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The effects of fetal neural cell conditioned medium on cell proliferation in the rat brain after traumatic brain injury

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ABSTRACT

Traumatic brain injury (TBI) is accompanied by an increase in the number of proliferating cells. However, the question of the nature, conditions of production and mechanisms of action of humoral factors secreted by fetal neural cells (FNCs) on reparative processes and neurogenesis in the brain after trauma and FNCs transplantation remains open.

*The **PURPOSE** of the study was to establish the possibility of the influence of the conditioned medium of fetal neural cell cultures on the proliferative activity of Ki-67-positive cells in the cortex and subcortical structures of the rat brain after TBI.*

***MATERIALS AND METHODS.** TBI was simulated by dropping a metal cylinder on the rat's head. Rats (E17-18) were used to obtain cultures of neural stem/progenitor cells. Conditioned media from cell cultures with high adhesive properties (HA-CM) and low adhesive properties (LA-CM) were used to treat the effects of experimental TBI in rats by intramuscular injection. The effect of conditioned media on the proliferative activity of Ki-67-positive cells in the cortex and subcortical structures of the brain after TBI was determined by immunohistochemical analysis using antibodies against Ki-67 protein.*

***RESULTS.** Immunohistochemical analysis of the brain sections showed that on the 5th day after traumatic brain injury in rats there was a statistically significant increase in the number of Ki-67-positive cells in the cortex, hippocampus and thalamus. It was found that the injection of HA-CM or LA-CM in animals with TBI increased the number of Ki-67-positive cells in the hippocampus compared with the TBI group and their value for the TBI+LA-CM group reached 59.6 ± 6.1, and for the TBI+HA-CM group - 47.2 ± 3.1 cells ($p < 0.05$ compared with the TBI group). In the cortex and thalamus, the number of Ki-67-positive cells in contrast decreased compared with the group of animals with TBI and for the group TBI+LA-CM was 20.2 ± 1.6 and 12.0 ± 1.7, respectively, and for the group TBI+HA-CM - 25.3 ± 2.1 and 13.3 ± 1.3, respectively.*

***CONCLUSIONS.** The administration of conditioned media from fetal neural cell cultures with high or low adhesive properties into animals with traumatic brain injury increases the number of Ki-67-positive cells in the hippocampus, possibly associated with increased neurogenesis, and decreases in the cortex and thalamus, which may be due to a weakening of reactive gliosis.*

***KEY WORDS**: traumatic brain injury; fetal neural cells; conditioned medium; immunohistochemistry; proliferative activity*

Traumatic brain injury (TBI) is accompanied by primary and secondary damage to brain cells, which leads to the development of neurological deficits, cognitive impairment and the possible development of disability [1]. In parallel with these pathological processes in the brain, the activation of neurogenesis is registered, which is manifested by an increase in the number of cells with signs of proliferation (expressing the Ki-67 marker) and differentiation (expressing specific markers of neural cells) [2, 3]. The activation of neurogenesis after TBI, which is manifested by an increase in the number of Ki-67- or PCNA-positive cells (PCNA - proliferating cell nuclear antigen), may persist for a year, especially in the hippocampus, despite progressive atrophy of brain tissue, neuronal death and ventricular dilation [4]. In intact animals, after one year of observation, the activation of neurogenesis in the hippocampus was not determined, which indicates long-term activation of neurogenesis after trauma [4].

In parallel with neurogenesis in the area of damage and adjacent areas of the brain, reactive astrogliosis and active angiogenesis, which are manifested by an increase in the number of Ki-67-positive cells of astroglia and endothelium, develop [2-4].

After TBI in mice, the number of new granular neurons derived from endogenous stem cells doubles every 3 days, but after 30-40 days this leads to the depletion and decrease in the number of stem cells in neurogenic niches of the brain such as the hippocampus and subventricular area, which promotes rapid aging or atrophy as in leukemia [5-7]. Increased formation of new neurons after TBI can not only cause their integration with brain neurons, but also lead to seizures, the development of epilepsy and inhibition of neurogenesis [2, 8, 9]. The inhibition of neurogenesis after trauma by various factors, such as growth factor inhibitor SU1498, causes a decrease in cell proliferation [2].

The above research results indicate the possibility of targeted correction and inhibition of increased proliferative activity of endogenous stem cells in neurogenic niches of the brain after trauma [10, 11].

To correct disorders after trauma, it is proposed to use different types of stem cells and their humoral factors (growth factors, cell exosomes) [12-14]. The therapeutic effect of stem cell transplantation after trauma is due not only to their replacement of damaged nerve tissue, but also to the fact that transplanted stem cells secrete factors that through paracrine effect or intercellular interaction can encourage proliferation of their own stem cells after brain trauma [15, 16].

The cells derived from fetal nerve tissue occupy a special place among the different types of neural stem cells. Fetal neural cells are quite easy to obtain in a sufficient therapeutic dose; they have low immunogenicity and are used in clinical trials in various pathologies of the nervous system [17, 18]. However, the question of the nature, conditions of production and mechanisms of action of humoral factors secreted by cultured fetal neural cells on reparative processes and neurogenesis in the brain after TBI remains open.

The **PURPOSE** of the study was to establish the possibility of the influence of the conditioned medium of fetal neural cell cultures on the proliferative activity of Ki-67-positive cells in the cortex and subcortical structures of the brain of rats after trauma.

MATERIALS AND METHODS

Nonlinear sexually mature (age - 4-5 weeks) rats of both sexes weighing 140-160 g (n = 37) from the vivarium of A. P. Romodanov State Institute of Neurosurgery of National Academy of Medical Sciences of Ukraine were used in the work. All work with experimental animals was carried out in compliance with the requirements of the Law of Ukraine №3447 IV "On protection of animals from cruelty ", "European Convention for the protection of vertebrate animals used for research and other scientific purposes" (Strasbourg, 1986), taking into account the principles of bioethics and biosafety standards. Animals were kept in standard conditions of the accredited vivarium. Euthanasia of experimental animals was performed by an overdose of ether for anesthesia. The study was approved by the Commission on Ethics and Bioethics of A. P. Romodanov State Institute of Neurosurgery of National Academy of Medical Sciences of Ukraine (Protocol № 26 of May 11, 2018).

Modeling of TBI in rats. Traumatic brain injury in rats was modeled according to the recommendations of Romanova G. O. and Biloshitsky V. V. [19, 20]. Before simulating TBI, rats were anesthetized intraperitoneally with 0.5 mL of a mixture of Ketamine (70.0 mg/kg) and Sedazine (15 mg/kg). Traumatic brain injury was simulated by dropping a metal cylinder (weighing 100 g) from the height of 120 cm on the rat's head. The animal's head was placed on a 5 cm thick foam cushion under a 120 cm high plastic tube 2.5 cm in diameter so that the metal cylinder hit the left hemisphere in the frontal and parietal areas of the brain. After the injury, the rats showed reflexively delayed breathing for a few seconds, minor cramps in the lower extremities and tail lift, which disappeared within 5-10 seconds. After 20-30 minutes the animals recovered from anesthesia and began to move and were returned to their cage.

Obtaining and cultivating NSC/NPC. Ether-anesthetized female rats (n = 14) during euthanasia (17-18 dpc) were euthanized by cervical dislocation under ether anesthesia. Under sterile conditions, brain tissue was obtained from 4-6 fetuses, washed in DMEM medium (*Sigma-Aldrich*, USA), freed from vessels and membranes, transferred to fresh nutrient medium and mechanically dissociated by repeated pipetting [21]. The resulting cell suspensions were pelleted by centrifugation for 5 min at 300 xg, washed in DMEM medium. Fresh DMEM medium was added to the cell pellet and resuspended. The obtained cells were transferred into plastic 50 mm Petri dishes at $4 \cdot 10^6$ cells and cultured in a CO_2 incubator (*Nuve*, Turkey) in humidified air (95 % humidity) with 5 % CO_2 at a temperature of +37 °C in a complete nutrient medium DMEM (*Sigma*, USA) with the addition of 10% fetal calf serum (*Sigma*, USA) and 80 μg/mL gentamicin (*Sigma,* USA). After 24 hours of incubation, the cell culture was divided into 2 groups. Cells with low adhesive properties were selected in separate tubes and precipitated by centrifugation. The pellet of these cells was resuspended in fresh DMEM medium, transferred to new plastic Petri dishes at $4 \cdot 10^6$ cells and cultured in a CO_2 incubator for 48 hours in 5.0 mL of DMEM medium with antibiotics, but without fetal calf serum. The second part of the cells that adhered to the plastic (cells with high adhesive properties) was cultured in 5.0 mL of fresh DMEM medium without fetal calf serum in a CO_2 incubator for 48 hours. *In vivo* observation of cell cultures with microphoto registration was performed twice a day during the entire time of cultivation using an inverted microscope TS-100 (*Nikon*, Japan).

After culturing, conditioned media (CM) were taken out of Petri dishes with cell cultures with high adhesive properties (HA-CM) and low adhesive properties (LA-CM). Cell debris was separated from the obtained media by centrifugation at 3000 rpm for 10 minutes; the protein level was determined by spectrophotometric method and frozen at -20 °C in cryovials for further use. In both types of conditioning medium (HA-CM and LA-CM) the protein content was determined by the conventional spectrophotometric method by the density of the solution at a wavelength of 260 nm and 280 nm on a spectrophotometer SF-24 (*Lomo*, Russia) in a cuvette 1.0 cm thick with a comparative nomogram [22]. HA-CM was found to contain 0.28 mg/mL and LA-CM – 0.26 mg/mL protein.

Defrosted conditioning media were used to treat the effects of experimental TBI in rats. CM was administered intramuscularly to animals at a dose of 1.0 mL on the 2nd, 3rd, 4th day after the injury. Animals were randomly divided into 4 groups: group 1 (n = 5) – intact animals, which did not undergo experimental interventions; group 2 (n = 6) – animals with TBI, which were treated with 1.0 mL of DMEM medium (comparison group); group 3 (n = 6) – animals with trauma, which were treated with LA-CM; Group 4 (n = 6) – animals with trauma, which were treated with HA-CM. On the 5th day after TBI, euthanasia of the animals was performed by an ether overdose and the brain was isolated for immunohistochemical study.

Immunohistochemical analysis of brain sections. The brain was fixed in 4 % formaldehyde and poured into paraffin blocks by standard histological methods. Sections 4-6 mm thick were made using a microtome Microm HM-430 (*Thermo Fisher Scientific*, USA). Sections were placed on Histobond+M adhesive slides (*Marienfield*, Germany) for further immunohistochemical staining. Restoration of antigenicity was performed in a DAKO PT Module in High pH buffer (*DAKO*, Denmark). The recovery time at a temperature of 98 °C was 40 minutes. Brain sections were incubated with monoclonal murine antibodies against Ki-67 clone MIB-1 (*DAKO*, Denmark) at a titer of 1: 500 for 30 minutes at room temperature. After washing the sections in buffer, the primary antibodies were visualized using the DAKO FLEX+ detection system (*DAKO*, Denmark). Sections were stained with diaminobenzidine for 3 minutes and then contrasted with Mayer's hematoxylin (*BioGnost*, Croatia). Stained sections were coated with Histofluid medium (*Marienfield*, Germany).

Counting Ki-67-positive cells. The number of Ki-67-positive cells was counted in 5 representative fields of view in every fifth frontal section of

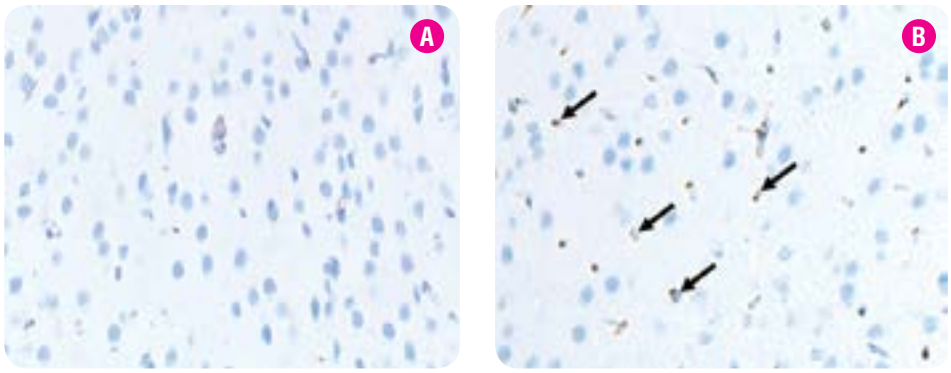

Fig. 1. Photomicrographs of Ki-67-positive cells in the cerebral cortex of an intact rat (**A**) and a rat on the 5th day after traumatic brain injury (**B**). Immunohistochemical staining for Ki-67 of nuclei (brown color); light microscopy, x200.

the brain: cortex (including parietal and pear-shaped areas), hippocampus (including CA1, CA3, hilus and dentate gyrus) and thalamus. Nonspecific staining outside the nuclei was not taken into account. A total of 5 sections per animal were examined. An AxioImager A2 microscope (*Carl Zeiss*, Germany) and an AxioCam MRc5 camera (*Carl Zeiss*, Germany) with a magnification of × 800 were used for the analysis of immunohistochemical preparations and cell counting.

Statistical analysis. To compare the data of the experimental groups, the analysis of variance ANOVA was used with Origin Pro 8.5 software (*Origin Lab. Corp.*, USA). The difference was considered significant at $p \leq 0.05$. The total number of Ki-67-positive cells in 5 representative fields of view on the frontal section of the brain is given as the mean value for 5 sections ± standard error of the mean value (M ± SEM).

RESULTS AND DISCUSSION

The marker Ki-67 is widely used to study the proliferative activity of cells [4]. Therefore, in our study, we performed immunohistochemical analysis of sections of the brain using antibodies against Ki-67 to determine the effect of traumatic brain injury on the proliferative activity of Ki-67-positive cells in different parts of the brain, as well as the possible neuroprotective effect of LA-CM or HA-CM animals with experimental TBI. To analyze the severity of traumatic brain injury and the depth of penetration of the lesion, the cortex and subcortical structures (hippocampus and thalamus) were examined.

The immunohistochemical analysis of the brain sections using antibodies against Ki-67 showed that in animals of the intact group (group 1) the total number of Ki-67-positive cells in the entire area of the frontal section of a single structure was: in the cortex - 11.2 ± 1.6 , in the hippocampus - 27.3 ± 2.1, in the thalamus - 8.7 ± 1.1 cells (**Fig. 1, 2**).

After experimental traumatic brain injury in rats, an increase in the number of Ki-67-labeled nuclei was observed and their value in the cortex reached 32.1 ± 2.2, in the hippocampus – 40.9 ± 2.3, in the thalamus – 17.4 ± 2.2 cells ($p < 0.05$ compared with the group of intact animals) (**Fig. 2**).

Administration of LA-CM or HA-CM to animals with traumatic brain injury increased the number of Ki-67-positive cells in the hippocampus compared with the TBI group and their value for the group of TBI + LA-CM animals reached 59.6 ± 6.1, and for the TBI + HA-CM group - 47.2 ± 3.1 cells ($p < 0.05$ compared with the TBI group) (**Fig. 2**). This increase in Ki-67-positive cells in the hippocampus after administration of LA-CM or HA-CM may be associated with increased neurogenesis in the subgranular zone of the dentate gyrus [23].

In the cortex and thalamus, the number of Ki-67-positive cells on the contrary decreased compared with the group of animals TBI and for the group TBI + LA-CM was 20.2 ± 1.6 and 12.0 ± 1.7, respectively, and for the group TBI + HA-CM – 25.3 ± 2.1 and 13.3 ± 1.3, respectively (**Fig. 2**). The decrease in Ki-67-positive cells in the cortex and thalamus after administration of LA-CM or HA-CM may be associated with a decrease in reactive gliosis and, consequently, a decrease in proliferating cells in these brain structures [24].

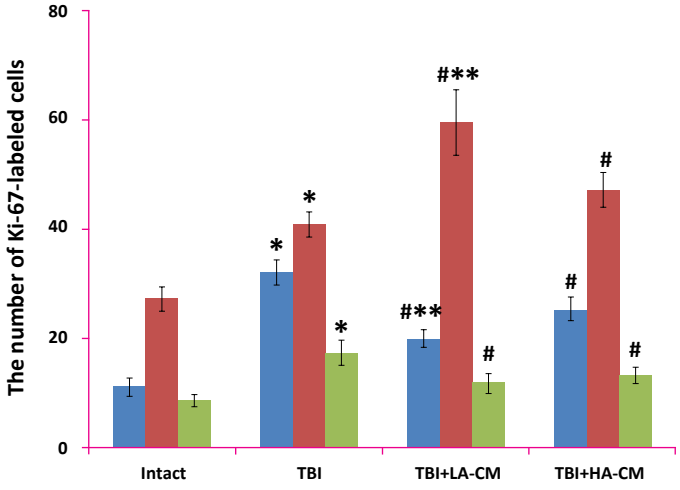

Fig. 2. The number of Ki-67-labeled cells in the frontal sections of the rat brain.
*Notes: * – $p < 0.05$ compared to the group of intact animals, # – $p < 0.05$ compared to the group of TBI animals, ** – $p < 0.05$ compared to the group TBI + HA-CM.*

Thus, immunohistochemical analysis of brain sections using antibodies against Ki-67 showed a positive effect of the administration of LA-CM or HA-CM to animals with traumatic brain injury. Such administration statistically significantly increased the possible neurogenesis in the hippocampus of animals with TBI and decreased the number of Ki-67-positive proliferating cells in the cortex and thalamus, which may be associated with a weakening of reactive gliosis and apoptosis.

In the study, we used antibodies against Ki-67 – one of the most well-known markers of proliferating cells. Antibodies against Ki-67 stain a specific nuclear structure (nucleolus) during the G1, S, and G2 cell cycle phases, but not in the G0 phase [25]. Given this pattern of immunostaining, it has been suggested that Ki-67 may be a good candidate for assessing cell proliferative status.

Comprehensive characterization of stem cells and their proliferative potential, the level of differentiation and purity of the population before their transplantation are crucial for the efficiency and safety of cell preparations. Therefore, it is important to study the various factors secreted by stem cells during their cultivation.

Studies of the proteome and secretion of human neural stem cells (NSCs) during their *in vitro* differentiation have shown that these cells express neuropilin-1 as well as catenin β-1, which are involved in the regulation of the signaling of vascular endothelial growth factor (VEGF), which induces proliferation and supports cell survival [26].

The mitogenic effect of NSCs has been shown to be associated with the secretion of growth factors such as platelet-derived growth factor AA

(PDGF-AA) and fibroblast growth factor 2 (FGF-2) [27]. These growth factors are also responsible for activating the proliferation of progenitor oligodendrocyte progenitor cells and promoting their maturation [27].

Thus, based on preclinical results, it can be concluded that the ability of various factors secreted by NSCs to activate endogenous neurogenesis, which may contribute to the recovery of damaged nerve tissue.

CONCLUSION

1. After traumatic brain injury in rats on the 5th day, a probable increase in the number of Ki-67-positive cells in the cortex, hippocampus and thalamus is registered; this may indicate the activation of neurogenesis and reactive gliosis.

2. Administration of conditioned culture of fetal neural cell cultures to animals with traumatic brain injury has different effects on brain structures: increases the number of Ki-67-positive cells in the hippocampus, which may be associated with increased neurogenesis, and reduces the cortex and thalamus, which may be caused by a weakening of reactive gliosis.

3. A more pronounced effect on the number of Ki-67-positive cells in the cortex and hippocampus after the administration of the conditioned medium of fetal neural cell cultures with low adhesive properties compared with high adhesive properties was detected.

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Вплив кондиційного середовища культур фетальних нервових клітин на проліферативну активність клітин головного мозку щурів після черепно-мозкової травми

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РЕЗЮМЕ

Черепно-мозкова травма (ЧМТ) супроводжується збільшенням кількості клітин, що проліферують. Проте відкритим залишається питання щодо природи, умов отримання та механізмів дії гуморальних чинників, що виділяються фетальними нервовими клітинами (ФНК), на репаративні процеси та нейрогенез у головному мозку після ЧМТ та трансплантації ФНК.

***МЕТОЮ РОБОТИ** було встановлення можливості впливу кондиційного середовища культур фетальних нервових клітин на проліферативну активність Ki-67-позитивних клітин у корі та підкоркових структурах головного мозку щурів після ЧМТ.*

***МАТЕРІАЛИ ТА МЕТОДИ**. ЧМТ моделювали шляхом падіння металевого циліндра на голову щура. Для отримання культур нейральних стовбурових/прогеніторних клітин використовували плоди щурів (E17-18). Кондиційні середовища з культур клітин з високими адгезивними властивостями (ВАКС) та низькими адгезивними властивостями (НАКС) були використані для лікування наслідків експериментальної ЧМТ щурів шляхом внутрішньом'язового введення. За допомогою імуногістохімічного аналізу з використанням антитіл проти білка Ki-67 визначали вплив кондиційних середовищ на проліферативну активність Ki-67-позитивних клітин у корі та підкоркових структурах головного мозку після ЧМТ.*

***РЕЗУЛЬТАТИ.** Імуногістохімічний аналіз зрізів головного мозку показав, що на 5 добу після ЧМТ головного мозку в щурів спостерігалося вірогідне збільшення кількості Ki-67-позитивних клітин у корі, гіпокампі і таламусі. Було встановлено, що введення НАКС або ВАКС тваринам із ЧМТ збільшувало кількість Ki-67-позитивних клітин у гіпокампі порівняно із групою ЧМТ і їх значення для групи тварин ЧМТ+НАКС сягало 59,6 ± 6,1, а для групи ЧМТ+ВАКС – 47,2 ± 3,1 клітин ($p < 0,05$ порівняно із групою ЧМТ). У корі і таламусі кількість Ki-67-позитивних клітин навпаки зменшувалася порівняно з групою тварин з ЧМТ і для групи ЧМТ+НАКС становила 20,2 ± 1,6 і 12,0 ± 1,7 відповідно, а для групи ЧМТ+ВАКС – 25,3 ± 2,1 і 13,3 ± 1,3 відповідно.*

***ВИСНОВКИ.** Введення кондиційних середовищ з культур фетальних нейральних стовбурових/прогеніторних клітин з високими або низькими адгезивними властивостями тваринам із черепно-мозковою травмою збільшує кількість Ki-67-позитивних клітин у гіпокампі, що, можливо, пов'язано із посиленням нейрогенезу, та зменшує – в корі і таламусі, що може бути спричинено послабленням реактивного гліозу.*

***КЛЮЧОВІ СЛОВА:** черепно-мозкова травма; фетальні нервові клітини; кондиційне середовище; імуногістохімія; проліферативна активність*