UDC 616-089.843: 616.831-009.11: 616-089.811

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EFFECT OF TRANSPLANTATION OF ADIPOSE-DERIVED MULTIPOTENT MESENCHYMAL STROMAL CELLS ON THE NERVOUS TISSUE AND BEHAVIORAL RESPONSES IN A MOUSE MODEL OF PERIVENTRICULAR LEUKOMALACIA

ABSTRACT

The study of opportunities to use stem cells of different origins in the treatment and rehabilitation of patients with perinatal pathology of the central neural system (CNS) is important.

The aim of our study was to evaluate the effects of transplantation of multipotent mesenchymal stromal cells (MMSCs) from adipose tissue in mice with experimental model of cerebral palsy – periventricular leukomalacia (PVL).

MATERIALS AND METHODS. *PVL was modeled by unilateral coagulation of common carotid artery in mice line FVB on sixth day after birth followed by exposure to hypoxia (6% O2) with intraperitoneal injection of the endotoxin lipopolysaccharide 1 mg/kg. For transplantation we used MMSCs from adipose tissue of the 2nd passage derived from mice FVB-Cg-Tg(GFPU)5Nagy/J. Syngeneic transplantation of GFP-positive MMSCs suspension into seven-day-old (P7) animals with a model of perinatal brain damage was performed stereotactically into right hemisphere in 24 hours after PVL. Corticospinal function of the control animals and the mice with PVL was assessed by testing reaching and retrieval of food rewards.*

RESULTS. *After modeling PVL operated animals lagged in development, had less weight, height and disorders of static and kinetic reflex compared to non-operated control mice. Animals with PVL had lower rates of successful attempts at obtaining food: the percentage of successful attempts in control animals was 58 ± 3% and in animals with PVL – 23 ± 4%. In the group of animals with MMSCs transplantation after PVL modeling corticospinal function recovery was observed and the number of successful attempts was 43 ± 4%.*

CONCLUSIONS. *Syngeneic stereotactic transplantation of multipotent mesenchymal stromal cells from adipose tissue contributes to the restoration of behavioral responses in animals after PVL and improves cytoarchitectonics in the focus of brain damage.*

KEYWORDS: *periventricular leukomalacia; adipose-derived multipotent mesenchymal stromal cells; cell transplantation*

Perinatal pathology of CNS is one of the most relevant medical and social problems of modern neurology and pediatrics. It amounts 49.8% of all neurological diseases in children. [1, 2] According to WHO every year 4-5% of children are born with birth defects, including 25-30% with CNS defects. Disability of child population due to perinatal CNS lesions, resulting in disorders of psychophysiological development of children, continues to grow and varies in the range from 0.5 to 4 cases per 1000 of total population (or per 200-300 child population) [3].

Children with perinatal CNS disorders have a high risk of physical, intellectual and emotional disorders. The group of syndromes resulting from underdevelopment or brain damage in the prenatal, intranatal and early postnatal periods is combined by the term "cerebral palsy". There are many possible causes of cerebral palsy; the main ones are hypoxic-ischemic brain damages, autoimmune mechanisms in the mother-fetus system, intrauterine infections, especially viral, etc. Recently, a significant role in the pathogenesis of cerebral palsy is given to neuro-immune conflict in the mother-fetus system, leading to disorders of both CNS and immune system of the fetus [4-6].

The largest renewable effect should be expected from rehabilitation activities to be held in the first months of such babies' life. The use of medications in the treatment of central neural system, especially at the early stages of the disease, can disrupt the complex relationship of compensatory-adaptive processes in the child's body, and often leads to complications, which prevent further drug therapy. Therefore it is important to search for non-drug methods of correction of the damaged CNS functions. Such methods would allow increasing the effectiveness of therapy by stimulation of natural recovery mechanisms, easy to combine with other traditionally used methods and do not cause undesirable effects.

Now there are actively studied the possibilities of applying cell therapy using stem cells of different origins in the treatment and rehabilitation of patients with perinatal CNS pathology [7, 8]. This pathology is interesting for studies with using stem cells for several reasons. Firstly, cell therapy in perinatal CNS pathology can be applied at the early stages of development when the immature brain is more receptive environment for the engraftment. Secondly, since several types of cells are affected at the perinatal CNS damage, stem cell therapy has a great potential.

The stem cells from different sources and varying degrees of differentiation, from embryonic and fetal cells to adult cells, will be to use as an active agent [9, 10]. At the same time there remains open the question not only about a supporting role of cells transplant in the regeneration, but about the possibility of transdifferentiation of others, different from neural stem cells, i.e. multipotent mesenchymal stromal cells (MMSCs), into neurons or glia [11, 12].

MMSCs have a tropism for a damaged area and can affect the progress of autoinflammation and its reparation. MMSCs have a special property to suppress excessive inflammatory processes and maintain homeostasis of the immune system due to physical and/or chemical interactions with the cells of the immune system [13]. They provide the recipient's immune system tolerance to allogeneic cells, actually for themselves. The use of autologous MMSCs would resolve the issues of immunological compatibility of transplant material and its testing for infection, and avoid ethical and legal problems concerning fetal donor material [14].

However, the introduction of stem cell transplantation techniques into clinical practice should be based on a thorough understanding of the mechanisms of their functioning and sufficient experimental data. The study of these issues is possible under experimental transplantation involving adequate disease models in laboratory animals. Perinatal brain damage in mice in the experiment allows simulating of human perinatal CNS pathology [15]. Transplantation of cells of various origins with different differentiation potential aims to study their regenerative potential and general laws of their participation in restorative processes after ischemic injury and inflammation.

MATERIALS AND METHODS

All animal 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 mice FVB "wild" type and FVB-C-Tg(GFPU)5Nagy/J, transgenic by green fluorescent protein (GFP). They were kept under standard conditions and diet with free access to water at the experimental clinic of Institute of Genetic and Regenerative Medicine NAMS. Mice FVB "wild" type six-day-old (P6) were randomized to one of three groups: control group (1) – shame-operated animals without periventricular leukomalacia (PVL) and without MMSCs transplantation; (2) – animals with PVL without MMSCs transplantation, which were only injected with MMSCs culture medium stereotactically; (3) – animals with PVL and MMSCs transplantation.

MODELING OF PERIVENTRICULAR LEUKOMALACIA IN FVB MICE

Modeling of periventricular leukomalacia was carried out by unilateral common carotid artery coagulation in six-day-old FVB mice (P6) that corresponds to the human perinatal period. During coagulation animals were anesthetized with isoflurane. Isoflurane provides excellent deep anesthesia, stable heart rate and fast waking of animals after anesthesia. Isoflurane was supplied in a mixture of oxygen and nitrogen: 3.0% for induction and 1.5% during surgery through a nose mask. Duration of anesthesia was less than 5 minutes. With such inhalation anesthesia with the use of isoflurane animal mortality was 3%. An hour after coagulation animals were placed in a hermetic chamber with 6.0% O_2 for 35 minutes. To create a hypoxic-ischemic injury combined with inflammation, the animals were injected with 0,015 ml endotoxin lipopolysaccharide (LPS, 1 mg/kg) intraperitoneally.

OBTAINING OF MULTIPOTENT MESENCHYMAL STROMAL CELLS FROM ADIPOSE TISSUE OF GFP-POSITIVE MICE

Adipose tissue of mice FVB-Cg-Tg(GFPU)5Nagy/J, transgenic by the GFP gene, was used to obtain MMSCs. The cell suspension was cultivated in complete nutrient medium *DMEM-LG*, which contained 15% FBS, antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml, (*Sigma-Aldrich*, USA)), 1: 100 non-essential amino acids (*Sigma-Aldrich*, St. Louis, MO, USA) in a CO_2 incubator under conditions of wet air with 5% CO_2 at +37 ^{0}C. The cells from 2-3 passages were used for transplantation. Passaging was carried out at reaching 80% confluence of a monolayer.

CHARACTERISTICS OF ADIPOSE-DERIVED MMSCs

Phenotypic characteristics of cultured MMSCs were determined by flow cytometry using cytofluorometer sorter BD FACSAria (Becton Dickinson, USA) with monoclonal antibodies CD44, CD73, CD90, CD34, CD45, CD117 fluorochrome-conjugated at a working concentration of 0.5 mg / ml. To compensate for spectral overlap of fluorochrome emission in multiparameter analysis we used cell control samples without introduction of antibodies (unstaining control), samples with each antibody alone (single staining control) and samples with a combination of several antibodies without one (fluorescence minus one – FMO control). As isotype control we used Ig of the same isotype of monoclonal antibody conjugated with appropriate fluorochrome. The percentage of dead and viable MMSCs was determined by 7-aminoactinomycin D.

To confirm multipotent properties the 2nd passage of MMSCs has been osteogenic and adipogenic differentiated for 21 days. Complete culture medium for osteogenic differentiation consisted of *DMEM-F12* medium (*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 consisted

of *DMEM-HG* medium (*Sigma*, USA) supplemented with dexamethasone (1 mM), indomethacin (200 mM), isobutyl methylxanthine (500 mM) and insulin (5 mg/ml).

Calcium in the extracellular matrix was detected by preparations staining with alizarin red, fixed in 4% formaldehyde solution. The products of alkaline phosphatase were detected by staining preparations with BCIP/NBT (*Sigma*, USA). Visualization of lipid granules in the cytoplasm of cells was performed by staining with solution of Oil Red S (*Sigma*, USA).

TRANSPLANTATION OF GFP-POSITIVE MMSCs

Syngeneic transplantation of GFP-positive MMSCs suspension into seven-day-old (P7) animals with the model of perinatal brain damage was performed stereotactically (coordinates: A: 1.5 mm caudal, L: 2.0 mm lateral to bregma, and V: 2.0 mm ventral to the skull surface) monolateral into the right hemisphere of the brain under intraperitoneal Calypsol-xylazine anesthesia 24 hours after PVL.

For transplantation we selected optimal volume of the culture medium and the dose of cells in it. The optimal dose was – $5x10^5$ cells in 2 ml medium. Cell viability after transferring of adhesive culture into suspension for transplantation was 92.4%. The animals of the control group were injected with only MMSCs culture medium in the relevant volume.

BEHAVIORAL TESTS

Corticospinal function of animals was assessed using reaching and retrieving test once a week for 4 weeks, starting from 28 days after birth (P28) [16]. Prior to testing the food was removed, while the water was left. The animals were placed in a plexiglass chamber with a slit of 1.5 cm × 0.5 cm, in which there was a crumb of food close as 1 cm, to ensure that the animal grabs the food using its legs, rather than its tongue. For 45 minutes animals were given 60 attempts to reach and remove the food. The percentage of successful attempts was calculated as following:

Successful reaches,% = (number of successful attempts / total number of attempts) ×100, if the animal has made 41-60 attempts in 45 minutes, or

Successful reaches,% = (number of successful attempts / 40) × 100, if the animal has made less than 40 attempts in 45 minutes.

Immunohistochemical staining of the brain sections

The identification of transplanted cells and assessment of the damage degree to the nervous tissue caused by PVL were carried out by immunohistochemistry using primary and secondary antibodies conjugated with fluorescent dyes AlexaFlour. Calypsol anesthetized animals were perfused transcardially with 4% paraformaldehyde solution (PF) in 0.1 M phosphate buffer saline (PBS). Front sections of the brain were made using vibratome VT1000A (*Leica*, Germany). The sections were blocked in a solution of 0.1 M PBS (pH 7.4) supplemented with 0.5% bovine serum albumin (BSA) and 0.3% Triton X-100. The vibratome sections were incubated in a solution of primary antibodies for 12 hours at + 4 0C. We used the following primary antibodies: anti-GFP (a marker of transplanted cells), 1: 7000 (*Novus Biologicals*, USA), anti-GFAP (a marker of astrocytes), 1: 1500 (*DakoCytomation*, Denmark), anti-Iba-1 (a marker of microglia), 1: 1000 (*Wako*, Japan), anti-MBP (myelin basic protein), 1: 1000 (*Sigma-Aldrich*, USA).The primary antibodies were visualized with relevant secondary antibodies, conjugated with fluorochrome AlexaFluor (*Invitrogen,* USA). The stained cell culture medium was covered with Immu-MOUNT (*Thermo Scientific*, USA). Immunohistochemically stained NPC culture was studied using confocal scanning microscope FV1000-BX61WI (*Olympus*, Japan).

STATISTICAL ANALYSIS

Statistical analysis of data was performed using software Statistic (v. 5, StatSoft). Values are given as a mean ± standard deviation of the mean. Nonparametric Kolmogorov-Smirnov test was used to assess differences between the values.

RESULTS AND DISCUSSION

After the modeling of periventricular leukomalacia operated animals lagged behind in development compared with the control non-operated mice. Animals with PVL model had less weight and height **(Fig. 1)** and disorders of statokinetic reflex.

To investigate the influence of stem cell transplantation on the nervous tissue after PVL we used multipotent mesenchymal stromal cells. Adhesive MMSCs culture was obtained from adipose tissue of mice FVB-Cg-Tg(GFPU)5Nagy/J.

At the 2nd passage there dominated fibroblast-like cells with high adhesiveness, diameter 80 mm, containing a significant number of vacuoles and granules **(Figure 2)**.

There has been demonstrated the ability of cells to differentiate into osteogenic, chondrogenic and adipogenic directions. Thus, the obtained cultures meet minimum criteria of MMSCs under the rules of the International Society for Cellular Therapy. Cell viability after transferring the adhesive culture into suspension for transplantation was 92.4%.

When phenotyping cultures of MMSCs from adipose tissue by flow cytometry we revealed a high level of markers CD44, CD73, CD90 expression, while the relative content of cells expressing hematopoietic markers CD45 and CD117 was less than 2% **(Fig. 3)**. Expression of CD34 marker in the early passages ranged from 8-12%, which is typical for adipose tissue MMSCs and can be a sign of a higher potential of cell differentiation in endothelial direction.

The reaching and retrieving behavioral test showed that compared to the control, the animals with PVL had lower rates of successful attempts. Success in the control animals was 58 ± 3% and in the animals with PVL – 23 ± 4% **(Fig. 4)**.

In the group of animals with MMSCs transplantation after PVL modeling corticospinal function recovery was observed and the number of successful attempts was 43 ± 4% **(Fig. 4)**.

Immunohistochemical analysis showed that on 30th day after transplantation GFP-positive MMSCs were localized over the body of the corpus in the vicinity of the injection site **(Fig. 5)**.

To assess the degree of damage caused by PVL we used immunohistochemical staining of the brain sections for myelin basic protein (MBP) and a scale from 0 to 5 [16].

Immunohistochemical analysis showed that in control animals the corpus callosum, which is formed by nerve fibers that connect the left and right hemisphere, was intensively stained with antibodies to myelin basic protein and on an evaluation scale corresponded to 0 **(Fig. 6 A)**. After periventricular leukomalacia the intensity of staining for MBP decreased

Fig. 1. The mice on the 3rd day after simulation of periventricular leukomalacia (PVL). lower – mouse with PVL, top – control animals.

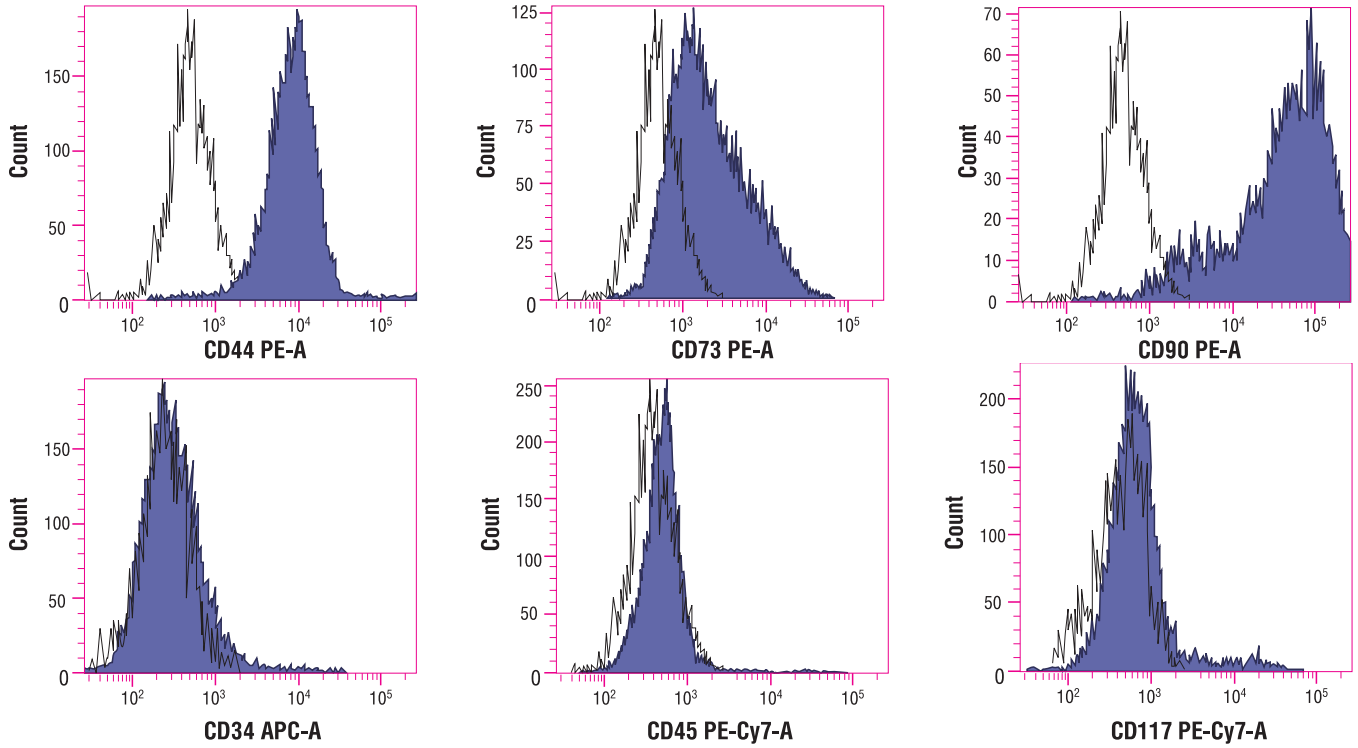

Fig. 3. FACS histograms of CD44, CD73, CD90, CD34, CD45, CD117 expression on MMSCs cell culture of adipose tissue, passage 2. Black contour – isotype-control, blue – incubation with monoclonal antibodies.

and on a damage evaluation scale corresponded from 3 to 4 **(Fig. 6 B)**, and after MMSCs transplantation – from 1 to 2 **(Fig. 6 B)**.

Transplantation of MMSC also hampered the development of both micro and astrogliosis **(Fig. 7)**.

Thus, modeling of periventricular leukomalacia may cause disorders of corticospinal function resulting from damage to the myelin sheath of nerve fibers (myelin basic protein degradation) and activation of astro- and microglia. Syngeneic stereotactic transplantation of multipotent mesenchymal stromal cells promotes restoration of corticospinal function in animals after PVL, reduces degradation of MBP and inhibits the development of gliosis.

The use of stem cells leads to a significant improvement of the animals' state after hypoxic-ischemic injury [17, 18]. The mechanism of the neuroprotective effect of transplanted stem cells consists in the release of a variety of factors that induce the migration of neural progenitors in the area of damage, stimulate the growth of dendrites and axons and reduce post ischemic inflammation [7, 18]. Mesenchymal stem cells modulate multiple signaling cascades during neurogenesis, angiogenesis, synaptogenesis and apoptosis using transmitters. There was found an increase of the expression of fibroblast growth factor (FGF-2), epidermal growth factor (EGF), glial cell-derived neurotrophic factor (GDNF) after MMSCs transplantation [18]. These factors play a key role in the proliferation of progenitor cells and in neurogenesis and cell differentiation [20, 21]. In addition, stem cells cause the formation

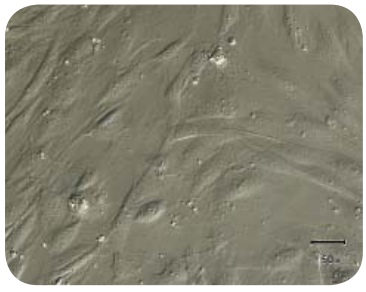

Fig. 2. Microphotographs of murine adipose-derived multipotent mesenchymal stromal cell culture, 2nd passage. Phase contrast.

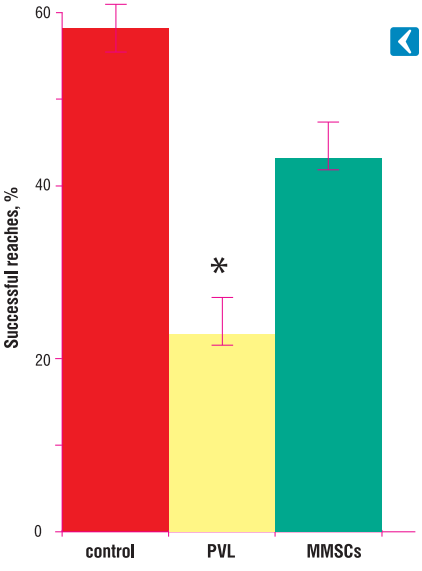

Fig. 4. Evaluation of corticospinal function using a reaching and retrieving test. PVL – periventricular leukomalacia, MMSCs - multipotent mesenchymal stromal cells. *Note: * - P < 0.05 compared to the control group.*

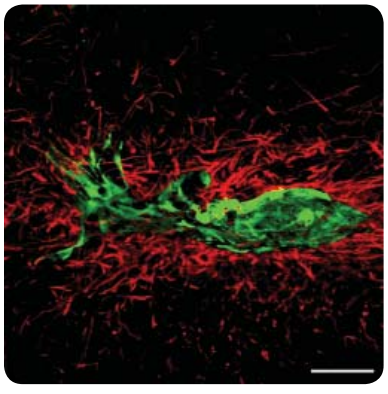

Fig. 5. Confocal micrograph of a frontal slice of a mouse brain on the 30th day after the transplantation of GFP-positive MMSCs. Transplanted MMSCs were located near the area of the injection. Green – GFP-positive MMSCs, red – GFAP-positive astrocytes.

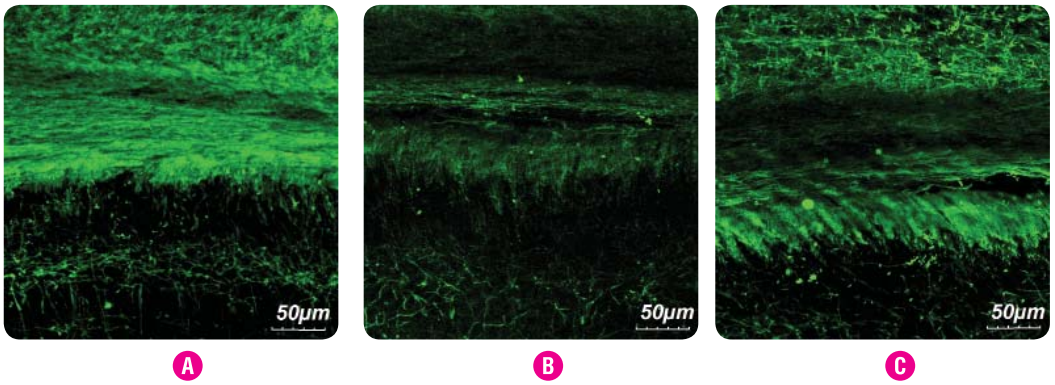

Fig. 6. Confocal micrographs of frontal sections of the mouse brain, stained for myelin basic protein (MBP).
A – control animals,
B – animal with PVL,
C – animal with PVL and MMSCs transplantation (30th day after transplantation).

of neuropilin-1 and 2, Neuregulin-1, and ephrin-B2 – messengers that play an important role in regulation of axons growth, synapse formation and integration in neural networks [19].

Mesenchymal stem cells stimulate proliferation of progenitor cells in the dentate gyrus of the hippocampus, their migration to the damaged area and differentiation into astrocytes, oligodendrocytes and neurons [17].

MMSCs also support the proliferation and differentiation of oligodendrocytes progenitor cells and, therefore, myelination of newly formed axons [17, 22]. In addition, mesenchymal stem cells reduce astroglial reaction, preventing the formation of glial scars that complicate the migration of axons and dendrites [23].

It is known that post ischemic inflammation is accompanied by activation of microglia and macrophages in the CNS [24]. During the brain damage there is an activation of local microglia and a migration of peripheral blood monocytes to the damaged area. [25] There are distinguished microglia type M1 and M2. M1-type microglia releases inflammatory cytokines, free radicals and neurotoxins, which leads to further tissue damage. Microglia M2, by contrast, has a neuroprotective effect and produces IL-10, insulin-like growth factor-1, transforming growth factor-β and other immunomodulating factors [25].

The use of mesenchymal stem cells reduces the amount of activated microglia M1 and, thus, slows the release of proinflammatory cytokines; and activates microglia M2, which synthesizes growth factors that support the regeneration of damaged tissue [19, 22, 26, 27].

Thus, a newborn brain, which is developing and after the birth, is more plastic compared to an adult brain and has a greater regenerative potential. Therefore, MMSCs transplantation may be an effective strategy to restore damaged brain during perinatal CNS pathology.

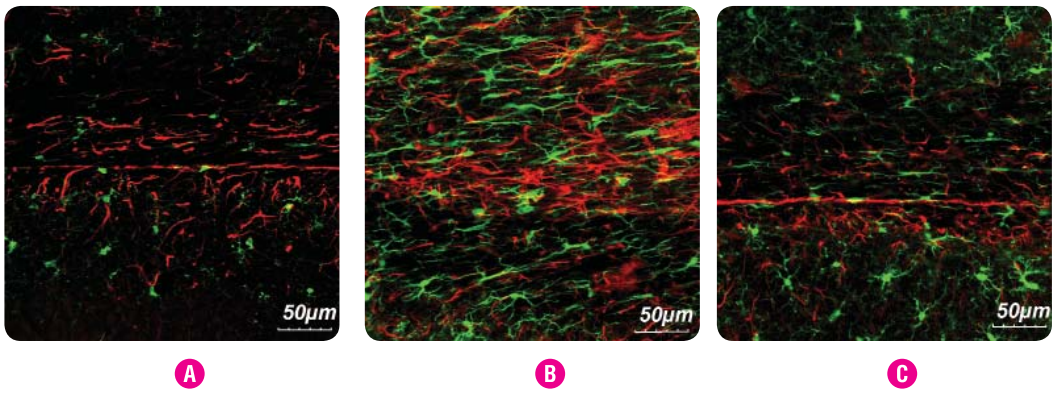

Fig. 7. Confocal micrographs of frontal sections of mouse brain stained for markers of microglia – Iba-1 (green) and a marker of astrocytes – GFAP (red).
A – control animals,
B – animal with PVL,
C – animal with PVL and MMSCs transplantation (30th day after transplantation).

CONCLUSIONS

Syngeneic stereotactic transplantation of adipose tissue MMSCs helps to restore behavioral responses in mice with modeled periventricular leukomalacia.

ACKNOWLEDGMENT

The research is carried out with support from the joint research project of NAS of Ukraine and Science and Technology Center in Ukraine № 5977.

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The authors indicate no potential conflicts of interest.

Received: February 09, 2015
Accepted: April 16, 2015