The effects of multipotent mesenchymal stromal cells on mouse brain slices at their co-culture in an in vitro model of periventricular leukomalacia

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Multipotent mesenchymal stromal cells (MMSCs) demonstrated a measurable therapeutic effect following transplantation into animal models of periventricular leukomalacia (PVL), brain white-matter degeneration resulting from hypoxic-ischemic incidents and/or inflammation. However, the mechanisms by which transplanted MMSCs promote cell survival and/or functional recovery remain indeterminate. In this work we used organotypic brain slices for PVL model in vitro (PVLmiv) subjecting cultures to oxygen-glucose deprivation (OGD) and endotoxin lipopolysaccharide (LPS). This approach allowed us to simulate important pathogenic factors both responsible for PVL, hypoxic-ischemic component and inflammation. Based on the cell viability and the glial reaction, we evaluated distant effects of MMSCs on brain slices with PVLmiv in the non-contact co-culture. Cell viability was assessed by the measurement of cytoplasmic enzyme lactate dehydrogenase (LDH) released into the culture medium. Glial reaction in the periventricular regions of slices was analyzed immunohistochemically using specific antibodies to glial markers of oligodendrocytes, astrocytes and microglia (Rip, GFAP and Iba-1, respectively). We showed that the PVLmiv resulted in a significant release of the cytosolic enzyme LDH into medium demonstrating substantial cell damage. A decrease of Rip-immunoreactivity indicated deterioration within oligodendrocytic population of cells, while an increase in GFAP and Iba-1 immunoreactivity reflected pronounced astro- and microgliosis. The presence of MMSCs in the co-culture diminished PVLmiv effects improving cell viability, preventing degradation of oligodendrocytes and extensive astro- and microgliosis in brain slices. Our data suggest that protective capacity of MMSCs can be executed distantly most likely via released biomodulatory compounds.

Key words: periventricular leukomalacia; brain slice culture; oxygen-glucose deprivation; lipopolysaccharide; multipotent mesenchymal stromal cells.

INTRODUCTION

Periventricular leukomalacia (PVL) is a form of brain injury provoked mainly by hypoxic-ischemic incidents and/or inflammatory response within white-matter of highly vulnerable brain of newborn, and tightly linked with the preterm childbirth, maternal infections and other labor/pregnancy complications. It is characterized by overt focal necrosis and death of oligodendrocytes - cells responsible for neuronal axon myelination - and astro- and microgliosis in the periventricular zones of the brain [1, 2]. PVL leads to distortion of cerebral signaling, and is responsible for the problems in motor control, delay in the physical, intellectual and emotional development, what often reveals as cerebral palsy or epilepsy later in life [1, 3, 4]. The exact pathogenic mechanisms of PVL and ways of their correction are studied insufficiently.

Contemporary neonatal pharmaceutical therapy of central nervous system (CNS) pathologies remains to be imperfect and hindered by persistent risk to disrupt complex
relationship of compensatory-adaptive processes at early stages of body development, and to cause additional complications interfering with further drug treatment [4]. Therefore, universal drug-free approaches in the therapy of CNS diseases, including PVL, are the focus of the great interest in extensive studies worldwide.

Stem cell (SCs) technology is considered to be a new promising tool for the effective treatment of CNS disorders [5, 6]. Numerous studies have demonstrated that SCs transplanted into the site of injury favored the recovery of damaged tissue via differentiation into specific cell type and production of the biomodulators (growth factors, cytokines and immunomodulators) essential for cell survival, reduction of inflammation and maintenance of tissue homeostasis [5, 7-9]. However, more research is required to better understand mechanisms of transplanted SCs effects.

Aimed to achieve higher fidelity of SCs therapy, many experiments are carried out to evaluate full potency of multipotent mesenchymal stromal cells (MMSCs). MMSCs not only have a tropism for the injured zone in the brain but also show strong immunomodulatory potential suppressing inflammation and favoring recovery of function [10]. Such promising neuroprotective effect from MMSCs transplantation was shown, particularly, in PVL animal model [6, 11]. However, it remains unclear if this positive effect is mediated by actual presence MMSCs in the tissue or by distant modulation via released bioactive factors.

In this study, we used PVL model in organotypic brain slices to evaluate the protective potential of adipose-derived MMSCs in non-contact co-culture.

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” (21.02.2006) and all norms of bioethics and biosafety.

Organotypic brain slice culture of newborn mice and co-culture with MMSCs

Brain slice cultures were obtained from FVB mice of postnatal day 7. After rapid decapitation of animal, the brain was removed, divided into two parts through the median line and using a tissue chopper (Mellwain, GB) cut into 350 microns thick frontal slices (350 mm), which included the corpus callosum (from bregma 1.10 to bregma 0.10). Slices from indicated zone of each brain hemisphere were chosen for culturing (Fig. 1). We analyzed the area of brain slice

Figure 1. Scheme of obtaining of organotypic brain slice culture and non-contact co-culture with MMSCs. Isolated brain of postnatal mouse was divided into two parts on the median line and cut into frontal slices. Brain slices were transfereed onto inserts with porous semitransparent membranes for culturing. For non-contact co-culture the inserts with brain slice cultures were placed into the 6-well plates with adherent culture of MMSCs. Analyzed area is shown by the dashed square
culture containing the cortex, corpus callosum and periventricular region.

Brain slices were cultured on the gas–liquid interface on porous Millicell CM inserts (Millipore, USA) placed into 6-well culture plates in a CO₂ incubator with 5 % CO₂ at 35 ºC. The culture medium contained 50 % MEM, 25 % Hanks balanced salt solution, 25 % inactivated horse serum, 10 mM Tris, 2 mM NaHCO₃, 12.5 mM HEPES, 15 mM glucose, 100 U/ml penicillin, 100 µg/ml streptomycin (all – Sigma-Aldrich, USA) at pH 7.2, and was changed on the second day of incubation and then twice a week. The slices were cultured for 12 days before PVLmiv and MMSCs co-culture.

For non-contact co-culture, immediately after PVLmiv, inserts with brain slices were transferred to other 6-well plates with earlier adhered MMSCs (4x10⁴ cells per well) The porous semitransparent membrane of insert was permeable to soluble factors, but prevented direct cell-to-cell contact. The co-cultures were incubated for 48 h at +35 ºC (Fig. 1).

Obtaining of multipotent mesenchymal stromal cells from adipose tissue of mice

Adipose tissue of FVB mice was used to obtain multipotent mesenchymal stromal cells (MMSCs). Subcutaneous inguinal fat pads were harvested after euthanasia of mice under sterile conditions. Isolated adipose tissue was cut into 1-2-mm³ pieces, washed with Ca-Mg-free phosphate-buffered solution (PBS) (HyClone, USA) and digested for 2.5 hours at 37 ºC with 0.1 % collagenase I (Sigma, USA). At the end of this procedure, collagenase was inactivated by adding Nutrient Medium (DMEM-HG, Sigma) containing 10 % of fetal bovine serum (FBS) (HyClon, USA). After centrifugation the whole suspension of cells 5 min at 300xg, the supernatant was discarded and the pellet (known as stromal vascular fraction-SVF) was resuspended in DMEM-HG medium containing 10 % FBS, penicillin 100 U/ml, streptomycin 100 µg/ml, 1:100 non-essential amino acids (all - Sigma-Aldrich, USA) and seeded to T25 culture flask (Sarstedt, USA) at density 4x10³ cells/cm² for cultivation. Cells grown in a CO₂ incubator at 37 ºC and 5 % CO₂ humidified atmosphere.

After 24 h non-adherent cellular debris were discarded and adherent adipose-derived stromal cells washed with PBS. Nutrient media was replaced every 2–3 days. Passaging was carried out at reaching 80 % confluence using 0.05 % trypsin-versene solution. At passage 2 cells were transferred into 6-well tissue culture plates (Sarstedt, USA) for further co-cultivation with brain slices onto cell culture inserts.

Phenotypic characteristics of cultured MMSCs were determined by flow cytometry using BD FACS Aria cell sorter (BD Bioscience, USA) with fluorochrome-conjugated rat anti-mouse monoclonal antibodies CD44 (BD Bioscience, Cat. #553134), CD73 (BD Bioscience, Cat. #550741), CD90 (BD Bioscience, Cat. #551401), CD34 (BD Bioscience, Cat. #560230), CD117 (BD Bioscience, Cat. #561074), CD45 (Thermo Scientific, Cat. #MA1-10233), at a working concentration of 0.5 µg/ml. To correct fluorescence spectral overlap compensation in multiparameter analysis we used control cell samples without of antibodies (unstained control), samples with each antibody alone (single-stained controls) and samples with a combination of several antibodies without one (fluorescence minus one – FMO controls). The isotype control was used to determine the level of non-specific background binding of antibodies. The viability of MMSCs was determined by 7-aminoactinomycin D. Data were analyzed using BD FACS Diva 6.2.1 software (BD Bioscience, USA).

To test the osteogenic capacity cultures were treated for 21 days with osteogenic differentiation medium containing 10⁻⁷ M dexamethasone (Sigma, USA), 0.1 mM ascorbic acid 2-phosphate (Sigma, USA), 10 mM β-glycerophosphate (Sigma, USA) and 10 % FBS in DMEM-HG. The osteogenic medium was replaced every 2–3 days. After differentiation, the cells were stained with Alizarin Red S to detect calcium deposits.
Modeling of periventricular leukomalacia on
organotypic brain slice culture of mice
Periventricular leukomalacia was modeled by oxygen-glucose deprivation (OGD) and reoxygenation of the brain slices, followed by addition of endotoxin lipopolysaccharide (LPS) into culture medium to mimic the process of inflammation. For the OGD, slices on inserts were placed into special chamber where the gas atmosphere contained 95 % nitrogen and 5 % CO₂, while liquid medium contained PBS, 12.5 mM Hepes and 15 mM D-sucrose substituting glucose. After 30 min of OGD slices were washed twice and returned to normal culture conditions with adding LPS for 48 hours (100 ng/ml) (L4130, Sigma-Aldrich, USA). This LPS concentration (100 ng/ml) was selected as the optimal of the three examined concentration (10, 100 and 1000 ng/ml) in the pilot LDH-testing of slices damage.

Lactate dehydrogenase (LDH) assay
Analysis of LDH relative level in the culture medium was performed by colorimetric method. During the injury of cell membrane, cytosolic enzyme LDH releases in the culture medium and indicating the degree of cell damage. The color intensity is directly proportional to the amount of the LDH in the culture medium and inversely proportional to cell viability in the culture.

To determine the changes in the relative amount of cytosolic LDH in culture medium in response to the PVL modeling or PVL and MMSCs co-culture, 200 µl of culture medium was collected into 24-well plate at 48 hours after impact. Samples were collected in duplicates. CytoTox Non-Radioactive Cytotoxicity Assay kit (Promega, USA) was utilized for the colorimetric method that was performed as follows: the reaction was initiated by adding 200 µl of substrate in each well and incubated at room temperature in the darkness for 30 minutes; the reaction was terminated by addition of 200 µl Stop Solution.

Optical density of the samples was measured with a spectrophotometer uniSPEC 2 (LLG, Germany) at wavelength of 492 nm. We determined the average values of duplicates for each well. As controls we used: 1 – culture medium from the wells without organotypic culture (optical density value of which was subtracted from that of obtained from experimental wells); 2 – culture medium from the well with untreated PVL and MMSCs cultures. Changes in the relative LDH amount in culture medium was expressed in arbitrary units that represent units of solution optical density normalized to the tissue area in the respective well. The values were normalized to the control.

Immunohistochemical staining of organotypic
brain slice cultures
Brain slice cultures were fixed with 4 % solution of paraformaldehyde in 0.1 M PBS. Fixed slices were kept in blocking non-specific binding of proteins solution containing 0.1 M PBS (pH 7.4), 0.5 % bovine serum albumin (BSA) and 0.3 % Triton X-100 (Sigma-Aldrich, USA). The incubation of slices in the primary antibodies solution lasted for 12 hours at +4 °C. We used the following primary antibodies: anti-GFAP (a marker of astrocytes) 1: 1500 (Dako Cytomation, Denmark), anti-Iba-1 (marker of microglia) 1: 1000 (Wako, Japan), Anti-Rip (marker of oligodendrocytes) 1: 200 (Abcam, USA). Relevant secondary antibodies, conjugated with fluorochrome AlexaFluor (Invitrogen, USA), visualized primary antibodies. Stained organotypic brain slice cultures were embedded in a drop of Immumount™ mounting medium (Thermo Scientific, USA). Immunohistochemically stained slice cultures were studied using confocal scanning microscope FV1000-BX61WI (Olympus, Japan).

Quantitative image analysis was carried out using the ImageJ software (National Institutes of Health, USA). The intensity and area of fluorescence marker were measured by automatic calculation of the average value of gray within the measurement threshold. The results were presented as integrated density of fluorescence in arbitrary units, which are equal to the fluorescence intensity multiplied by area of fluorescence (excluding the integrated density of the background fluorescence).
Statistical analysis
Statistical analysis was performed using the Origin Pro 8.5 software (OriginLab Corp., USA). Data sampling included the results obtained from 3 experiments. The results are shown as mean from four values (n = 4) in each experimental group ± standard error of the mean (SEM). The data were characterized by normal distribution, the statistical probability of differences was determined by paired Student t-test; the differences were considered significant at P < 0.05.

RESULTS
According to the minimal criteria of the International Society for Cellular Therapy (ISCT) mesenchymal stem cells must meet the following properties: plastic-adherent in standard culture conditions; high expression of CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules; must differentiate to osteoblasts, adipocytes and chondroblasts in vitro [12]. In our study the phenotypic analyses of adipose-derived MMSCs showed a high level of mesenchymal markers (CD44, CD73, CD90) expression, while the relative content of cells expressing hematopoietic markers CD45 and CD117 was less than 2 % (data non shown). Multilineage differentiation capacity of adipose-derived MMSCs was confirmed by effective directed osteogenic differentiation.

Viability assessment by LDH
Spectrophotometric analysis showed that 48 hours after 30 min OGD treatment and LPS adding in the culture medium (PVLmiv) the relative LDH level increased by 4.3 times comparing to the control slice cultures (Fig. 2). In contrast, in the presence of MMSCs, LDH level in co-culture medium was considerably lower than under PVLmiv (namely, 2.5 times).

Thus, the data revealed the PVLmiv resulted in a significant release of the cytosolic enzyme LDH in culture medium, and these effects were ameliorated to a significant extent following non-contact co-cultivation of slices with MMSCs.

Evaluation of Rip, GFAP and Iba-1 positive glia in brain slice culture
The next step in the study was the estimation of oligodendrocytes in the zone of lateral ventricles with corpus callosum of brain slices culture. Immunohistochemical analysis was performed 48 h after treatments, when the impact on the viability (by the LDH levels) was the strong pronounced. It was found that the non-contact co-culturing the brain slices with MMSCs after PVLmiv increased Rip-immunoreactivity of the brain slices compared to PVLmiv group (1.9 ± 0.4 a.u. vs. 4.0 ± 1.1 a.u., respectively), but did not reach control values – 5.9 ± 1.1 a.u.) (Fig. 3, A1-A4).

Evaluation of GFAP and Iba-1 positive immunostaining (markers of astrocytes and microglia, respectively) in the brain slice cultures revealed that PVLmiv resulted in an increase of the integrated density of corresponding fluorescent signal (GFAP+, Iba-1+) comparing to control group. In case of astrocytes, the integrated density of GFAP-positive signal

![Figure 2. The relative amount of LDH enzyme in the culture medium of brain slices 48 hours after PVLmiv alone or in combination with non-contact MMSCs co-culture. Note: * – statistically significant difference compared with control (P < 0.05), # – statistically significant difference compared with PVLmiv (P < 0.05)](image-url)
The effects of multipotent mesenchymal stromal cells on mouse brain slices at their co-culture in an in vitro model

Organotypic brain slices culture is useful object for modeling PVL and exploring the response of nervous tissue to potential neuroprotective agents including stem cells. The cultured brain slices retain tissue organization, cell-to-cell contacts and synaptic organization similar to the in vivo environment [16, 17]. The organotypic culture provides an easy access to the extracellular space allowing better control over experimental conditions and direct impact on tissue by various substances of desired concentration [18]. This experimental system can be useful during the research of contact as well as non-contact (humoral) interaction of stem cells with tissue.

Previously we showed that, in addition to the hypoxic-ischemic injury of neural tissue, neuroinflammation is a required component in the development of PVL model in vivo [11]. For PVL modeling in vitro on brain slices we applied OGD and endotoxin (LPS) to imitate hypoxic-ischemic conditions and neuroinflammation. As revealed cell viability assay based on estimation of cytosolic LDH in culture medium, combined effect of these harmful factors resulted in the significant cellular damage in 48 hours after the PVLmiv (Fig. 2). MMSCs showed significant protective effect on brain slices at non-contact co-culture increasing the cell survival.

As is well known, the periventricular leukomalacia is characterized by injury of myelinated nerve fibers mostly of the corpus callosum [19]. Therefore, in our study the immunohistochemical analysis of oligodendrocytes in organotypic brain slices culture in the zone of lateral ventricles with corpus callosum was carried out. We have shown that Rip-immunoreactivity of oligodendrocytes was increased in the co-culture brain slices and MMSCs in comparison to PVLmiv group.

It has been shown that glial cells become activated in response to many CNS pathologies, such as trauma, stroke, etc. [20]. Activation of glial cells has been recognized as the primary component and hallmark of neuroinflammation [21]. Astrocytes and microglia are major players in the inflammatory response. Upon activation,
glial cells respond to pro-inflammatory cytokines with an increase in proliferation, change of phenotypes, phagocytosis and release of a battery of pro-inflammatory molecules like iNOS, COX-2, interleukins (IL-1, IL-6) and pro-inflammatory cytokines like TNF-α [22]. Up-regulation of glial intermediate filament is an important step in glial activation and the

![Confocal images of brain slices](image)

Figure 3. Confocal images of brain slices in control (A1, B1, C1), 48 hours after PVLmiv (A2, B2, C2) and PVLmiv and MMSCs co-culture (A3, B3, C3). A1-A4 - oligodendrocytes (marker Rip - green); B1-B4 - astrocytes (marker GFAP - red) and C1-C4 - microglia (marker Iba-1 – violet/pseudocolor) in brain slice culture of mouse. Scale bar – 500 µm. A4, B4, C4 – histogram of integrated fluorescence density. a.u. – arbitrary units. Note: * – statistically significant difference compared with control (P < 0.05), # – statistically significant difference compared with PVLmiv (P < 0.05)
enhancement of these proteins is the best-known hallmark of reactive gliosis [23, 24].

In PVL model in vivo, we have shown that it is accompanied by a pronounced reactive gliosis [11]. Therefore, to assess astro- and microgliosis in PVLmiv, we performed immunohistochemical study of the brain slices using antibodies to markers of astrocytes (GFAP) and microglial cells (Iba-1). We found that after 48h after PVLmiv astrocytic GFAP and microglial Iba-1 immunoreactivity were significantly increased compared to the control brain slices. The non-contact co-cultivating of murine brain slices with MMSCs following the PVLmiv resulted in decrease of reactive gliosis.

Earlier studies were based on the hypothesis that the neuroprotective effects of MMSCs are caused by the replacement of damaged cells by differentiation of transplanted MMSCs into neurons or glial cells [25, 26]. However, the current studies prefer a model of regeneration induced by the secretion of a variety of cytokines, chemokines, growth and trophic factors [27-30]. There is evidence that the MMSCs has neuroprotective potential via a modulatory mechanisms by secreting anti-inflammatory molecules [31-33]. Our data are consistent with previous reports, suggesting that the observed effects were mediated by MMSCs-secreted protective factors, as brain slices and MMSCs did not have direct contact.

CONCLUSIONS

In the current study, we demonstrate that the PVLmiv resulted in a significant release of the LDH into the culture medium, decrease of Rip-immunoreactivity and pronounced gliosis that imitate the damage of the white matter and neuroinflammation of the brain. These effects were ameliorated to a significant extent following non-contact co-culture of brain slices with MMSCs. Our data suggest that protective capacity of MMSCs can be executed distantly, evidently via released biomodulatory compounds.
Вплив мультипотентних мезенхимальних клеток на срези головного мозга в моделі перивентрикулярної лейкомаляції in vitro при їх кокультуванні

Мультипотенційні мезенхимальні стромальні клетки (ММСК) продемонстрували терапевтичний ефект після їх трансплантації на in vivo моделі перивентрикулярної лейкомаляції (ПВЛ). Однак механізми, з допомогою яких трансплантовані ММСК спосібствують виживанню клеток і/або їх функціональному відродженню, останні не визначеними. В цій роботі була використана in vitro модель ПВЛ, в якій органотипічні срези головного мозга миші подвергалися кислород-глюкозній депривації, а її індукувала рецидивна гіпоксична зони, створена через секрецію біологічно активних речовин.

Висновки: ММСК у кокультурі уменьшав ушкоджуючі ефекти ПВЛ, підвищуючи життєздатність клітин, запобігаючи деградації олігодендроцитів і значно-му астро- та мікрогіаліозу. Присутність ММСК в кокультурі уміщено впливає на віддалені ефекти клітин, запобігавши деградації олігодендроцитів і значному астро- та мікрогіаліозу, який зводиться до відповідного відродження клітин.

Ключові слова: перивентрикулярна лейкомаляція; культура срезів головного мозку; кислород-глюкозна депривація; мультипотенційні мезенхимальні стромальні клітини.

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