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MORPHOLOGICAL CHANGES IN THE SOMATOSENSORY CORTEX OF A RAT BRAIN AFTER ACUTE ISCHEMIA-REPERFUSION MODELLING FOLLOWED BY ADMINISTRATION OF MESENCHYMAL STEM CELLS OF VARIOUS ORIGINS, RAT EMBRYONIC FIBROBLASTS AND WHARTON'S JELLY MSCS LYSATE

Aim. *To evaluate the effect of mesenchymal stem cells of various origins, rat embryonic fibroblasts and Wharton's jelly MSCs lysate on the dynamics of destructive changes in the somatosensory cortex of rats with artificially induced acute cerebrovascular injury.* ***Materials and methods.*** *Modelling of transient bilateral ischemia-reperfusion (IR) in rats by ligation of the internal carotid arteries. Transplantation of mesenchymal stem cells (MSCs) of different origins into the femoral vein of experimental animals. Cytological study on 7th and 14th days after IR with counting the total number of neuronal nuclei in 1 mm^2 in the somatosensory cortex of animals and the ratio of the intact neuronal nuclei to the pathologically altered nuclei.* ***Results.*** *Transplantation of MSCs, rat embryonic fibroblasts, MSC lysate from human umbilical cord Wharton's jelly (hUC-WJ) and administration of citicoline led to an increase in the number of intact neurons and nuclei without pathological changes in the somatosensory cortex of rats. The most effective in terms of*

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cytomorphology was the transplantation of hUC-WJ MSCs. ***Conclusions.*** *The MSC transplantation is effective in the treatment of cerebral ischemia. The most effective prevention of destructive changes was observed in the somatosensory cortex of rats that were transplanted with MSCs from human umbilical cord Wharton's jelly after IR.*

Keywords: *ischemic stroke, cytomorphology, somatosensory cortex, mesenchymal stem cells, rats.*

Introduction

The vascular disease was the basic cause of disability and mortality in the working-age people worldwide for the past decades. Every year an acute cerebrovascular accident happens in 100,000—110,000 ukrainians, and a third of them are working, half of these people are expected to become disabled and only one of ten will fully return to the previous life mode [1].

Ischemia in acute cerebrovascular accident frequently leads to the infarct zone formation, in which nervous cells quickly necrotize and surrounding penumbra area appears, where cells remain functionally suppressed but capable to live for a limited time [2]. The ischemic zone suffers extremely strongly from oxygen and glucose deficiency, resulting in cell death due to necrosis, which affects the neurons, neuroglia cells and vascular endothelium; while in the penumbra area cellular destruction slows down and develops primarily by apoptosis [3].

Recently, promising experimental data have been obtained that mesenchymal stromal cells (MSCs) therapy can enhance the brain tissue recovery after ischemia. In this study, we evaluated the influence of different origin MSCs, rat embryonic fibroblasts, human Wharton's jelly (hWJ-MSC)-derived cells lysate and citicoline on the destructive changes dynamics in the somatosensory cortex of rats with artificially induced acute cerebrovascular casualty.

Materials and Methods

Wistar male rats (190 animals aged 4 months, with a body weight of 160—190 g) were used in the experiment. Experimental ischemia-reperfusion of brain by bilaterally applying ligatures to the internal carotid arteries was performed with subsequent transplantation of hWJ-MSC lysate, rat embryonic fibroblasts, MSCs of various origins or citicoline administration. The animals were bred in the Vinnytsia National Medical University (VNMU) vivarium, housing circs are standard, with free water and food accession. When conducting the research, all procedures complied with ethical standards and normative demands for the humane animal handling [4, 5]. The experiment protocol was approved by the Bioethics Committee of National Pirogov Memorial Medical University (protocol No. 2 dated January 31, 2024).

Propofol anesthesia (Propofol-novo medication, Novofarm-Biosintez LLC production, Ukraine, at a dose of 60 mg/kg) was used to execute rats' brain ischemia. 20-minute cerebral ischemia was performed by applying ligatures to the internal carotid artery. In 20 minutes, the ligatures were removed (reperfusion beginning) and a single injection of 0,9% NaCl solution at a rate of 2 ml/kg was administered to the femoral vein in the control pathology group, while the remaining experimental groups were administered citicoline, rat embryonic fibroblasts or mesenchymal stem cells suspended in 0,2 ml of physiological solution (10^6 cells/animal). We injected mesenchymal stem cells of various origins, rat embryonic fibroblasts and Wharton's jelly lysate into the femoral vein immediately after IR, since early MSC transplantation led to better neurological recovery and reduced infarct volume, and also required a smaller number (1×10^6) of donor cells to have a favourable effect [6, 7]

The chosen experimental IR model reflects the clinical situation of cerebral infarction and is appropriate to study experimentally an action of potential neuroprotective substances [8].

The rats in the experiment were devided into 8 groups:

- group 1 (n = 10, a group of sham-operated animals).

• group 2 (n = 40) was control, the rats under experimental IR modelling without any therapy.

• group 3 (n = 20) underwent experimental IR of the brain followed by one-time administration of hWJ-MSCs at a dose of 10^6 cells/rat.

• group 4 (n = 20) was transplanted with rat embryonic fibroblasts once after the IR (at a dose of 10^6 cells/rat).

• group 5 (n = 25) immediately after the experimental IR received 10^6 cells/rat of human adipose tissue MSCs.

• group 6 (n = 25) was treated with rat adipose tissue MSCs with a dose of 10^6 cells/rat.

• group 7 (n = 25) immediately after the experimental IR was injected once with hWJ-MSC lysate (0.2 ml/rat).

• group 8 (n = 25) was injected with citicoline («Neuroxon» medication, Arterium Corporation production, Ukraine) with a dose of 250 mg/kg. Citicoline was chosen due to its capability to enhance neuroregenerative processes in brain effectively and improve cognitive processes and memory in persons with cerebral ischemia [9—12].

The method details for cells isolation were described previously [13]. Briefly, hUC-WJ MSCs were isolated from umbilical cord by the explant method, cultivated in DMEM mediun with 10% of fetal calf serum (FCS) and after two passages *in vitro* were characterized by morphology and minimal surface markers (CD34, CD45 — negative and CD73, CD90, CD105 — positive). Human adipose-derived MSCs were obtained from liposuction material by fermentation with collagenase type 1, cultivated for two passages in DMEM/F12, supplemented with 10% FCS and also characterized by minimal criteria for MSCs; the same method was used for isolatiom of MSCs from abdominal adipose tissue of rats. Rat embryonic fibroblasts were got from muscle tissue of 15-day-old rat embryos by keeping them in mixure of 0.25% solution of trypsin and 0.02% Versen in a ratio of 1:1 at 37 °C overnight. DMEM medium with 10% of FCS was added to the resulting cell suspension which was seeded on Petri dish and passaged twice *in vitro*. Cells were identified by spindle-shaped morphology and hematoxylin-eosin staining. To receive hUC-WJ MSCs lysate 10^6 cells in DMEM medium were subjected to ultrasound disintegration with further centrifugation at 12000xg for 10 min, supernatant was used for next stage. Injection of all type of the cells as well as cell lysate was performed in 0,2 ml of 0,9% NaCl into the femoral vein of the animals.

Cytological examination described earlier was performed in 7 days after IR (this time period corresponds to the ischemia subacute period) and in 14 days (corresponds to the recovery period) [14]. For this purpose, animals were decapitated (using propofol anesthesia, at the dose like earlier mentioned). The rat brain was removed, fixed with 4% formaldehyde solution (for 24 hours), then washed in running water, held through alcohols with increasing concentration and xylene, and after classic histological wiring, embedded in Paraplast Plus© (Leica Scientific (McCormick©), USA). Sections (three in number) of 5 μm thick were prepared on a rotary microtome. Deparaffinized sections were dyed in accordation to Nissl standard technique.

Using BX-51 microscope (Olympus production, Japan), digital images of frontal brain sections for all rats' groups were analyzed (exploiting the ImageJ computer program, 1.48v, free license, Rasband production, USA, 2015). Statistical analysis of the data was executed using Microsoft© Exel©-2010 and "Statistica 6.0" (StatSoft® Snc, USA) software. The credibility of differences was assessed by the Student's t-test and unpaired nonparametric Mann-Whitney U test. Differences between the indexes were considered statistically credible at $p < 0.05$.

Results and Discussion

Group 1 (a group of sham-operated animals). When analyzing the somatosensory area paraffin sections, no pathological changes were detected (Fig. 1*a*). Pyramidal neurons in the somatosensory cortex layers were of variable size, with a small euchromatin nucleus and basophilic Nissl granules

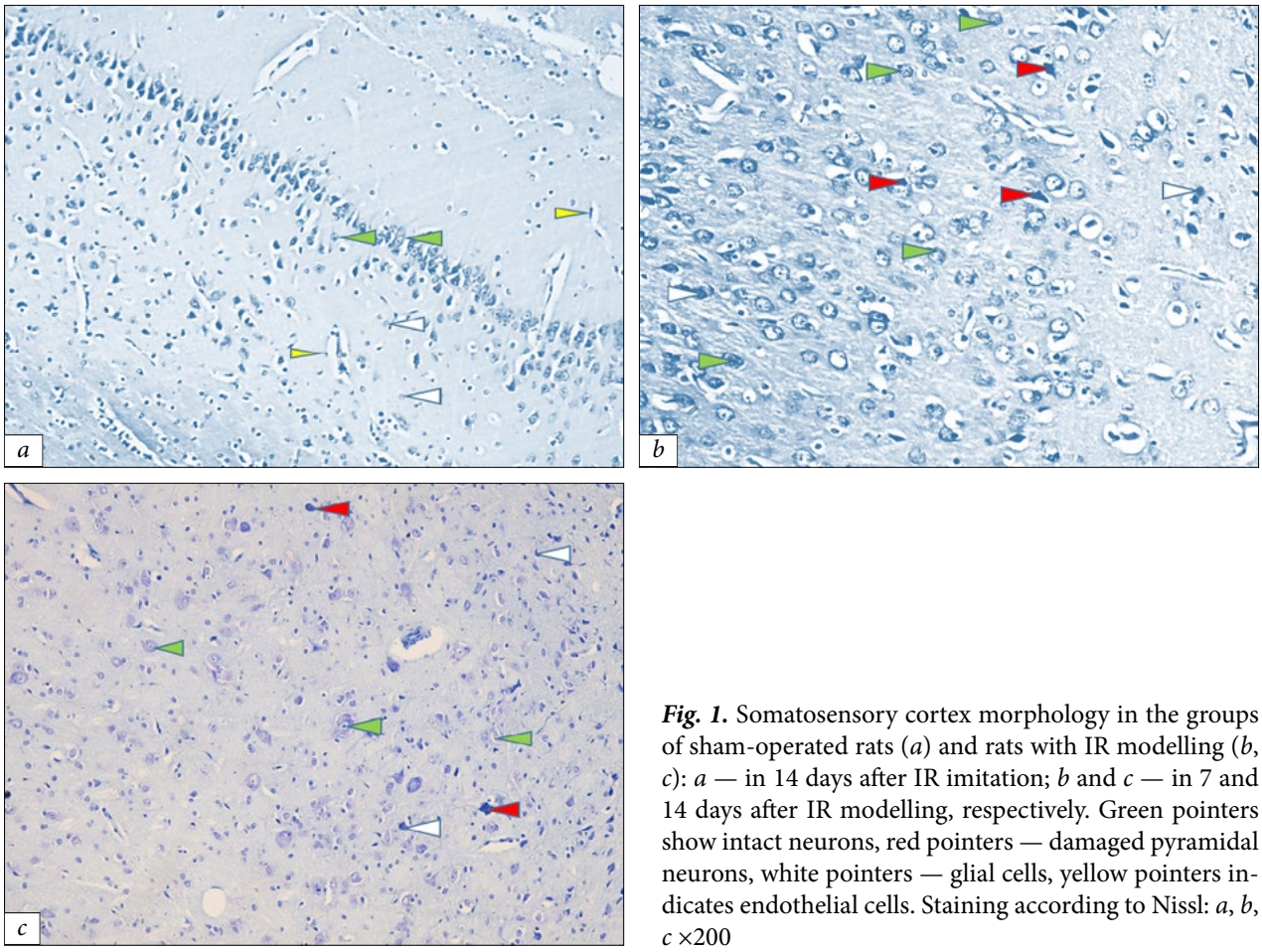

Fig. 1. Somatosensory cortex morphology in the groups of sham-operated rats (*a*) and rats with IR modelling (*b*, *c*): *a* — in 14 days after IR imitation; *b* and *c* — in 7 and 14 days after IR modelling, respectively. Green pointers show intact neurons, red pointers — damaged pyramidal neurons, white pointers — glial cells, yellow pointers indicates endothelial cells. Staining according to Nissl: *a*, *b*, *c* ×200

and a single apical short processes directed to the cortical surface and several basal ones (Fig. 1*a*). Stellate cells were smaller (compare to the pyramidal cells), with an euchromatin nucleus, a clearly visible nucleolus and processes diverging at different planes. Stellate neurons were present in all cortical layers except for the superficial one. Endothelial cells having heterochromic nuclei nicely visualized in the vascular wall.

Group 2 (the rats under an experimental IR modelling without any therapy). In rats with experimental IR modelling the following cytomorphological changes are observed: first, pyramidal cells are shrunken, hyperchromic, but nuclei and nucleoli in them aren't visualized (Fig. 1*b*, *c*). Second, there is pronounced pericellular edema, which forms light «halos» around pyramidal neurons, as well as vacuolization of the neuropil. Perivascular edema draws attention, while the blood vessels lumen is straiten. Glial cells acquire a dark color, reactive gliosis manifests itself. Instead, the stellate cells do not show structural changes and they look similar t vo stellate neurons of the somatosensory cortex in the control group. In 14 days after IR modelling, the nature of the ruinous changes in the somatosensory cortex is similar to the cytomorphological changes observed in 7 days, also there is (according to morphometric analysis) a slight rise in the number of pyramidal neurons with intact nuclei and decrease in reactive gliosis (Fig. 1*b*, *c*, Tabl. 1).

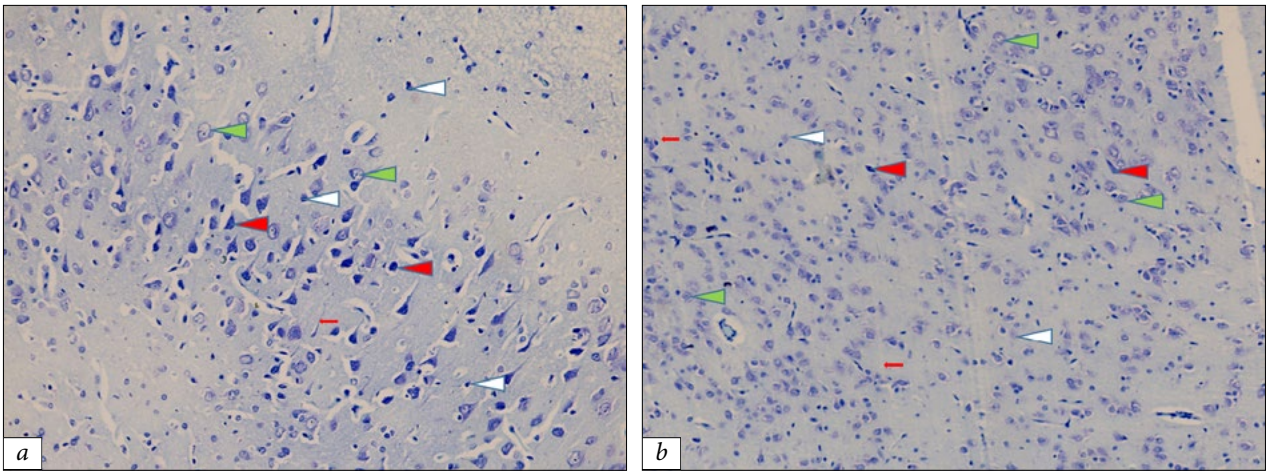

Fig. 2. Somatosensory cortex morphology in 7 days (*a*) and 14 days (*b*) after experimental IR modelling and subsequent transplantation of hWJ-MSCs. Green pointers show intact neurons, red pointers — affected neurons, white pointers — neuroglial cells, red sticks — apical dendrites. Staining according to Nissl: *a* ×200, *b* ×100

Table 1. **The comparison of the intact and affected neuron nuclei number (with karyorrhexis and karyopyknosis) in the somatosensory cortex (in 1 mm² of the rats brain cortex) in 7 and 14 days after experimental IR and administration of mesenchymal stromal cells, rat embryonic fibroblasts or citicoline**

Group	Intact nuclei, absolute number/mm², M ± SD		Damaged nuclei (karyopyknosis, karyorrhexis), absolute number/mm², M ± SD	
	7 days	14 days	7 days	14 days
Sham-operated rats	128,25 ± 9,36	128,25 ± 9,36	0	0
Ischemia-reperfusion (control group)	1,00 ± 0,89	3,17 ± 1,17	22,67 ± 7,28	34,17 ± 5,42
IR + hWJ-MSCs	90,20 ± 12,01*	105,20 ± 7,36	15,60 ± 3,51	16,40 ± 7,40*
IR + rat embryonic fibroblasts	60,00 ± 4,30*#	66,00 ± 6,10*#	23,60 ± 2,88*#	22,67 ± 3,67*#
IR + MSCs of human adipose tissue	70,20 ± 1,92*#	75,00 ± 1,87*#	25,20 ± 13,70*#	24,80 ± 2,77*#
IR + MSCs of rat adipose tissue	70,50 ± 12,44*#	76,50 ± 14,01*#	21,0 ± 5,83*#	20,33 ± 6,02*#
IR + cell lysate from hWJ-MSC	55,33 ± 8,59*#	58,67 ± 4,89*#	33,67 ± 1,97*#	31,17 ± 5,67*#
IR + citicoline	37,67 ± 6,41*#	45,50 ± 3,27*#	30,83 ± 5,74*#	33,83 ± 2,86*#

Notes: * — $p < 0,05$ relative to the indicator of the control group. # — $p < 0,05$ relative to the group 3 (rats which passed IR and hWJ-MSCs administration).

Group 3 (the rats having experimental IR followed by one-time administration of hWJ-MSCs at a dose 10^6 cells/rat). Transplantation of hWJ-MSCs after experimental IR modelling may be considered the most effective compared to transplantation of other MSCs for the brain ischemia treatment. Thus, in 7 days only single pyramidal neurons were observed, they were shrunken and hyperchromic. At the same time somatosensory cortex cytoarchitectonics was analogous to that

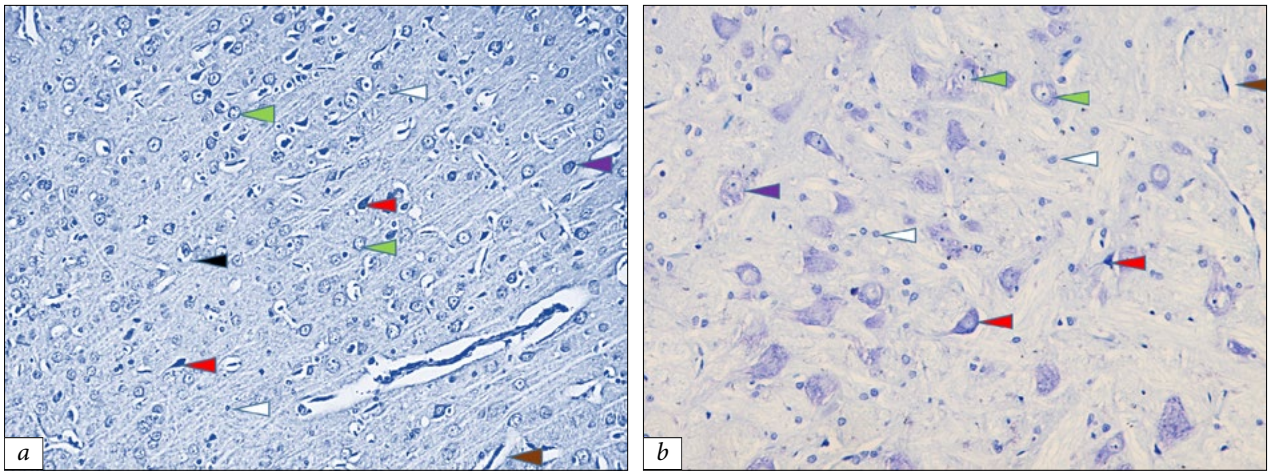

Fig. 3. Somatosensory cortex morphology in 7 days (*a*) and 14 days (*b*) after experimental IR modelling and subsequent rat embryonic fibroblasts transplantation. The white arrow shows vasoconstriction, the black — intercellular oedema, the purple — heterochromic nuclei, while the red one indicates pyknotic nuclei with obvious perinuclear oedema, green pointers show unaffected neurons, Staining according to Nissl: *a* ×200, *b* ×400.

in the group 1 (Fig. 2*a*). Most pyramidal neurons have a euchromatin nucleus, the morphology of stellate cells is not changed. In the neuropil typical parallelism of apical dendrites is saved. Endothelial cells in the vascular wall contained hetechromic nuclei. Single neuroglial cells are dark-colored. On the day 14 after IR, the nature of destructive changes in the somatosensory cortex was similar to that on the day 7, but a slight increase (by 1.2%, $p > 0.05$) in the number of pyramidal neurons having intact nuclei and a decrease in reactive gliosis took place (Fig. 2*b*, Tabl. 1).

In the group 4 (the rats transplanted with rat embryonic fibroblasts (at a dose 10^6 cells/rat) once after the IR) in 7 days after both pyramidal cells with an euchromatin nucleus and damaged pyramidal neurons with signs of karyopyknosis were observed in a section of the cortex of the somatosensory area (Fig. 3*a*). There is intercellular swelling in the neuropil as well as perivascular swelling, vasoconstriction. In 14 days heterochromic and pyknotic nuclei (with obvious perinuclear edema) are observed between pathologically unchanged euchromic nuclei of pyramidal neurons (Fig. 3*b*). However, the cytoarchitectonics of the somato-sensory cortex in rats did not undergo significant changes.

Group 5 (the rats which immediately after the experimental IR received 10^6 cells/rat of human adipose tissue MSCs). This group showed the following cytomorphological changes in the somatosensory cortex: in 7 days the morphology of most pyramidal cells was unchanged, the nucleus was euchromatin, and hyperchromic pyramidal neurons were occasionally found (Fig. 4*a*). Obvious parallelism of the apical dendrites was saved. Endothelial cells having heterochromic nuclei are present in a vessel wall. There is no microglial infiltration. In 14 days, no significant changes were observed in the somatosensory cortex: similarly, pyramidal cells demonstrated euchromic nuclei with one nucleolus, single heterochromic and pyknotic nuclei were also visualized (with visible perinuclear edema). (Fig. 4*b*).

Group 6 (the rats treated with rat adipose tissue MSCs at a dose of 10^6 cells/rat). Similar changes (to the group 5) occurred in rats which, after experimental IR, received stem cell trans-

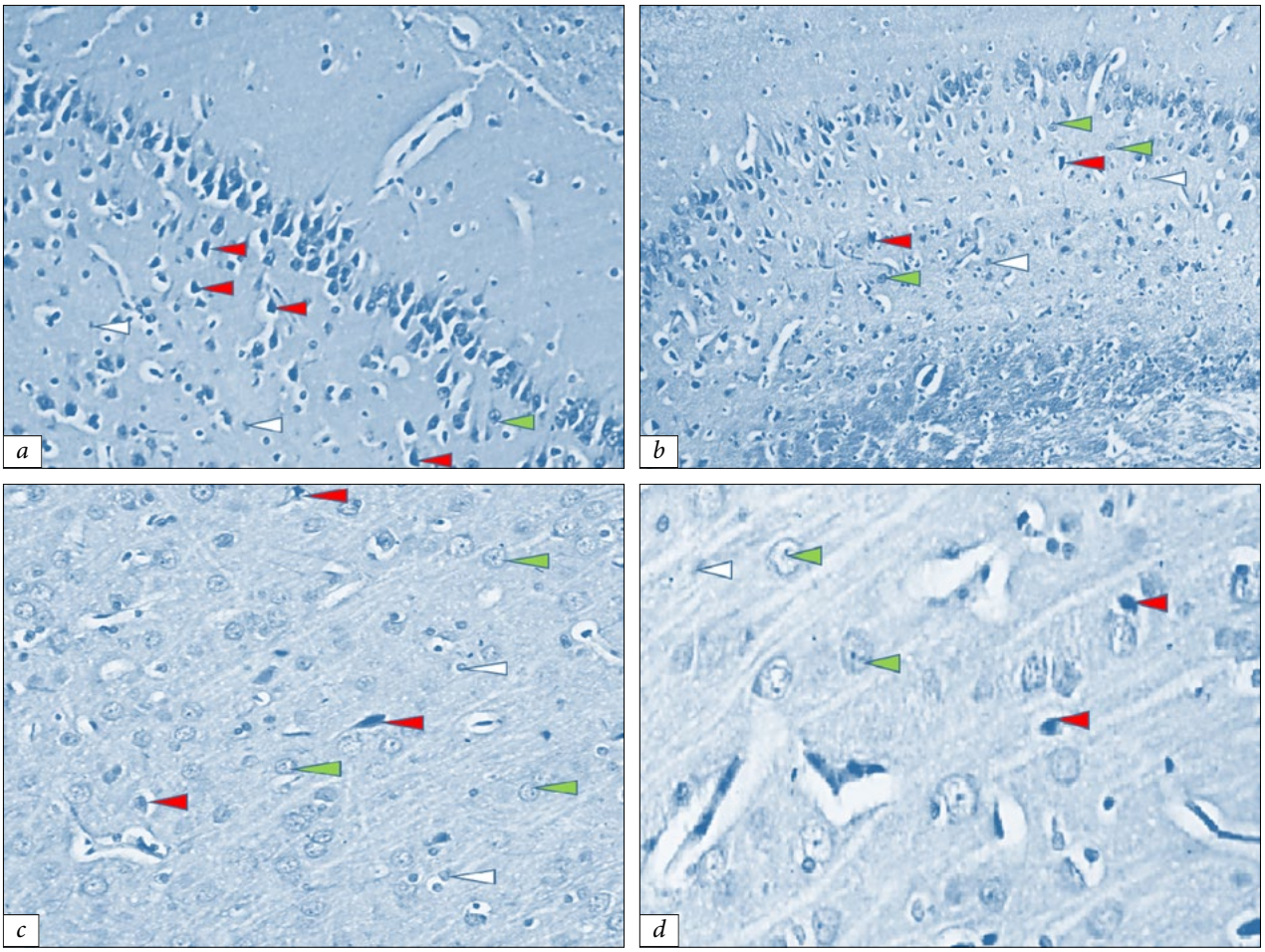

Fig. 4. Somatosensory cortex morphology in 7 days (*a*, *c*) and 14 days (*b*, *d*) after experimental IR and subsequent one-time transplantation of stem cells from human adipose tissue (*a*, *b*) and rat adipose tissue (*c*, *d*). Green pointers show intact neurons, red pointers — affected neurons, white pointers — neuroglial cells. Staining according to Nissl: *a*, *c* ×200, *b* ×100, *d* ×400

plantation from rat adipose tissue (Fig. 4*c*, *d*). Thus, in 7 days in the somatosensory cortex neurons having unchanged morphology were mostly observed (see Fig. 4*c*), only few pyramidal neurons were hyperchromic, pericellular edema was observed around such the cells. The areas both with microvacuolization and with perivascular edema were isolated, and microglial infiltration appeared insignificant. In 14 days, a decrease in the affected pyramidal neurons number was observed (see Fig. 4*d*). There were areas with well-visible beams of apical dendrites. Stellate cells in both 7 and 14 days were almost without structural changes.

Group 7 (the rats which were once injected with hWJ-MSC lysate (0.2 ml/rat) immediately after the experimental IR). In 7 days, the mixture of intact pyramidal cells having euchromic nuclei with one nucleolus and affected neurons containing pyknotic and heterochromic nuclei with apparent perinuclear edema took place in the somatosensory cortex of rats (Fig. 5*a*). Apical dendrites were hardly visible. Neuroglial cells were stained dark. The stellate cells did not

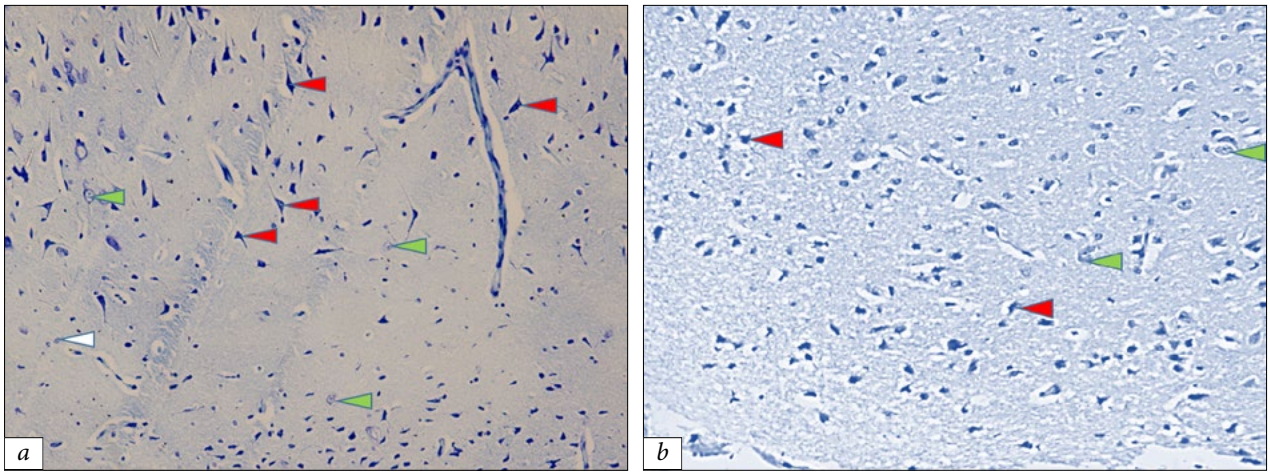

Fig. 5. Somatosensory cortex sections in 7 days (*a*) and 14 days (*b*) after IR modelling followed by cell lysate from hWJ-MSC introduction. Green pointers show intact neurons, red pointers — affected pyramidal cells, white pointers — neuroglial cells. Staining according to Nissl ×200

demonstrate any structural transformations and looked like those in the control group. Intracellular and perivascular edema also were observed. In 14 days, the somatosensory cortex cytomorphology was similar to that which was fixed in 7 days, but there is a trend towards an increase in the number of unaffected pyramidal cells (3,1%, $p > 0.05$), a decrease in intracellular edema, and also perivascular edema (Fig. 5*b*, Tabl. 1).

Group 8 (the rats which were injected with citicoline at a dose of 250 mg/kg immidiatelly after IR). In 7 days after the model IR and citicoline application, in the somatosensory cortex the neuropil vacuolation was clear visible, pyramidal cells were hyperchromic and shrunken (Fig. 6*a*). Glial cells looked dark coloured. A specific pericellular edema was also observed, forming light «aureole» around the pyramidal cells, as it was in the group 2 (see Fig. 6*a*). Apical dendrites were visualized with difficulty. In 14 days, an unreliable rise in the intact pyramidal neurons number took place (from 55.0 to 57.4%, $p > 0.05$) (Fig. 6*b*, Tabl. 1). Stellate neurons almost did not change both in 7 days and in 14 days.

It is worth emphasizing that no significant differences in the morphological features of tissues within one study group were found.

The morphometric analysis notices that MSCs transplantation, lysate from hWJ-MSC or rat embryonic fibroblasts usage as well as citicoline administration caused an increase in the neurons number, nuclei of which were not pathologically changed (Table 1).

Comparing the micromorphometry results of neurons in the somatosensory cortex, has shown (see Table 1) that IR modelling is accompanied by a sharp decrease in the number of neurons having intact nuclei, both on the observation day 7 (on average 1.0 ± 0.89 n umber/mm^2) and on the day 14 (3.17 ± 1.17 number/mm^2, in comparison to the sham-operated rats where this figure averaged 128.25 ± 9.36 number/mm^2 ($p < 0.05$)). At the same time, the number of neurons with karyopyknosis and karyorrhexis increased relatively. The use of intravenous hWJ-MSC transplantation as an experimental therapy for acute ischemic stroke contributed to a better neuroprotective effect than other mesenchymal stem cells, Wharton's jelly MSC lysate, and citicoline. This group of rats demonstrated the highest number of intact

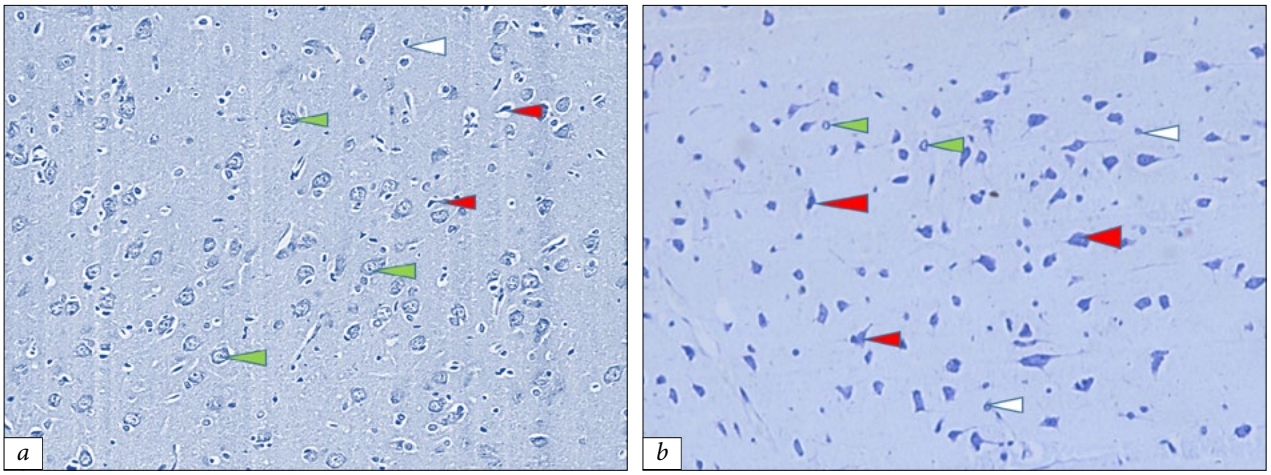

Fig. 6. Somatosensory cortex morphology in 7 days (*a*) and 14 days (*b*) after IR and citicoline usage. Unaffected neurons are indicated by green pointers, red pointers show changed pyramidal cells, white pointers — neuroglial cells. Staining according to Nissl ×200

neuronal nuclei on both days 7 and 14 of the experiment, on average 90.20 ± 12.01 number/mm^2 and 105.20 ± 7.36 number/mm^2 ($p < 0.05$) and the lowest number of neurons in which nuclear destruction was observed during the studied periods, on average 15.60 ± 3.51 number/mm^2 and 16.40 ± 7.40 number/mm^2 ($p < 0.05$), compared to other groups of animals receiving experimental treatment.The worst result (manifested with the lowest intact neuron nuclei percentage) among all the experimental rat groups was obtained in animals administrated with citicoline after IR.

The outcome of our study indicate that intravenous transplantation of MSCs obtained from both human and rat adipose tissue, hWJ-MSCs and their cell lysate, as well as rat embryonic fibroblasts and citicoline medication after experimental ischemia of the rat brain is able to minimize the ischemic damage area in the somatosensory cortex, and also contributes to the neuro- and astrocytic apoptosis reduction after cerebral infarction. This is in agreement with the results of our previous studies [15—17].

A number of studies have demonstrated the efficacy of MSC transplantation after ischemic stroke, their contribution to the improvement of brain function, effective protection of ischemic neurons and stimulation of regenerative processes in damaged areas of the brain [18]. This can be explained by the fact that MSCs secrete a wide range of trophic and immunomodulatory cytokines, the so-called "MSC secretome", which triggers and stimulates endogenous neuroprotection, namely neurogenesis and angiogenesis, which is extremely important for the ischemic brain damage therapy [19, 20].

According to some researcher hWJ-MSCs are able to provide effective neurogenesis through a paracrine mechanism, as they express the genes involved in angiogenesis and neurogenesis, and the genes responsible for the synthesis of secretory factors through which these processes are regulated [21].

Conclusions

1. 20-minute ischemia of rat brain followed by restoration of the blood supply causes pronounced degeneration of the somato-sensory cortex, which is manifested by the shrinkage and hyperchromia of pyramidal neurons, karyopyknosis and karyorhexis of their nuclei, evident pericellular edema

forming light «aureole» around pyramidal cells, neuropil vacuolization.

2. In the groups of rats transplanted with MSCs of various origins, cell lysate from hWJ-MSC, rat embryonic fibroblasts or citicoline medication after experimental IR, the somatosensory cortex cytomorphology is significantly closer to normal, and the number of neuron nuclei without pathological changes in the somato-sensory cortex is rather higher.

3. The transplantation of hWJ-MSCs, carried out immediately after one-time experimental IR, is the most effective in terms of restoration the cellular structure of the somatosensory cortex of rats (compared to the transplantation of MSCs of other origin).

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МОРФОЛОГІЧНІ ЗМІНИ В СОМАТОСЕНСОРНІЙ КОРІ ГОЛОВНОГО МОЗКУ ЩУРІВ ПІСЛЯ МОДЕЛЮВАННЯ ГОСТРОЇ ІШЕМІЇ-РЕПЕРФУЗІЇ З НАСТУПНИМ ВВЕДЕННЯМ МЕЗЕНХІМАЛЬНИХ СТОВБУРОВИХ КЛІТИН РІЗНОГО ПОХОДЖЕННЯ, ЕМБРІОНАЛЬНИХ ФІБРОБЛАСТІВ ЩУРА ТА ЛІЗАТУ МСК ВАРТОНОВИХ ДРАГЛІВ

Мета. Оцінити вплив мезенхімальних стовбурових клітин різного походження, ембріональних фібробластів щура та лізату МСК Вартонових драглів на динаміку змін деструктивного характеру у соматосенсорній корі щурів зі штучно викликаним гострим цереброваскулярним ураженням. ***Матеріали та методи.*** Моделювання транзиторної двосторонньої ішемії-реперфузії (ІР) у щурів шляхом двостороннього накладання лігатур на внутрішні сонні артерії. Трансплантація МСК різного походження у стегнову вену експериментальним тваринам. Цитологічне дослідження через 7 та 14 днів після ІР з підрахуванням у соматосенсорній корі тварин загальної кількості ядер нейронів у 1 мм^2 і співвідношення кількості інтактних ядер нейронів до патологічно змінених ядер. ***Результати.*** Трансплантація МСК, ембріональних фібробластів щура, лізату МСК з Вартонових драглів пуповини людини та введення цитиколіну призводили до збільшення в соматосенсорній корі головного мозку щурів кількості неушкоджених нейронів і ядер без патологічних змін. Найефективнішою в плані цитоморфології була трансплантація МСК з Вартонових драглів пуповини людини. ***Висновки.*** Трансплантація МСК є ефективною при лікуванні ішемії мозку. Найефективніше попередження деструктивних змін спостерігалося в соматосенсорній корі щурів, яким після ІР трансплантували МСК з Вартонових драглів пуповини людини.

***Ключові слова*:** ішемічний інсульт, цитоморфологія, соматосенсорна кора, мезенхімальні стовбурові клітини, щури.