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EFFECTS OF BONE MARROW MULTIPOTENT MESENCHYMAL STROMAL CELLS ON THE NEURAL TISSUE AFTER ISCHEMIC INJURY IN VITRO

ABSTRACT

Stem cells application in neural system injuries is an actual and prospective scientific field of modern regenerative medicine. In recent years much attention has been paid for study of regenerative effects of multipotent mesenchymal stromal cells (*MMSCs*) from different sources on injured tissues.

The aim of our study was to determine the level of tissue damage in hippocampus after *in vitro* model of ischemia and to investigate the effect of bone marrow *MMSCs* in non-contact co-culture with ischemic neural tissue. The ischemic injury of neural tissue *in vitro* was modeling in organotypic hippocampal slice culture (*OHCs*) by oxygen-glucose deprivation (*OGD*). Immunohistochemical analysis after 24 hours of *BM-MMSCs* co-cultivation with *OHCs* after ischemia showed a significant reduction of caspase-3-positive dead neural cells, as compared to those in ischemic damage without *BM-MMSCs* co-cultivation, and reducing of glial cells activation. After co-cultivation of *OHCs* after *OGD* with *BM-MMSCs* there remained cytoarchitectonics of the neural tissue.

Analyzing of our data, the neuroprotective effects of *BM-MMSCs* in non-contact co-cultivation with ischemic hippocampal organotypic slice culture is shown.

KEYWORDS: multipotent mesenchymal stromal cells, hippocampal organotypic slice culture, oxygen-glucose deprivation, co-cultivation, immunohistochemical staining.

Ischemic brain lesions are the third most common cause of death in developed countries. It may be the result of both, focal circulatory disorders and transitory cerebral ischemia, caused by a temporary cardiac dysfunction. Ischemic brain injury – ischemic stroke or cerebral infarction – is the result of vessels occlusion with microthrombus after atherosclerotic plaques disruption. Ischemic damage of brain tissue is a result of a number of interrelated processes that develop across time and space [1]. Development of effective treatment methods of this pathology requires further study of molecular and cellular mechanisms of processes, resulting from cerebral blood flow disorders. In the early developmental stages they are largely associated with changes in the biophysical characteristics of the mechanisms that provide the integrative function of neurons: synaptic transmission and functioning of ion channels, including responsible for electrical excitability [1, 2]. These mechanisms play an important role in both natural and artificial neuroprotective effects.

There are many treatments for a stroke, but they are not perfect. At present, much attention is paid to cell therapy. In particular, since the late 20th century there is implemented an international program for the study of stem cells potential, including bone marrow origin. This may result in a significant progress in the treatment of neurodegenerative diseases [3-5].

Multipotent mesenchymal stromal cells (*MMSCs*) are a population of cells with high adhesive ability *in vitro*. They are characterized by a significant proliferative activity and maintenance their stemness. They also can differentiate *in vitro* into chondrocytes, osteocytes, adipocytes; and other types of cells in conditions for induction a specific differentiation way [6, 7, 8].

The most common *MMSCs* sources are bone marrow, cord blood, adipose tissue, Wharton's jelly, placenta, umbilical vein, amniotic fluid, amniotic membrane, synovial fluid, skeletal muscles, liver and, even, cord

or peripheral blood [9]. *MMSCs* subpopulations are usually heterogeneous by self-renewal capacity and potential to differentiation [8, 9].

Using flow cytometry with a large number of surface markers we demonstrated that cultured population of human bone marrow *MMSCs* is homogeneous for more than 98%. Under certain conditions *in vitro* these cells are easily differentiated. *MMSCs* co-cultivation with hematopoietic stem cells (*HSCs*) showed that *MMSCs* can maintain viability or even division of *HSCs*, herewith *MMSCs* form functional stroma [10]. In the culture of stromal progenitor cells and *HSCs*, *MMSCs* serve as a feeder for the survival of all hematopoietic cell clones. In addition, *MMSCs* can synthesize cytokines to support vitality of hematopoietic cells.

Around the world much attention is paid to study of *MMSCs* properties, carrying out fundamental and clinical researches. This interest is due to many factors, including relative ease of cultivation, existence or possibility of finding specific markers, immune suppression at the use of allogeneic material and so on. Unique potential of *MMSCs* has already been used to restore the functioning of damaged organs [11].

In terms of tissue niches formation an interesting fact is an interaction of stem cells of various origins among themselves and with differentiated cells of a microenvironment. Such interaction determines self-support of progenitor cells and/or direction of their further differentiation. However, the mechanisms and peculiarities of this intercellular interaction in the modified oxygen content are not completely understood. In the model of co-cultivation of hematopoietic and stromal progenitor cells there has been shown that, under conditions of low oxygen content *in vitro*, *MMSCs* actively support hematopoiesis and enhance the formation of hematopoiesis site with subsequent differentiation of hematopoietic precursors. There increases a part of stromal cells, in which an adhesion molecule *VCAM-1* is expressed and the synthesis of interleukins (*IL-6*, *IL-8*) activates.

In studies *in vitro*, it was found that *MMSCs* have unique immunomodulatory properties, due to absence of their immunogenicity, and capacity to inhibition of proliferation and activation of lymphocytes. At co-cultivation of lymphocytes with *MMSCs* there was a change of population composition of immunocompetent cells by reducing the proportion of embryonic cells, increasing the proportion of *CD34⁺*-cells and suppression of *T*-cells activation. Reducing the oxygen content additionally inhibits the ability of *T*-cells to *HLA-DR* antigen presentation [12]. *MMSCs* have immunosuppressive effect and therefore may provide tolerogenic effect in allogeneic transplantation. *In vitro* system *MMSCs* prevent maturation of dendritic cells, block proliferation of *T*-lymphocytes, inhibit chemotaxis and differentiation of *B*-lymphocytes, and induce the reproduction of regulatory *T*-cells [13]. Proceeding from this, mechanisms of contact and indirect interference are involved in interaction of *MMSCs* and immunocompetent cells from different subpopulations. Dynamic changes in levels of cytokines, growth factors and oxygen create unique niche in terms of such interaction [12].

During co-cultivation of murine *MMSCs* with cells from the fetal murine midbrain, a greater number of *MMSCs* with markers of neurons (*NeuN*) and astroglia (*GFAP*) was shown. The results of these experiments confirmed the hypothesis that direct contact between cells (in addition to transmitting signals by trophic factors and cytokines) is important for differentiation of *MMSCs*. Apart from retinoic acid as a factor of *MMSCs* differentiation into neuron-like cells, there is used dimethyl sulfoxide, butylated hydroxyanisole, butylated hydroxytoluene and β -mercaptoethanol in a serum-free medium. Other ways of inducing neural differentiation *in vitro* are also applied. For example, the use of 5-azacytidine – demethylating substance that can modify gene expression in a medium that contains a mixture of nerve growth factor (*NGF*), brain-derived neurotrophic factor (*BDNF*) and neurotrophic factor (*NTF*). Also there was investigated an effect of neural inducer noggin, an agent capable to diffusion, which mediates neural induction at early stages of embryogenesis and neurogenesis in adults. These data indicate that neurons formed processes; there were found specific markers and «neural» genes in them; cells began to respond to the action of depolarizing stimuli as functionally mature neurons [14].

Therefore, regenerative properties of *MMSCs* in ischemic brain damage can be considered as one of the most effective recovery methods of damaged tissue morphological and functional state.

Thus, the aim of this study was to determine the level of damage to the hippocampal tissue cells in a model of ischemia *in vitro* and to investigate the effect of *BM-MMSCs* at their co-cultivation with ischemic tissue.

MATERIALS AND METHODS

All operations with experimental animals were conducted in accordance with the Law of Ukraine «On protection of animals from cruelty,» «European Convention for the protection of vertebrate animals used for experimental and other scientific purposes», as well as the principles of bioethics and biosafety regulations. [15]

Isolation of hippocampal organotypic slice culture (OHCs).

Hippocampal slices were prepared from 8-9-day-old *FVB* wild-type mice. The animals were decapitated, and the brain was isolated from the brainpan. Isolation and cultivation of hippocampal slices were performed by *L. Stoppini*: hippocampi were isolated from the brain in the cooled medium (50% *MEM*, 5 mM *Tris*, 12,5 mM *Hepes*, 25% of 10x salt solution *HBSS*, *pH* = 7,3) [16]. Slices were cut by an automatic chopper (*McIlwain*, UK), perpendicular to the longitudinal axis of the hippocampus, 350-375 μ m thick. Then they were cultured for 5-7 days. Cultivation of organotypic slices was carried out on semipermeable membranes located on gas (a mixture of air with 5% *CO₂*) and liquid (50% *MEM*, 25% Hanks balanced salt solution, 15 mM D-glucose, 25% inactivated horse serum, *pH* = 7,2) phase at 37 °C. Culture medium was changed on the second day of incubation, and then two or three times a week. Within 5-7 days of culturing, hippocampal slices were completely cleaned from cells, damaged during allocation, and reached stable condition. During this time slices smoothed, their thickness decreased from 300-400 μ m to 200-250 μ m.

To detect damaged cells we used staining of cultured slices with propidium iodide (*PI*), a stable fluorescent dye, which penetrates into cells with damaged membrane, binds to *DNA* molecule and acquires red fluorescence. *PI* at a concentration of 2 μ l/ml was added to the culture medium prior to the oxygen-glucose deprivation. Cultures were analyzed by fluorescent microscope *XSP-139A-TP*. For the experiment there were selected slices without any *PI* staining detected.

Modeling of ischemic hippocampal damage in vitro

Oxygen-glucose deprivation (*OGD*) was created by keeping slices in a special chamber, in which oxygen in the air was replaced by nitrogen, and glucose in the culture medium was replaced with sucrose. *OGD* period lasted for 10 minutes. Further slices were returned to normal cultivation conditions for 2 hours – normoxic reoxygenation.

Isolation and cultivation of BM-MMSCs

Bone marrow cells were obtained from *FVB*-mice (3 months old) by washing them from the femurs with medium *RPMI-1640* (*Sigma*, USA) in sterile conditions. We plated $4 \cdot 10^5$ cells/cm² and cultured for 2 weeks, changing the culture medium every 2-3 days. Cultivation was carried out in *CO₂*-incubator under conditions of humidified air with 5% *CO₂* at +37 °C [17]. Nutrient medium *RPMI-1640:DMEM* (1:1) contained 15% fetal bovine serum (*Sigma*, USA) and 2 mM L-glutamine.

The first passage was performed at 80% confluence of monolayers removing cells with 0.05% trypsin solution. Then they were transplanted into a new bottle with a density $2 \cdot 10^4$ cells/cm².

At second passage *BM-MMSCs* were plated in 6-well plates at $1.5 \cdot 10^5$ into each well and cultured for seven days.

Phenotyping of BM-MMSC cell cultures

Phenotyping of cell cultures for markers *CD34*, *CD44*, *CD45*, *CD73*, *CD90*, *CD117* was performed using fluorochrome-labeled monoclonal

antibodies to mouse membrane antigens. We added monoclonal antibodies to $2 \cdot 10^6$ cells in 50 ml of suspension at the rate of $0.5 \mu\text{g}/10^6$ cells and incubated them for 20 minutes at 4°C . After incubation, cells were washed twice in buffer *CellWash* (*Becton Dickinson*, USA). Then we carried out analysis with the cell sorter *BD FACSAria* (*Becton Dickinson*, USA) using the *BD FACSDiva 6.1.2* software. To set up compensation of overlapping emission spectra of fluorochromes in multivariable analysis, there were used control samples of cells without antibodies (*unstained control*); samples with each of the antibodies separately (*single stained control*); and samples with a combination of several antibodies without one of them (*fluorescence minus one control*). The level of surface markers expression was measured in percentages and statistically calculated using Mann-Whitney U-test.

The percentage of dead and viable *BM-MMSCs* was determined with the *BD FACSAria* cell sorter by level of entry into cells with damaged membrane *7-AAD*. The percentage of viable cells in the culture was $93.6 \pm 0.5\%$.

The resulting cell cultures have satisfied the minimal criteria of *MMSCs* by phenotype and ability to directed multilinear differentiation [18, 19].

Co-cultivation of *BM-MMSCs* with ischemic neural tissue and its immunohistochemical evaluation

Cultured on semipermeable membranes hippocampal slices after short-term *OGD* were placed in 6-well plates, where *BM-MMSCs* were previously (7 days) cultivated. *BM-MMSCs* culture medium was replaced prior to co-cultivation. Within 24 hours after co-cultivation *OHCs* were fixed and immunohistochemical analysis was performed.

To identify neurons and glial cells there was used a double immunohistochemical staining with antibodies to astrocyte marker *GFAP* (chicken polyclonal, 1:1500, *Dako*, Denmark); neuronal marker *NeuN* (mouse monoclonal, 1:1000, *Chemicon*, UK); microglia cells marker *Iba-1* (rabbit polyclonal, 1:750, *Molecular Probes Inc.*, USA); a marker of apoptotic nuclei *caspase-3* (rabbit polyclonal, 1:200, *Molecular Probes Inc.*, USA).

Organotypic hippocampal cultures were fixed with 4% formaldehyde solution, washed with 0.1 M phosphate buffered solution (*PB*), treated with a solution of 0.3% *Triton X-100*, 0.5% bovine serum albumin

(*BSA*) for better penetration of antibodies and prevention an excessive nonspecific combining. Within 24 hours hippocampal cultures were incubated in a mixture of primary antibodies. After washing in 0,1 M *PB*, slices were treated for 1 hour with a compound of secondary anti-mouse *Alexa Fluor-555-conjugated* (1:1000, *Invitrogen*, USA), anti-rabbit *Alexa Fluor-488-conjugated* and anti-chicken *Alexa Fluor-647-conjugated* antibodies (1:1000, *Invitrogen*, USA).

After washing in 0.1 M *PB* cultures were fixed with cover glass in a special medium for fluorescent preparations (*Dako*, Denmark). Hippocampal slices were studied using confocal microscopy *FluoView™ FV1000* (*Olympus Inc.*, USA) with a digital camera combined with a computer.

RESULTS AND DISCUSSION

One of the advanced research methods in cell and molecular biology is the cultivation of cells and tissues. Systems *in vitro* are a convenient experimental tool for precise control, subtle manipulations and long-term monitoring of normal and pathological processes in different tissues and, particularly, in the nervous tissue.

Therefore, an object of our research was organotypic hippocampal cultures. The hippocampus is a structure of the brain responsible for learning, memory and spatial orientation. Along with cerebral cortex and striatum, it is extremely sensitive to the damaging effects, in particular, the lack of oxygen and glucose [20-22]. The hippocampus is one of the most sensitive parts of the brain during ischemic injury with selective damage of pyramidal neurons in *str. pyramidale CA1* area [23-24]. Glia actively participates in the control of neuronal activity and synaptic transmission in normal and different pathological conditions [25].

Microscopic analysis of hippocampal organotypic cultures in control samples without *OGD* showed that hippocampal tissue retained the typical topography of cell layers and zones that are characteristic of the hippocampus *in vivo*, such as neurons in *CA1* area located in the middle of slice (4-8 layers of pyramidal cells) and have a traditional pyramidal shape (**Fig. 1**). Glial cells evenly localized in all layers of the *CA1* area of the hippocampus. A layer of glial cells adjoins to the surface of semipermeable membrane with thin spikes, providing fixation

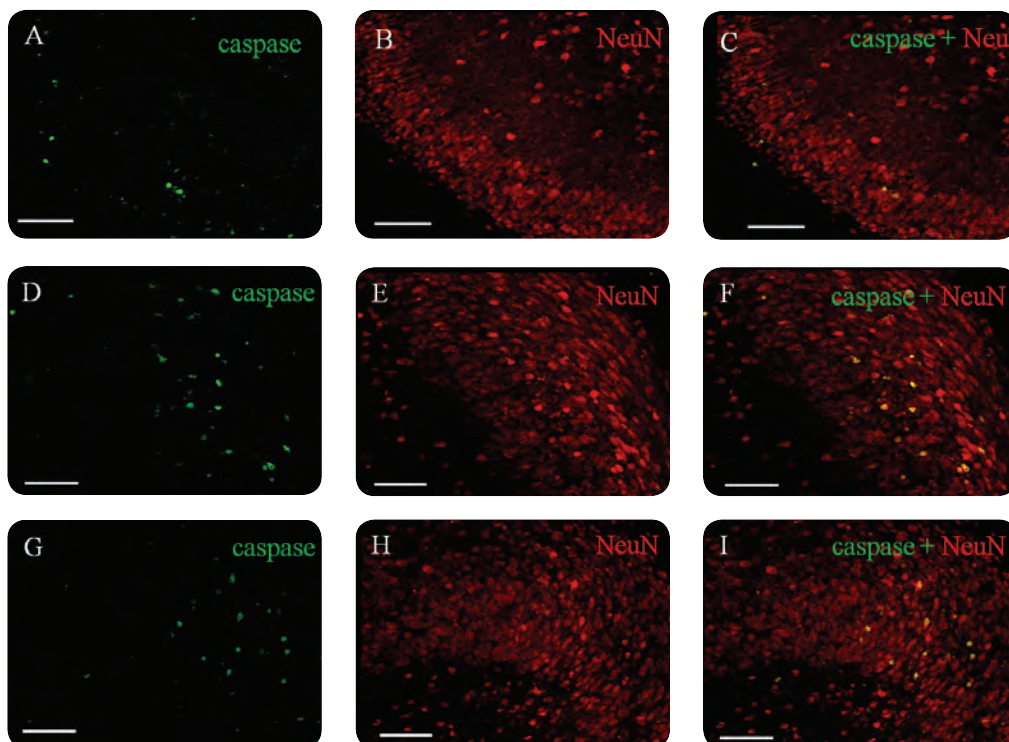


Fig. 1. Immunohistochemical staining of organotypical hippocampal slices for caspase-3-positive nuclei of neuronal cells (green) and *NeuN*-positive nuclei of neurons (red), *CA1* area. **A-C** – control group; **D-F** – 24 hours after *OGD*; **G-I** – 24 hours after *OGD* + *BM-MMSCs*. Scale – 50 μm .

of cultured slices on the membrane, performing trophic and protective functions. In the organotypic hippocampal culture, there are stored all the cell types, which can be found in the hippocampus *in vivo*: pyramidal and granule neurons, interneurons and glial cells (astrocytes, microglia, oligodendrocytes) [26, 27].

Thus, for a long time during the period of cultivation cytoarchitectonics, type specificity of cells, intercellular communication and other features characteristic of the living tissue remain in this organotypic culture of nervous tissue *in vitro*. At the same time it is much easier to manipulate experimentally than the model *in vivo* [28].

After the experimental oxygen-glucose deprivation in organotypic hippocampal culture of mice we observed damage of pyramidal neurons from CA1 hippocampal zone along with activation of glial cells (Fig. 1, 2). At the first hours after ischemic modeling in CA1 hippocampal area we found out an increase of glial cells immunoreactivity and structural reorganization of neurons.

In the hippocampal organotypic slice culture, undamaged neurons are uneven and lose their compact arrangement (Fig. 1). Between neurons we observed blanks and increased intercellular space. The emergence of numerous blanks in *str. pyramidale* and restructuring of compact arrangement of the pyramidal neurons is directly related to the death of neurons above (Fig. 1). At double immunohistochemical staining with markers caspase-3 and *NeuN* there was found that ischemic damage significantly increased the number of caspase-3-positive nuclei of neurons. These results indicate that some neurons die by apoptosis mechanism. Mechanism of the pyramidal neurons death in CA1 area at ischemia occurs by necrotic and apoptotic phenotype [29, 30].

Also, within 24 hours of the *OGD* process we observed reactive gliosis (Fig. 2). Microglial cells transformed from a state of rest, which is indicated by extensive net of processes that depart from the small soma, into amoeboid shape. Processes of such cells shortened and thickened, and size of soma significantly increased. In astrocyte cell phenotype we observed hypertrophy of the soma and processes. Such changes in the structure of glial cells are an indicator of the peak of reactive astrogliosis [31, 32].

Immunohistochemical analysis after 24 hours of *BM-MMSCs* co-cultivation with *OGD* after ischemia showed a significant reduction

of caspase-3-positive nuclei of neural cells, as compared to those in ischemic damage without co-cultivation with *BM-MMSCs*, and reducing of hippocampal glial cells activation (Fig. 1, 2). After co-cultivation of hippocampal slices after *OGD* with *BM-MMSCs*, there remained cytoarchitectonics and type specificity of neural tissue cells. Neurons were located tightly and compactly, extracellular space decreased.

Co-cultivation of the *OGD*-treated slices with *BM-MMSCs* significantly improve the morphological status of the ischemic neural tissue. Bone marrow multipotent mesenchymal stromal cells in our model have shown neuroprotective effects. The direct mechanism of *BM-MMSCs* action in ischemic brain is still unknown. But we assume that such an improvement of morphological and functional state of the ischemic tissue takes place through activation of synaptogenesis, neurogenesis and neuroprotection due to growth factors, as noted by several authors [12, 33-35].

Thus, based on published data and our results, the use of stem cells in the treatment of ischemic effects of brain injury can take a leading position. At present this area of cell transplantation is under thorough experimental study. It is possible that in the future obtained positive results will be confirmed in clinical studies of the treatment effectiveness of ischemic brain injury.

CONCLUSIONS

IN THE CONDITIONS OF SHORT-TERM *OGD* THE NUMBER OF NEURONS IN ORGANOTYPIC HIPPOCAMPAL CULTURE REDUCES; AND ACTIVATION OF ASTROGLIAL AND MICROGLIAL CELLS IS OBSERVED. AT CO-CULTIVATION OF *BM-MMSCs* WITH ISCHEMIC NEURAL TISSUE, MORPHOLOGICAL STATE OF THE LATTER SIGNIFICANTLY IMPROVES AND NEUROPROTECTIVE EFFECT OF *BM-MMSCs* IS SHOWN.

ANALYSING OF OUR FINDINGS AND THE LITERATURE DATA, WE CAN ASSUME THAT IMPROVING OF MORPHOLOGICAL AND FUNCTIONAL STATE OF THE ISCHEMIC TISSUE TAKES PLACE THROUGH ACTIVATION OF SYNAPTOGENESIS, NEUROGENESIS AND NEUROPROTECTION DUE TO GROWTH FACTORS.

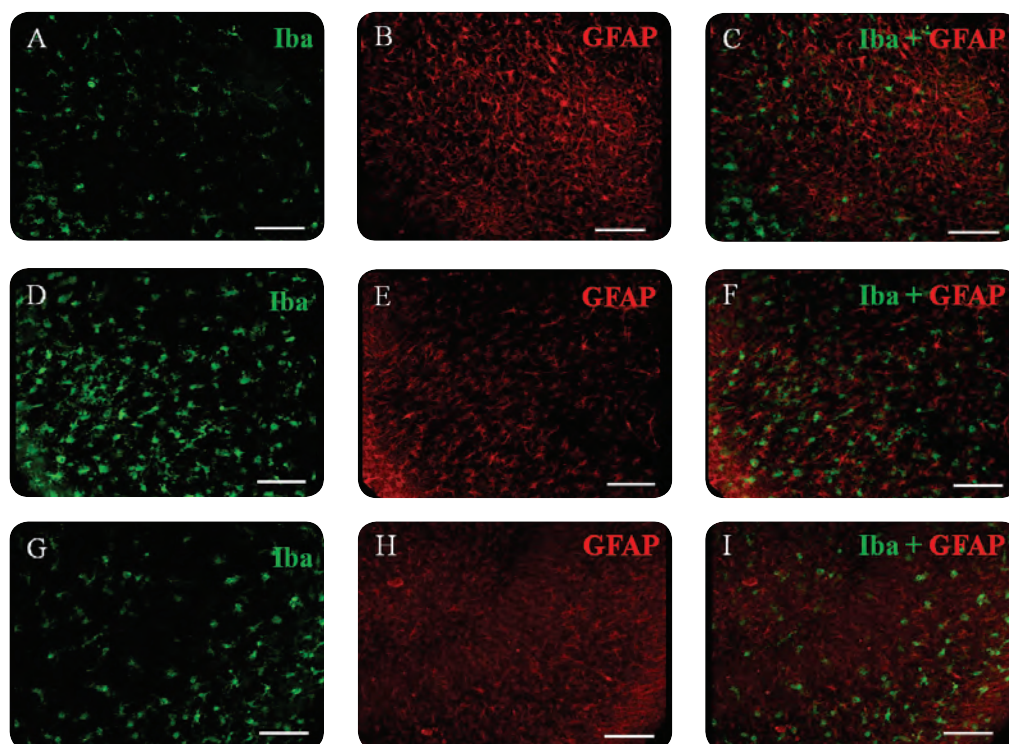


Fig. 2. Immunohistochemical staining of organotypical hippocampal slices for *Iba* – microglia (green) and *GFAP* – astroglia (red), CA1 area. A-C – control group; D-F – 24 hours after *OGD*; G-I – 24 hours after *OGD* + *BM-MMSCs*. Scale – 50 μ m.

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