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Mechanisms of Positive Effects of Transplantation of Human Placental Mesenchymal Stem Cells on Recovery of Rats after Experimental Ischemic Stroke

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Mesenchymal stem cells isolated from human placenta and *in vitro* labeled with fluorescent magnetic microparticles were intravenously injected to rats 2 days after induction of focal cerebral ischemia (endovascular model). According to MRT findings, transplantation of mesenchymal stem cells led to an appreciable reduction of the volume of ischemic focus in the brain. Two or three weeks after transplantation, labeled cells accumulated near and inside the ischemic focus, in the hippocampus, and in the subventricular zone of both hemispheres. Only few human mesenchymal stem cells populating the zone adjacent to the ischemic focus started expressing astroglial and neuronal markers. On the other hand, transplantation of mesenchymal stem cells stimulated proliferation of stem and progenitor cells in the subventricular zone and migration of these cells into the ischemic zone. Positive effects of transplantation of these cells to rats with experimental ischemic stroke are presumably explained by stimulation of proliferation of resident stem and progenitor cells of animal brain and their migration into the ischemic tissue and adjacent areas. Replacement of damaged rat neurons and glial cells by transplanted human cells, if it does take place, is quite negligible.

Key Words: *mesenchymal stem cells; ischemic stroke; differentiation; paracrine effect; substitute therapy*

Acute ischemic stroke is one of the most incident severe diseases of the central nervous system (CNS) in countries with well-developed economy, in many cases leading to disability or death. Therapy aimed at resumption of circulation and protection of the brain tissue from destructive effects of hypoxia and its consequences should be started within the first hours after stroke, during the so-called "therapeutic window" [6], but for many patients qualified care within so short a period is unavailable for technological or other reasons. Stimulation of regeneration processes is also more effective if started early. One of the most promising trends of anatomical and functional repair of the CNS after injury is the use of methods of regenerative medicine, for example, transplantation of stem, progenitor, or differentiated cells (cell therapy).

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The processes underlying functional rehabilitation after stroke are neoangiogenesis, neoneuronogenesis, regeneration of damaged axons, and synaptic plasticity [5,10]. All these processes are induced after stroke, but the degree of their stimulation is usually insufficient for tissue regeneration and functional rehabilitation. Transplantation of stem cells of different origin intensifies these processes [5].

We previously showed that transplantation of mesenchymal stem cells (MSC) isolated from human placenta or umbilical cord carried out soon after infliction of experimental dosed injury stimulates recovery of neurological functions and behavioral reactions in rats with experimental ischemic stroke [1] and in spinal injury [13,14]. The mechanisms of favorable effects of MSC transplantation in experimental cerebral ischemia are little studied. Two mechanisms, substitution and paracrine, are most probable [3,7]. The former mechanism can be realized only if transplanted MSC are capable of concentrating in the ischemic focus and the adjacent brain areas with subsequent differentiation into normally functioning neurons and glial cells replacing the cells dead as a result of stroke. The second mechanism is realized if transplanted cells not only incorporate into the brain tissue, but also produce the specific paracrine factors stimulating resident stem cells [10].

We studied homing of human MSC labeled with magnetic microparticles in the brain of rats with experimental ischemic stroke soon after surgery and evaluated possible differentiation of donor cells in the recipient brain and/or their stimulation of the resident stem cells.

MATERIALS AND METHODS

MSC were isolated from human placenta by the enzymatic method with some modification [8]. The cells were cultured in complete DMEM-F12 with PBS (10%), streptomycin (100 μg/ml), penicillin (100 U/ml), L-glutamine (2 mM) in culture flasks (75 cm^2 with a filter; Greiner) in a CO_2 incubator at 37°C. The initial cell concentration was 2.5×10^5/flask. After attaining 80-90% confluence the cells were removed with trypsin-versen (1:1, 0.25% trypsin:0.02% versen; PanEco), washed twice in Hanks solution for 5 min, centrifuged at 300*g*, and inoculated into fresh flasks in the initial concentration.

MSC were labeled with magnetic particles carrying Dragon Green fluorescent label (d=0.96 μ; Bangs Laboratories) according to manufacturer's instruction. After attaining 80-90% confluence, a suspension of particles (5 μl stock suspension/ml culture medium) was added to MSC culture. The cells were incubated with the particles for 24 h in a CO_2 incubator under common conditions. The medium was replaced after incubation. The efficiency of label was evaluated on an EPICS Coulter XL flow cytofluorometer (Beckman Coulter). At least 10^4 events were registered for each sample. The results were processed using WinMDI software.

The study was carried out on male Wistar rats initially weighing 260-300 g (from Stolbovaya Breeding Center). Focal cerebral ischemia was induced as described previously [11] by occlusion of the middle cerebral artery (MCA) with a Nylon thread with a silicone tip. Controlled duration of MCA occlusion was 1 h. Physiological parameters were recorded during the operation: blood pressure, heart rate, body temperature. Experimental animals (n=20) received intravenous injection of MSC labeled with magnetic microparticles (2×10^6 cells in 1 ml saline into the femoral vein) on day 2 after surgery. Controls (n=24) after MCA occlusion were injected with saline.

The protocol of animal experiments was discussed and approved by the Ethic Committee of the Russian State Medical University.

Magnetic imaging of the brain was carried out on days 1, 7, 14, 30 after surgery on a BioSpec 70/30 device (Bruker) at 7 T magnetic field induction and 105 mT/m gradient system. RF signal was transferred through a linear transmitter with an inner diameter of 72 mm and detected using a surface acceptor coil for the rat brain. The ischemic focus was evaluated and labeled MSC were visualized at the following pulse sequences: for obtaining T2-weighted images: RARE (Rapid Acquisition with Relaxation Enhancement) spin echo pulsed sequence with the following parameters: TR (repetition time)=6000 msec, TE (echo time)=63.9 msec, section thickness 0.5 mm, resolution 0.164×0.164 mm/pixel; for obtaining T2*-weighted images (high sensitivity to local heterogeneity of magnetic field): SNAP (sensory nerve action potential) gradient echo pulsed sequence with the following parameters: TR=113 msec, TE=13 msec, section thickness 1 mm, resolution 0.156×0.156 mm/pixel. The total duration of scanning was 25 min for each animal. The animals were narcotized with chloral hydrate (300 mg/kg) intraperitoneally and fixed in a positioning device with stereotaxis and thermoregulation systems. ECG, respiration rate, and rectal temperature were measured during MRT by Small Animal Monitoring and Gating System device (SA Instruments, Inc.). The magnetic images were analyzed using Image J software.

Experimental animals were decapitated under chloral hydrate narcosis on days 5, 12, and 19 after transplantation of human MSC. The brain was removed directly after sacrifice and frozen in liquid nitrogen vapor for 20 min. The samples were stored at -80°C.

Frontal serial sections of the rat brain (10 μ) were sliced on a Microm HM560 (Carl Zeiss). Cryosections

were fixed in 4% paraformaldehyde (Sigma-Aldrich). Fixed sections were post-stained with DAPI (1 μg/ml; Sigma-Aldrich). The tissues were visualized and images were obtained in an Axioplan 2 fluorescent microscope using AxioCam HRc digital camera (Carl Zeiss).

In order to evaluate possible differentiation of transplanted human cells in rat ischemic brain, frontal cerebral sections were stained with antibodies specific to human neuronal (NeuN) and glial (GFAP) proteins (Chemicon; antibody dilution 1:100) by the method recommended by the manufacturer. Antispecies antibodies labeled with rhodamine (λ_{ex}=550 nm, λ_{em}=570 nm; Chemicon; antibody dilution 1:200) served as the second antibodies. The nuclei were post-stained with DAPI (1 μg/ml). The resultant preparations were embedded in medium for fluorescence protection (DakoCytomation).

The rat brain sections were also stained with antibodies to Ki-67 nuclear protein (proliferating cell marker; Chemicon; antibody dilution 1:100). Antispecies antibodies labeled with horseradish peroxidase served as the second antibodies. Staining was carried out according to the instruction using the DAB visualization systems.

RESULTS

The efficiency of MSC labeling was evaluated by two methods: cytofluorometry and immunocytochemistry. According to cytofluorometry data, about 90% cells were charged with microparticles. Immunocytochemical analysis showed that microparticles were primarily located in the perinuclear space of the cell (data not shown). We previously showed that MSC labeling did not affect their viability and capacity to osteogenic [2] and neurogenic [15] differentiation in culture.

The volume of the ischemic focus after MCA occlusion was evaluated by MRT in all animals on days 1, 7, 14, and 30 after surgery. Typical dynamics of the the volume of ischemic focus in control and experimental rats is shown on Figure 1. Injection of human MSC appreciably reduced the size of ischemic focus. Significant differences between the experimental and control groups were detected starting from day 14 postoperation (12 days after cell transplantation; Table 1). One month after MCA occlusion, the volume of ischemic focus in experimental rats was 2-fold less (23 mm^3) than in the controls (64 mm^3; Table 1; Fig. 1).

MSC labeled with magnetic particles were detected in the ischemic hemisphere on T2*-weighted images (T2*-WI). Two weeks after surgery, these cells were concentrated in the ischemic focus (Fig. 2). T2*-weighted images (SNAP) 24 h after MCS occlusion and later revealed no signs of hemorrhagic transformation in the experimental animals.

Immunohistochemical analysis was carried out 7, 14, and 21 days after MCA occlusion (Table 2). Analysis of serial frontal sections of experimental rat brain showed predominant accumulation of human cells around the vessels mainly in the ischemic hemisphere 5 days after intravenous injection of labeled MSC (7 days postoperation). Some labeled cells were detected in the contralateral hemisphere, also near the vessels. Twelve days after injection of labeled MSC these cells formed tracks, which started from the perivascular accumulations of human MSC in both hemispheres of experimental animals. Small groups of human MSC also appear in the ischemic zone during this period. Solitary labeled cells were diffusely scattered in the parenchyma of both hemispheres. Labeled cells positively stained with antibodies to human NeuN (nuclear marker protein of mature neurons) were detected in

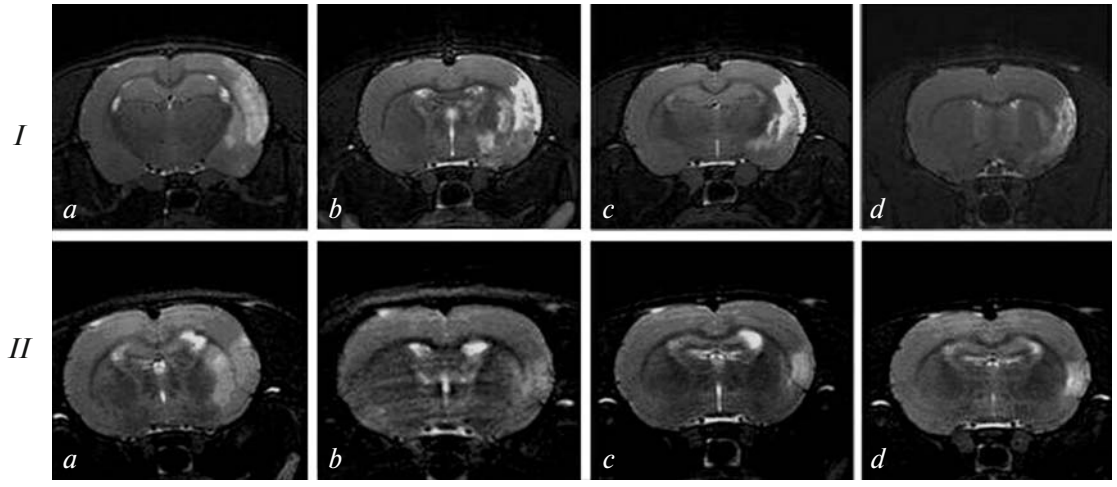

Fig. 1. MR images of the brain of rats with experimental stroke. *I*: control animal intravenously injected with saline; *II*: experimental animal intravenously transplanted human MSC. *a*) one day after MCS occlusion; *b*) 7 days; *c*) 14 days; *d*) 30 days.

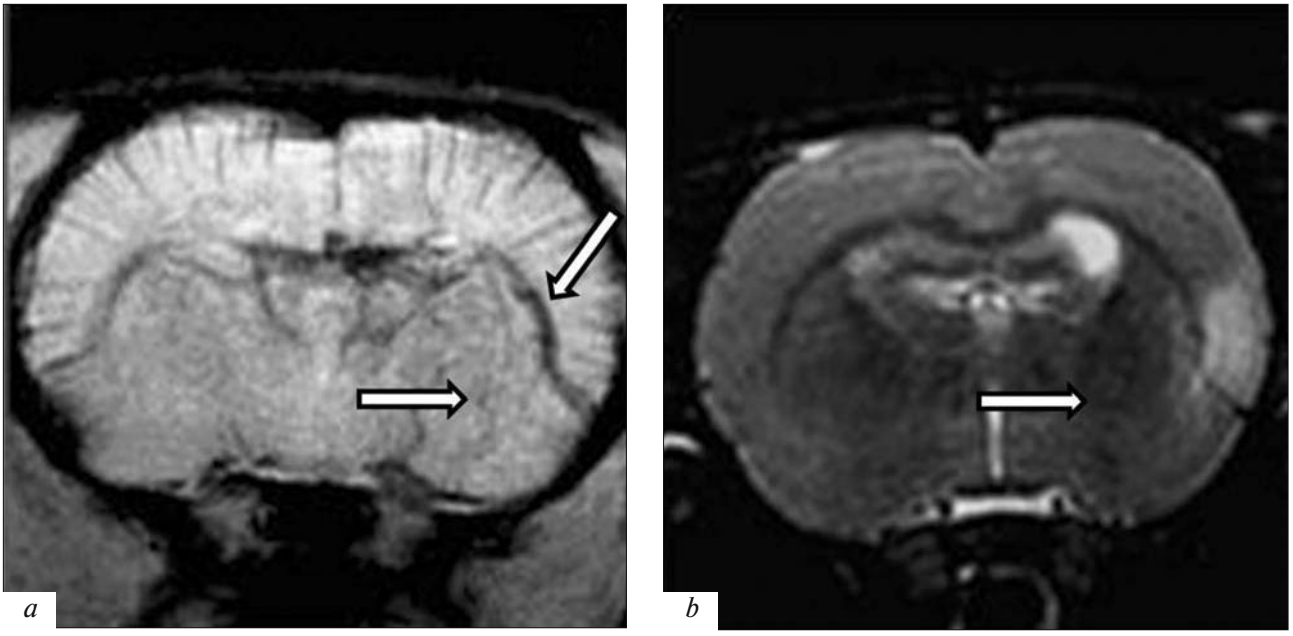

Fig. 2. MR images of the brain of a rat with experimental ischemic stroke on day 14 after MCA occlusion (day 12 after MSC transplantation). Arrows show accumulation of transplanted MSC. *a*) SNAP T2*-WI; accumulation of labeled MSC around the ischemic zone; *b*) RARE T2-WI; brain ischemia, visualization of the involved zone of the cortical/subcortical location.

the zone adjacent to the ischemic focus. Three weeks after transplantation of human MSC their accumulations were seen mainly in the ischemic focus and near it, as well as in the subventricular zone of the lateral ventricles and in the hippocampus of the ischemic and contralateral hemispheres. Some labeled cells in the ischemic zone were positively stained with antibodies to human GFAP (astroglia marker protein). NeuN-positive cells were detected only near the stroke focus, but not inside it. Some of the data presented in Table 2 are shown as digital photographs in our recent on-line publication [15].

In our previous experiments, cells positively stained with antibodies to human neuron marker proteins (βIII-tubulin, NeuN) and human astrocyte marker proteins (GFAP) were detected around the ischemic focus 45 and 64 days after MCA occlusion. However, cells expressing these markers had no typical morphological features due to which they could be classified as mature neurons or astrocytes.

Our findings indicate that some transplanted cells start differentiation into neurons and glia, but this cannot fully explain the positive effects of MSC transplantation during the first month postoperation, because differentiation is incomplete, involves just a small portion of cells, and starts late. The first signs of neuronal differentiation are observed only 2 weeks after the operation, when the volume of infarction is significantly reduced (according to MRT). According to our findings, the neurological status of rats also improves by this time [1,15].

It is known that stem cells (including MSC) are characterized by a potent paracrine effect due to the factors they release [3,4]. We found that intravenous injection of human cells to rats with ischemic stroke leads to active stimulation of resident neuronal stem

TABLE 1. Dynamics of Shrinkage of the Focus of Ischemia Induced by MCA Occlusion in the Control and Experimental Groups of Rats

Group	MRT mode	Volume of ischemic focus, mm^3			
		Day 1	Day 7	Day 14	Day 30
MCA occlusion MCA occlusion+human MSC	T2-WI (RARE)	190±33	123±19	75±11	64±12
MCA occlusion+human MSC	T2-WI (RARE)	172±36	112±24	38±7*	23±3*

Note. *$p<0.05$ compared to MCS occlusion without treatment.

TABLE 2. Immunohistochemical Analysis of Cryosections of the Brain of Rats with Ischemic Stroke after Transplantation of Human MSC

Day after operation	*n*	Method of analysis	Result
Day 7	6	Immunohistochemical analysis of serial cryosections of the brain	Accumulation of labeled cells around vessels mainly in ischemic hemisphere. Separate cells near the vessels in the contralateral hemisphere
		Staining of brain sections by antibodies to human GFAP, NeuN	No positive staining
Day 14	7	Immunohistochemical analysis of serial cryosections of the brain	Small accumulations of labeled cells in ischemic zone; some labeled cells diffusely scattered in the parenchyma of both hemispheres. Clearly seen tracks formed by labeled cells in both hemispheres
		Staining of brain sections by antibodies to human GFAP, NeuN	Solitary cells expressing human NeuN protein and carrying magnetic particles in the zone adjacent to ischemic focus. No staining for human GFAP
Day 21	7	Immunohistochemical analysis of serial cryosections of the brain	Large foci of labeled cells in ischemic zone and foci in subventricular zone and hippocampus of both hemispheres; separate cells around the vessels
		Staining of brain sections by antibodies to human GFAP, NeuN	Small groups of cells expressing human NeuN protein and carrying magnetic particles in the zone adjacent to ischemic focus. No cells of this kind in ischemic focus. Solitary cells with clear-cut co-localization of human GFAP and magnetic particles in ischemic focus

cell proliferation in the subventricular zone, especially in the hemisphere on the side of ischemia (Fig. 3). The number of dividing cells in the subventricular zone of experimental rats was significantly higher than in control animals (Fig. 3, *a*, *b*). Moreover, Ki-67-positive cells (proliferating cells) formed tangetial tracks in experimental animals; nothing of this kind was seen in controls (Fig. 3, *c*, *d*). In addition, judging from staining with antibodies to Ki-67 (proliferation marker), proliferating cells accumulated in the focus of ischemia and around it in experimental animals (Fig. 3, *e*, *f*).

These results confirm our published data indicating that early transplantation of human placental MSC to rats with experimental ischemic stroke stimulates recovery of damaged brain areas [1,15]. During the first week after intravenous transplantation to rats, MSC penetrate into brain tissue. First, human cells can be seen near vessels, and then they form chains (tracks). The formation of tracks is presumably a manifestation of MSC migration from the perivascular sites of their penetration from the blood stream into cerebral tissue towards the ischemic focus and towards the subventricular zones and hippocampal dentate nuclei in both hemispheres, where they accumulate during week 3 after transplantation. The resident neural stem and progenitor cells of the brain are located in the subventricular zones and in the hippocampus [10]. These cells start active proliferation and migration towards the ischemic focus under the effect of transplanted MSC. The stroke to a certain measure stimulates proliferation of stem cells in the subventricular zones and hippocampus [10], but injection of MSC several-fold enhances this process. On the other hand, the majority of human MSC migrating into the ischemic focus die. Just a small portion of them starts expressing neuronal and glial cell markers, but these cells do not transform into true neurons and astrocytes over 1.5-2 months after the transplantation. Hence, stimulation of postinfarction recovery of cerebral tissue of rats after transplantation of human placental MSC is presumably realized through stimulation of resident stem cells of rat brain.

We demonstrated homing of human placental MSC into the ischemic stroke focus and in zones of location of the resident stem and progenitor cells of the brain. Invasion of these cells into the neurogenic zones of the brain is of particular importance for understanding of the mechanism of the effect of transplanted cells.

The available data on the tropism of transplanted stem cells for these zones are extremely scanty. It was shown that after stereotaxic injection of human bone marrow MSC to rats with ischemic stroke, the transplanted cells were immunohistochemically detected in the hippocampus on day 7 after transplantation and along the lateral ventricle in the subventricular zone of the ischemic hemisphere on day 14 after transplatation [9]. On the other hand, according to MRT, the main

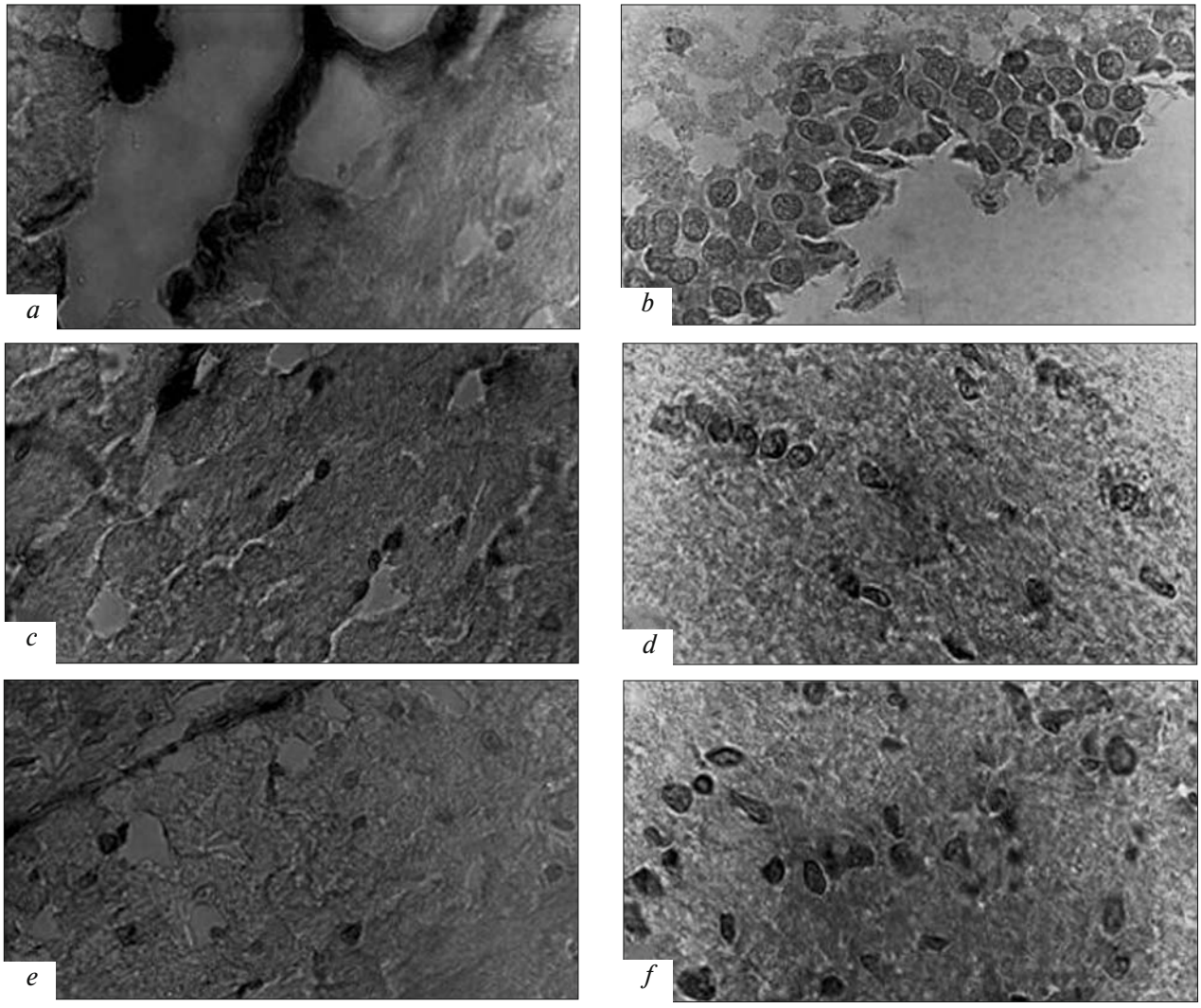

Fig. 3. Effect of human MSC transplantation to rats with experimental stroke on proliferation, migration, and homing of subventricular zone cells. Staining with antibodies to Ki-67. *a*, *c*, *e*: control animal; *b*, *d*, *f*: experimental animal. *a*, *b*) subventricular zone; *c*, *d*) area between subventricular zone and ischemic focus; *e*, *f*) ischemic focus. 40× objective.

accumulation foci of human MSC were detected in the peri-infarction region and in the *corpus callosum*. After stereotaxic transplantation into the brain of immunodeficient mouse fetuses, genetically modified human fetal neural stem cells were detected in the neurogenic and other cerebral compartments of adult animals [12].

Our results also indicate that MCA occlusion in rats is an adequate model for studies of the cellular and molecular mechanisms of the effects of transplantation of MSC and other stem cells in ischemic stroke.

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