuclei in cross sections skeletal muscles was performed (Table 1).
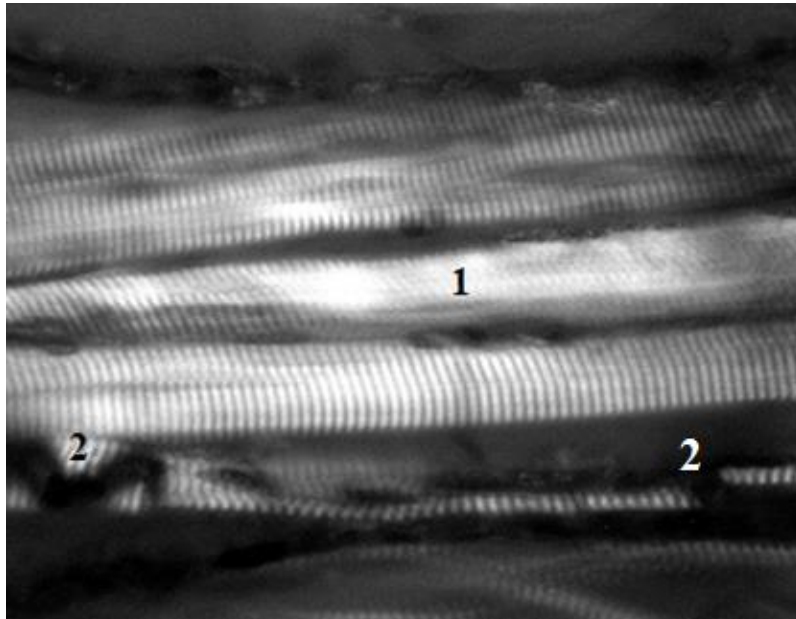
Figure 4. Muscle fibers of femoral area. Reperfusion after 1 day. Polarization microscopy, magnification × 300. Symbols: 1 – area of significant muscle fiber anisotropy; 2 – muscle fiber crack

Figure 5. Ultrastructure of the myosimplast of the skeletal muscle of the thigh area. Reperfusion after 1 day, magnification × 15000. Symbols: 1 – swollen hypertrophied polygonal shape mitochondria; 2 – mitochondrial lysis; 3 – pyknotic osmophilic nucleus of the myosimplast; 4 – caveola

Table 1.

Changes in average diameter (d), area (s_s) and area of nuclei (s_n) of muscle fibers, as well as the nuclear-cytoplasmic ratio (s_n/s_s) of the quadriceps femoris muscle of rats at different times after removal of the tourniquet (M ± m)
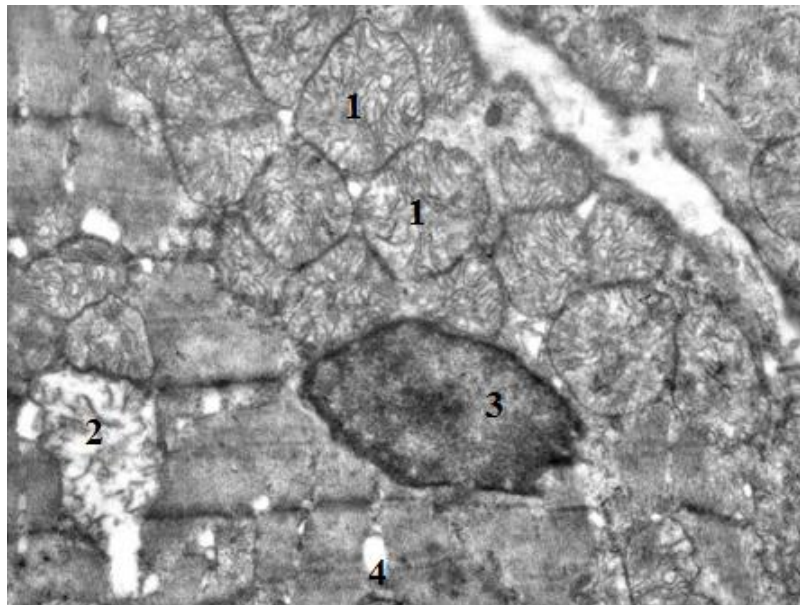
Indicator	Control	Reperfusion 1 hour	Reperfusion 2 hours	Reperfusion 1 day	Reperfusion 7 days	Reperfusion 14 days
d, mcm	40.37±0.56	40.77±0.79	41.67±0.35*	41.73±0.41*	41.58 ±0.43	40.81±0.44**
s_n, mcm²	63.96 ±0.08	63.98±0.08	63.99±0.12	64.03±0.08	64.01±0.08	63.78±0.10
s_s, mcm²	1282.9±34.6	1312.3±52.4	1364.7±22.7*	1369.2±26.6*	1359.2±28.2	1309.8±28.0**
s_n/s_s	0.050±0.001	0.050±0.002	0.047±0.001*	0.047±0.001*	0.047±0.001	0.049±0.001

Note 1. * – p<0,05 in comparison with control group.
Note 2. ** – p<0,05 in comparison with control group.

The average diameter of muscle fibers after 1 day of reperfusion was higher by 3,37 % (p<0,05) than the indicator of the control group. There was a statistically significant decrease in the nuclear-cytoplasmic ratio by 6,0 % in groups of animals with reperfusion after 2 hours and 1 day in comparison with the corresponding indicator of the control group (0,050 ± 0.001, p<0,05), which may indicate a decrease in nuclear activity as a result of ischemic injury. On the cross sections of the skeletal muskules a decrease in the average number of nuclei (2,67±0,09) per one muscle fiber by 17,34 % (p<0,00005) after 1 day of reperfusion was observed.

In all five study groups of white rats a comparative analysis of the average content of products of LPO and AOP in the blood serum was performed (Table 2).

Table 2.

Indicators LPO and AOP in the blood serum of rats in different terms of ischemia-reperfusion (M ± m)

Reperfusion term	DC, c.u./ml	TC, c.u./ml	TBA-AP, mmol/l	SOD, c.u./l	Catalase, cat/l
Control group	0.42±0.11	0.28±0.04	2.98±0.27	54.84±3,92	32.44±1.83
1 hour	0.62±0.13*	1.33±0.37	3.21±0.23*	54.52±4.61	32.72±1.86
2 hours	0.53±0.06	2.54±0.23*	4.34±0.40*/**	64.72±4.54*	51.60±1.08*/***
1 day	0.70±0.11	1.36±0.12*/**	4.93±0.22*	72.08±1.70*	50.64±1.68*
7 days	0.53±0.16	0.35±0.10***	3.93±0.30*/**	59.96±2.92*/**	40.22±1.04*/**
14 days	0.49±0.10	0.51±0.15	3.41±0.24	54.92±2.79**	33.46±2.05

Note 1. * – p<0,05 in comparison with control group.
Note 2. ** – p<0,05 in comparison with control group.
Note 3. *** – p<0,005 in comparison with control group.

The dynamics of changes in the LPO indicates a significant increasing the concentration of free oxygen radicals in the blood serum of experimental animals. The maximum growth in the content of triene conjugates after 2 hours of reperfusion in 8,2 times (p<0,05), the increase in content of diene conjugates after 1 hour of reperfusion by 47,17% (p<0,05) and after 1 day by 66,04% (p>0,05), as well as an increase in the concentration in the serum of rats TBA-AP by 65,79% (p<0,05) after 1 day of reperfusion confirms the activation LPO in experimental animals in the early reperfusion period.

In the study of AOP in the blood serum there was statistically significant increasing in the activity of SOD (by 31,44%, p<0,05) and catalase (by 59,06%, p<0,05) in the groups of animals of the early reperfusion period with the reperfusion after 2 hours and 1 day, with decreasing activity of these enzymes in the late reperfusion period after 7 days, and returning to the level of control values after 14 days after removal of tourniquet.

In order to predict the severity of development of ischemic-reperfusion changes of soft tissues of the limbs in the early reperfusion period, a neural network clustering on the base of the LPO and AOP indicators and morphological changes was applied. The following indicators were taken to analyze the combined changes in their values: DC - diene conjugates (1), TC – triene conjugates (2), TBA-AP – TBA-active products (3), SOD – superoxide dismutase (4), CAT – catalase (5). The clustering was also subjected to the indicator of severity of morphological changes in different periods after removal of the tourniquet S (6), which was defined for each experimental animal as "4" in the case of taking material for study after 1 day after applying the tourniquet, "3" – after 2 hours, "2" – after 1 hour and "1" – without imposing a tourniquet (control). For the neural network clustering algorithm, the parameters proposed by the program and the number of clusters equal to three were selected.

Figure 6 (both fragments) shows the results of the program clustering of indicators of the study of animals of different groups. The first cluster includes 45,83% of experimental animals, the second cluster – 25,00%, and the third cluster – 29,17% of animals.

The lowest values of the morphological changes in the hind limbs of rats with the development of ischemic-reperfusion tissue damage (S) was found in the first cluster, and the highest – in the third cluster. The cluster portrait can be used to determine that the third cluster has the highest levels of diene conjugates (DC, 1), TBA-active products (TBA, 3), superoxide dismutase (SOD, 4) and catalase (CAT, 5).
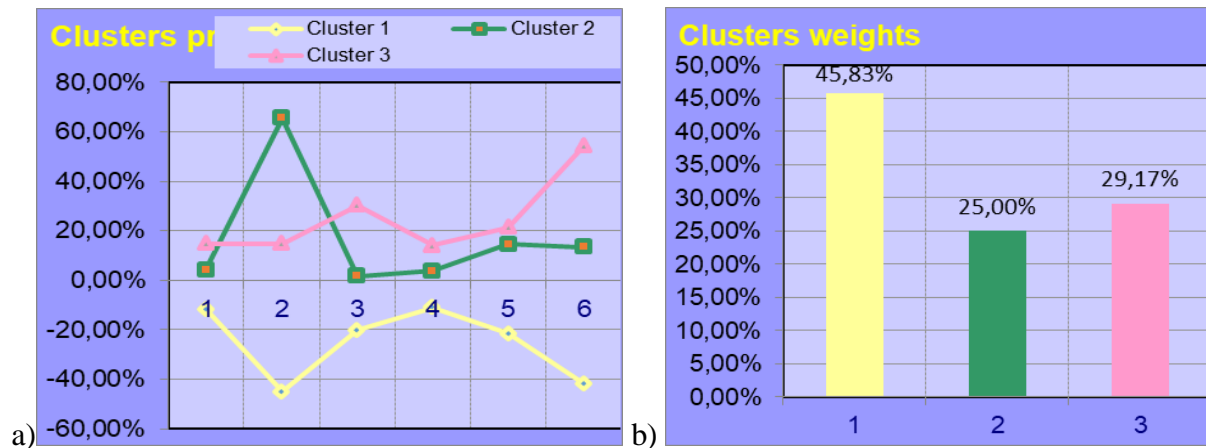
Figure 6. The results of clustering of the LPO and AOP in the blood serum of experimental animals in different periods of ischemia-reperfusion:

a) cluster portrait – the value of parameters, including indicators of the LPO, AOP and the severity of morphological changes in different periods after removal of the tourniquet;

b) cluster shares – the percentage of experimental animals that fell into a particular cluster.

Conclusions

To optimize the prediction of morphological ischemic disorders in experimental acute ischemia-reperfusion, a method of analyzing the results of experimental studies based on the average values of morphometric parameters, indicators of lipid peroxidation and antioxidant protection, with using neural network clustering is proposed.

Histological examination of skeletal muscles of the hind limbs reveales, that the most significant morphological changes increase by the end of the first day of reperfusion and are manifested by disorganization of structural components of myosimplasts, reduction of striation, edema, fragmentation and ruptures of muscle fibers, nuclei migration outside the sarcolemma and leukocyte infiltration in the endomysium.

Structural disorders of muscular tissue in the early reperfusion period were unidirectional and progressive, as evidenced by morphometry. The average cross-sectional area of muscle fibers after 1 day of reperfusion increased by 6,72% (p<0,05) compared with the control group. There was a decrease in the average nuclear-cytoplasmic ratio (by 6,0% (p<0,05)) and the number of nuclei per one muscle fiber (by 17,34% (p<0,00005)).

Submicroscopic reorganization of the hemomicrocirculatory bed with the development of ischemia-reperfusion is manifested by destructively altered precapillary arterioles and hemocapillaries, parietal microthrombosis, protein and erythrocyte extravasates. Structural changes of myosimplast are characterized by severe edema, damage to their organelles (especially mitochondria), activation of phagosomes, pyknosis of nuclei, fragmentation of

myofibrils. At the end of the late reperfusion period, the histological and submicroscopic changes in the hemomicrocirculatory bed vessels and skeletal muscles decreased sharply, and their ultrastructure was significantly restored.

Morphological damage to the vascular bed and muscle tissue of the hind limbs of rats is accompanied by activation of LPO and AOP in the early reperfusion period. An increase in serum SOD and catalase activity was detected in the early reperfusion period after 2 hours and 1 day of reperfusion, followed by a decrease in the activity of these enzymes in the late reperfusion period after 7 days and return to control values 14 days after femoral artery decompression.

Analysis of cluster portraits by using neural network clustering based on the indicators LPO and AOP of sera of white rats in different periods after removal of the tourniquet revealed that in predicting the development of morphological disorders in ischemia-reperfusion, the most significant are combined changes in diene conjugates, TBA-active products, superoxide dismutase and catalase, among which the most important are changes the TBA-active products and catalase. The growth of these indicators may indicate the greatest severity of skeletal muscle damage in ischemia-reperfusion injury.

It seems promising to introduce the use of correlation analysis when working with a large amount of digital information to predict the development of pathological processes for the purpose of their further application in practice.

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