MRI evaluation of frequent complications after intra-arterial transplantation of mesenchymal stem cells in rats

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Abstract. Intra-arterial transplantation of mesenchymal stem cells (MSCs) is an effective delivery route for treatment of ischemic brain injury. Despite significant therapeutic effects and targeted cells delivery to the brain infraction, serious adverse events such as cerebral embolism have been reported and may restrict potential clinical applications of this method. In current study, we evaluate potential complications of intra-arterial MSCs administration and determine the optimum parameters for cell transplantation. We injected SPIO-labeled human MSCs via internal carotid artery with different infusion parameters and cell dose in intact rats and in rats with the middle cerebral occlusion stroke model. Cerebrovascular complications and labeled cells were visualized in vivo using MRI. We have shown that the incidence of cerebral embolic events depends on such parameters as cell dose, infusion rate and maintenance of blood flow in the internal carotid artery (ICA). Optimal parameters were considered to be 5x10⁵ hMSC in 1 ml of PBS by syringe pump with velocity 100 µ/min and maintenance of blood flow in the ICA. Obtained data should be considered before planning experiments in rats and, potentially, can help in planning clinical trials in stroke patients.

1. Introduction
Ischemic stroke is one of the leading causes of death and disability worldwide. The only currently available and effective treatment is reperfusion methods, that are restricted by “narrow” therapeutic window and have many contraindications [1]. Transplantation of mesenchymal stem cells (MSCs) has shown promising therapeutic results in different animal models of stroke and in humans according to the results of clinical trials [2]. MSCs have several practical advantages for clinical application, including proven safety, lower risks of malignancies and availability. Therefore, transplantation of MSCs can be a promising treatment strategy to reduce cerebral infarction and neurological deficit [3]. However, the cellular and molecular mechanisms of MSCs’ beneficial action, as well as the optimum therapeutic time window, route of administration and cell dose still have to be determined.
Intracerebral, intravenous and intra-arterial routes of MSCs transplantation are all effective in ischemic stroke models [4]. Specifically, intra-arterial administration provides targeted delivery of cells to the brain lesion bypassing the peripheral organs and ensuring better functional recovery after stroke [5]. Unfortunately, intra-arterial administration can trigger serious adverse events restricting potential clinical use, such as cerebral embolism [6, 7]. It has been argued that parameters of cell administration such as cell dose, infusion volume, velocity and injection technique are related to the severity and frequency of complications after intra-arterial MSCs delivery [7, 8].

In this study, we evaluate potential complications of intra-arterial MSCs administration and determine the optimum parameters for cell transplantation. We injected SPIO-labeled (superparamagnetic iron oxide microparticles) human MSCs via internal carotid artery with different infusion parameters and cell dose in intact rats and in rats with the middle cerebral occlusion stroke model. Cerebrovascular complications and labeled cells were visualized in vivo using MRI. Obtained data provided recommendation for safety intra-arterial MSCs transplantation and should be considered before planning experiments in rats and, potentially, in stroke patients in clinical trials.

2. Materials and Methods

2.1 Cell culture and labelling
Mesenchymal stem cells (MSCs) were isolated from normal human placenta (gestation age 38-40 weeks). Human placental samples were washed with Hanks solution (PanEko) and after gentle mechanical stimulation incubated with 0.1% solution of type I collagenase (Gibco) for 30 min at 37°C. The suspension was centrifuged (300 × g, 10 min) resuspended in Hanks Solution (Gibco, Invitrogen), and processed for mononuclear fraction separation. The cells were cultured in DMEM-F12, supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco) and 10% FBS at 37°C, in a humidified atmosphere containing 5% CO2 in T75 culture flask. Cells were allowed to adhere for 3 days and non-adherent cells were removed by replacing the media. Upon reaching 80% to 90% confluence, adherent MSCs were harvested by trypsinization. Human mesenchymal stem cells (hMSCs) were labeled with SPIO-containing microparticles (MC03F, Bang Laboratories, Inc., d=0.90 μm) carrying Dragon Green fluorescent dye (λex=480 nm, λem=520 nm) according to manufacturer’s instructions. A suspension of microparticles was added to hMSCs culture when it attained 80-90% confluence (2.5 μl stock suspension per 1 ml culture medium) and incubated for 12h in a CO2 incubator. After incubation, hMSCs were washed twice with PBS to remove the particles that had not been taken up and then harvested by trypsinization.

 Additionally, in part of animals to confirm the localization of SPIO microparticles we transplanted hMSCs co-labeled with lipophilic membrane cell tracking red fluorescent dye PKH26 (Sigma-Aldrich) according to the manufacturer's protocol.

2.2 Animals
All manipulations with experimental animals were carried out in accordance with the European Communities Council Directive of 24 November 1986 regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC) and were approved by the local Ethical Committee of the Pirogov Russian National Research Medical University. Adult male Wistar rats (n=46) weighing 250–300 g were used for the experiment. Animals were randomly divided into the following experimental groups depending on the parameters of intra-arterial hMSCs administration: group 1 (n=5) – intact rats with transplantation of 5x10^5 hMSC in 1 ml of PBS by manual syringe injection with the velocity 300 μ/min and no blood flow in the internal carotid artery (ICA); group 2 (n=6) – intact rats with transplantation of 5x10^5 hMSC in 1 ml of PBS by syringe pump with velocity 100 μ/min and no blood flow in the ICA; group 3 (n=5) – intact rats with transplantation of 5x10^5
hMSC in 1 ml of PBS by syringe pump with velocity 100 µ/min and maintenance of blood flow in the ICA; group 4 (n=3) – intact rats with transplantation of 1x10^6 hMSC in 1 ml of PBS by syringe pump with velocity 100 µ/min and maintenance of blood flow in the ICA; group 5 (n=27) – rats with transplantation of hMSCs 24h after ischemic stroke modelling with previously determined optimal parameters of cell administration (1x10^5 hMSC in 1 ml of PBS by syringe pump with velocity 100 µ/min and maintenance of blood flow in the ICA).

2.3 Transient middle cerebral artery occlusion

Transient middle cerebral artery occlusion (MCAO) was performed in animals of group 5 according to the intraluminal suture method [9–11]. For the surgery rats were anesthetized with 3% isoflurane and maintained at artificial ventilation with the mixture of 2-2.5% isoflurane and 97.5-98% atmospheric air supplied by E-Z-7000 animal anesthesia system (E-Z Anaesthesia® Systems), temperature was maintained at 37°C with a heating pad during anesthesia. Midline incision of the skin and dissection of superficial fascia and muscles were performed to expose the right common carotid artery (CCA), the external carotid artery (ECA) and internal carotid artery (ICA). The vagus nerve was anaesthetized additionally with 0.2 ml of 0.5% bupivacaine and sharply separated from the CCA and ICA. After ligation of the pterygopalatine artery (PPA), cauterization of superior thyroid artery (STA) and occipital artery (OA), and after placing the microsurgical clip on CCA, the ECA was then cut with microscissors and silicon rubber-coated 4-0 monofilament (Doccol corporation, diameter 0.19 mm, length 30 mm; diameter with coating 0.37+/−0.02 mm; coating length 3-4 mm) was inserted into the stump of the ECA down toward the lumen of ICA for 18-20 mm to the middle cerebral artery (MCA) till mild resistance was felt. Since that time the occlusion of MCA started. The silk suture around the ECA stump was tightened to prevent bleeding, the microclip from the CCA was removed and the incision was closed. After 90 min the rat was re-anesthetized, afterwards the incision was reopened, filament was withdrawn, the stump of the ECA was ligated and the operating wound was closed.

2.4 Intra-arterial hMSCs transplantation

Cell transplantation was performed under isoflurane anaesthesia as described above. The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were exposed (or re-exposed 24 h after MCAO in case of group 6). Microsurgical clips were placed on CCA and ICA, while the PPA was ligated (not ligated in part of the animals). The ECA was then cut and a polyurethane microcatheter (Doccol corporation, outer diameter 0.635 mm, inner diameter 0.305 mm) filled with saline was inserted into the stump of the ECA and advanced into the extracranial branch of ICA. The microsurgical clip from CCA was removed to maintain the blood flow in the ICA (for animal group 3-5) of left in CCA till the end of cells infusion (for animal groups 1-2). The catheter was then connected to a 1 ml syringe, for groups 2-6 syringe was placed in the microinjector, and SPIO-labeled hMSCs suspended in 1 ml of PBS were delivered into the ICA over different time intervals. After cell transplantation the stump was electrocoagulated, and incision was closed.

2.5 Magnetic resonance imaging

Before and immediately after hMSCs transplantation, animals of all experimental groups underwent brain MRI examination with the 7T ClinScan system (Bruker BioSpin, USA) with phased array four elements rat brain coil to evaluate cerebrovascular complications. During the procedure rats were maintained at inhalation anaesthesia as described in the previous section. The following MRI protocols were used:
- T2 weighted imaging (T2WI; Turbo Spin Echo pulse sequence with restore magnetization pulse; TR/TE = 4000/46 ms; spectral fat saturation; FOV = 37 x 29.6 mm; slice thickness = 0.5 mm; matrix size = 320 x 256);
- Susceptibility Weighted Imaging (SWI; 3D Gradient Echo with RF spoiling and flow compensation; TR/TE=50/19.1 ms; flip angle = 15; spectral fat saturation; FOV = 30 x 20.6 mm; slice thickness = 0.5 mm; matrix size = 256 x 176).
- Diffusion weighted image with calculation of apparent diffusion coefficient maps (DWI with ADC; echo planar pulse sequence; TR/TE=5000/22 ms; 14 b factors from 0 to 2000 s/mm²; spectral fat saturation; FOV = 32 x 20 mm; slice thickness = 1 mm; matrix size = 80 x 52).

2.6 Histology
The animals were sacrificed with a lethal dose of Chloral hydrate immediately after MRI and transcardially perfused with 100 ml phosphate buffered saline (PBS, pH 7.4, 0.1 M) followed by 100 ml of ice-cold 4% paraformaldehyde in PBS. Series of coronal 40 μm-thick sections were obtained with a HM 650 v vibratome (Microm GmbH, Berlin, Germany). Staining of cell nuclei was performed by incubating slide-mounted tissue sections for 10 min with 2 μg/mL DAPI solution (Sigma). Fluorescence confocal micrographs were captured with the Nikon A1R MP+ laser scanning confocal microscope equipped with a 405, 488, 561, 638 lasers and Plan Apo20x/0.75 Dic N, Apo IR 60x/1.27 WI and Apo TIRF 60x/1.49 oil Dic objective lenses. To visualize the iron microspheres, Perls’ Prussian blue staining was performed and bright-field images were acquired using the Keyence BZ-9000E microscope.

3. Results and Discussion
We found that frequent complications after intra-arterial administration of hMSCs included extracranial distribution of transplanted cells and cellular embolism of the intracranial arteries, followed by the development of cerebral infarctions.

3.1 Extracranial MSCs distribution
Internal carotid artery has a large extracranial branch, the pterygopalatine artery (12 in Figure 1). Consequently, part of cells injected via ICA can be transferred to the PPA blood supply area.

Figure 1. Arterial system of rat brain. Numbers on figure indicate:
1. Common carotid artery
2. Internal carotid artery
3. External carotid artery
4. Basilar artery
5. Posterior cerebral artery
6. Middle cerebral artery
7. Anterior cerebral artery
8. Posterior communicating artery
9. Anterior communicating artery
10. Occipital artery
11. Superior thyroid artery
12. Pterygopalatine artery
13. Hypothalamic artery
14. Ventral thalamic artery
15. Anterior choroidal artery
16. Olfactory artery
17. Superior cerebellar artery

Figure 2 demonstrates the distribution of SPIO-labeled hMSCs not only in the right brain hemisphere, but also in the masticatory muscles. To prevent extracranial “cell leakage”, the PPA should be ligated prior to cell infusion.
3.2 Cerebral embolism

We investigated the relationships between cell dose, infusion velocity, injection technique and cerebral embolic events after intra-arterial transplantation of hMSCs. In MRI, embolic stroke areas are visualized as zones of low apparent diffusion coefficient (ADC) on ADC maps in acute stage.

3.2.1 Intact rats. The optimum parameters of transplantation were determined in the experiments with intact rats. Animals were divided into 4 groups, each representing sequential change of the infusion parameters, as described in the Materials and Methods section.

The first studied parameter was the rate of MSCs infusion. The cell concentration of $5 \times 10^5$ hMSCs in 1 ml of PBS chosen for this experiment has been previously evaluated as relatively safe and sufficient to provide therapeutic effect [8]. In group 1 hMSCs were injected via ICA using a manual syringe at the rate of about 300 µ/min, and in group 2 – by a syringe pump at the rate of 100 µ/min. In both groups no blood flow in ICA around the catheter was maintained. As can be seen in Figure 3, in the first two groups severe cerebral embolism followed by ischemia and the development of cerebral infarction was observed (in group 1 in 4 out of 5 rats, and in group 2 in 5 out of 6 rats). In group 3 with the slow infusion rate of 300 µ/min and the opening of blood flow around ICA no embolic complications at all were observed. Probably, the maintenance of blood flow in ICA prevents the hMSCs aggregation and improves their distribution.

SPIO-labeled cells are visualized in the brain tissue as single hypointense spots with SWI, which was confirmed by histological study (displayed in Figure 4 (a) and (b)).

Figure 2. MRI of rat brain after intra-arterial injection of SPIO-labeled hMSCs (immediately after injection). The white dotted box delineates the area of hMSCs distributions in the right brain hemisphere, while the red dotted box shows the area of extracranial cell scattering in the masticatory muscles (zone of PPA blood supply). SPIO-labeled hMSCs could be visualized as hypointense spots with SWI. On ADC and T2wi no embolic complications are visualized.

Figure 3. MRI of rat brains immediately after intra-arterial injection of SPIO-labeled hMSCs in groups 1-3. Detailed description of each group is given in the “Materials and Methods” section. White dotted box indicates areas of hMSCs distribution in the right brain hemisphere, and red arrow indicates infarction zone due to cell embolism. SPIO-labeled hMSCs visualized as hypointense spots on SWI.
Figure 4. Comparison of MRI data and histology.
(a) – brain images taken after intra-arterial injection of SPIO-labeled hMSCs in a group 3 rat without cellular embolism. Confocal microscopy image shows fluorescently labeled SPIOs (Dragon green - DRGN) and nuclear counterstain with DAPI (blue).
(b) – another example of brain images of a rat from group 3 without cellular embolism, hMSCs have additional label: membrane dye PKH26 is red and SPIO microparticles in cytoplasm are green.
(c) – example of the images of brain taken from a group 5 rat with transplantation of hMSCs 24h after MCAO and optimal parameters of cell administration). Confocal microscopy images showing fluorescent labeled SPIOs (Dragon green – DRGN) and nuclear counterstain with DAPI (blue). Brightfield microscopy (Perl's Prussian blue staining) images showing SPIO label in brain tissue.
White dotted boxes indicate areas of cell distribution in the right brain hemisphere. SPIO-labeled hMSCs visualized as hypointense spots with SWI. Blue arrow indicates infarction zones formed after MCAO. The scale bars represent 20 µm for confocal microscopy and 100 µm for brightfield microscopy.
At the next stage, in the animal group 4 the number of transplanted hMSCs was increased from 5x10^5 hMSCs to 1x10^6 hMSCs in 1 ml of PBS. The other injection parameters were left the same as for group 3. However, as can be seen from Figure 5, cerebral embolis was again observed in all rats in that group, suggesting that the usage of high doses of hMSCs in potentially dangerous even combined with slow infusion rate and maintenance of blood flow in ICA.

**Figure 5.** MRI of the brain of a rat from group 4 immediately after intra-arterial injection of 1x10^6 SPIO-labeled hMSCs by syringe pump at the rate of 100 µ/min and maintenance of blood flow in the ICA. Red arrow points to an infarction zone formed due to cellular embolism.

3.2.2 Rats with MCAO. After determining optimal injection parameters for intact rats, we assessed their applicability in MCAO cerebral infarction model. In experimental group 5 24h after MCAO 1x10^5 hMSCs in 1 ml of PBS were administrated by syringe pump with the speed of 100 µ/min and maintenance of blood flow in the ICA. The 24h time window appears to be the most reasonable according to the literature data and possible clinical application [4].

No cerebral embolic events were observed in the majority of rats (in 22 out of 27 rats). As seen in Figure 6, no increase of the infarction zone was detected. SPIO-labeled hMSCs in the right hemisphere were appropriately visualized with SWI as hypointense spots, which was confirmed by histology, as shown in Figure 4 (c).

**Figure 6.** MRI of the brain of a rat from group 5 (transplantation of hMSCs 24h after MCAO, optimal parameters of cell administration) before and immediately after intra-arterial injection of SPIO-labeled hMSCs. Stroke zone remains unaltered after injection. Blue arrows indicate the infarction zone after MCAO. White dotted box indicates the area of hMSCs distribution in the right brain hemisphere. SPIO-labeled hMSCs are visualized as hypointense spots with SWI.

In 5 rats of this group the spread of the cerebral infarction zone after cells administration was revealed, suggesting cellular embolism (Figure 7). Embolic complications observed after the
optimization of the parameters of intra-arterial transplantation can be the result of changes in intracerebral hemodynamics in the area around brain infarction or too deep insertion of the catheter inside ICA and lack of blood flow around it.

Figure 7. MRI of the brain of a rat from group 5 (transplantation of hMSCs 24h after MCAO, optimal parameters of cell administration) before and immediately after intra-arterial injection of SPIO-labeled hMSCs. New areas of ischemia due to cellular embolism can be observed. Blue arrows show the infarction zone after MCAO, red arrows indicate new infarction areas due to cellular embolism. White dotted box indicates the area of hMSCs distribution in the right brain hemisphere. SPIO-labeled hMSCs are visualized as hypointense spots with SWI.

Summarized data related to cerebral embolic complications after intra-arterial injection of SPIO-labeled hMSCs for all groups of rats are presented in Figure 8.

Figure 8. Percentage of rats with and without complications (cerebral embolism) after intra-arterial injection of SPIO-labeled hMSCs in different experimental groups. Detailed description of each group is given in the Materials and Methods section.

4. Conclusions
In summary, our results provide the optimal parameters for safety intra-arterial administration of hMSCs in intact rats and after MCAO. We have shown that the incidence of cerebral embolic events depends on such parameters as cell dose, infusion rate and maintenance of blood flow in ICA. Moreover, the correct ligation of intracerebral branches of ICA can improve target cell delivery to the brain. Obtained data should be considered before planning experiments with intra-arterial administration of cells in laboratory animals and can help in further clinical trials with intra-arterial transplantations of stem cells in ischemic stroke patients.
Acknowledgements
The authors acknowledge the support by the Russian Foundation for Basic Research, grant No 16-29-07116 (cell culturing and labelling, animal surgery, MRI examination, histology and microscopy) and by the Russian Science Foundation, grant No 14-45-00040-II for the manuscript preparation and publication.

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