DOI 10.1007/s10517-023-05822-1

CELL TECHNOLOGIES IN BIOLOGY AND MEDICINE

Therapeutic Efficacy and Migration of Mesenchymal Stem Cells after Intracerebral Transplantation in Rats with Experimental Ischemic Stroke

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Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 1, pp. 3-13, March, 2023
Original article submitted November 22, 2022

We studied therapeutic efficacy and migration characteristics of mesenchymal stem cells isolated from the human placenta after their intracerebral (stereotactic) administration to rats with the experimental ischemic stroke. It was shown that cell therapy significantly improved animal survival rate and reduced the severity of neurological deficit. New data on the migration pathways of transplanted cells in the brain were obtained.

Key Words: *cell therapy; mesenchymal stem cells; ischemic stroke; middle cerebral artery occlusion model; intracerebral administration*

Cell therapy with mesenchymal stem cells (MSC) is a promising way of treatment of many neurological diseases, including ischemic stroke [1-3]. The significant effect of MSC transplantation on the recovery of neurological functions and regenerative processes in the brain has been proven on biological models of cerebral infarction [4-6]. The results of the first clinical trials demonstrating the safety of the cell therapy using MSC are promising [7-9]. For further introduction of cell therapy into clinical practice, the necessary condition is to study the mechanisms of action of MSC that will help to determine the optimal parameters of their transplantation (the therapeutic "window", the way of transplantation, the dosage and multiplicity of injections).

One of the key points for understanding the mechanisms of action of MSC is the study of their distribution and migration after administration. Currently, various methods of administration have been developed and studied: intravenous, intra-arterial, intracerebral (stereotactic), intraventricular, intranasal, and others [2,10,11]. It should be noted that MSC produced a positive therapeutic effect, regardless of the administration route, however, the optimal route in cerebral infarction is still not determined [11,12]. Each administration route has its advantages and disadvantages.

Intravenous administration is quite effective and one of the least invasive routes [13-15]. However, most of the intravenously administered MSC are retained in parenchymal organs (lungs, liver, spleen and others), and only single cells reach the brain (about 2-4% of

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the injected dose) [16-19]. Small number of MSC in the brain after intravenous injection significantly limits the study of their migration after transplantation. After intra-arterial administration, transplanted cells in greater quantities enter the cerebral vessels (about 21% of the administered dose), bypassing the reticuloendothelial system organs [20,21]. Intra-arterial administration of MSC showed a high therapeutic efficiency, but this method requires great technical precision and compliance of transplantation parameters (the dosage of transplanted cells, the rate of administration, and the maintenance of cerebral blood flow) to prevent cell embolism [22]. Preclinical studies showed that after intra-arterial administration, the cells are relatively quickly eliminated from the cerebral vessels and brain tissue, which also makes it rather difficult to observe their migration [23]. Targeted delivery of the entire dose of cells can be ensured by their intracerebral (stereotactic) administration [24]. However, it should be taken into account that this method of administration requires highly precise neurosurgical access [25]. In addition, the cells can be transplanted in a limited volume (no more than 20 μl of cell suspension can be administered into rat brain) to avoid mass effect: compression and displacement of brain structures by the transplant [12]. Modern stereotactic systems ensure cell delivery into almost any brain zone, but the optimal area has not yet been determined. Thereby, MSC can be injected directly into the area of damage [26,27]. This makes it possible to deliver MSC very close to the infarction focus, however, this creates an unfavorable microenvironment in the area of damage, including for transplanted cells. According to the available data, the closer to the damaged area the cell transplantation occurs, the less their survival after administration [28]. In addition, MSC migration in the brain cannot be studies in this case, because they are already at the site of injury.

An important property of MSC is migration to the damaged area. This phenomenon is based on the ability of cells to move along the gradient of chemoattractants that accumulate in the area of injury [29]. The latter allows cell transplantation to the periphery of the focus of cerebral infarction or even to the opposite hemisphere [30].

Our aim was to study the features of migration and therapeutic efficacy of MSC isolated from the human placenta after their intracerebral (stereotactic) administration to rats with modeled endovascular occlusion of the middle cerebral artery.

MATERIALS AND METHODS

MSC. Cells were isolated from placenta of healthy woman after term labor (38-40 weeks of gestation) [31,32]; written informed consent was obtained. MSC of placenta were phenotyped by the main immunophenotypic markers by flow cytometry ($CD34^-$, $CD45^-$, $HLA\text{-}DR^-$, $CD105^+$, $CD29^+$, $CD73^+$, and $CD90^+$) and their multipotency was verified through differentiation to chondrogenic, osteogenic, and adipogenic lineage cells. Passage 3-5 cells were used for transplantation. To study MSC migration, the cells were labeled with superparamagnetic microparticles based on iron oxide (SPIO) (MC03F, diameter 0.9 μm; Bang Laboratories, Inc.) with Dragon Green fluorescent label (λ_{ex}=480 nm, λ_{em}=520 nm) according to the manufacturer's protocol. The dose of transplanted MSC was 3×10^5 cells in 15 μl of saline.

Laboratory animals. The experiments were carried out on male Wistar rats (n=76) weighing 230-300 g. The animals were obtained from certified nursery and kept in the vivarium of Pirogov Russian National Research Medical University. During the whole experiment, rats were kept 5 animals in a cage, under a 12-h light regime, room temperature (22±2°C), humidity 45-65% and with free access to water and briquetted feed. The study was approved by the Committee of Maintenance and Use of Laboratory Animals of Pirogov Russian National Research Medical University (Protocol-Application No. 24/2021; December 10, 2021). All surgical manipulations and MRI examination of animals were performed under inhalation anesthesia with isoflurane mixed with atmospheric air (Aerran, Baxter Healthcare Corporation): 3.5-4% isoflurane for induction of anesthesia and 2-2.5% isoflurane to maintain anesthesia. At the end of the experiment, as well as for histological studies in dynamics, the animals were euthanized by inhalation of a lethal dose of isoflurane and additional intraperitoneal injection of a lethal dose of tiletamine (Zoletil, Virbac).

Study design. Experimental ischemic stroke was simulated by the method of transient endovascular occlusion of the middle cerebral artery. In 24 h after the procedure, the animals were randomly divided into 3 groups. Group 1 (control, n=35) included rats with experimental stroke model. Group 2 (n=31) consisted of rats with experimental stroke and intracerebral transplantation of MSC (3×10^5 cells in 15 μl of saline); 13 rats of this group underwent dynamic observation for 14 days to assess the therapeutic efficacy of cell therapy; in 8 rats, MSC migration was assessed by MRI and histological examination after 1 h (n=1), 1 day (n=1), 11 days (n=1), and 14 days (n=4). Group 3 included rats (n=10) with experimental stroke and intracerebral injection of 15 μl of physiological saline at coordinates similar to those in group 2 rats.

Modeling of acute focal cerebral ischemia. Transient (90 min) endovascular occlusion of the right middle cerebral artery was performed using a mono-

filament as described previously [33] in modification [34] with MR-control of intravascular position of the filament according with [35]. The nylon monofilament with a silicone tip (diameter 0.19 mm, length 30 mm; coated diameter 0.37±0.02 mm; coating length 3-4 mm; Doccol Corporation) was inserted into the lumen of the internal carotid artery up to the origin of the middle cerebral artery. Then, MRI control of the filament position was performed. From the moment of occlusion of the middle cerebral artery, the countdown began for the formation of a focus of cerebral infarction. After 90 min, the monofilament was removed, the surgical wound was sutured, and antibiotic (gentamicin sulfate 4%, Dalkhimpharm) was administered. Then, the animals were placed into a heated cage where they recovered from anesthesia.

Intracerebral cell transplantation. Stereotactic transplantation of MSC was performed under inhalation anesthesia. A gel with dexpanthenol (Cornere-gel, Dr. GERHARD MANN Chem.-Pharm. Fabrik) was placed in the conjunctival sacs to prevent the sclera and cornea from drying out. After shaving off the hair on the head, the skin was disinfected with solutions of betadine and 70% ethanol, then local anesthesia was performed with 0.1 ml of 0.5% bupivacaine solution (Markain, RECIPHARM MONTS). A sagittal 1-cm skin incision was made with a scalpel, the skull was scalped, and a hole ~1 mm in diameter was drilled at coordinates AP=+0.6 mm and ML=3.5 mm to the bregma. MSC or saline were injected 24 h after pathology modeling into the region of the left striatum (contralateral area of cerebral infarction) at VD=-4.5 mm using a 500 μl Hamilton syringe fixed in an injector (Leica Microsystems GmbH) at a rate of 3 μl/min. After cell injection, the needle was left in the brain for 5 min to ensure cell diffusion at the injection site, after which it was slowly removed, the wound was sutured with an interrupted suture, antibiotic (gentamycin sulfate 4%) was administered. The skin was treated with chloramphenicol ointment (Levomekol, Nizhpharm) and dexpanthenol spray (AEROPHARM). Then, the animals were placed in a heated cage, where they recover from anesthesia.

Assessment of therapeutic efficacy of cell therapy. In order to study the effectiveness of cell therapy over time (within 14 days), we assessed survival rate, total neurological deficit, and the volume of the focus of cerebral infarction according to MRI data 24 h after the modeling of acute focal ischemia (before cell injections) and on the 7th and 14th days. The neurological deficit was assessed by the modified Neurological Severity Score (mNSS); the maximum score for this scale is 18) [36]. At the end of the observation period (day 14), the motor function of forelimbs was also assessed in the "Cylinder" test (OpenScience) [37]. The percentage of the left forelimb use on the side of the paresis (contralateral area of cerebral infarction) was determined.

MRI. The study was carried out on a 7T ClinScan tomograph for small laboratory animals (Bruker BioSpin) under inhalation anesthesia with isoflurane. The volume of the focus of cerebral infarction was estimated 24 h after modeling of experimental stroke and on days 7 and 14. The MR protocol consisted of obtaining T2-weighted images (T2-WI) in axial projection (Turbo Spin Echo pulse sequence with restore magnetization pulse; turbo factor 9; TR/TE=4000/46 msec; averages=2; spectral saturation of fat; FOV=37×29.6 mm; section thickness 0.5 mm; matrix size 320×256; breath synchronization). Morphometric analysis of the cerebral infarction focus was performed using ImageJ software. The area of the hyperintense zone was measured on each slice on T2-WI, and then, the total volume of the infarction focus was calculated. Prior to intracerebral administration of SPIO-labeled MSC and in dynamics on days 1 and 14 after transplantation, high-resolution susceptibility WI (SWI) were obtained (3D Gradient Echo with RF cleansing and flow compensation; TR/TE=50/19.1 msec; flip angle=15; averages=1; with frequency suppression signal from adipose tissue; FOV=30×20.6 mm; section thickness 0.5 mm; matrix size 256×176).

Histological examination. The animals were sacrificed 1 and 24 h, 11 and 14 days after the stereotaxic administration of MSC. After euthanasia, transcardial perfusion with 4% paraformaldehyde in 0.01 M PBS (pH 7.4) was performed. After decapitation, the brain was removed and stored in 4% paraformaldehyde in PBS at 4°C for 24 h. Then, frontal sections of the brain 50 μm thick were obtained using a Microm HM 650V Vibrating-Blade Microtome (Thermo Scientific). To visualize SPIO by classical histological method, Perls staining was performed. To this end, sections on glass slides were rinsed in distilled water and placed for 10 min in Perls' solution, which consisted of 2% aqueous solution of potassium ferrocyanide and 2% aqueous solution of hydrochloric acid. Then, the preparations were again rinsed in distilled water and stained in a neutral red solution. For immunohistochemical staining, the sections were incubated for 1 h in a mixture of 0.3% Triton X-100, 5% normal goat serum (Sigma-Aldrich), 0.01 M PBS (pH 7.4). Then, the nuclei were washed in 0.01 M PBS (pH 7.4), stained with DAPI (2 μg/ml; Sigma-Aldrich), and placed under coverslips in glycerol. Microphotographs of preparations were obtained using a Keyence BZ 9000E digital fluorescent microscope and a Nikon A1R MP+ laser scanning confocal microscope. The distance of cell migration was determined by the distance (in μm) between the initial injection site and the cells locat-

ed at the greatest distance from it using the ImageJ software package.

Statistical analysis. The obtained results were processed in SPSS Statistics 23.0 (IBM). The perception level for all experiments was $p<0.05$. Survival rate was based on Kaplan–Meier curves using the Log Rank test and amendments to multiple comparisons. To assess the dynamics of the neurological deficit, the volume of the focus of cerebral infarction, we used the total linear model with repeated measurements. Differences between groups according to the results of test "Cylinder" and also the distance of MSC migration were estimated using the Mann–Whitney *U* test with multiple comparison adjustment (FDR).

RESULTS

Comprehensive assessment of the therapeutic efficacy of MSC after intracerebral administration to rats with experimental cerebral infarction was performed. The survival rate, overall neurological deficit, forelimb motor function, and volume of cerebral infarction by MRI were assessed. The "therapeutic window" between focal ischemia modeling and cell injection (24 h) was chosen on the basis of published data [38,39], as well as based on the maximum values of the "therapeutic window" for reperfusion therapy [40]. The dose of transplanted cells and injected volume of cell suspension (3×10^5 MSC in 15 μl of saline) were also selected based on the data of safety and efficiency of MSC transplantation [12,30,41,42]. Intracerebral infusion of more than 20 μl is traumatic for rats and can lead to the development of brain edema, displacement of the median structures, and increase the mortality. According to other studies, the optimal dosage of transplanted stem cells in a relatively small volume for intracerebral administration varied from 3×10^5 to 7.5×10^5 cells [12,43].

Strict adherence of the selected parameters for intracerebral administration of MSC made it possible to prevent the development of complications in experimental animals: none of the animals showed a significant mass effect or brain edema according to the MRI results.

The survival rate. The survival rate of animals was evaluated by the Kaplan–Meier method within 14 days after intracerebral injection of MSC. The study included two control groups: rats with experimental stroke and rats with experimental stroke and intracerebral injection of saline along coordinates similar to the infusion of MSC. The last group was included in the experiment to simulate brain microtrauma that occurs during stereotaxic cell transplantation. Thus, the survival rate of 76 rats was evaluated. At the same time, the rats sacrificed for histological study were also taken into account.

During the observation period, the maximum number of animal deaths in all experimental groups was observed within the first 2-4 days after acute focal ischemia modeling (1-3 days after intracerebral administration) (Fig. 1), which can be related to the development of vasogenic edema of the brain substance with the subsequent occurrence of dislocation syndromes, as well as the appearance of hemorrhagic transformations in the area of infarction [44]. The groups were compared using the Log Rank test adjusted for multiple comparisons. Significant differences were found between all groups ($p<0.05$). The best survival rate was observed in the group with intracerebral injection of MSC, the worst was in the group with intracerebral injection of saline against the background acute focal cerebral ischemia. Therefore, intracerebral transplantation of MSC contributed to a significant improvement of the survival rate of animals with the ischemic stroke model. It should be noted that the improvement of survival rate occurred even despite the invasiveness of the intracerebral method of introducing cells. At the same time, the survival rate in the group with solution infusion was so low that we excluded it from further analysis of the therapeutic efficacy due to insufficient number of survived animals (more than a half of animals died by day 7).

The neurological deficit. Changes in the neurological deficit according the mNSS scale was evaluated in dynamics after stereotactic MSC transplantation. The measurements were carried out directly 24 h after modeling of acute focal ischemia (before cell

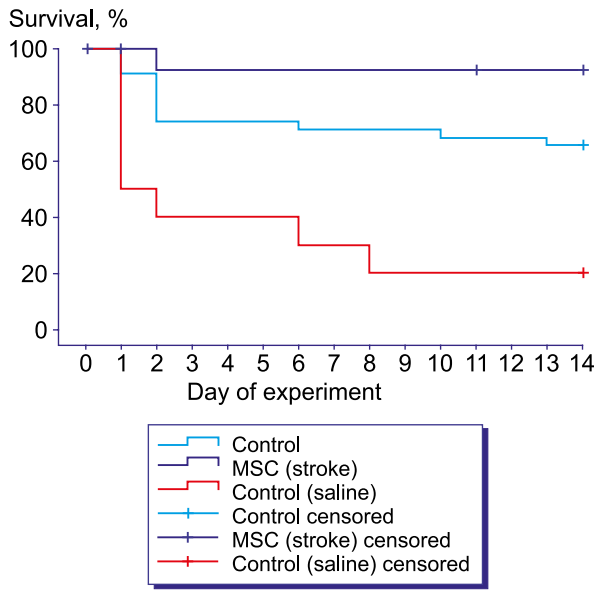

Fig. 1. The Kaplan—Meier survival curves. Censored data refer to animals sacrificed at the end of the observation period and earlier for histological studies. Statistically significant differences were found between all study groups.

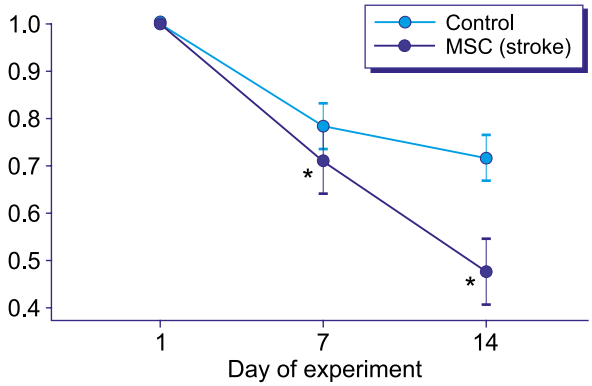

Fig. 2. Dynamics of neurological deficit (normalized to the first day) according to mNSS score. *p<0.05 in comparison with the control.

transplantation for the group of MSC) and on days 7 and 14. The dynamics of changes in neurological deficit was estimated by the method of the general linear model with repeated measurements (Fig. 2). The dynamics of normalized (relative to day 1) neurological deficit in the control group and MSC group significantly differed (p<0.05) throughout the observation period. When indicators of neurological deficit were separately compared on days 7 and 14 using the method of confidence intervals, significant differences between the groups were revealed only on day 14. Thus, intracerebral transplantation of MSC caused a significant decrease in the severity of the general neurological deficit by day 14.

The motor function of the forelimb. The forelimb motor function was assessed using the "Cylinder" test. The test implies active behavior and movement of the animal to orientate in space and explore the walls of the installation, therefore, measurements performed only at the end of the observation period (on day 14). At the earlier terms, the motor function was not assessed due to severe neurological deficit. The percentage of use of the front left limb on the side of the paresis (contralateral side of the area of cerebral infarction) was calculated and pairwise comparison of these values was carried out using the Mann–Whitney test, adjusted for multiple comparisons (Fig. 3). The significant differences (p<0.05) between the control group and MSC group were established. Thus, intracerebral transplantation of MSC significantly accelerated recovery of the motor deficit of the forelimb on day 14 after transplantation. These findings are consistent with the mNSS scores that showed a decrease of neurological deficit at this time point.

Stroke volume. The volume of the focus of cerebral infarction was assessed by MRI data (T2-WI) 24 h after experimental stroke modeling and days 7 and 14. Using the method of general linear model with repeated measurements, the dynamics of changes in the stroke volume normalized to day 1 was evaluated. The volume of the cerebral infarction gradually decreased in both groups, however, there were no significant differences in the dynamics of regression (Fig. 4). Published data on changes in the size of the cerebral infarction zone after MSC transplantation are rather contradictory. Some investigators reported more rapid decrease in the lesion volume after cell therapy [45,46], while others did not observe significant differences from the control [12,14,21]. The reason for these differences is not fully understood, but it can be associated to different study protocols and sources of MSC.

Thus, intracerebral administration of MSC from the human placenta to rats 24 h after modeling of experimental cerebral infarction showed a significant improvement in the survival rate and neurological status of animals during the entire observation period. These results are consistent with published data that MSC after stereotaxic administration have a positive effect on the recovery of neurological functions, especially motor deficit [6,12,47,48]. It is interesting to note that systemic (intravenous and intra-arterial)

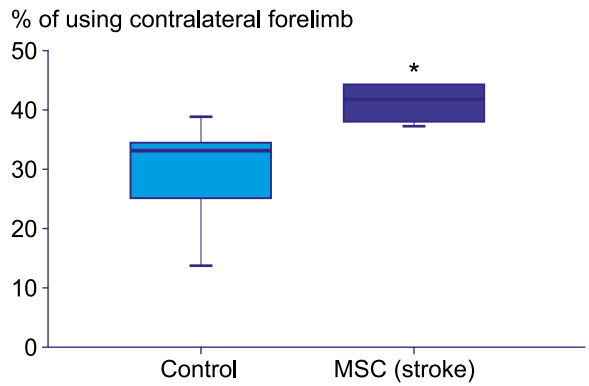

Fig. 3. The results of the "Cylinder" test on day 14 of the observation period. *p<0.05 in comparison with the control.

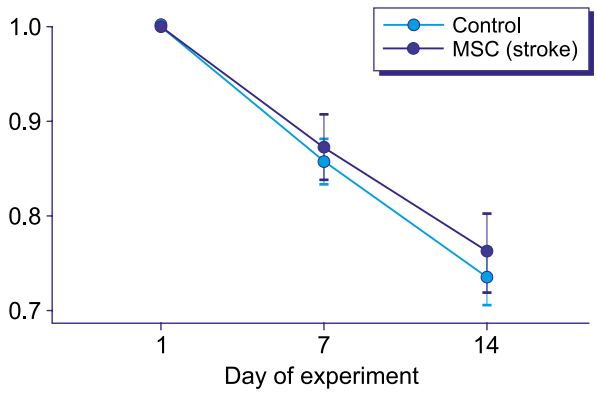

Fig. 4. Dynamics of changes in the volume of the infarction focus according to MRI data (normalized to the first day). The focus of infarction was measured by T2-WI.

transplantation of MSC also contributes to the significant regression of the neurological deficit in laboratory animals, however, this effect is recorded earlier, starting from day 7 [14,21].

Based on the comprehensive assessment of the therapeutic efficacy, it can be concluded that intracerebral transplantation of MSC is a promising experimental approach to the treatment of ischemic stroke. To facilitate introduction of this technology into clinical practice, further studies of the mechanisms of action of MSC and the nature of their distribution and migration are needed, which will help in the design of clinical trials and increase the effectiveness of cell therapy.

MSC migration in the brain. Stereotactic transplantation was performed in the striatum region in the left (contralateral zone to infarction) hemisphere of the brain. The injection coordinates were chosen taking into account the possibility of assessing the range of MSC migration towards the ischemic focus. To visualize MSC, MRI was performed followed by histological verification of the obtained data. To this end, MSC were labeled with SPIO conjugated with the Dragon Green fluorescent label before the transplantation. For the detection of transplanted cells, the SWI pulse sequence was used, which is the most sensitive to changes in the local inhomogeneity of the magnetic field among all T2-weighted MR pulse sequences and this makes it possible to visualize even single SPIO-labeled stem cells in the brain [32,49]. Additionally, T2-WI was also used to visualize the anatomy of brain structures. After transplantation, SPIO-labeled cells were visualized on SWI and T2-WI as zones of reduced MR signal intensity (hypointense, "dark" areas), because SPIO microparticles inside the MSC cytoplasm create a local inhomogeneity of the magnetic field around the cells and reduce T2* relaxation time [50]. It is important to note that due to the pronounced distortion of the local magnetic field in the areas of SPIO accumulation, the size of the areas of signal intensity reduction on MRI can significantly exceed the real size occupied by transplanted MSC in the brain [32].

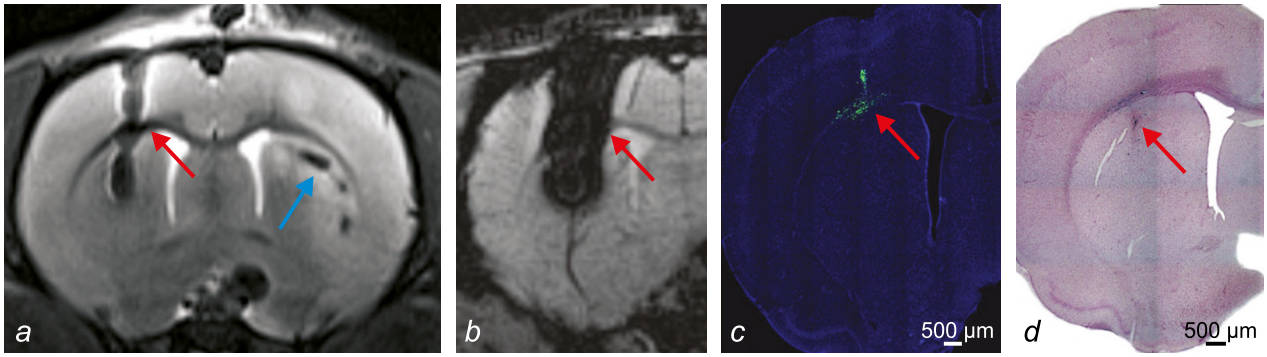

Fig. 5. Migration of MSC after intracerebral injection in the brain of rats with the model of acute focal ischemia. MR images (*a*, *b*) and microphotographs (*c*, *d*) of the rat brain on day 14 after MSC transplantation in the region of the left striatum. *a*) T2-WI for visualization of the injection track (red arrow) and cerebral infarction zone, hemorrhagic transformations in the area of infarction (blue arrow). *b*) SWI, hypointense zone corresponds to the place of accumulation of SPIO-labeled MSC. *c*) Localization of SPIO-labeled cells (green fluorescence, arrow). *d*) Localization of SPIO-labeled cells (blue Perls staining, arrow).

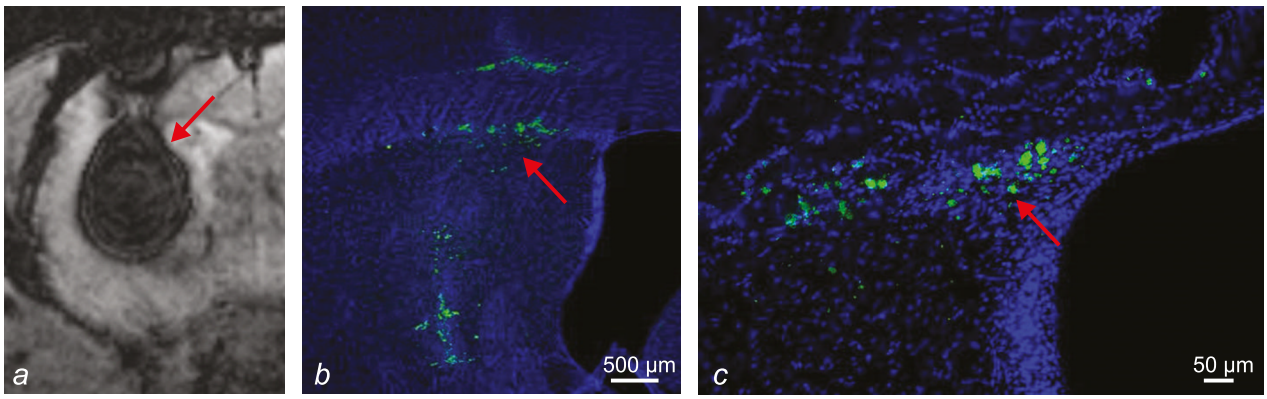

Fig. 6. Migration of MSC through the corpus callosum after intracerebral injection in rats with an acute focal cerebral ischemia. MR image (*a*) and micrographs (*b*, *c*) of the brain rats 14 days after transplantation of MSC into the region of the left striatum. *a*) SWI, the hypointense zone corresponds to the accumulation of SPIO-labeled MSC (arrow). *b*, *c*) Localization of SPIO-labeled cells (green fluorescence, arrow): labeled MSC migrate along corpus callosum to the area of the left lateral ventricle.

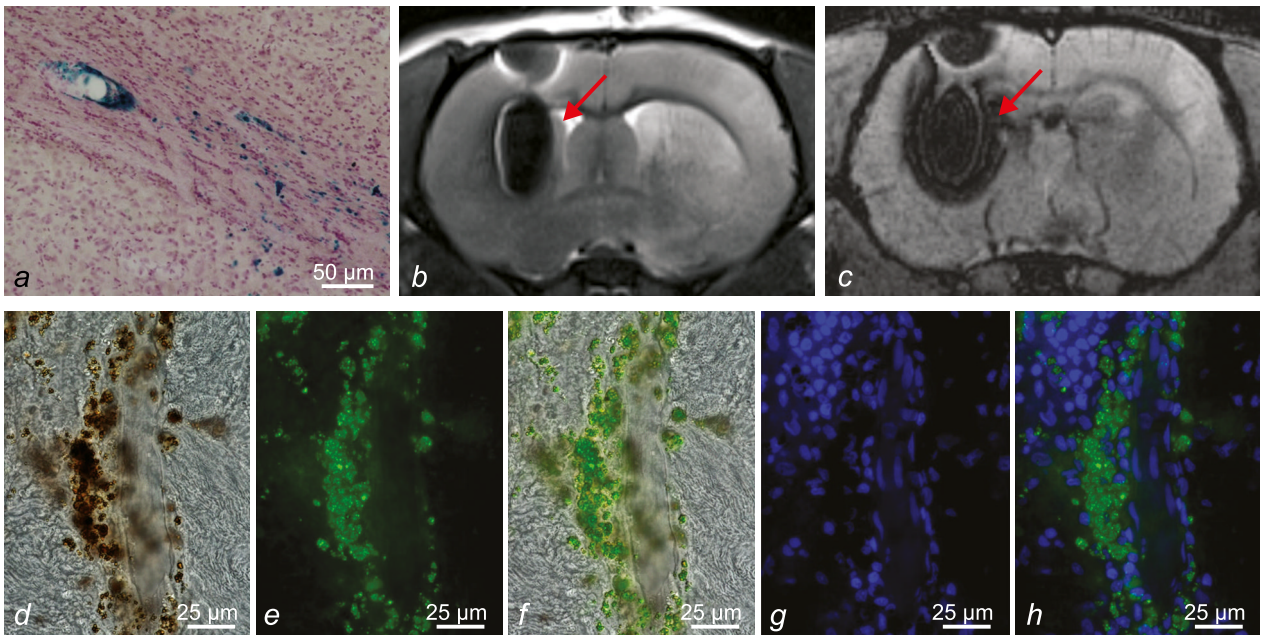

Fig. 7. Migration of MSC along cerebral vessels after intracerebral administration to rats with the model acute focal cerebral ischemia. Microphotographs (*a*, *d-h*) and MR images (*b*, *c*) of the rat brain on day 14 after MSC transplantation into the region of the left striatum. *a*) Localization of SPIO-labeled cells in the area of the corpus callosum (blue staining according to Perls). *b*) T2-WI for visualization of the injection track (arrow) and brain infarction zone. *c*) SWI, hypointense zone corresponds to the site of accumulation of SPIO-labeled MSC. *d-h*) Transplanted SPIO-labeled MSC around the cerebral vessel. Light microscopy: SPIO microparticles are brown (*g*); fluorescence microscopy: localization of SPIO-labeled cells (green fluorescence) (*e*); combined image (*f*); fluorescence microscopy: nuclei labeled with DAPI (blue fluorescence) (*g*); combined image: blue fluorescence — DAPI, green — SPIO (*h*).

Immediately after intracerebral injection into the region of the left striatum, transplanted MSC were visualized along the needle track. Within 14 days after transplantation, labeled MSC migrated to insignificant distances from the track into the brain substance in the striatum area and to longer distances along the corpus callosum in the medial and lateral directions from the transplantation area (Fig. 5). Migration to the maximum distance towards the opposite hemisphere occurred medially along the corpus callosum to the border with the left lateral ventricle (Fig. 6), however, SPIO-labeled cells were not visualized in the opposite hemisphere.

It is known that the subventricular zone in the mammalian brain is a neurogenic region [51]. It cannot be ruled out that transplanted MSC move along a gradient of chemoattractants that can be secreted by endogenous neuronal stem cells. For some animals (n=9), the maximum distance of cells migration was determined: the mean distance of maximum migration in the lateral and medial directions along the corpus callosum on day 14 was 985±435 and 912±383 μm, respectively. There were no significant differences in the distance of cell migration along the corpus callosum between these directions. Migration of stem cells through the corpus callosum after transplantation has also been described in other studies [52,53], including after transplantation into healthy animals [54,55]. It can be assumed that the white matter axons may guide the movement of transplanted stem cells. In addition, we were able to show that in rats with a model of acute focal ischemia, a significant part of the transplanted cells was visualized around the cerebral vessels from their outer side (Fig. 7) both in the striatum around the injection track and in the corpus callosum at all periods of the study. The affinity of MSC to vessels after intracerebral transplantation was also noted in the case of injection of stem cells into the brain of healthy rats [55].

It is also important to note that during 2 weeks of follow-up, we did not detect MSC migration to the opposite hemisphere of the cerebral infarction zone. At the same time, the transplanted cells had a pronounced therapeutic effect, improved animal survival, and reduced the neurological deficit.

The obtained data on MSC migration in the brain after stereotaxic administration suggest that the positive effect of transplanted cells is mediated by paracrine mechanisms and interactions with cerebral vascular cells and other components of the neurovascular niche and lead to activation of the “trigger” mechanisms resulting in sustained functional recovery of animals after modeling of acute focal cerebral ischemia.

The work was carried out within the framework of State Assignment No. 056-00019-20-00 (registration No. AAAA-A20-120020590123-5, February 5, 2020).

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