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ABSTRACT

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CEREBROPROTECTIVE POTENTIAL OF TRANSPLANTATION OF RAT EMBRYONIC FIBROBLASTS IN THE TREATMENT OF EXPERIMENTAL ACUTE ISCHEMIC STROKE

Introduction. Currently, ischemic stroke is one of the most common neurological diseases, characterized by high mortality and disability rates. Stem cell-based therapies, particularly embryonic stem cells, is a promising direction in the modern treatment of ischemic stroke.

Objective: To evaluate the cerebroprotective effect of rat embryonic fibroblast transplantation in acute ischemic stroke.

Methods. The study was conducted on 74 Wistar rats using a model of transient bilateral 20-minute ischemia-reperfusion by bilaterally ligating the internal carotid arteries. The animals were divided into three research groups: 1 – sham-operated animals, 2 – a control pathology group (intravenous injection of 0.9% NaCl solution post-ischemia-reperfusion), and 3 – a treatment group (intravenous transplantation of rat embryonic fibroblasts at a dose of 10^6 cells per animal, suspended in 0.2 ml of physiological saline post-ischemia-reperfusion). The effects of fibroblast transplantation were assessed based on mortality rates and neurological deficit dynamics using the McGraw Stroke-index. Hippocampal damage in the rats' brains was evaluated immunohistochemically using specific anti-NeuN antibody markers, while DNA fragmentation in hippocampal neuron nuclei was measured via flow cytometry.

Results. The obtained results showed that with subtotal cerebral ischemia (bilateral occlusion of the internal carotid arteries) in rats, first of all, significant neurodegenerative processes occur in the dentate gyrus in the CA1 area, and this leads to significant disturbances in the neurological status of the experimental animals and their high lethality. Therapy by intravenous transplantation of rat embryonic fibroblasts significantly reduced mortality rates, improved neurological status, and decreased neuroapoptosis in the hippocampus. Additionally, NeuN-

positive neuron fluorescence intensity in the CA1 hippocampal region increased more than twofold compared to the control pathology group.

Conclusions. Experimental therapy with intravenous transplantation of rat embryonic fibroblasts preserved cytoarchitectonic integrity in the pyramidal layer of the CA1 hippocampal region and significantly reduces the level of DNA fragmentation in hippocampal neuron nuclei. These effects, along with decreased mortality rates and improved neurological outcomes, highlight the neuroprotective potential of embryonic fibroblasts in ischemic stroke. These findings indicate the promising use of embryonic fibroblasts for neuroprotection in ischemic stroke, which may provide a foundation for further research in this field.

Keywords: cerebral ischemia, hippocampus, stem cells, cerebroprotection, rats.

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ЦЕРЕБРОПРОТЕКТОРНИЙ ПОТЕНЦІАЛ ТРАНСПЛАНТАЦІЇ ЕМБРІОНАЛЬНИХ ФІБРОБЛАСТІВ ЩУРА ПРИ ТЕРАПІЇ ЕКСПЕРИМЕНТАЛЬНОГО ГОСТРОГО ІШЕМІЧНОГО ІНСУЛЬТУ

Вступ. На сьогоднішній день одним із найпоширеніших неврологічних захворювань, що характеризуються високим рівнем смертності та інвалідизації, є ішемічний інсульт. Перспективним напрямком сучасного лікування ішемічного інсульту є клітинна терапія стовбуровими клітинами, зокрема ембріональними.

Мета: оцінити церебропротекторний ефект трансплантації ембріональних фібробластів щура при гострому ішемічному інсульті.

Методи. Дослідження проведено на 74 щурах лінії Вістар із використанням моделі перехідної двобічної 20-хвилинної ішемії-реперфузії шляхом білатерального накладання лігатур на внутрішні сонні артерії. Тварин було розподілено на 3 експериментальні групи: 1 – псевдооперовані тварини, 2 – контрольна патологія (внутрішньовенне введення 0,9 % розчину NaCl після ішемії-реперфузії) і 3 – група, що отримала лікування (після ішемії-реперфузії внутрішньовенно трансплантували ембріональні фібробласти щура в дозі 10^6 клітин/тварину суспендованих в 0,2 мл фізіологічного розчину). Проведено оцінку впливу трансплантації ембріональних фібробластів щура на рівень летальності та динаміку неврологічного дефіциту в щурів, який визначали за шкалою Stroke-index McGraw. Пошкодження гіпокампа головного мозку в щурів оцінювали імуногістохімічно з використанням специфічних маркерів антитіл anti-NeuN, а рівень фрагментації ДНК в ядрах нейронів гіпокампа щурів із використанням методу проточної цитометрії.

Результати. Отримані результати показали, що при субтотальній церебральній ішемії (білатеральна оклюзія внутрішніх сонних артерій) у щурів, перш за все, значні нейродегенеративні процеси виникають у зубчастій звивині в ділянці CA1 і це призводить до значних порушень у неврологічному статусі піддослідних тварин і великої їх летальності. Терапія шляхом

внутрішньовенної трансплантації ембріональних фібробластів щура сприяла достовірному зниженню рівня смертності, покращенню неврологічного статусу та зменшенню нейроапоптозу в гіпокампі щурів з ішемією-реперфузією, а також демонструвала збільшення інтенсивності флуоресценції NeuN-позитивних нейронів у CA1 ділянці гіпокампа в середньому більше ніж у 2 рази, в порівнянні з групою тварин із контрольною патологією.

Висновки. Експериментальна терапія з внутрішньовенною трансплантацією ембріональних фібробластів щура сприяє збереженню цитоархітектоніки в пірамідному шарі CA1 ділянки гіпокампа, значно зменшує рівень фрагментації ДНК в ядрах нейронів гіпокампа, що поряд із зниженням показника летальності, створює позитивну динаміку змін у неврологічному статусі тварин із церебральною ішемією. Ці результати вказують на перспективність застосування ембріональних фібробластів для нейропротекції при ішемічному інсульті, що може стати основою для подальших досліджень у цьому напрямку.

Ключові слова: церебральна ішемія, гіпокамп, стовбурові клітини, церебропротекція, щури.

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ABBREVIATIONS

DNA – deoxyribonucleic acid; RNA – ribonucleic acids; IR – ischemia-reperfusion; AU – arbitrary units; CNS – central nervous system

INTRODUCTION

Ischemic stroke is one of the major neurological diseases with high disability and mortality rate [1]. The development of ischemic brain injury involves a cascade of complex pathological processes that lead to reduced oxygen and nutrient supply to the brain, causing an energy deficit in neurons, followed by oxidative stress, inflammation, and apoptosis. However, current approaches to the treatment of ischemic stroke are based on intravenous thrombolysis, surgical thrombectomy, and neuroprotection, but only a limited number of patients have the opportunity to receive these urgent procedures [2-4]. Therefore, the development of new methods in the treatment of ischemic stroke is considered a pressing issue in modern medicine.

In recent years, a new therapeutic strategy using stem cells has gained attention in stroke treatment [5]. Current approaches to cell therapy for stroke are mostly limited to the transplantation of autologous bone marrow or adipose-derived stem cells. Embryonic stem cells demonstrate the ability to differentiate into various types of somatic cells, in particular into certain neuronal lineages, which may serve as a new cell source for cell therapy of degenerative nervous system diseases [6]. Additionally, they secrete extracellular vesicles containing DNA, RNA, and proteins [7]. The neuroprotective and neuroregenerative potential of stem cells in ischemic stroke is characterized by neurogenesis, angiogenesis, and synaptic plasticity, which positively affects functional recovery following cerebral ischemia and ischemic-reperfusion injuries [8]. However, according to experimental studies conducted to date, transplantation of stem cells in ischemic stroke still raises unresolved questions regarding its efficacy depending on cytological origin, optimal delivery routes, dosage, timing of administration after stroke onset, and transplantation frequency.

The aim of this study is to investigate the cerebroprotective effect of transplantation of rat embryonic fibroblasts on hippocampal neurons, neurological deficit dynamics and lethality in rats with acute ischemic stroke.

MATERIALS AND METHODS

The study was carried out on 74 sexually mature Wistar rats with a body weight of 160-190 g, bred in the vivarium of the National Pirogov Memorial Medical University, Vinnytsya (NPMMU). The experimental animals were housed under standard vivarium conditions, maintaining a 12-hour light/dark cycle, a temperature range of 20-26°C, and a relative humidity

of 40-70%, with unrestricted access to food and water. All procedures adhered to ethical standards and regulatory requirements for the humane treatment of laboratory animals [9, 10].

The research protocol received approval from the Bioethics Committee of NPMMU (protocol No. 2, dated January 31, 2024). Rat embryonic fibroblasts were provided by the Institute of Molecular Biology and Genetics (IMBG) of the National Academy of Sciences of Ukraine (NAS) under a Scientific Cooperation Agreement between IMBG of the NAS and the NPMMU dated September 22, 2017. The cerebroprotective properties of rat embryonic fibroblasts were studied using a model of transient bilateral 20-minute ischemia-reperfusion (IR) by bilaterally ligating the internal carotid arteries (ICAs) under propofol anesthesia (Propofol-Novo, LLC "NovoFarm-Biosynthesis", Ukraine, 60 mg/kg). The distribution of rats into experimental groups is shown in Figure 1.

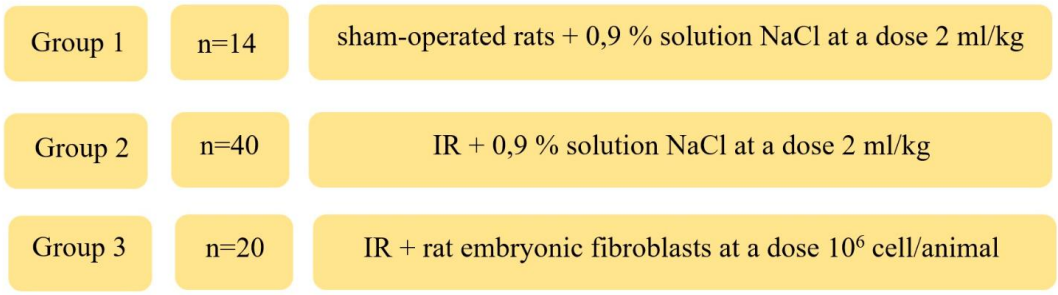


Figure 1 – *Distribution of rats in the experiment*

Immediately after IR, a single intravenous transplantation of rat embryonic fibroblasts was performed at a dose of 10^6 cells per animal, suspended in 0.2 ml of saline, as early transplantation had a better cerebroprotective effect and required fewer donor stem cells (1×10^6) [11, 12]. The effect of rat embryonic fibroblast transplantation on rat survival in a model of acute cerebral IR was assessed. On the 7th (subacute period of ischemia) and 14th (recovery period) days after the performed IR, the dynamics of neurological deficit in rats was determined using the Stroke-index McGraw scale. The experimental animals were euthanized from the experiment by humane decapitation using pentobarbital anesthesia ("Penbital", Bioveta a.s., Czech Republic, 100 mg/kg) [13]. The rats' brains were extracted and DNA fragmentation levels in the nuclei of rat hippocampal neurons was determined using the flow cytometry with a "Partec PAS" flow cytometer (Partec, Germany). A total of 20,000 events were assessed for each nuclear suspension sample. DNA fragmentation was analyzed via flow cytometry using FloMax software (Partec, Germany) by detecting Sub-G1 regions in DNA histograms. In order to carry out histoimmunochemical and morphological studies, in the corresponding observation periods (7th and 14th days post-IR), the rats' brains were fixed with 4% formaldehyde solution for 24 hours. After fixation, the brain was rinsed under running water, processed through ascending concentration of alcohols and xylenes, and embedded in Paraplast Plus© (Leica Scientific (McCormick©), USA) following standard histological procedures. Sections with a thickness of 5 μm were prepared using a YD-315 rotary microtome (Nanbei, China).

Before conducting immunohistochemical analysis, paraffin-embedded brain sections from experimental animals were first deparaffinized using xylene, followed by rinsing in a 0.1 M phosphate buffer (PB) solution. In addition, for brains that had been fixed in formaldehyde but not embedded in paraffin, 40-μm-thick coronal sections were prepared using a VT1000A vibrotome (Leica, Germany). Both paraffin and vibratome sections were incubated in a blocking solution composed of 0.1 M PB (pH 7.4), 0.5% bovine serum albumin, and 0.3% Triton X-100. Primary antibody incubation was performed at 4°C for 48 hours, followed by visualization using secondary antibodies conjugated with Alexa Fluor® 488, 594, or 647 fluorescent dyes (dilution 1:1000, Invitrogen, USA). After staining, the sections were mounted using Immu-MOUNT medium (Thermo Scientific, USA).

The labeled brain sections were analyzed under a FV1000-BX61WI confocal laser scanning microscope (Olympus, Japan) equipped with multiple laser lines, including a 543-nm helium-neon (HeNeG) laser for green fluorescence, a 633-nm helium-neon (HeNeR) laser for red fluorescence, and a multi-line argon laser emitting at 458, 488, and 515 nm.

For detecting the Mouse anti-NeuN (NEUronal Nuclei) marker (clone A60, Sigma-Aldrich, St. Louis, MO, USA; Cat. No. MAB377), sections from the CA1 hippocampal region were analyzed using the same

magnification, exposure time, and laser settings to ensure consistency. The acquired images were used to assess the fluorescence intensity of the marker, with five sections per animal being evaluated. The fluorescence signal's intensity and spatial distribution were quantified by automatically measuring the average gray value within the defined threshold. The results were expressed as the integrated fluorescence density in arbitrary units (AU), calculated by multiplying the fluorescence intensity by the fluorescence-positive area, excluding any background fluorescence signal.

Statistical processing of the study results was carried out using the Statistica 7.0 software (*StatSoft Inc.*, USA). Statistical analysis was performed using nonparametric (Mann-Whitney U-test) statistics. Differences in mean values of indicators between comparison groups were considered statistically significant at $p \leq 0.05$.

RESULTS

In the group of sham-operated rats, in which ICAs preparation was performed under propofol anesthesia and ligatures were applied without further vessel ligation, no lethal cases were recorded throughout the entire follow-up period (96 hours).

Intravenous injection of 0.9% NaCl solution to rats with modeled IR (control pathology) after ICA ligation led to a progressive rise in mortality rates (Fig. 2).

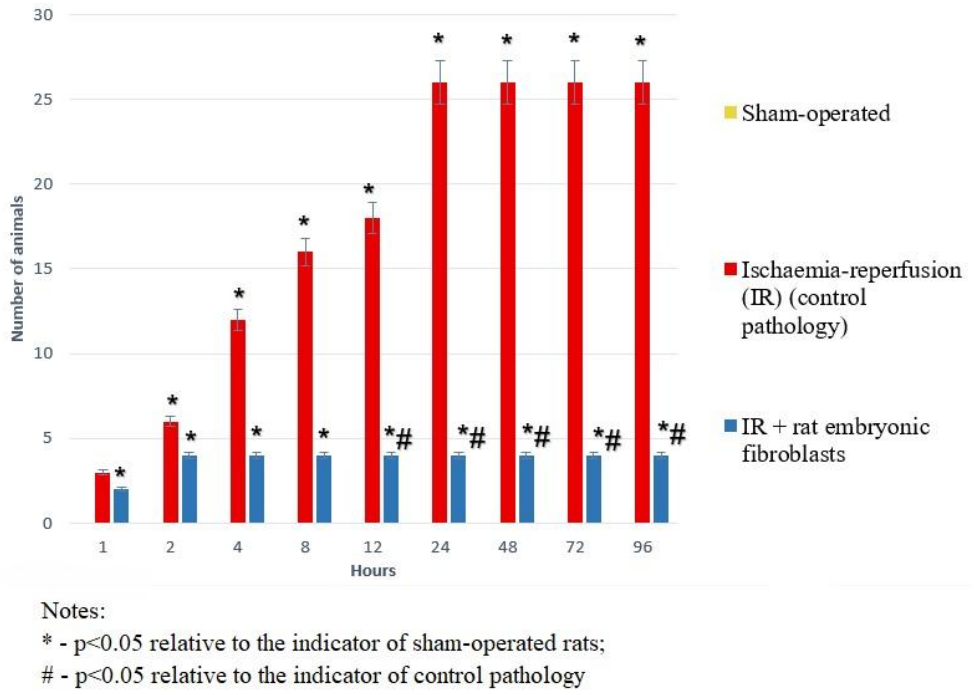
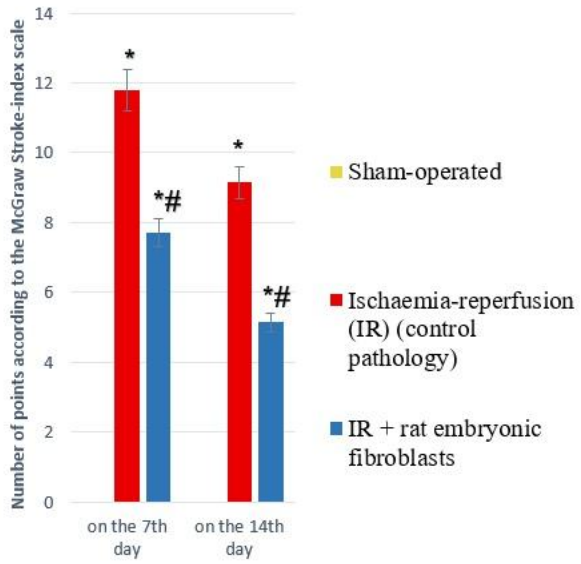


Figure 2 – *Dynamics of the mortality rate in animals with induced IR and against the background of intravenous transplantation of rat embryonic fibroblasts*

The first fatal cases of rats with induced IR were observed within the first hour post-injury. Over the next 24 hours of follow-up, the lethality of rats with acute ischemic stroke against the background under intravenous injection of 0.9% NaCl solution increased sharply and reached 65%. This negative mortality dynamics in rats with induced IR during the early hours of acute cerebral ischemia indicates the intensification of the biochemical cascade processes in brain neurons and the formation of an ischemic focus. Subsequently, over the next 3 days of the experiment, no further fatalities were recorded in this group of experimental animals.

Experimental therapy of rats with acute ischemic stroke by intravenous transplantation of rat embryonic fibroblasts provided brain protection throughout the entire follow-up period. Specifically, within the first hour, the mortality was 10%, during the second hour – 20%, and remained unchanged during the following experimental periods, being significantly lower compared to control pathology group.

An integral indicator for assessing the cerebroprotective effect of rat embryonic fibroblasts on the brain in IR-injured rats, alongside a reduction in mortality rates, is the positive progression of neurological status changes (Fig. 3).



Notes:
* - p<0.05 relative to the indicator of sham-operated rats;
# - p<0.05 relative to the indicator of control pathology

Figure 3 *– The dynamics of neurological deficit in rats according to the Stroke-index McGraw scale with modeled IR and against the background of intravenous transplantation of rat embryonic fibroblasts*

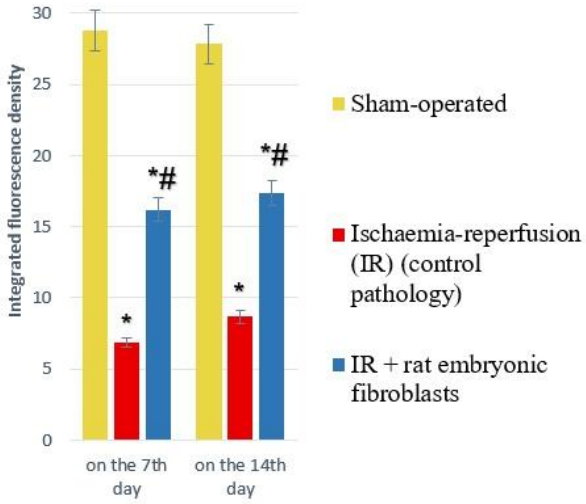
The study found that modeled IR in rats caused severe neurological changes, including paralysis, paresis, ptosis (Fig. 3). Thus, by day 7 of follow-up, in the untreated IR group, the average score on the C. P. McGraw scale was 11.8 ± 0.48 points, corresponding to a severe degree of neurological deficit. Observations on day 14 showed that this group did not exhibit complete recovery of lost CNS functions, with an average score of 9.1 ± 0.30 points on the Stroke-index McGraw scale. However, intravenous transplantation of rat embryonic fibroblasts significantly reduced neurological deficits in the subacute period to an average of 7.7 ± 0.28 points ($p < 0.05$) and in the recovery period to an average of 5.1 ± 0.19 points ($p < 0.05$), compared to control pathology group.

During the immunohistochemical examination utilizing neuronal markers (anti-NeuN antibodies), it was found that the frontal sections of sham-operated animals exhibited NeuN-positive pyramidal neurons in the hippocampus with strong fluorescence. These neurons were arranged in a compact layer within the *stratum pyramidale*, comprising 3 to 5 neuronal layers.

Assessment of the cytoarchitectonic structure of the rat brain following IR through immunohistochemical analysis demonstrated that the CA1 hippocampal region was particularly susceptible to ischemic-reperfusion injury. By the 7th and 14th days post-IR, a marked reduction in fluorescence intensity of NeuN-positive neurons in the CA1 hippocampal region was observed.

Most NeuN-positive neurons in the pyramidal layer of the CA1 region exhibited markedly reduced fluorescence intensity than in the group of sham-operated animals. The number of intact NeuN-positive pyramidal cells demonstrating maximal fluorescent staining was minimal.

On days 7 and 14 after IR, therapeutic transplantation of rat embryonic fibroblasts enhanced the fluorescence intensity of NeuN-positive neurons compared to group 2 (IR), but it did not reach the levels observed in the sham-operated group 1 (Fig. 4).



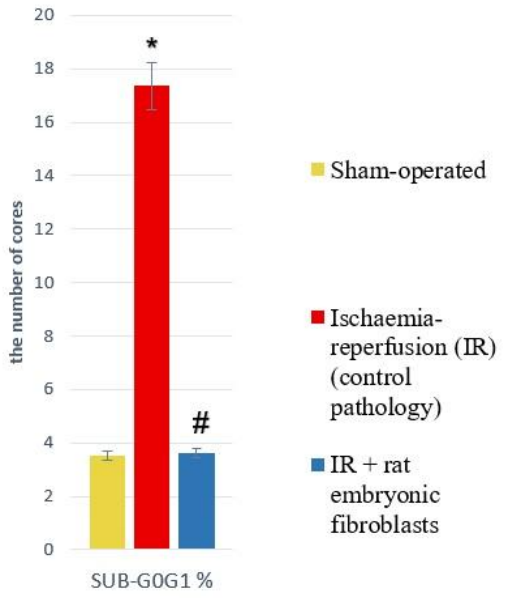
Notes:
* - p<0.05 relative to the indicator of sham-operated rats;
# - p<0.05 relative to the indicator of control pathology

Figure 4 *– Integral density of fluorescence of NeuN-positive neurons on frontal sections of the CA1 hippocampal region. The x-axis represents the experimental groups (1-3) on days 7 and 14 following IR*

The statistical evaluation of NeuN-positive neuron fluorescence intensity revealed that IR led to a reduction in fluorescence by a factor of 4.2 on day 7 and 3.2 on day 14 compared to group 1 (sham-operated animals), with values of $6.86 \times 10^6 \pm 0.96$ AU and $8.67 \times 10^6 \pm 0.82$ AU, respectively (Fig. 5). Experimental treatment with intravenous transplantation of rat embryonic fibroblasts resulted in a statistically significant enhancement of fluorescence intensity compared to group 2 (IR group).

Specifically, in this experimental group, the integral fluorescence density of NeuN-positive neurons reached $16.21\times10^6\pm1.50$ AU (day 7) and $17.36x10^6\pm1.18$ AU (day 14), but it did not reach the values registered in the sham-operated rat group: $28.79x10^6\pm2.17$ AU (day 7) and $27.84x10^6\pm1.64$ AU (day 14) (Fig. 4).

Earlier research [14] demonstrated that intravenous transplantation of rat embryonic fibroblasts stimulates hippocampal neuron protection and attenuates destructive changes, which allows to reduce the secondary ischemic focus. A key indicator of neuroapoptosis is nuclear DNA fragmentation. Therefore, in conditions of post-reperfusion cerebral ischemia using the IR model, it was relevant to characterize the influence of transplantation of rat embryonic fibroblasts on neuroapoptotic changes in the rat hippocampus. Flow cytometry, a widely recognized method for assessing neuronal DNA fragmentation, was utilized for this purpose. The findings indicated that in the untreated pathology control group (IR without intervention), the level of DNA fragmentation in hippocampal neuron nuclei significantly increased 4.9 times on day 7 after the modeling of cerebral IR (Fig. 5).



Notes:
* - p<0.05 relative to the indicator of sham-operated rats;
# - p<0.05 relative to the indicator of control pathology

Figure 5 – *Dynamics of nuclear DNA fragmentation of hippocampal neurons in rats with cerebral IR and under therapeutic transplantation of rat embryonic fibroblasts*

This may indicate an intense formation process of the ischemic focus due to neurons undergoing necrosis and those in the ischemic penumbra, where neurons are in a state of apoptotic death. Experimental therapy with intravenous transplantation of rat embryonic fibroblasts in animals with modeled IR significantly reduced DNA fragmentation in hippocampal neuron nuclei to an average of 3.63±0.56%, compared to the control pathology group of rats – 17.35±1.97% ($p < 0.05$) and almost reached the DNA fragmentation level in sham-operated animals – 3.52±0.14% (Fig. 5). Thus, the suppression of the intensity of neuroapoptosis in the hippocampus of rats under the influence of rat embryonic fibroblasts indicates a decrease in the ischemia focus due to the preservation of morphologically intact neurons in the ischemic penumbra zone. This effect is one of the leading mechanisms of the cerebroprotective action of rat embryonic fibroblasts in post-reperfusion brain injury.

DISCUSSION

Numerous studies on animal models and a limited number of clinical trials have been devoted to assess the therapeutic effect of various stem cells types, particularly embryonic stem cells, as a treatment option for ischemic-reperfusion brain injury [15]. Stem cell therapy of cerebral ischemia is a promising strategy due to their neuroprotective and regenerative potential [16]. However, the mechanisms by which transplanted stem cells exert their influence the brain with ischemic-reperfusion injury remain unknown and require further investigation. Embryonic stem cells are regarded as an optimal candidate for cell-based therapy in treating neurodegenerative conditions. Thus, in this study, it was established that intravenous transplantation of rat embryonic fibroblasts provided brain protection in rats with modeled IR throughout the entire study period, particularly, increasing the survival rates with a reduction in the dynamics of neurological disorders in experimental animals compared to rats subjected IR without specific treatment. Morphological study [14] and immunohistochemical analysis confirmed that the CA1 hippocampal region was most vulnerable to ischemic-reperfusion injury. During the studied observation periods following IR, the fluorescence intensity of NeuN-positive neurons in the CA1 hippocampal region significantly decreased by 4.2 (on day 7) and 3.2 times (on day 14) compared to the group of sham-operated rats. Findings from the study by N. Yavari et al. (2022) [17] and the experiment by R. Montoya-García et al. (2023) [18] indicated that hippocampal neurons exhibit a distinct susceptibility to ischemia, particularly in the context of impaired cerebral circulation, compared to other brain cell types. In instances of global cerebral ischemia (occlusion of a

major artery, cardiac arrest, etc.) or under conditions of severe hypoglycemia, significant neurodegenerative processes primarily affect the dentate gyrus in the CA1 hippocampal region.

Therapeutic intravenous transplantation of rat embryonic fibroblasts demonstrated a statistically significant increase in the intensity of fluorescence of NeuN-positive neurons in the CA1 hippocampal region, more than doubling in comparison to the IR group that did not receive treatment. These results suggest that rat embryonic fibroblasts possess neuroprotective properties and can protect hippocampal neurons from ischemic-reperfusion injury.

The next stage of our study established that after 20-minute cerebral ischemia followed by reperfusion, the intensity of neuronal death in the hippocampus of rats increased sharply. It is well-established that acute disturbances in cerebral circulation lead to the formation of an ischemic focus, characterized by destructive-degenerative alterations in neural tissue architecture. This process results in the development of an infarct core, where cells undergo rapid necrosis, and a surrounding penumbra, where cells experience milder ischemia and remain viable for a limited period, albeit with impaired functionality [19]. In the ischemic core, a rapid decline in blood flow occurs within minutes of an ischemic event, leading to irreversible damage and necrotic cell death affecting neurons, glial cells, and endothelial cells. At the same time, the penumbral zone undergoes a more gradual process, which may extend over hours or days, culminating in cell death primarily through apoptosis [20]. One key indicator of neuroapoptosis is nuclear DNA fragmentation, which we assessed using flow cytometry. Thus, on day 7 after the modeled IR, the intensity of DNA fragmentation in hippocampal neuron nuclei in the control pathology group of rats increased by 4.9 times. Therapeutic intravenous transplantation of rat embryonic fibroblasts significantly decreased the level of DNA fragmentation in the nuclei of rat hippocampal neurons by an average of 4.8 times. The reduction in neuroapoptosis intensity in the rat hippocampus after intravenous transplantation of rat embryonic fibroblasts indicates the prevention of destructive-degenerative changes in the cytoarchitecture of nervous tissue. This serves as further evidence of the cerebroprotective effect of rat embryonic fibroblasts in acute ischemic stroke therapy.

CONCLUSIONS

A 20-minute bilateral reversible occlusion of the ICAs was accompanied by destructive-degenerative changes in the cytoarchitectonics of the rat hippocampus, leading to subsequent neuronal death due to necrosis and apoptosis, as well as severe neurological disorders, ultimately resulting in animal mortality. Cell therapy with intravenous transplantation of rat embryonic fibroblasts was effective in protecting the nervous tissue of the rat hippocampus from ischemic-reperfusion injury, as evidenced by flow cytometry and immunohistochemical analysis. The use of rat embryonic fibroblasts for therapeutic purposes in a modeled IR led to the normalization of the neurological status of the experimental animals and contributed to their survival.

PROSPECTS FOR FUTURE RESEARCH

Given the experimental effectiveness of embryonic stem cells in the therapy of cerebral ischemia, transplantation of these cells (perhaps in combination with other stem cells) may be utilized in future clinical programs for the ischemic stroke treatment.

AUTHOR CONTRIBUTIONS

Serhii Konovalov – conception and design, the acquisition, analysis and interpretation of data for the work, manuscript writing, agreement to be accountable for all aspects of the work.

Vasyl Moroz – substantial contributions to the conception or design of the work, critical review of manuscript, final approval.

Mykhaylo Yoltukhivskyi – drafting the work and revising it critically for important intellectual content, critical review, final approval.

Nataliia Gadzhula – analysis and interpretation of data, drafting the work, review, correspondence with journal.

Olena Cherepakha – data analysis, manuscript preparation, review of literature.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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