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EFFECT OF NEURAL STEM CELL TRANSPLANTATION ON COGNITIVE FUNCTIONS OF MICE AFTER CEREBRAL ISCHEMIA-REPERFUSION

ABSTRACT

This study is aimed to determine the effect of transplantation of neural progenitor cells (NPCs) isolated from fetal hippocampus on cognitive functions of experimental animals after short-term global cerebral ischemia. NPCs were isolated from hippocampus of FVB-Cg-Tg(GFPU)5Nagy/J mice, transgenic by the GFP. Ischemic brain injury in FVB «wild» type mice was modeled by bilateral occlusion of the common carotid arteries for 20 min. GFP-positive NPCs were stereotaxically transplanted into the hippocampus of experimental animals in 24 hours after ischemia-reperfusion. Cognitive functions were evaluated using Morris water maze. Results of this study showed that global short-term cerebral ischemia resulted into cognitive impairments in mice. Stereotaxic transplantation of NPCs promoted the cognitive function recovery in experimental animals after ischemic brain injury. Thus, the data indicates that transplantation of NPCs may have a therapeutic effect in treating of ischemic stroke.

KEYWORDS: cerebral ischemia, hippocampus, cognitive functions, neural stem cells, transplantation.

Ischemic brain lesions are one of the leading causes of disability and mortality in the elderly. According to WHO, stroke is the second most frequent, after cardiovascular diseases, cause of death. Due to the increased level of morbidity from atherosclerosis, hypertension, diabetes mellitus, stroke is more common in people of working age [12]. Severe hypoxic-ischemic encephalopathy in newborns can cause the development of cerebral palsy [22]. It is known that cerebral stroke leads to a number of emotional disorders (irritability, increased excitability and anxiety, etc.) [3]. In experimental models of global cerebral ischemia has been shown that ischemic brain damage in animals causes the memory and learning ability disorders, hyperthermia, and a significant increase in locomotor activity [1, 14, 23].

It is believed that the effects of ischemic damage on nervous tissue can be compensated by activation of their own reparative mechanisms or correction through various therapeutic methods [18]. Recently the possibility of using stem cell therapy for the treatment of ischemic and degenerative diseases of the neural system has been actively studied [28]. It is planned to use stem cells from different sources and various differentiation potential (from embryonic and fetal cells to the cells of an adult organism), as an active agent [19, 25]. The basis of these studies is the theoretical and experimental developments that indicate the ability of stem cells to differentiate into neuronal or glial cells, compensating

for lost tissue function. For example, the use of fetal neural stem cells (NSCs) suggests their differentiation into specialized cells of the central neural system [8, 13]. During embryogenesis, NSCs are located in the ventricular zone of the neural tube, and are able to differentiate into all cell types, which are necessary for the formation of the CNS. It was thought that neurogenesis occurs only during embryonic development of an organism. Recent studies have shown that new neurons are continuously generated from the NSCs throughout life [7, 29]. This neurogenesis in adult mammals occurs in certain areas of the brain, namely subgranular zone (SGZ) of hippocampal dentate gyrus and subventricular zone (SVZ) of lateral ventricles.

Also we know that stem cells are able to recognize areas of damaged tissue, migrate toward these areas, and differentiate into cell type needed to restore lost function [6, 11, 30, 31]. To investigate the possible regenerative potential of NSCs there are widely used various models of ischemic brain damage with subsequent transplantation [7, 16, 21].

However, the introduction of stem cell transplantation methods into clinical practice should be based on a profound understanding of their functioning mechanisms, and sufficient experimental data. The study of selected range of issues is possible under experimental transplantation involving adequate models of pathology in laboratory animals. Experimental ischemic brain lesions in mice allow simulating

the effects of ischemic stroke in humans [4, 9, 10]. Transplantation of cells of different origin with various differentiations potential aims at study of their regenerative potential, and general functioning principles in restorative processes after ischemic damage.

Considering all of the above, the aim of this study was to investigate the effect of fetal neural progenitor cells transplantation on cognitive function of experimental animals after short-term global cerebral ischemia.

MATERIALS AND METHODS

All experiments were performed in accordance with international principles of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (European convention, Strasburg, 1986), article 26 of the Law of Ukraine «On protection of animals from cruelty» (№ 3447-IV, 21.02.2006), and all norms of bioethics and biosafety.

In our study, we used *FVB* «wild» type and *FVB-C-Tg(GFPU)5Nagy/J* mice, transgenic by the green fluorescent protein gene (GFP). The mice were kindly provided by the *European Molecular Biology Laboratory* (*Monterotondo*, Italy).

Pubertal male *FVB* «wild» type mice (12 weeks) were randomly assigned to one of three groups: A – control (n = 5) – sham-operated animals without ischemia/reperfusion injury and neural progenitor cells transplantation (*NPCs*); B – mice (n = 13) with *NPCs* transplantation after ischemia/reperfusion; and C – animals (n = 5), which after ischemia/reperfusion were injected with the *NPCs's* cultivation medium.

ISOLATION AND CULTURE OF NPCs

The *FVB-Cg-Tg(GFPU)5Nagy/J* mice, transgenic by *GFP* gene, were used as donors of neural progenitor cells. The source of *NPCs* was hippocampus isolated in sterile conditions from the fetus brain on 17-18 days of embryonic development. Fetal nervous tissue was mechanically dissociated in the *Neurobasal* medium with Pasteur pipettes of different diameters. After the dissociation, a cell suspension was passed through nylon cell strainer (*Falcon*, USA) with a pore diameter of 40 µm. A purified fraction of *NPCs* was obtained by centrifugation of cell suspension in density gradient (22% solution of Percoll). The percentage of viable cells in suspension was determined by flow cytometry, using a cell sorter *FACSAria* (*Becton Dickinson*, USA), after incubation of cell suspension with 7-amino-actinomycin D (*7-AAD*).

To analyze the phenotype of *NPCs* there was used an immunocytochemical staining of cells in culture. For this purpose, freshly isolated cells were seeded on cover glasses coated with *Matrigel* (4•10^5 cells per 35-mm cultural dish). *NPCs* were cultured in *Neurobasal* medium (*Invitrogen*, USA) with 20 ng/ml human recombinant *FGF-2* (*R&D*). On the third day of cultivation, the culture medium was replaced with cold of 4% paraformaldehyde (*PFA*), and after fixation immunocytochemical staining of cell culture was performed.

IMMUNOCYTOCHEMICAL STAINING OF NPCs CULTURE

After washing off the PFA, cells culture was blocked in a 0,1 M phosphate buffer (pH = 7,4) supplemented with 0,5 % bovine serum albumin (*BSA*) and 0,3 % *Triton X-100*. Marker of neural progenitor cells nestin was used to characterize the phenotype of the cell culture. For this purpose cell culture was incubated in a solution of primary mouse anti-nestin monoclonal antibodies (*Chemicon*, USA) for 12 hours at 4 ^{0}C. Primary antibodies were visualized by anti-mouse secondary antibodies conjugated with fluorochrome *Alexa Fluor 555* (*Invitrogen*, USA). The nuclei of cells were contrasted with fluorescent dye *Hoechst33342* (*Invitrogen*, USA). Dyed cell culture was covered with medium *Immu-*

MOUNT (*Thermo Scientific*, USA). Immnocytochemicaly stained culture of NPCs was analyzed using a confocal scanning microscope *FV1000-BX61WI* (*Olympus*, Japan).

MODELING OF HIPPOCAMPAL ISCHEMIC INJURY

Ischemic injury of the hippocampus in *FVB* «wild» type mice was modeled by bilateral occlusion of the common carotid arteries for 20 min. The animals were anesthetized with 2,2,2-tribromoethanol (125-240 mg/kg, intraperitoneally). Then, under the surgery stereomicroscope, the common carotid arteries were isolated; and atraumatic elastic vascular micro-clamps were applied on them. After 20 minutes the clips were removed and the wound was sutured in layers under aseptic condition. In sham-operated animals of the control A group only artery preparation was done and they were under anesthesia for 20 minutes without application of clips.

MEASUREMENT OF REGIONAL CEREBRAL BLOOD FLOW

To assess a regional cerebral blood flow (*rCBF*) and confirm ischemic conditions after bilateral occlusion of the carotid arteries there was used a laser Doppler flowmeter (*moor-VMS-LDF-1*, *Moor Instruments*, UK). *rCBF* was controlled before and during occlusion, then straight after reperfusion. The data were analyzed using the software *moorLAB* (*Moor Instruments*, UK). In next studies, we used only animals, which *rCBF* fell below 15 % of normal baseline before occlusion.

TRANSPLANTATION OF HIPPOCAMPAL NPCs

Syngeneic transplantation of 2-2,5•10^5 *GFP*-positive *NPCs* in volume of 2 µl of medium were injected into the hippocampus of animals with ischemic model stereotaxically (coordinates from the bregma: lateral ± 1,5 mm, posterior – 2,0 mm, dorsoventrally 1,7 mm) under combined 2,2,2-trybromoethanol anesthesia (125 mg/kg, intraperitoneally). Sham-operated animals were injected with the *NPC's* culture medium in the same location.

BEHAVIORAL TEST

To assess cognitive functions (spatial memory) there was used the Morris water maze (*MWM*). The role of *MWM* performed a black round pool (150 cm in diameter and 60 cm height), containing transparent plexiglass platform [24]. During the experiment the platform remained in a constant location. Water temperature was 28°C. The walls of the pool were marked with high contrast geometric images (squares, stars, triangles). Such external visual cues contributed to the formation of the idea about spatial location of platform in animals. The trajectory and time of animal swimming were monitored with a digital video camera. Testing of animals in the   to check their ability of executing the tasks related to spatial memory was performed according to the procedure adapted for mice [20]. For four consecutive days (from 12 to 15 days after ischemia) there were performed behavioral tests. During these tests the mice were trained to find the platform, oriented on the external visual cues. Each session consisted of 2 cycles (4 attempts) per day, during which the mice were placed into the pool, and had 60 seconds to find the platform. For each animal of all experimental groups there was determined a parameter «time of platform searching» (an average of 8 attempts at the time, in seconds), spent by the mouse to find the platform in a particular day of the study. If the mouse did not find the platform within 60 seconds, it was placed on the platform for memorizing its location. On the fifth day of testing (16 days after ischemia), transparent platform was submerged for a few millimeters from the water surface; and each mouse was placed into the pool to find a hidden platform.

STATISTICAL ANALYSIS

Statistical data analysis was performed, using the software *Statistica* (version 5, *StatSoft*). Values are presented as mean value ± standard error of the mean. Nonparametric Kolmogorov-Smirnov test was used to assess differences between the values.

RESULTS AND DISCUSSION

To study the phenotype of hippocampal neural progenitor cells culture, we used the immunocytochemical method. Immunocytochemical analysis showed that at the third day of cultivation *NPCs* were mostly rounded or had bipolar shape and thin spines. The majority of cells (95,2%) expressed marker of immature cells – nestin **(Fig. 1)**, which is typical for type-2 hippocampal progenitor cells [17]. These data indicate that the cells, which we used for transplantation, had properties of neural progenitor cells.

The effect of ischemic brain injury on cognitive function and possible neuroprotective effect of neural stem cells transplantation in our study were assessed, using behavioral tests – Morris water maze.

The animals from each experimental group were tested for 5 days from the 12th day after surgery. The time spent on searching the platform during the first 4 days of testing decreased in animals of all experimental groups. Despite the reduction in time spent to search the platform in all groups, this parameter in the group of animals with ischemia was significantly greater than that in sham-operated mice (control) and in the group of animals with ischemia plus *NPCs* transplantation. In animals of the control group time of platform search was 35,3 ± 3,4 sec on the 12th day, and then decreased to 20,3 ± 2,1 sec on the 15th day **(Fig. 2)**. Compared with the control, ischemic animals had higher scores, indicating a lack of spatial memory and learning ability. The time of platform search in this animal group also decreased from 12th day to 15th day and reached 48 ± 2,7 sec and 32 ± 2,3 sec, respectively **(Fig. 2)**.

In the group of animals with *NPCs* transplantation after ischemic brain damage more rapid regression of cognitive impairment was observed, including reduce of the deficit of spatial memory and learning ability. Search time in this group decreased from 12th day to 15th day and was 42 ± 1,5 sec and 22 ± 1,0 sec, respectively **(Fig. 2)**.

On the 16th day, when the platform was submerged under the water, the time of platform search increased in all groups of animals, and amounted 24,3 ± 2,7, 36 ± 2,3 and 28,8 ± 1,4 sec in control, ischemic and the group with ischemia plus *NPCs* transplantation, respectively. There were no statistically significant differences between control animals and a group of «transplant ischemia plus *NPCs*» at all time intervals.

To assess cognitive function of experimental animals there have been developed many behavioral tests, but the water maze, designed by Richard Morris, is one of the most widely used behavioral tests for studying spatial learning and memory [24, 27]. Successful execution of the *MWM* test by animals is associated with long-term potentiation (*LTP*) and *NMDA*-receptor function, making this test the basic approach in the study of hippocampal neural networks [2, 15]. Our results indicate that short-term global cerebral ischemia resulted in a disorder of spatial learning in experimental animals. We observed recovery of cognitive functions in two weeks after *NPCs* transplantation. Even if two weeks is sufficient for differentiation of transplanted progenitor cells into neurons and glial cells this term is not sufficient for donor cells integration into existing neural hippocampal networks of the recipient [26]. We consider that recovery of spatial learning and memory in experimental animals may be due to the secretion of trophic and mitogenic factors of the transplanted cells, which contributed to the survival of damaged cells and restoration of their lost functions. Several studies have shown that restoration of ischemic neurological status of animals in the early period after stem cell transplantation is likely not due to the replacement of damaged cells with new transplanted ones but to donor cell secretion of various growth factors [5, 32].
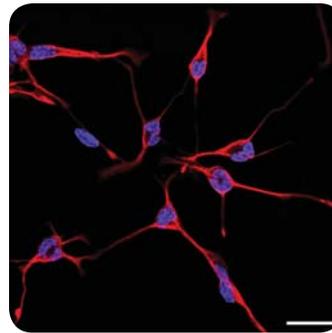

Fig. 1. Immunocytochemical staining of neural stem cells culture for nestin. Most cells were positive for nestin (red). Nuclei were contrasted by Hoechst 33342 (blue). Scale = 20 µm.
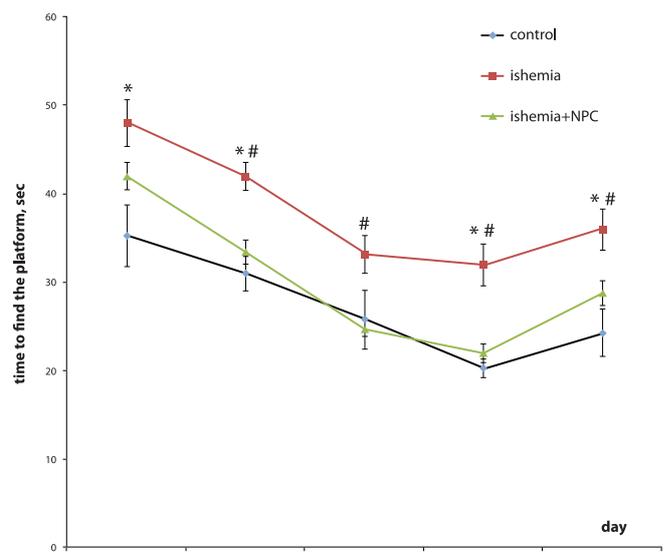

Fig. 2. The time of platform search in the Morris water maze. The chart shows the average time of platform search by sham-operated animals (control), ischemic mice (ischemia), and group of ischemic animals with transplanted NPCs (). * – p <0.05 compared with control group, # – p<0,05 compared with the group of animals with ischemia plus NPCs transplantation.

CONCLUSIONS

THUS, STEREOTAXIC TRANSPLANTATION OF NEURAL PROGENITOR CELLS CONTRIBUTES TO THE RESTORATION OF COGNITIVE FUNCTION (SPATIAL MEMORY) IN ANIMALS AFTER ISCHEMIC BRAIN INJURY, MODELED BY BILATERAL OCCLUSION OF THE COMMON CAROTID ARTERY.

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