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Morphofunctional Characteristic of Edema-Swelling of the Cerebral Cortex of White Rats After Severe Traumatic Brain Injury Without the Use of L-Lysine Escinate and In the Course of Its Use

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AIM OF STUDY The study is devoted to a morphometric assessment of the manifestations of edema-swelling of the somatosensory cortex (SSC) of the brain of white rats after severe traumatic brain injury (TBI) without using L-lysine escinate and when using it as a therapeutic effect.

MATERIAL AND METHODS We stained sections with hematoxylin-eosin and performed morphometric methods. On thin (4 μm) serial frontal sections of SSC, neurons and microvessels in the control (intact animals, n=5) were examined in 1 (n= 5), 3 (n=5), 5 (n=5), 7 (n=5) and 14 (n=5) days after injury without treatment (n=25, comparison group) and with treatment (n=25, main group). In color raster images (lens x100), using the plug-in filter "Find Maxima", maximum brightness areas (MBA) were determined , which were then analyzed using the "Analyze Particles" program from ImageJ 1.52 s. MBA corresponded to SSC sites with a high degree of hydration of nerve tissue — edema-swelling. Statistical hypotheses were tested using nonparametric criteria.

RESULTS AND DISCUSSION In control animals, a low degree of hydration of SSC tissue was noted (relative area 3–8%). In the comparison group, 1 and 3 days after STBI, foci of edema-swelling covered up to 30% of SSC, in 5 days — up to 15%, in 7 days — up to 20%, in 15 — up to 18%. Significant heteromorphism and heterogeneity of changes in the neuropil around neurons and blood vessels was noted. In the dynamics of the post-traumatic period, the proportion of large foci of edema-swelling (intra- and perineuronal, perivascular) decreased. In the main group, one day after STBI, there was a statistically significantly smaller number of foci of edema-swelling and their total relative area. The values range of these variables significantly decreased. L-lysine escinat affected the water balance most effectively in the acute post-traumatic period (day 1 and 3). The drug "smoothed out" the manifestation peaks (number, focal area) of edema-swelling: the values of the studied morphometric indicators were statistically significantly different. Consequently, morphometric signs of hydropic dystrophy after STBI were detected in both studied groups during the 15 days of observation.

CONCLUSION The degree of SSC nervous tissue hydration increased after STBI. L-lysine escinate statistically significantly reduced manifestations of hydropic dystrophy. The drug significantly affected the degree of hydration of neural tissue observed in the early post-traumatic period.

Keywords: traumatic brain injury; neocortex; edema-swelling; morphometry; Wistar rats, L-lysine escinate

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Conflict of interest Authors declare lack of the conflicts of interests

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AMB – areas of maximum brightness
CE – cerebral edema
ICH – intracranial hypertension
ICP – intracranial pressure
SSC – somatosensory cortex
STBI – severe traumatic brain injury

INTRODUCTION

Severe traumatic brain injury (STBI) is one of the most serious types of injury in terms of both fatality and long-term consequences for survivors. Of the total number of cases of traumatic brain injury in Russia, the proportion of victims with TBI is relatively small and amounts to about 3.5–7% [1–3]. However, the mortality rate for this type of injury ranges from 41 to 85%, and the disability rate of people of working age who have undergone STBI remains extremely high [2, 4].

The most important reason for the development of unfavorable outcomes in patients with TBI is progressive intracranial hypertension (ICH), which occurs due to the development of post-traumatic cerebral edema (CE), which is one of the morphological forms of acute damage to the central nervous system, manifested by an increase in the volume of brain tissue [5]. It, in turn, leads to the development of acute dislocation syndrome, and then to disruption of vital functions and possible death [6–8].

Cerebral edema in TBI is characterized by a stage-by-stage development, the greatest pathogenetic significance in which the vascular component is attached [9, 10]. In the acute period of injury, vasogenic edema prevails, which develops due to an increase in the permeability of the vascular wall due to a violation of the function of the blood-brain barrier. At the same time, fluid in the brain tissue accumulates unevenly: in the white matter, predominantly interstitially, in the gray matter - intracellularly, mainly in the neuroglia. Then, in connection with the transfer of fluid from the outside to the intracellular space, a cytotoxic CE develops [11, 7, 12].

Currently, there are both surgical and conservative methods for correcting ICH, the purpose of which is to reduce the intracranial volume and the volume of cerebrospinal fluid [13]. At the same time, the search for a drug that can act directly on CE, providing protection of neurons and stabilization of the microcirculatory vascular bed of the brain, remains relevant [9].

In this regard, L-lysine escinat is noteworthy. This drug helps to normalize vascular permeability, thereby providing antiexudative and anti-inflammatory effects [14]. There is clinical evidence of the effectiveness of L-lysine escinat in reducing edema-swelling of the brain, which in turn reduces intracranial pressure (ICP) and eliminates the dislocation of brain structures [9, 8].

Experimental modeling of TBI allows for a quantitative morphometric study of the dynamics of manifestations of edema-swelling of the nervous tissue, which is of particular importance for assessing the effectiveness of treatment methods [15]. In this regard, the effect of the use of L-lysine escinat under conditions of experimental traumatic brain injury using histological and morphometric methods has not been studied. There is no data confirming the effectiveness of the drug in the dynamics of the postischemic period.

MATERIAL AND METHODS

The study was carried out on the basis of the Federal State Budgetary Educational Institution of Higher Education "Omsk State Medical University", approved by the ethical committee of the university (protocol No. 107 of October 2, 2018). The experimental animals were 55 outbred mature male Wistar rats weighing 270–350 g (Federal Research Center Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences). The animals were kept in standard laboratory conditions with timely intake of food and drinking water. The experiment was carried out in accordance with the "Rules for work with experimental animals" (Appendix to the Order of the Ministry of Health of the USSR dated Aug 12, 1977 No. 755) and the recommendations of the International Committee for the Science of Laboratory Animals, supported by WHO, Directive of the European Parliament No. 2010 / 63 / EU dated Sept. 22, 2010 "On the protection of animals used for scientific purposes."

For the histological study of the somatosensory cortex (SSC) of the brain in the dynamics of the post-traumatic period, STBI was simulated against the background of moderate sedation. We used Zoletil 100 solution (at a dosage of 5-7 units).

During the 12 hours prior to TBI, the animals did not receive food, but they were not restricted in water. Five minutes after the introduction of Zoletil 100 and the onset of relaxation of the skeletal muscles, the rat was fixed on its stomach on a wooden table by the front and hind legs. A polyester pad measuring 5.0x5.0 cm and a height of 2 cm was placed under the lower jaw in order to exclude a fracture of the lower jaw. The fixed animal was placed on a rail rack platform 1.5 meters high. A load weighing 200-250 grams (calculated according to the weight of animals 270-320 g) was placed over the head of the rat at a height of 50 cm so that the blow fell on the parieto-occipital region.

After STBI, all animals (n = 50) were randomly (number generator) divided into two groups. In the main group (n = 25), immediately after injury and then once a day, a solution of systemic angioprotector (L-lysine escinate) in a dilution of 0.1 ml of the drug in 0.2 ml 0 was injected with an insulin syringe into the lateral vein of the rat's tail, 9% NaCl solution (dosage was calculated in accordance with the average weight of the rat, 295 ± 25 g). The animals of the comparison group (n = 25) did not receive the drug. Intact rats (n = 5) served as control.

The withdrawal of the animals of the main group and the comparison group from the experiment was carried out 1, 3, 5, 7 and 14 days after injury by bloodletting followed by perfusion of the fixing solution. Manipulations were performed under general anesthesia (Zoletil 100). After thoracotomy, 100–125 ml of a solution of 0.9% NaCl and Fragmin (5000 units) was injected through a puncture into the left ventricle, which flowed out through the incision of the right atrium. Then the wound in the atrium was clamped, and 30 ml of 4% paraformaldehyde solution in phosphate buffer (pH 7.2-7.4) was injected into the left ventricle. After that, decapitation and extraction of the brain from the cranial cavity were performed. The extracted brain was stored at a temperature of 3-5 ° C in a similar fixative. Then the material was embedded in homogenized paraffin (HISTOMIX®) using an STP 120 automatic carousel for histological guiding. Serial frontal sections with a thickness of 4 μm were performed at the level of SSC (-) 2.40 - (-) 3.36 mm from Bregma [16]. The sliding microtome HM 450 (Thermo) with electronic control of the section thickness

was used. The preparations were prepared from each 10th serial section (5 - for a case, 25 - for a period): they were stained according to Nissl, as well as with hematoxylin and eosin.

The preparations were photographed using a Leica DM 1000 microscope (x100 objective, GXCAM-DM800 Unique Wrap-Around 8MP AUTOFOCUS USB camera, pixel size 1.4x1.4 μm), the image was saved in files with the *tiff* suffix (2592x1944 pixels). To achieve maximum contrast and clarity of the image, the correction was performed using the Camera Raw filter (contrast, white balance, clarity) in Photoshop CC. Further morphometric research was carried out using the ImageJ 1.52s software. Used color images presented from each section in the form of stacks (field of view: 50x50 μm, or 2500 μm^2). To identify areas of edema-swelling (maximum brightness of pixels - Maxima) of the nervous tissue, we used the plug-in filter "Find Maxima" M. Schmid of the ImageJ 1.52s program (https://imagej.nih.gov/ij/docs/menus/process. html # find-maxima). The image masks obtained as a result of the operation of this filter were used to determine ("Analyze Particles") the relative area (S,%), the numerical density (N, per 2500 μm^2), and the area of one zone of maximum brightness (S_{max}, μm^2). The non-uniformity of the distribution of the areas of maximum brightness (AMB) was evaluated using the "Segmented Particles" watershed algorithm applied to the values of the image brightness.

The formation of samples (randomization) of visual fields (n = 100, for each period) for each experimental group was carried out from the obtained data arrays using a random number generator in the Statistica 8.0 program. This made it possible to avoid systematic errors when testing statistical hypotheses. The distribution of the variation series was assessed using the Lilliefors and Shapiro-Wilk tests (Statistica 8.0) and quantile graphs (R environment). Statistical hypotheses were tested using the nonparametric Mann-Whitney, ANOVA (one-way analysis of variance), and Kruskal-Wallis tests (StatSoft Statistica 8.0). Quantitative data in the work are presented as a median (50% quartile is a measure of the central trend) and 25−75% quartiles, ranges with outliers, without outliers are measures of the spread of the studied values. The median is an outlier-independent robust characteristic and is most suitable for this study [17]. During the statistical analysis, the null hypothesis was rejected at $p \leq 0.05$.

RESEARCH RESULTS

Normally (control group), on sections of SSC stained with hematoxylin-eosin, the neuropil was represented by a homogeneous substrate without structural manifestations of edema-swelling of its components (dendrites, synapses, astrocyte processes), typical normochromic neurons without signs of hydropic dystrophy prevailed (Fig. 1). The AMB of the image pixels (maximum hydration and free water) corresponded mainly to the vascular lumens (Fig. 1 A, C) and were rarely found in the perivascular spaces (Fig. 1 B).

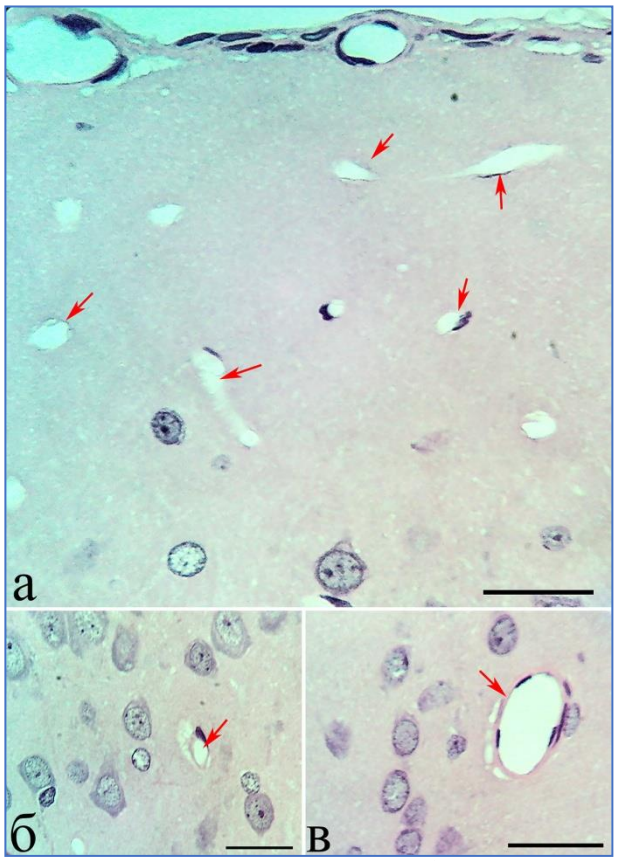

Fig. 1. Layers I (A) and II (B, C) Somatosensory cortex of a white rat brain: maximum brightness areas correspond to lumen (arrows), signs of edema-swelling are absent. Hematoxylin-eosin stain; x40 magn; scale — 25 microns

All this indicated a low degree of hydration (the ability of water to chemically associate with hydrophilic substances - swelling) of the SSC nerve tissue and an almost complete absence of free water (a mechanical form of communication with surrounding structures - edema) is normal. On raster images of preparations of nervous tissue fixed in formalin and stained with hematoxylin and eosin, the degree of hydration of its components was determined by the brightness of pixels (from 0 to 255). The maximum value (230−245) was reached by areas containing free water; in other areas, the degree of hydration (chemical bond) of the neuropil in terms of pixel brightness changed from 190 to 230.

After STBI, typical structural signs of edema-swelling of neurons, dendrites, neuropil, edema of pericellular and perivascular processes of astrocytes appeared (Fig. 2). This indicated changes in the strength of water binding by structures of nervous tissue. The degree of hydration of neuron molecules and, especially, neuropil, as well as the amount of free water increased.

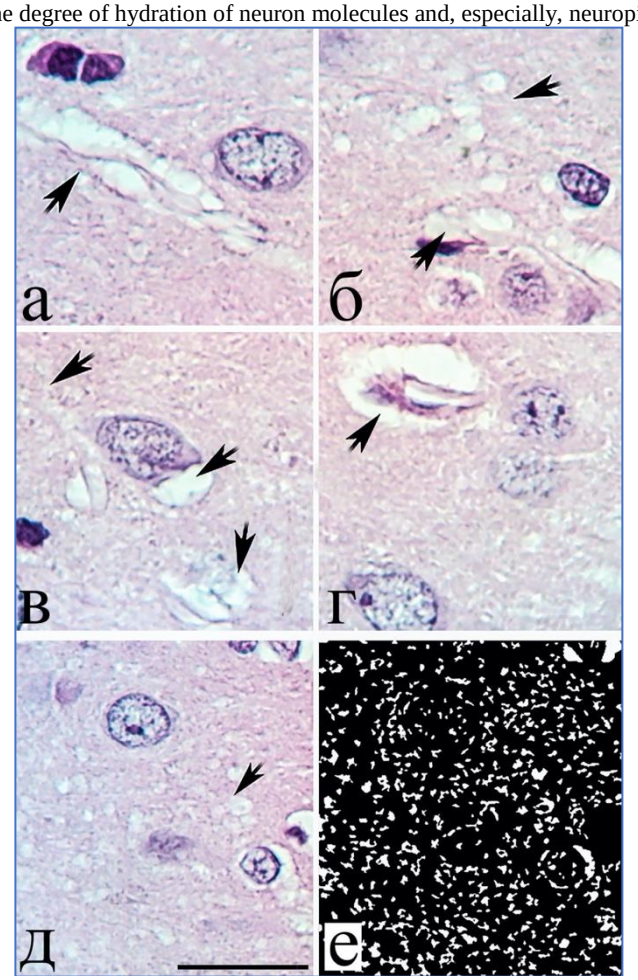

Fig. 2. Somatosensory cerebral cortex of a white rat after traumatic brain injury, layer III, day 1: A–E — randomly selected field of view and various manifestations of localized edema-swelling areas of the nerve tissue, F — binary field picture "D" (white — areas of maximum brightness — edema-swelling, black — hematoxylin-eosin-stained structures with a low degree of hydration). Hematoxylin-eosin stain; x100 lens; scale — 25 microns

Evaluation of changes in the content of bound and free water was carried out by the number of bright pixels in color images, identified using the Find Maxima plug-in filter in the ImageJ 1.52S program (Fig. 3).

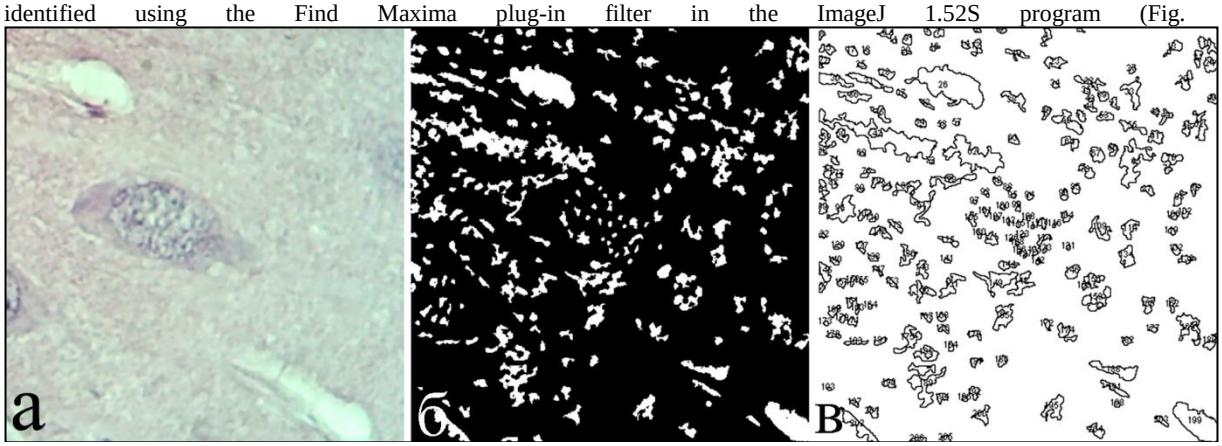

Fig. 3. The main stages of the technology for morphometric research of the zone of maximum image brightness : A — standard randomly selected area of interest (50x50 microns); B, C — the results of applying the filter "Find Maxima" and the plug-in "Analyze Particles". Areas of brightness have a complex shape, numbered

In control animals, the amount of AMB in the field of (2500 μm2) SSC varied within 20–100 (Min-Max), that is, 8,000–40,000 per 1 mm^2 of SSC slice (Fig. 4 A). In this case, the relative area of all AMB did not exceed 8% (Fig. 4B), and the area of one AMB

varied from 1 to 4.5 μm2 (Fig. 4C). Large AMB corresponded to capillary lumens (Fig. 1 A, C). We did not take into account arterioles and venules.

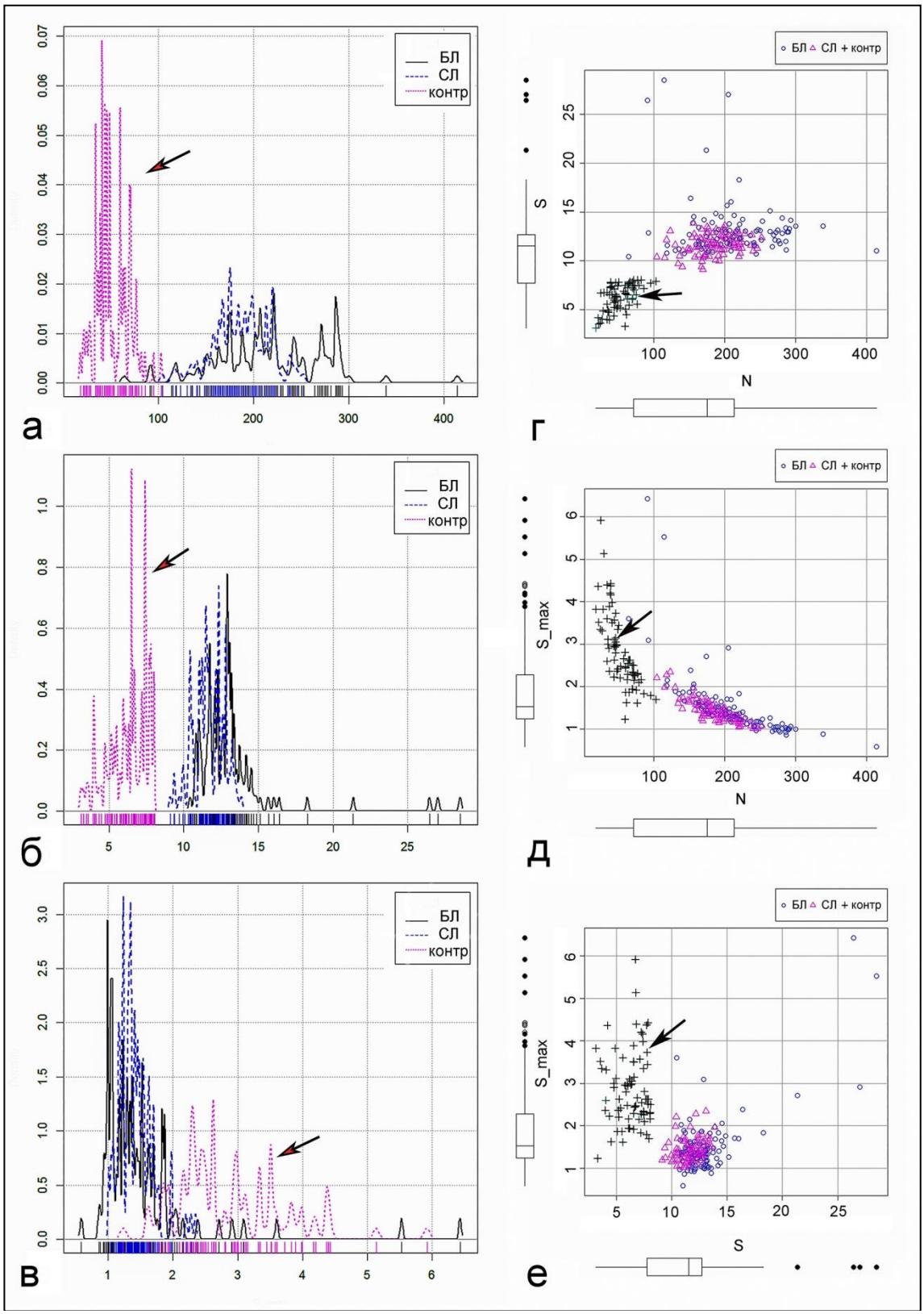

Fig. 4. Histograms of the frequency distribution of the visual fields in the control, in the main group and the comparison group in a day after the STBI by the number of maximum brightness areas (A), the relative area of all areas of maximum brightness (B), the area of one zone of maximum brightness (C) and the spatial distribution of these variables relative to each other (D–F). N is the number of areas of maximum brightness in the field of view (by 2500 μm^2), S is the relative area of the areas of maximum brightness (%), S_{max} is the area of one zone of maximum brightness (μm^2). Boundary "box and whisker plots" — values of the independent variables: median (50%), 25%–75% th quartiles, non-outlier range and outliers

In the acute post-traumatic period (1st and 3rd days) after STBI, significant manifestations of edema-swelling were noted (Fig. 2). As a result, the structure of the variational series of the studied variables changed: the ranges with outliers and the number of extreme values increased. This was especially evident for the relative area of all AMB and the area of one AMB (Fig. 4 B, C; 5).

On the dot plots of the spatial distribution of the studied variables relative to each other, it can be seen that the accumulations of values in the control were located autonomously from that one day after ischemia. The spatial distributions of the variables in the untreated and treated groups differed significantly in outliers (Fig. 4 D – F). In the main group, the distribution of the values of the variables was more compact and close to the control (Fig. 4 D – F). In addition, the distribution of the variation series one day after injury in the comparison group significantly differed from the normal according to the test data ($p < 0.01$), which was confirmed when constructing quantile graphs. Consequently, the range without outliers (Max-Min), with outliers and extreme (extreme) points of the variation series were important indicators in assessing the effect of the drug on the morphometric structural manifestations of edema-swelling after STBI. That is, when comparing close values of central trends (median, mean), in view of their implicit changes, it is advisable to more fully use the indicators of variation of variables.

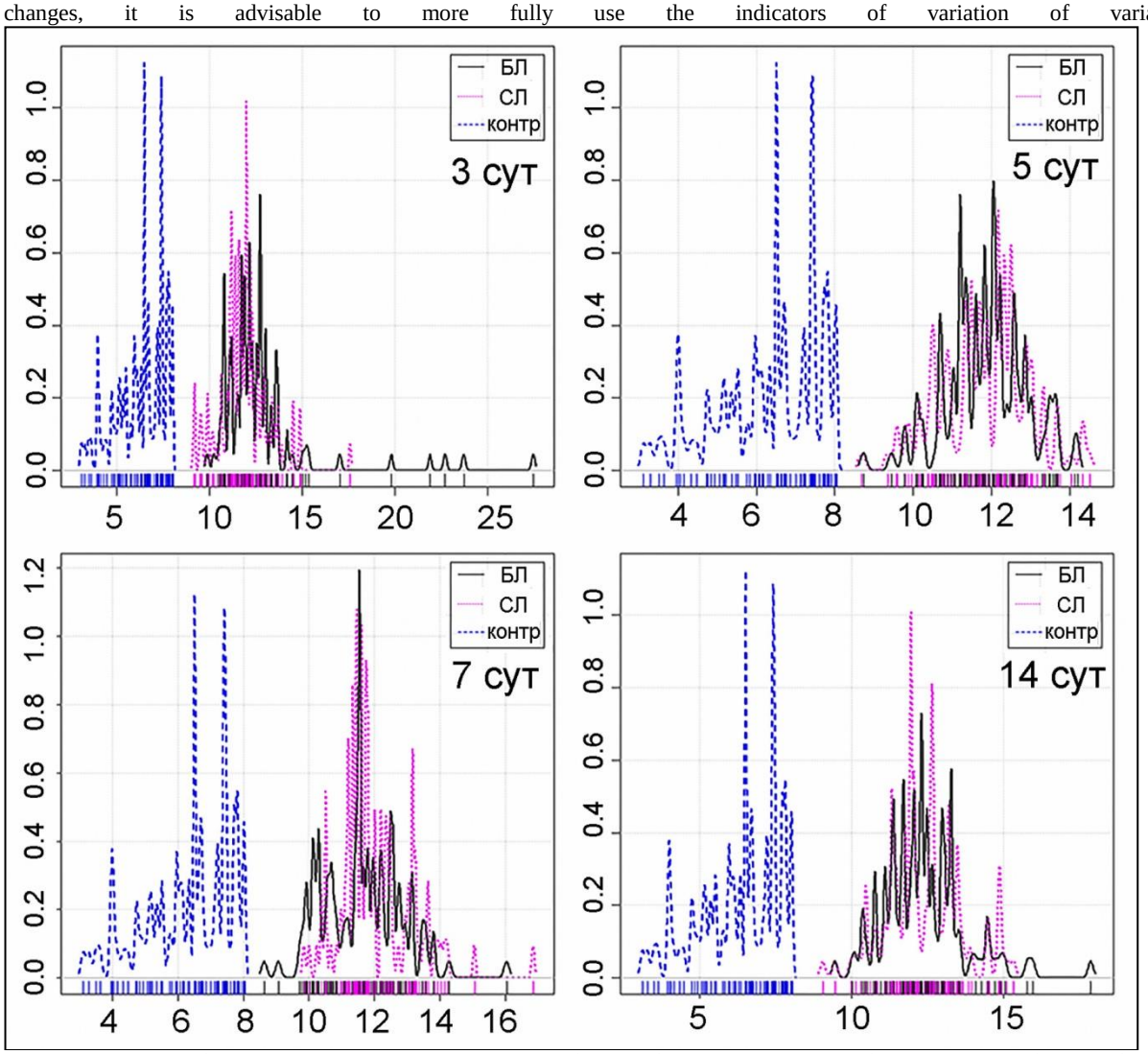

Fig. 5. The dynamics of changes in the distribution density (ordinate axis) of the visual fields according to the value of the relative area (%) of the areas of maximum brightness (abscissa axis)

In the group without treatment and with treatment, all studied variables (N, S_{rel}, S_{max}) on day 1, 3, 5, 7, and 14 after STBI statistically significantly differed from those in the control group ($p < 0.001$; Mann-Whitney and Kolmogorov-Smirnov test).

One day after STBI in the comparison group, the amount of AMB per unit of visual field increased 4 times compared to the control (Fig.2A), while their total relative area varied from 10 to 28%, and the area of the bulk of one AMB - from 1 to 6 μm2 (Fig. 6). Similar sizes in the neuropil corresponded to sections of small and medium processes of astrocytes, synaptic terminals, and dendrites. This indicated that precisely these structures in the acute period underwent maximum hydropic changes. Larger AMB - pericellular and perivascular edema - are represented by a set of conglomerates of the corresponding astrocytic processes.

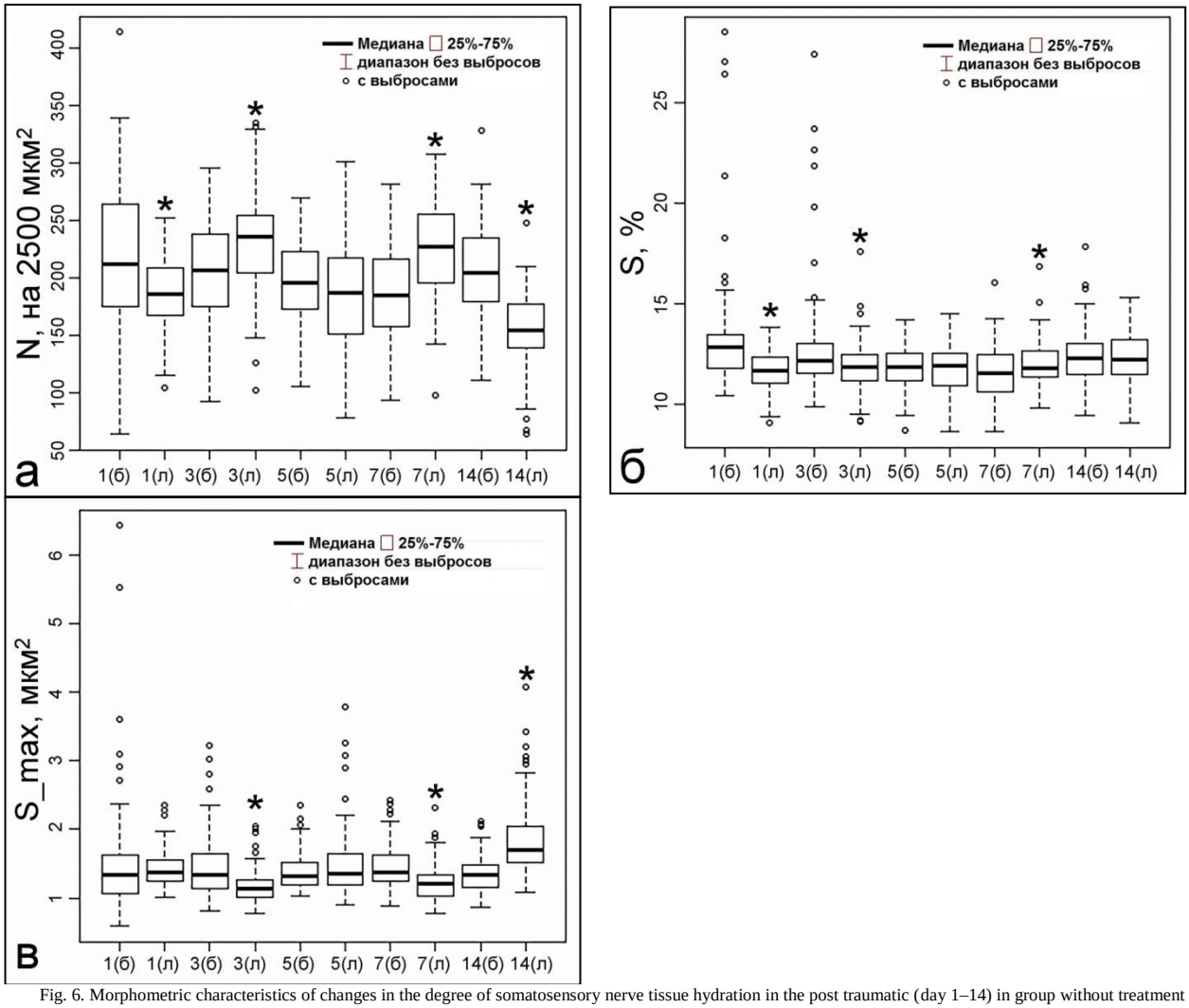

Fig. 6. Morphometric characteristics of changes in the degree of somatosensory nerve tissue hydration in the post traumatic (day 1–14) in group without treatment and with treatment: A — number of areas of maximum brightness in the field of view, B — relative area of all the areas of maximum brightness, C — the area of one zone of maximum brightness; * — differences between groups are statistically significant ($p<0.001$, Kolmogorov–Smirnov test). The material is presented as the median (50%), 25–75% th quartile, the non-outlier range and outlier range

On day 3, 5, 7 and 14 after STBI, the values of the median and 25−75% quartiles of the studied variables in the comparison group remained at the level of the first day. However, their maximum and extreme values decreased 5 days later, and this was especially evident for the relative area of AMB (Fig. 6). Probably, this indicated a decrease in the degree of hydration of the nervous tissue of the SSC 5 days after the injury. The variables reflecting the degree of hydration of the SSC nervous tissue did not reach the control values during the entire observation period. The relative area of AMB ranged from 9 to 18% 14 days later. That is, 9-18% of the area / volume of SSC tissue remained in a state of increased hydration, with zones of perivascular and pericellular edema, however, the proportion of large foci of edema-swelling (intra- and perineuronal, perivascular) decreased, and less vessels were compressed by edema.

DISCUSSION

Thus, after STBI, there was a significant increase in the degree of hydration of the nervous tissue (swelling) in SSC, when the part of the water chemically bound to macromolecules passed into a free form (edema). This process was accompanied by small-focal changes in tinctorial properties of perikaryons, neuronal processes, synaptic terminals, and astrocytes. As a result, it became possible, using the “Find Maxima” filter (from the ImageJ 1.52s program), to identify the AMB corresponding to the areas of maximum hydration of the nervous tissue on color images, and evaluate them morphometrically (the “Analyze Particles” plugin). It was found that within 14 days after STBI, the studied variables (the number and relative area of AMB) in the compared groups statistically significantly exceeded the control value. That is, after TBI during the entire observation period, a high overall level of hydration of the SSC nervous tissue was maintained.

The use of L-lysine escinat had a statistically significant effect on the studied morphometric parameters. One day after STBI in the main group, there was a smaller number of AMBs and their total relative area. At the same time, the spread of the values of the variables narrowed. A similar trend persisted for the relative area of the AMB after 3 days. After 5 and 14 days, there were no statistically significant differences in the median of this indicator. However, during treatment, the relative area of the AMB in different fields of view varied from 9 to 15%, and without treatment, from 9 to 20% (Fig. 6 B).

The data obtained indicated that L-lysine escinate most effectively influenced the water balance in the acute post-traumatic period (days 1 and 3), contributing to a decrease in the number of edema-swelling foci and their total relative area / volume. This was superimposed on the natural process of reducing the hydration of the nerve tissue of the SSC after injury. However, even with

the use of L-lysine escinat, a high degree of hydration of neurons, astrocytes, and neuropil was maintained. The drug only "smoothed" the extreme peaks (jumps) in the number and area of edema-swelling foci in the acute period after injury. At the same time, the pathobiochemical process of hydropic dystrophy itself developed in both groups studied.

CONCLUSION

1. After a severe traumatic brain injury, the degree of hydration of the nervous tissue of the somatosensory cortex increased. One day after the injury, the relative area of edema-swelling zones in the untreated group varied from 10 to 30% of the field of view of the somatosensory cortex.

2. The introduction of L-lysine escinat led to a statistically significant decrease in the manifestations of hydropic dystrophy. The most pronounced effect of the drug on the degree of hydration of the nervous tissue of the somatosensory cortex was noted in the early post-traumatic period: in a day, the relative area of edema-swelling zones in the treatment group did not exceed 15%. There was also a decrease in the number density of edema-swelling foci (especially with regard to emissions): the maximum value without treatment reached 350, and with treatment - 250 by 2500 μm2. However, despite the presence of the drug's effect, hydropic changes in the somatosensory cortex after severe traumatic brain injury persisted throughout the observation period.

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