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ASTROCYTE RESPONSE TO INTRACEREBRAL HEMORRHAGE IN VARIOUS BRAIN REGIONS AND PHARMACOLOGICAL CORRECTION

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Astrogliosis is a typical astrocyte response to intracerebral hemorrhage. The purpose of the study was to analyze morphological changes in astrocytes in the cerebral cortex, hippocampus, and striatum of rats with intracerebral hemorrhage and after administration of dexamethasone, granulocyte colony-stimulating factor and their combination. Astrocyte morphology, changes in projection perimeter and their density on days 1, 3, 10, 30 and 60 in three brain regions were studied based on the results of immunohistochemical staining for glial fibrillary acidic protein in astrocytes. Dexamethasone stimulated astrocyte hypertrophy in the cerebral cortex on days 3 and 10, and the granulocyte colony-stimulating factor caused an increase in astrocyte density on day 3 of the study. The morphological features of astrocyte response in the study regions of the brain were different: active hypertrophy and gliosis in the striatum, and predominantly hypertrophy of processes in the cerebral cortex and hippocampus.

Key words: intracerebral hemorrhage, astrocytes, dexamethasone, granulocyte colony-stimulating factor.

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ВІДПОВІДЬ АСТРОЦИТІВ У РІЗНИХ ДІЛЯНКАХ МОЗКУ НА ВНУТРІШНЬОМОЗКОВИЙ КРОВОВИЛИВ ТА ФАРМАКОКОРЕКЦІЮ

Астрогліоз є типовою реакцією астроцитів на внутрішньомозковий крововилив. Мета дослідження – проаналізувати морфологічні зміни астроцитів кори головного мозку, гіпокампу та смугастого тіла щурів за внутрішньомозкового крововиливу та введення дексаметазону, гранулоцитарного колонієстимулюючого фактора та їх комбінації. За результатами імуногістохімічного виявлення гліального фібрилярного кислого білка вивчали морфологію астроцитів, зміни периметра проекції та їх щільності на 1, 3, 10, 30 і 60 добу в трьох структурах мозку. Дексаметазон стимулював гіпертрофію астроцитів у корі головного мозку на 3 та 10 добу, а гранулоцитарний колонієстимулюючий фактор спричиняв збільшення щільності астроцитів на 3 добу дослідження. Морфологічні особливості астроцитарної відповіді досліджуваних структур головного мозку були різними: активна гіпертрофія та гліоз у смугастому тілі та переважно гіпертрофія відростків у корі головного мозку та гіпокампі.

Ключові слова: внутрішньомозковий крововилив, астроцити, дексаметазон, гранулоцитарний колонієстимулюючий фактор.

The study is a fragment of the research project "The study of restorative processes in the brain and nerve trunk during modulation of accumulation and differentiation of mesenchymal stem cells", state registration No. 0123U101051.

Intracerebral hemorrhage (ICH) is a severe injury causing direct damage to the brain tissue which results in the formation of a hematoma. The process includes reactive changes in astrocytes, astrogliosis, or glial scarring in the perifocal area of hemorrhage [3]. Though the scar may be necessary as a demarcation zone to stop the spread of brain necrosis, it can be an insurmountable obstacle for regenerating nerve fibers, which make it impossible for axons to sprout through the damaged region [6]. Astrocyte morphology outside the scar can also change, which depends on the distance to the center of the damaged region (astrocyte hypertrophy), while some cells respond by elongation their processes towards the lesion (polarization) [4].

The hypothesis is that there is a specific functional niche for astrocytes in the perifocal area (or penumbra in case of ischemia) of the damaged brain. Astrocyte response and its consequences vary depending on the microenvironment in the lesion area. The mechanisms of astrocyte fate in a specific microenvironment have not been studied completely; hence, recruitment of astrocytes to sites of injury remains debatable. It is difficult to detect the microenvironment trigger activating astrocytes. On the other hand reactive changes in astrocytes could be used as a marker or parameter to assess the effect of drugs for the treatment of ischemic brain damage, similar to attempts to determine how GFAP serum levels influence stroke and brain injury [13].

This study investigated astrocyte reactivity in the brain after ICH as an indicator of response to dexamethasone, granulocyte colony-stimulating factor (G-CSF) and their combination. Stimulation of mesenchymal stromal cells with a growth factor in the hemorrhage site was considered. Accumulation of $CD44^+$ cells in a traumatic neuroma of a peripheral nerve during G-CSF suppression of the inflammatory reaction [1] was shown in pilot studies. The hypothesis is that astrocytes in various brain structures may

respond to G-CSF and dexamethasone, which may be indicative of their effects on the development of cellular responses to injury.

The purpose of the study was to investigate the reactive changes in astrocytes in the sensorimotor cortex, hippocampus, and striatum of rats after intracerebral hemorrhage modeling and with the use of granulocyte colony-stimulating factor, dexamethasone, and their combination.

Materials and methods. Unilateral ICH was modeled in the internal capsule in males of Wistar rats (an average weight of 210.2±4.6 g). The animals were anesthetized intraperitoneally with sodium thiopental (50 mg/kg). The skulls were fixed, a parietal incision was made for craniotomy (1.0 mm in diameter). The needle of a 1.0 ml syringe was inserted to a depth determined from a stereotaxic atlas (capsula interna dextra, L=3.5–4.0; H=6.0; AP=0.6–1.0). 0.02 ml of autologous blood containing no anticoagulants was injected. The needle was fixed and left for 10 minutes after which time the same amount of blood was reinjected. This is a sequence of two-stage blood injection is necessary for a blood clot formation at the injection site. The needle was then removed, and the wound was sutured with 2 USP polyamide filament and irrigated with povidone-iodine.

The animals were randomized into the following study groups after ICH modeling: ICH, dexamethasone (Dex), granulocyte colony-stimulating factor (G-CSF), and Dex+G-CSF. Dexamethasone was administered subcutaneously at a dose of 10 mg/kg once a day for 3 days (Lekhim, Ukraine). G-CSF was administered at a dose of 50 μg/kg once a day for 3 days (Sanofi Winthrop Industria, France).

The control group included intact rats with penetrating traumatic brain injury (pTBI). Craniotomy and needle insertion, with no blood injection, were performed in the pTBI group. pTBI is a control for traumatic brain injury. Each group included 8 animals for every study period.

The rats were withdrawn from the study on days 1, 3, 10, 30 and 60. For this purpose, the animals were anesthetized intraperitoneally with sodium thiopental (50 mg/kg), and intracardial perfusion was performed (first with 200 ml of saline, then with 200 ml of 10% formalin solution diluted in saline).

3 mm frontal sections with the needle track and hematoma were cut from the rat brain. The brain samples were dehydrated in isopropanol and embedded in paraplast (Leica Surgipath Paraplast Regular). 4 μm sections were made for immunohistochemistry. Astrocytes were studied based on expression of glial acidic fibrillary protein (GFAP). Rabbit anti-GFAP, ab-7260 (Abcam, UK) was used at a 1:200 dilution. The reaction products images were made using a diaminobenzidine-based detection system (EnVision FLEX; Dako, Glostrup, Denmark). The antibody sections were incubated at 24°C (with primary and secondary antibodies for 20 min and 10 min, respectively). Rat brain sections with positive protein expression were used as a positive control; for negative controls all procedures were performed except for the use of primary antibodies. The preparations were examined using Olympus BX51 microscope, their photos were taken with Olympus C3040ZOOM digital camera using Olympus DP-Soft 3.2 software (Olympus, Tokyo, Japan).

Morphometric measurements were performed using ImageJ (ver. 1.54 Java 1.8.0_172). Morphometry included the measurement of the projected astrocyte processes perimeter. The end points of astrocyte processes were located on the micrographs, all selected points were connected, the region covered by an astrocyte was determined as the projection perimeter (μm). Astrocyte density was measured by number of cells on micrographs (×200, 2048×1536 px), with the area of 0.35 mm^2 (700×500 μm).

Statistical data were processed using StatPlus (version 7.0). One-way ANOVA with the Bonferroni post hoc test was used for statistical analysis. The group differences were considered statistically significant at $P<0.05$.

Each procedure with laboratory animals was performed in compliance with the bioethical norms adopted by the Bioethical Review and Scientific Research Ethics Committee (Minutes No. 160 of September 26, 2022).

Results of the study and their discussion. Immunohistochemistry detected GFAP in the soma and processes of astrocytes, which decrease in thickness as they branch. Morphology of astrocytes, particularly of their processes, is extremely diverse, still certain degree of morphological heterogeneity was detected for astrocytes in the cortex, hippocampus, and striatum. Astrocyte density was generally identical in the intact sensorimotor cortex and dorsolateral striatum, though astrocytes had more branched processes, and some cells showed a more intense positive response to GFAP in the striatum. In the hippocampus astrocytes had a distinct morphological phenotype and clear stratification in the hippocampal layers. Astrocyte bodies were mainly detected in the stratum radiatum and stratum oriens, and processes were polarized in the stratum pyramidale and only single astrocytes were detected in this layer. In the intact hippocampus, no visual difference was found in the density or morphology of astrocytes in CA1, CA2, CA3 and dentate gyrus. The situation was completely different after brain injury.

In the pTBI group, no significant changes were seen 1 day after brain injury; astrocytes with elongated processes were detected in the cerebral cortex, particularly in the striatum, on day 3. Focal astrogliosis along the track of needle insertion, and elongation of astrocyte processes in the striatum were detected on day 10. On days 30 and 60, mild hypertrophy of the processes of some astrocytes was seen in the three study regions; morphometry showed a decrease in the size of cells, though their specific density in the hippocampus and striatum increased versus the previous study periods. Fig. 1 shows astrocytes detected both in the intact brain and after pTBI or ICH injury.

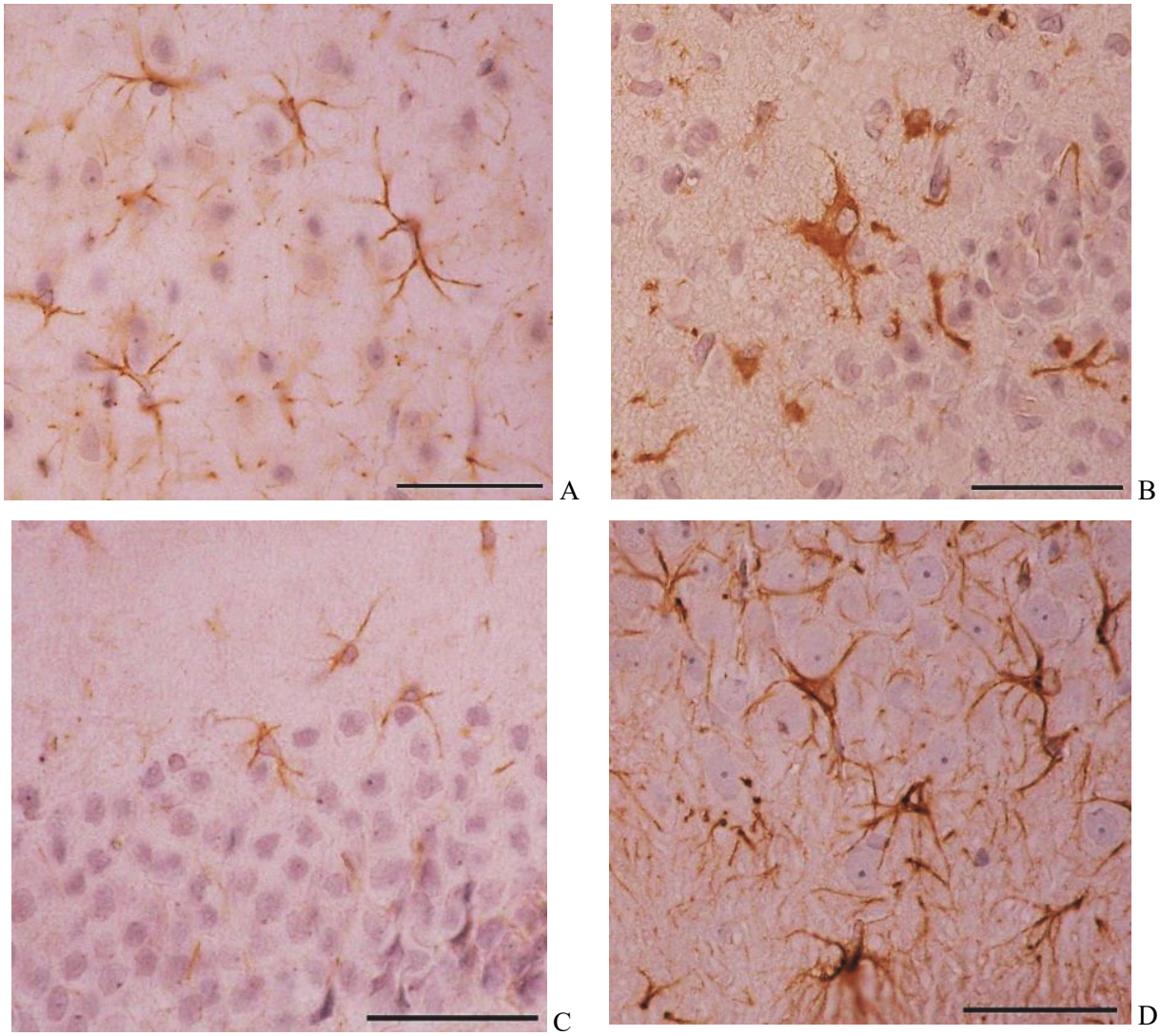

Fig. 1. Reactive changes of astrocytes in sensorimotor cortex and CA1 sector of the hippocampus of the ipsilateral brain hemisphere of rats following ICH simulation.

Note: a, b – cerebral cortex; c, d – hippocampus; a, c – intact astrocytes; b – astrocytes with soma hypertrophy; d – astrocytes with elongated processes with mild or no soma hypertrophy. Immunohistochemistry for GFAP. Scale bar 50 μm.

An attempt was made to differentiate reactive transformation of astrocytes into cells with hypertrophy of the soma and cells with significant elongation and branching (hypertrophy) of their processes. Astrocytes with both morphologies were found in the pTBI and ICH groups, i.e., they were not specific to the brain injury mechanism and could be morphological signs of the dynamics of astrocyte hypertrophy.

Figures 2 and 3 show the results of morphometric measurements of astrocytes and changes in their specific density in the study brain structures. A significant increase in the projected astrocyte processes perimeter was detected in the three study regions of the brain on 3 day after pTBI modeling. This parameter decreased on day 30 after pTBI modeling.

An increase in intensity of the immunohistochemical reaction was seen in the cortical astrocytic processes on days 3 and 10 in the ICH group. On day 30, hypertrophy was combined with an increase in the specific density of astrocytes (astrogliosis) in the dorsolateral striatum, while astrocytes with long hypertrophied processes were detected in the hippocampus. Astrocytes got smaller and their processes were significantly shorter on day 60. This means that reactive changes in astrocytes did not include any

significant increase in the specific density of astrocytes in the study regions, though severe astrogliosis was detected around the hematoma on day 10 and next terms.

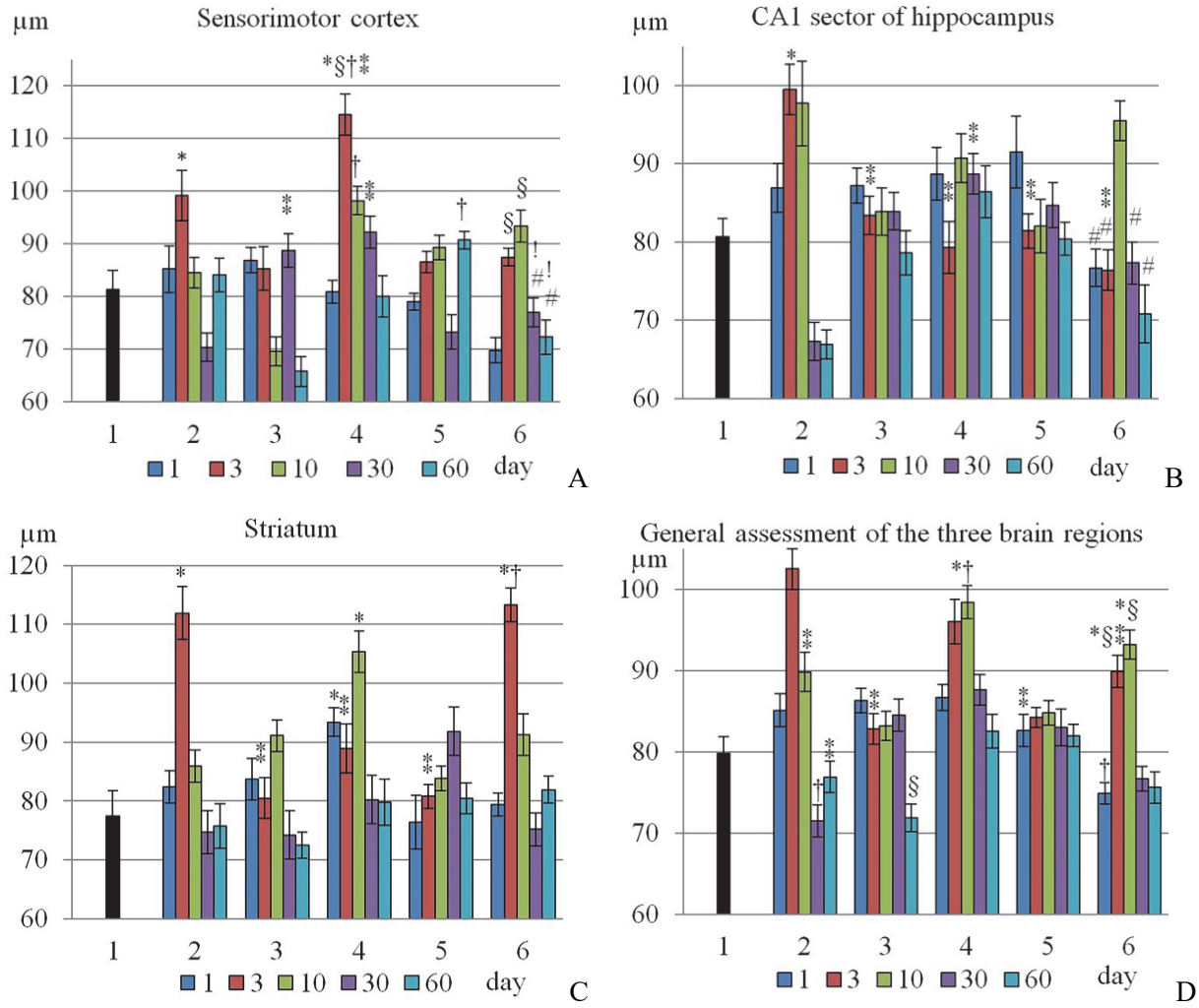

Fig. 2. Changes of astrocyte perimeter in sensorimotor cortex, CA1 sector of the hippocampus and dorsolateral striatum, and overall assessment of parameter in the ipsilateral brain hemisphere of rats following ICH simulation (M±m). Note (henceforward): 1 – control; 2 – pTBI; 3 – ICH; 4 – Dex; 5 – G-CSF; 6 – Dex+G-CSF; * – P<0.05 to control; ⁑ – P<0.05 to pTBI; † – P<0.05 to ICH (within one period); § – P<0.05 to day 1 (within one group); ‡ – P<0.05 to G-CSF on day 3; # – P<0.05 to Dex+G-CSF on day 3; ! – P<0.05 to Dex+G-CSF on day 10.

Significant astrocytes hypertrophy was detected on day 3 and 10 after ICH modeling in the Dex group. In the following periods, the projected astrocyte processes' perimeter decreased; however, no statistically significant changes in the specific density of astrocytes were found, while there was severe astrogliosis in the perifocal region.

The histological condition was different in the G-CSF group. There was no significant change in the astrocyte perimeter, with a tendency to some increase in the striatum only. The specific density of astrocytes increased in the cerebral cortex and striatum on day 3.

Astrocyte hypertrophy on days 3 and 10 and an increase in the specific density of astrocytes in the striatum on days 10 and 30 were detected in the Dex+G-CSF group. A significant elongation of astrocytic processes with higher intensity of immunoreaction to GFAP was seen in the cerebral cortex and hippocampus, however, did not affect the significant changes in the perimeter of astrocytes. More specifically, in the Dex+G-CSF group, astrocytes response was slightly different: astrogliosis decreased in the cerebral cortex (as described for the G-CSF group). In the striatum, astrocyte hypertrophy was similar to that described for the Dex group.

Thus, changes in reactive astrocytes were detected in the study regions of the cerebral cortex, hippocampus, and striatum outside the hematoma. Astrocytes had heterogeneous morphology in the study regions, which could potentially be an oligemia zone. Astrocytic hypertrophy mainly developed in the striatum on day 3 to 30 after ICH, and dexamethasone and G-CSF effects were not identical. G-CSF increased the specific density of astrocytes in the cerebral cortex, while dexamethasone activated

hypertrophy of single astrocytes. Reactive changes of astrocytes decreased during a separate period (days 30 and 60), which may be a sign of some stabilizing processes occurring in the damaged brain around the hematoma.

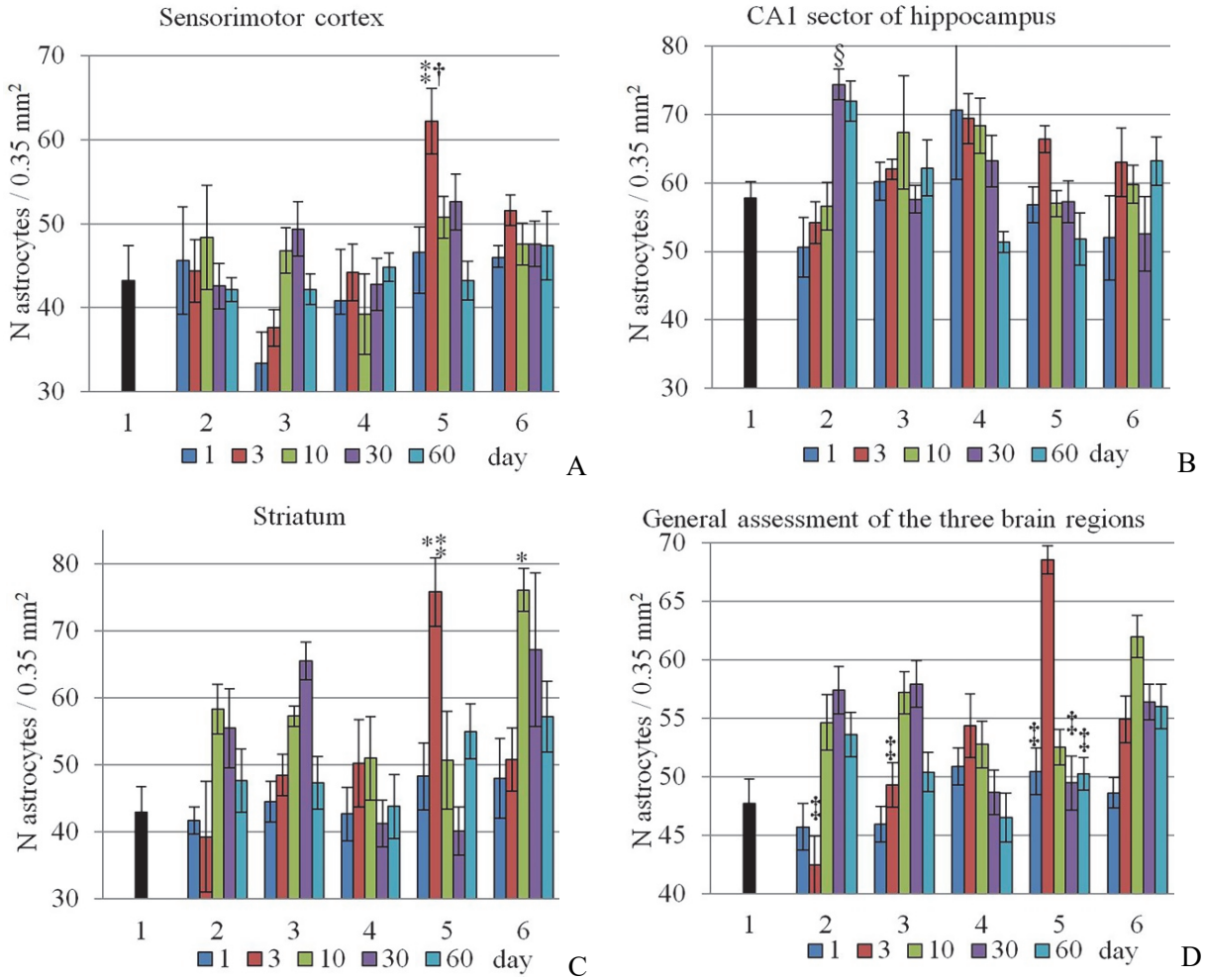

Fig. 3. Changes in specific density of astrocytes in sensorimotor cortex, CA1 sector of the hippocampus and dorsolateral striatum in the ipsilateral brain hemisphere of rats following ICH simulation.

Astrocytes morphology varies depending on the anatomical structure of the brain, and neuronal organization of different brain regions is somewhat specific. Astrocytes migrate to the injury site and appear to proliferate and form a glial scar. Generation of new astrocytes in the scar can also vary from local brain progenitor cells or ependymal progenitor cells [11]. This kind of astrocyte response seems to have a protective effect: it limits damaged tissue, supports other cells via the secretion of some trophic factors, protects cells from secondary damage, suppresses inflammation, stabilizes the microenvironment [7] and has effects on microcirculation after stroke [2]. Some studies show the integral involvement of astrocytes in the restoration of some brain structures. For example, hemorrhage in the striatum had effects on the damage caused to neurons of the striatum and sensorimotor cortex, and astrocytes as mediators in corticostriatal units can influence plasticity and motor recovery [10]. The intercellular communication between astrocytes and neurons, vascular cells and other gliocytes at the scar boundaries remains unclear. A change in astrocytes morphology could potentially be used to predict some abnormality. Evaluation of astrocytes specific markers such as GFAP could be used for this purpose. GFAP expression is known to be closely connected with structural brain abnormalities after ischemic stroke [7], intracerebral hematoma [10], herpes virus infection [5]. Astrocytes respond in the form of GFAP hyperexpression and often become hypertrophic.

Dexamethasone can have effects on astrocytes response and GFAP expression in the hippocampus in animal models of epilepsy, in the neocortex after brain injury, and in various brain regions in a dose-dependent manner if administered chronically [9, 12]. This study showed that dexamethasone had a stimulating effect on astrocyte hypertrophy, while G-CSF caused hypertrophy and an increase in the number of astrocytes. In the Dex+G-CSF group, the response of astrocytes was slightly modified, though hypertrophy generally developed in the brain on days 3 to 30 after ICH. The relationship between the

astrocyte response, the scar morphology around the hemorrhage, and the immunophenotypic characteristics of migration of non-resident cells to the hemorrhagic site due to dexamethasone and G-CSF exposure is a promising area of hemorrhagic stroke studies.

The prospects for further research lie in studying the role of cells with a pro-inflammatory phenotype and mesenchymal stem cells, expressing CD44, CD68, CD90, CD146 [8], and their potential role in the regenerative processes in the brain.

Conclusions

1. Astrogliosis after ICH varied depending on the brain region: it was more intensive in the striatum compared to the sensorimotor cortex and the hippocampus. Astrocyte hyperplasia began in the acute period after brain injury and continued until the end of the study. Astrocyte hypertrophy was detected in the acute period, and then astrocyte reactivity gradually decreased.
2. Dexamethasone increased astrocyte hypertrophy, while G-CSF contributed to an active increase in their specific number during the acute period after injury. Dexamethasone and G-CSF combination caused the inhibition of astrogliosis in the sensorimotor cortex of the brain.
3. The morphology and reactive changes in astrocytes can serve the basis for a comprehensive assessment of drug effects on the development and prognosis of structural changes in the brain after injury, particularly of traumatic or ischemic origin.

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