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Protective effects of adipose-derived multipotent mesenchymal stromal cells of mice on periventricular leukomalacia model *in vitro*

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ABSTRACT

Periventricular leukomalacia (PVL) is a form of white matter lesions of the brain that results from hypoxic-ischemic injury and/or inflammation of nervous tissue, and is one of the causes of cerebral palsy. On PVL models in vivo, we have demonstrated neuroprotective effect of transplantation of adipose-derived multipotent mesenchymal stromal cells (MMSCs). However, the mechanisms, which realize neuroprotective effect of transplanted MMSCs, remain unexplored.

THE AIM *was to assess the influence of adipose-derived MMSCs on cultured mouse brain slices at their contact co-culturing on PVL models in vitro.*

METHODS. *Periventricular leukomalacia in vitro was modelled by a 30-minute oxygen-glucose deprivation (OGD) of mouse brain slices, followed by the addition of 100 ng/ml LPS in culture medium. For co-cultivation we used adipose-derived MMSCs obtained from mice FVB-Cg-Tg (GFPU) 5Nagy/J, transgenic for green fluorescent protein (GFP). The viability of cultured sections cells was evaluated by analysing the level of lactate dehydrogenase (LDH) in the culture medium. Probable MMSCs differentiation into neurons and glial cells was studied using immunohistochemical staining of slices using specific antibodies to neurons and oligodendrocytes (NeuN and Oligodendrocytes, respectively).*

RESULTS. *Modelling of PVL in vitro on organotypic culture of brain slices led to a significant increase in level of cytosolic enzyme LDH in the culture medium. Co-cultivation of slices with MMSCs at PVL reduced the amount of this enzyme. Furthermore, it is shown that under conditions of PVL in vitro, MMSCs are able to differentiate into cells of nervous tissue.*

CONCLUSIONS. *Adipose-derived MMSCs have protective effect when they are co-cultivated with the mice brain slices on PVL model in vitro.*

KEYWORDS: *periventricular leukomalacia; lipopolysaccharide; brain organotypic tissue culture; multipotent mesenchymal stromal cells*

Periventricular leukomalacia (PVL) is a form of the brain white matter lesions, characterized by oligodendrocytes death - cells responsible for myelination of axons and astro- and microgliosis in the periventricular area of the brain [3, 9]. Damage to the myelination of axons results in disorders of brain signaling that manifests itself in the deterioration of motor control, delayed physical, intellectual and emotional development of children [3, 6, 21]. Because of underdevelopment or brain damage in the prenatal, intrapartum and early postnatal periods may occur a group of syndromes, which are combined by a term «cerebral palsy» (CP).

There are many possible reasons for PVL; the main ones are hypoxic-ischemic brain damage, autoimmune mechanisms in the mother-fetus system, intrauterine infections, especially viral, etc. Recently, an important role in the PVL pathogenesis is given to neuroimmune conflict in the mother-fetus system that leads to violation of both central nervous system and the immune system of the fetus [11, 12, 24].

Current pharmacological therapy of perinatal pathology of CNS is imperfect and can disrupt the complex relationship compensatory-adaptive processes in the body of the child, which often leads to complications that

prevent the continuation of drug therapy [23]. Rehabilitation measures in such cases should be held in the first months of life, when the largest renewable effect can be expected.

Cell therapy with stem cells is a promising approach to the treatment of many CNS diseases, including PVL [8, 14, 18]. Despite numerous studies in this area, the question remains open not only about supporting roles of transplant cells in regeneration, but also the possibility of trans-differentiation of other non-neural stem cells such as multipotent mesenchymal stromal cells (MMSCs) into neurons or glial cells [7, 19]. MMSCs have a tropism for the damage area and can affect the progress of inflammation and repair it. [1] They provide the recipient immune system tolerance to allogeneic cells, actually themselves. The use of autologous MMSCs would solve the problem of immunological compatibility of transplant material and its testing for infection, and avoid ethical and legal restrictions on fetal donor material [4]. It was shown that in the transplantation of adipose-derived MMSCs on a model of hemorrhagic stroke in rats the neurological deficits are weakened, brain atrophy is reduced, myocardial ischemic area is significantly reduced and a number of small vessels is growing [10]. In previous work on periventricular leukomalacia model *in vivo*, we also demonstrated the neuroprotective effect of transplantation of adipose-derived MMSCs [20]. However, the disclosure of mechanisms by which transplanted MMSCs promote cell survival and functional recovery of animals requires further research.

In this paper, we studied the effect of adipose-derived MMSCs on the cultured mouse brain slices at their contact co-culturing on the PVL models *in vitro*.

MATERIALS AND METHODS

All animal experiments were performed in compliance 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 mice FVB «wild» type and FVB-C-Tg(GFPU)5Nagy/J, transgenic for green fluorescent protein (GFP), which were kept under standard conditions at the vivarium of SI IHRM with free access to water and food.

Organotypic culture of mice brain slices. Organotypic culture of the brain slices was obtained from 7-day-old FVB mice [21]. After a quick decapitation under ether anesthesia, the brain was isolated, divided into two parts by the median line; and we made 350 μm thick cuts with automatic chopper (*McIllwain*, England). The sections were cultured on porous semipermeable nitrocellulose membranes Millicell-CM (*Millipore*, USA), placed in a CO_2 incubator at the border of gas (a mixture of air with 5 % CO_2) and liquid medium (pH = 7.2) containing 50 % MEM, 25 % balanced salt solution Hanks, 25 % inactivated horse serum, 10 mM Tris, 2 mM $NaHCO_3$, 12.5 mM HEPES, 15 mM glucose, 100 U/ml penicillin, 100 μg/ml streptomycin (all – *Sigma*, USA) in 6-well plates at 35 ^{0}C. The culture medium was changed on the second day of incubation and then twice a week.

Obtaining of multipotent mesenchymal stromal cells from adipose tissue. Adipose tissue of FVB-Cg-Tg(GFPU)5Nagy/J mice, transgenic for the GFP gene, was used to obtain MMSCs by the previously described method [20]. Fragments of crushed subcutaneous adipose tissue from the groin area were fermented in 0.1% collagenase 1A solution (*Sigma*, USA) for 90 min at 37 ^{0}C in a shaker at 100 rpm. The resulting suspension was washed in culture medium DMEM (*Sigma*, USA) by centrifugation at 400x g. We collected supernatant, which contained mature adipocytes, resuspended the precipitate in DMEM medium containing 15 % fetal bovine serum (FBS) and passed through a cell filter with pore diameter of 100 microns. The cells were seeded in 25 cm^2 culture flasks at a a density of $5•10^5$ cells/cm^2 and cultured in complete nutrient medium DMEM-HG (*Sigma*, USA) containing 10 % FBS (HyClon, USA), antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml, (*Sigma-Aldrich*, USA), 1:100 nonessential amino acids (Sigma-Aldrich, USA) in a CO_2 incubator under conditions of moist air with 5 % CO_2 at +37 ^{0}C. Passaging of the cultures was carried out at reaching 80 % confluency of a monolayer using 0.25 % trypsin solution (*Sigma*, USA).

Phenotyping of cells from the resulting cultures was performed on the 2nd passage by flow cytometry using a rat anti-mouse IgG1 monoclonal antibody against the markers CD44-PE, CD73-PE, CD90-PE, CD34-APC, CD45-PE and CD117-Pe-Cy7 (all – *Becton Dickinson*, USA) in the work concentration of 0.5 μg/ml. The percentage of dead and viable MMSCs was measured in terms of penetration of 7-amino actinomycin D (*Becton Dickinson*, USA) into cells with damaged membrane. Phenotyping of adipose-derived MMSCs culture confirmed a high level of expression of markers CD44, CD73, CD90 (84-96 %), while the relative content of cells expressing hematopoietic markers CD34, CD45 and CD117 was less than 2 %.

To confirm multipotent properties of the MMSC culture of the 2nd passage, we conducted directed differentiation for 21 days in osteogenic and adipogenic directions. Complete culture medium for osteogenic differentiation medium consisted of DMEM-F12 (*Sigma*, USA) supplemented with 10 % FBS, and contained L-ascorbic acid 2-phosphate (0.05 mM), dexamethasone (100 nM) and β-glycerophosphate (10 mM). Complete culture medium for adipogenic differentiation medium consisted of DMEM-F12 (*Sigma*, USA) with the addition of 1 μM dexamethasone, 200 μM indomethacin, 500 μM isobutyl methylxanthine and 5 μg/ml insulin (all – *Sigma*, USA).

The postponement of calcium in the extracellular matrix of cultured cells was detected by staining preparations fixed in 4 % formaldehyde solution with Alizarin Red S. The production of alkaline phosphatase was confirmed by staining with BCIP/NBT (*Sigma*, USA). Visualization of lipid granules in the cytoplasm of cells was performed by staining with Oil Red O (*Sigma*, USA).

According to morphological characteristics, immunophenotype and potential for directed differentiation obtained adipose cell cultures, which were used in further experiments complied with the minimal criteria for defining MMSCs [5].

For contact co-culturing with slices of FVB mice brain we used GFP-positive MMSCs of 2-3 passages. Viability of cells for transplantation was 93.6 %.

Periventricular leukomalacia modelling on organotypic culture of mice brain slices. Periventricular leukomalacia was modelled by oxygen-glucose deprivation (OGD) of the brain sections, followed by addition in culture medium of endotoxin lipopolysaccharide (LPS) to simulate the process of inflammation. OGD was created in a special chamber with an oxygen-free gas medium, which contained 95 % nitrogen (N2) and 5 % CO_2. For OGD, normal culture media was replaced for PBS, 12.5 mM Hepes adding 10 mM D-sucrose instead of glucose. OGD duration was 30 minutes, after which the sections were washed twice and returned to normal culture conditions (normoxic reoxygenation for 24 and 48 hours). After OGD, the culture medium was added with 100 ng/ml LPS (L4130, *Sigma-Aldrich*, USA).

MMSCs were applied in suspension directly on cultured brain slices ($25•10^3$ cells per one slice) 2 hours before PVL modelling.

In the study we had the following experimental groups: 1 – intact cultured brain slices; 2 – slices after PVL; 3 – intact cultured MMSCs; 4 – cultured MMSCs after PVL; 5 – slices + MMSCs; 6 – slices + MMSCs after PVL.

Quantitative assessment of lactate dehydrogenase (LDH) in the culture medium. Determination of the changes in the relative amount of the cytosolic enzyme LDH in the culture medium was performed by colorimetric method using a test kit G1780 (*Promega*, USA). While

damage to the cell membrane LDH is released into the culture medium and characterizes the degree of cell damage. The color intensity is directly proportional to the amount of the enzyme LDG in the culture medium and inversely proportional to the viability of cells in culture [2].

After conducting experiments on organotypic brain sections, we took 200 µl culture medium in 24-well plates. In each well we added 200 µl of substrate to determine LDH. The samples were incubated at room temperature in the dark for 30 minutes. Then we added 200 µl solution, which stopped the reaction (G1780 kit). Optical density of the samples was measured with a spectrophotometer uniSPEC 2 (*LLG*, Germany) in a cuvette at wavelength 492 nm. Samples were analyzed at 24 and 48 hours after experimental influences in doubles and determined the average value for each hole. Changes in the relative amount of LDH in the culture medium was expressed in conventional units. Conventional units corresponded the optical density units of the solution, which correlated to the area of tissue in the corresponding hole, and normalized to control.

Immunohistochemical analysis of organotypic culture of brain slices. Identification of transplanted cells and assessment of the damage degree to the nervous tissue caused by PVL, was conducted by immunohistochemistry using primary and secondary antibodies conjugated with fluorescent dyes Alexa Flour 14 days after PVL and application of MMSCs on organotypic culture. Sections of the mice brain were fixed with 4 % solution of formaldehyde in 0.1 M phosphate buffer saline (PBS). Observed sections were blocked in a solution of 0.1 M PBS (pH = 7.4) supplemented with 0.5% bovine serum albumin and 0.3% Triton X-100. The incubation of the sections in a solution of primary antibodies lasted within 12 hours at +4 °C. We used the following primary antibodies: anti-GFP (a marker of transplanted cells), dilution 1:7000 (*Novus Biologicals,* US); anti-NeuN (marker of neuronal nuclei), 1:1000 (*Millipore*, USA) and anti-Oligodendrocytes (oligodendrocyte marker), 1:1000 (*Sigma-Aldrich*, USA). Primary antibodies were visualized with corresponding secondary antibodies conjugated with fluorochrome AlexaFluor (*Invitrogen*, USA). Further the organotypic brain culture slices were covered with medium Immu-MOUNT (*Thermo Scientific*, USA). Immunohistochemically colored culture was studied using confocal scanning microscope FV1000-BX61WI (*Olympus*, Japan).

Statistical analysis. Statistical analysis was performed using the software Origin Pro 8.5 (*OriginLab Corporation*, USA). Sample data included the results obtained from three experiments. The results are shown as the arithmetic mean of the four values (n = 4) in each experimental group ± standard error of the mean (SEM). These were characterized by normal distribution, statistical significance of differences was determined by paired Student t-test, the differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

VIABILITY EVALUATION OF ORGANOTYPIC BRAIN SLICES AND MMSCs BY LDG.

In this work we used our previously developed model of PVL *in vitro*, in which modelling of the brain white matter lesions was achieved by oxygen-glucose deprivation of cultured brain sections and the addition of endotoxin lipopolysaccharide in the culture medium [21].

About 10 % of the GFP-positive cells applied to the cultured cut attached to brain slice (**Fig. 1**). The rest MMSCs distributed on the surface of a porous membrane.

After 24 and 48 hours from the start of PVL *in vitro* modeling, the relative amount of LDH in the culture medium increased compared to control slices 3.1 times and 4.3 times, respectively (**Fig. 2**). Co-culturing of MMSCs significantly reduced the amount of LDH compared to PVL, both after 24 and after 48 hours (2.4 times and 1.9 times, respectively).

In addition, we investigated the effects of components that provide modelling PVL *in vitro*, the viability of MMSCs in the culture without

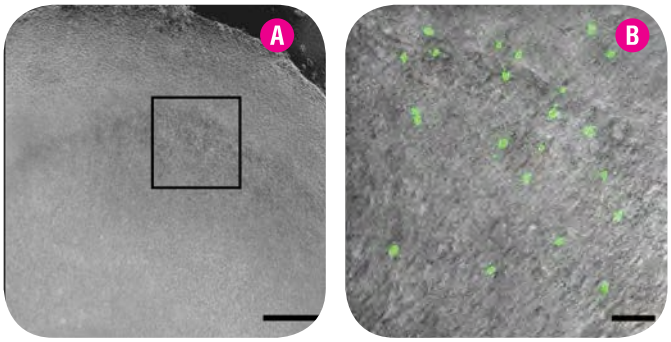

Fig. 1. Microphotographs of the cultured mouse brain slice **(A)** – area marked with square is increased in **Fig. B.** GFP-positive MMSCs (green) after application on a cultivated slice. Phase contrast, scale: **A** – 500 µm, **B** – 50 µm.

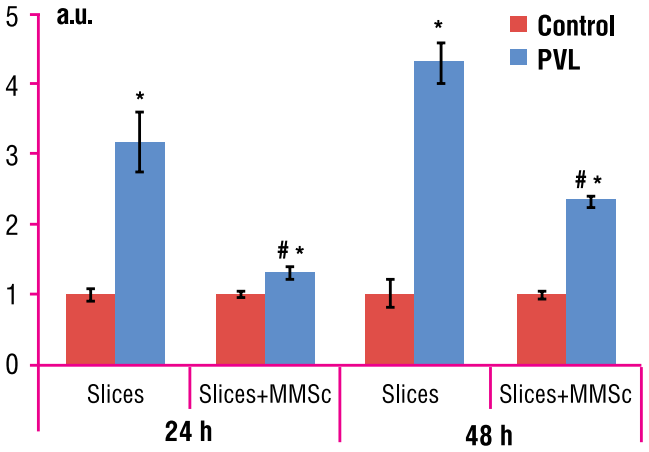

Fig. 2. The relative amount of enzyme LDH in culture medium of organotypic brain slices 24 and 48 hours after the start of the simulation PVL *in vitro* (PVL) under culturing slices separately or in co-culture with MMSCs.

*Notes: * – statistically significant difference compared to control, # – statistically significant difference compared to PVL in the respective sections without MMSCs group ($p < 0,05$).*

cultured slices. At the 2nd passage in MMSCs culture, fibroblast cells with high adhesiveness, diameter 80 µm, containing a large number of vacuoles and granules dominated (**Fig. 3**). 24 and 48 hours after modelling PVL *in vitro*, relative amount of LDH in MMSCs culture medium did not change significantly compared with control values (without modelling PVL *in vitro*). That is, the presence of components (damaging factors) that simulate PVL *in vitro*, did not significantly affect the MMSCs viability in culture, indicating a high resistance of MMSCs to damaging factors used for PVL modelling in this experiment (including 30 min OGD and 100 ng LPS).

Thus, the results of spectrophotometric analysis demonstrated that *in vitro* modelling of PVL increased the relative amount of LDH in the culture medium compared to control slices, and co-culturing with MMSCs significantly reduced the amount of LDH compared to PVL and had neuroprotective character.

IMMUNOHISTOCHEMICAL ANALYSIS OF ORGANOTYPIC CULTURE OF THE BRAIN SLICES.

Immunohistochemical analysis showed that on the 14th day of co-culturing of GFP-positive MMSCs with organotypic slices of the brain after PVL a significant number of MMSCs survived and retained the phenotypic

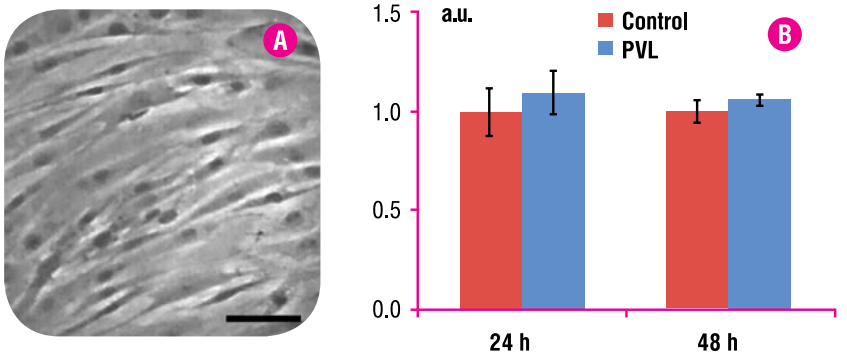

Fig. 3. Impact of PVL *in vitro* on the viability of MMSCs in culture without cultured slices, 2nd passage. **A** – microphotographs of MMSCs culture. **B** – histogram showing the relative amount of enzyme LDH in culture medium of MMSCs 24 and 48 hours after modelling PVL *in vitro*. Scale – 300 μm.

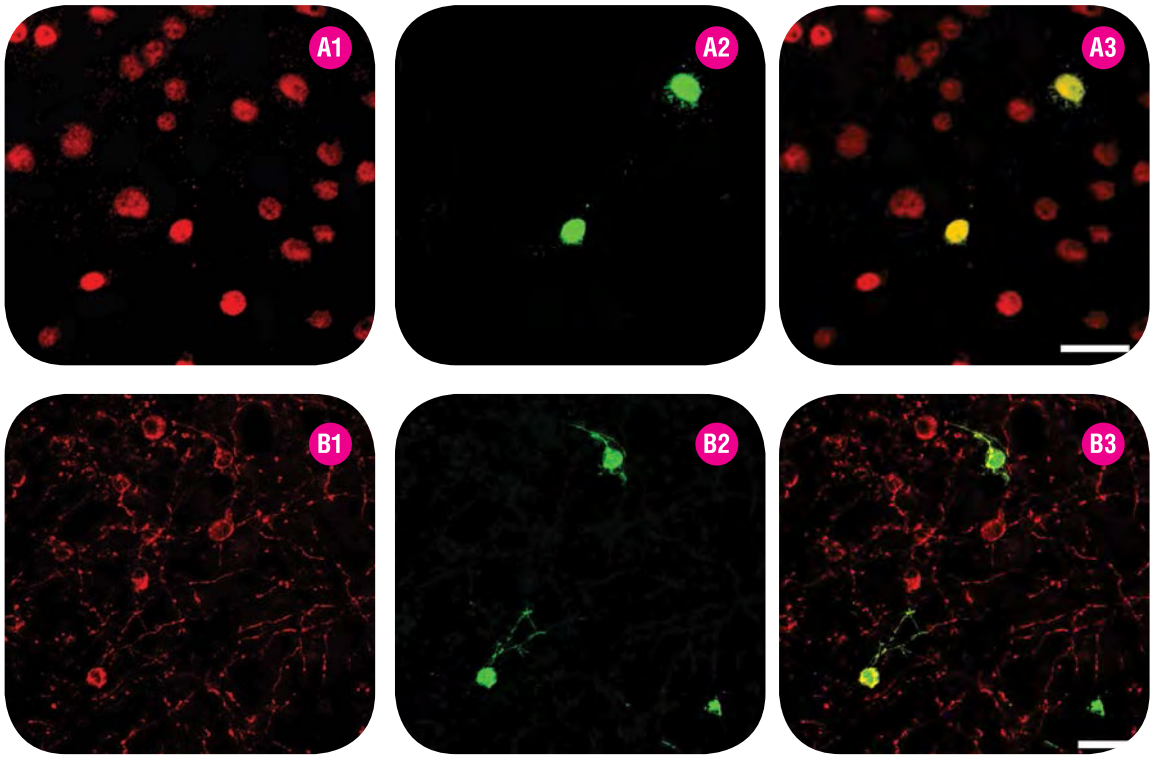

Fig. 4. Immunohistochemical analysis of joint culture of GFP-positive MMSCs and mouse brain slices on the 14th day of co-cultivation. **A1** – confocal image of slices stained on neuronal marker NeuN (red color), **A2** – GFP-positive MMSCs (green color), **A3** – merge of **A1** and **A2** images (yellow coloring of GFP-positive cells expressing NeuN); **B1** – confocal image of slices stained on oligodendrocyte marker O4 (red color), **B2** – GFP-positive MMSCs (green color), **B3** – merge of **B1** and **B2** images (yellow coloring of GFP-positive cells expressing oligodendrocytes). Scale – 100 μm.

traits. Approximately 5 % GFP-positive MMSCs that were adherent to cultured slice, differentiated into mature NeuN-positive neurons (**Fig. 4A1-A3**) or oligodendrocytes (**Fig. 4B1-B3**).

Thus, immunohistochemical analysis showed that a small number of MMSCs differentiated into neural direction and formed mature neurons and oligodendrocytes on the 14th day of co-culturing of GFP-positive MMSCs with organotypic brain slices after PVL.

Possible mechanisms of MMSCs neuroprotective action can be connected with both the replacement of damaged cells by differentiation and integration of transplanted cells or with bioactive factors that can modulate the development of damage at PVL. It is believed that the MMSCs neuroprotective properties are not mostly implemented directly through differentiation and paracrine due to various factors inducing migration of endogenous neural progenitors in the zone of an injury, stimulate the growth of dendrites and axons and reduce post ischemic inflammation [13, 15, 22]. It was shown that conditioned medium with MMSCs protected neurons culture from induced apoptosis [25]. MMSCs modulate multiple signaling pathways during neurogenesis, angiogenesis, apoptosis and synaptogenesis using transmitters by secretion of fibroblast growth factor (FGF-2), epidermal growth factor (EGF), glial cell derived neurotrophic factor (GDNF) and others. [22]. MMSCs also produce high levels of cytokines, which are involved in the processes of cell proliferation and tissue regeneration, such as IGF-1 (insulin-like growth factor-1), VEGF-a (vascular endothelial growth factor-a), SDF-1 (stromal cell -derived factor-1) and erythropoietin [16].

We know about the influence of MMSCs transplanted on recipient tissue by differentiating into cells specific to the place of transplantation [17]. There is evidence showing that MMSCs of adipose tissue contributed morphological and functional recovery of damaged spinal cord. They expressed glial marker GFAP, β-tubulin-3 and neural filaments

NF160 [17]. Our research showed that under PVL at contact co-culturing of MMSCs with brain slices, a certain number of transplanted MMSCs differentiated into neurons and oligodendrocytes as they expressed markers of mature neurons and oligodendrocytes (**Fig. 4**).

Despite the large amount of data, which indicate a positive effect of MMSCs transplantation in various diseases of the CNS, introduction of stem cell transplantation techniques into clinical practice require additional research and should be based on a thorough understanding of the mechanisms of their functioning and sufficient experimental material. The study of indicated range of questions is possible under experimental transplantation involving adequate models of this disease. Used *in vitro* model of PVL is adequate to study the mechanisms and means of neuroprotection in this condition.

CONCLUSIONS

Thus, our results indicate that MMSCs have a protective effect on cultured brain slices and are able to differentiate into neural cells at modelling PVL in vitro.

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