Tracking of transplanted neural stem cells labeled with superparamagnetic iron oxide in ischemic stroke

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Abstract

Clinical treatments for ischemic stroke are limited. At present, neural stem cell (NSC) therapy is considered a promising method for treating ischemic stroke. However, the behavior of transplanted NSCs remains ambiguous. Herein, we report the feasibility of superparamagnetic iron oxide labeling for long-time magnetic resonance imaging to track transplanted NSCs in a transient middle cerebral artery occlusion model. According to our studies, in vivo monitoring of transplanted NSCs during ischemic stroke in the current work may provide another insight into the tracking strategy of transplanted NSCs, thereby advancing NSC-based therapy toward clinical application.

Keywords: Neural stem cell; Ischemic stroke; Superparamagnetic iron oxide; Magnetic resonance imaging; Tracking

1. Introduction

Ischemic stroke causes significant mortality and morbidity, affecting millions of people worldwide[1-3]. Given the potential risk factors and the aging population, the prevalence of ischemic stroke is expected to increase[4]. Therapeutic strategies for stroke have made significant progress during the past several years. Current pharmacological treatments for ischemic stroke are primarily to reduce brain damage through the use of thrombolytic agents, antithrombotic agents, or neuroprotective agents[5-7]. However, the current therapies cannot be applied to all ischemic stroke patients and adequately improve the outcomes.

Studies have shown that the use of cell therapy following ischemic stroke increases neuroplasticity, improves the structure and physiological functions of brain tissue, and promotes the recovery of the nervous system[8]. Neural stem cells (NSCs) play key roles in brain homeostasis and exhibit potential therapeutic function in ischemic stroke[5,8-11]. Our previous study has demonstrated that si-circHIPK2 regulates NSC differentiation, and NSCs transduced with si-circHIPK2 promote neuroprotection and functional recovery after stroke[12]. However, the clinical application of stem cell therapy remains a big challenge because the biodistribution and fate of transplanted stem cells in the brain need to be determined in this therapy[13,14]. Tracking transplanted NSCs in vivo to eval-
Long-term in vivo MRI tracking of SPIO-labeled NSCs

In recent years, a number of methods have been used to track NSCs, such as bromodeoxuridine (BrdU) \(^{15}\). However, this method has its limitations because BrdU signal becomes weak when transplanted NSCs proliferate and differentiate, making it difficult to monitor the cells over a long time. Researchers are now making efforts to develop appropriate methods for long-term tracking of stem cells after transplantation. Here, superparamagnetic iron oxide (SPIO) nanoparticles are considered to be the practical application of stem cells because nano iron oxide is the only inorganic nanomaterial that has been approved by the Food and Drug Administration (FDA) as a nanodrug \(^{16}\). SPIO can be used as a clinical magnetic resonance imaging (MRI) contrast agent \(^{17,18}\). Studies have shown that SPIO has been successfully used for tracking different cell types by MRI in vivo because of its characteristics, which include easy synthesis, superparamagnetic, high saturation magnetization, good biocompatibility, and low toxicity \(^{19}\). To monitor the migration and survival of transplanted NSCs in vivo, SPIO can be used as an MRI tracer to non-invasively monitor in vivo.

In the present study, SPIO nanoparticles were used to detect the migration and distribution of transplanted NSCs in (transient middle cerebral artery occlusion [tMCAO]) mice. We sought to demonstrate the authenticity of SPIO-labeled NSCs tracking in tMCAO mice and the capacity of NSCs for migration, thus providing a visual basis for evaluating their therapeutic effect on ischemic stroke.

2. Materials and methods

2.1. Synthesis of SPIO nanoparticles

SPIO was synthesized based on previous reports \(^{16,20}\). First, polyglucose sorbitol carboxymethyl ether (PSC, 200 mg) aqueous solution (10 mL) was aerated with nitrogen to remove oxygen for 5 min. Second, ferric chloride (FeCl\(_3\), 60 mg, 0.37 mmol) and ferrous chloride (FeCl\(_2\), 30 mg, 0.236 mmol) were dissolved in 15 mL of double distilled water (ddH\(_2\)O). The mixture was then added into the PSC solution. Third, 28% of ammonium hydroxide (1 g) was added into the solution with the mixture, and then mechanically stirred in a water bath for 30 min (80°C). Next, the iron oxide was collected by an ultrafiltration centrifuge tube (30 kD) and washed with ultrapure water 3 times. The technique was based on the preparation process of ferumoxyl, which has been approved by the FDA as an inorganic nanodrug.

2.2. Inductively coupled plasma mass spectrometry (ICP-MS)

NSCs were digested into single-cell suspension by enzyme and cultured at a density of 5 \(\times\) 10\(^4\)/well in 500 \(\mu\)L of medium into 24-well microplate. Then, the cells were harvested and counted. The cell suspension was dissociated by hydrochloric acid (HCl). The concentration of iron in cell lysates was measured by ICP-MS according to Perkin Elmer's operating procedures.

2.3. Ethics approval and consent to participate

All animal experiments were performed in accordance with the standard guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the Medical School of Southeast University.

2.4. Cell cultures

NSCs from C57BL/6J or enhanced green fluorescent protein (eGFP) transgenic mice were isolated from embryonic day 14 mouse hippocampus and sterilized with 75% of alcohol. Fetal mice were taken out from pregnant mice and placed in a 50 mL centrifuge tube with sterile phosphate-buffered saline (PBS). Brains were removed from the fetal mice and dissociated to obtain the hippocampus. Next, the hippocampus was placed in a medium supplemented with PBS, and the tissues were digested with trypsin (25200056, Gibco, USA). Then, the cells were plated on cell culture flasks containing basic fibroblast growth factor (bFGF, 20 ng/mL, Stem Cell, Canada), epidermal growth factor (EGF, 20 ng/mL, Stem Cell, Canada), and B-27 supplement (2%, Gibco, USA). The culture was maintained in a humidified chamber (5% carbon dioxide [CO\(_2\), 37°C]. The NSCs (3\(^{rd}\) - 8\(^{th}\)) were used in the study.

2.5. Cell Counting Kit-8 (CCK-8) assay

NSCs were digested into single-cell suspension by enzyme and cultured at a density of 1 \(\times\) 10\(^3\)/well in 100 \(\mu\)L of medium into 96-well microplate for 24 h. Then, the cells were treated with different concentrations of SPIO (0, 25, 50, 100, 200, and 300 \(\mu\)g/mL). After treatment for 48 h, 10 \(\mu\)L of CCK-8 reagent was added to each well, and then cultured for 4 h. The absorbance was analyzed at 450 nm.

2.6. tMCAO

tMCAO was processed according to a previous study \(^{12,21,22}\). Briefly, anesthesia was induced with 3% of isoflurane, 30% of oxygen, and 70% of nitrous oxide in an anesthetic chamber and maintained with 1.5% of isoflurane through a facemask. A temperature-controlled heating pad was used to maintain the temperature at 37.0 \(\pm\) 0.5°C during the surgery and recovery period. A 1 cm midline skin incision was made over the neck, and the right common carotid artery (CCA) was carefully dissected free from the surrounding nerves under a stereo dissecting microscope and tied off, exposing the right external carotid artery (ECA).
and isolating from its small artery branches. The ECA was ligated 3 mm distal to its origin, and an arteriotomy was then performed on the ECA. Following that, a silicone-rubber-coated nylon filament (602356PK5Re, Doccol Co., USA) was inserted into the ECA and advanced for 9 – 10 mm to the carotid bifurcation along the internal carotid artery (ICA) and the origin of the middle cerebral artery (MCA). The incision in the neck was subsequently sutured. After 1 h of occlusion, the silicone-rubber-coated nylon filament was removed to restore blood flow. In sham mice, the filament was not inserted.

Using the Moor full-field laser perfusion imager (moorFLPI-2, Moor Instruments, Axminster, UK), the cortical cerebral blood flow was measured in both cerebral hemispheres and recorded 15 min before MCAO and throughout the ischemic period until 15 min after the onset of reperfusion. Animals that did not show a reduction in cerebral blood flow (CBF) of at least 75% of the baseline level or died after ischemic induction (<10%) were excluded from further experimentation [24]. A total of 33 mice were included in this study; however, based on the aforementioned criteria, seven mice were excluded due to death (n = 4) or failed stroke induction (n = 3).

### 2.7. Microinjection of neural stem cells

The lateral ventricles of mice were microinjected with NSCs at 7 days after tMCAO at the following coordinates: 0.3 mm behind the bregma and 1.0 mm lateral from the sagittal midline, at a depth of 2.2 mm from the skull surface. A total of 2 μL of cell suspension (1 × 10^6 cells) was injected into the left lateral ventricle at a rate of 0.1 μL/min by microinjection pump.

### 2.8. Magnetic resonance imaging scanning

*In vivo* MRI was performed using a 7-Tesla small animal MRI scanner (BrukerPharmaScan, Karlsruhe, Germany). The mice were anesthetized with 2% of isoflurane delivered through a nose cone. The respiratory rate and body temperature of the mice were monitored through a physiology monitor. All sequences were performed on the MRI scanner (BrukerPharmaScan, Karlsruhe, Germany). In brief, tissue sections were incubated with Perls stain for 30 min at room temperature. Then, the tissue sections were washed with distilled water for 5 min. The sections were covered with counterstaining solution for 10 min and subsequently washed with distilled water for 5 min. The tissue sections were sealed with neutral resin.

### 2.12. Bromodeoxyuridine (BrdU) labeling

NSCs were prepared on poly-L-lysine-coated glass coverslips. 10 μL of Dulbecco's PBS containing 10 mM BrdU was added in the 1 mL cell culture medium. After 4 h, the cells were fixed overnight in 4% of paraformaldehyde (PFA) at 4°C. The cells were then denatured with HCl for 30 min at room temperature and washed with PBS 2 times. Subsequently, the cells were blocked for 1 h in PBS containing 10% normal goat serum and 0.1% Triton X-100 at room temperature. The blocked cells were incubated with mouse anti-BrdU antibody (1:200; SC-32323, Santa Cruz, CA) that were diluted in PBS containing 10% normal goat serum and 0.1% Triton X-100 at 4°C overnight. The samples were washed with PBS 3 times before being incubated with donkey anti-mouse (conjugated to Alexa-Fluor 576) secondary antibody for 1 h at room temperature. After washing with PBS for another 3 times, the samples were mounted using ProLong.
Gold Antifade Reagent with 4’,6-diamidino-2-phenylindole (DAPI) (0100-20, SouthernBiotech, USA). The samples were imaged by microscopy (Olympus, Japan) and analyzed by Image J software.

2.13. Immunofluorescence (IF) staining

Before staining, for tissue samples, coronal slices (30 μm) were prepared from brain tissues and incubated with Triton X-100 (0.25% in PBS) for 15 min, and subsequently blocked for 1 h in PBS containing 10% of normal goat serum and 0.1% of Triton X-100 at room temperature. For cell samples, the NSCs were fixed for 20 min in 4% of PFA in PBS and washed 3 times with PBS. Then, the cells were incubated with Triton X-100 (0.25% in PBS) for 15 min and blocked for 1 h in PBS containing 10% of normal goat serum and 0.1% of Triton X-100 at room temperature. The blocked sections and cells were incubated with primary antibodies that were diluted in PBS containing 10% normal goat serum and 0.1% Triton X-100 at 4°C overnight. The primary antibodies used in this study contained GFAP (16825-1-AP, Proteintech) and TUJ1 (ab18207, Abcam). The samples were then washes with PBS three times before being incubated with secondary antibodies, including Alexa Fluor 594 goat anti-mouse IgG (A-11005, Invitrogen) and Alexa Fluor 594 goat anti-rabbit IgG (A-11012, Invitrogen) for 1 h at room temperature. The samples were washed with PBS three times and mounted using ProLong Gold Antifade Reagent with DAPI (Southern Biotech, USA). The samples were imaged by microscopy (Olympus, Japan) and analyzed by Image J software.

2.14. Statistics

The data were presented as mean ± standard error of the mean (SEM). Significance was established using t-test for paired values. One-way analysis of variance (ANOVA) followed by Holm-Sidak post hoc test was used for comparisons of three groups. Statistical analysis was performed using GraphPad Prism 8.0.

3. Results

3.1. Characterization of SPIO-labeled neural stem cells

To determine the characterization of transplanted NSCs labeled with SPIO, we first evaluated the viability of SPIO-labeled NSCs. CCK-8 assay was performed with NSCs that were incubated with SPIO nanoparticles at concentrations of 0, 25, 50, 100, 200, and 300 μg/mL for 48 h. As shown in Figure 1A, SPIO nanoparticle concentrations of <300 μg/mL showed low cytotoxicity (90%). The viability decreased by 9.64% when the concentration was 300 μg/mL. We then explored the uptake of SPIO nanoparticles, in which a PB staining assay was performed. The results showed that the intake of SPIO by NSCs was directly proportional to the concentration of SPIO (Figure 1B). Subsequently, MRI was used to test the signal of SPIO nanoparticles at different concentrations (Figure 1C). With the increase in SPIO nanoparticle concentration, the MRI signal increased gradually, and markedly enhanced signals were observed in samples with concentration over 200 μg/mL. Moreover, we assessed the concentration-related internalization of SPIO by ICP-MS (Figure 1D). We found that the amount of iron increased along with the concentration of SPIO, demonstrating that NSCs internalized SPIO nanoparticles in a dose-dependent manner. As shown in Figure 1E and F, SPIO-labeled NSCs did not affect cell proliferation. In conclusion, at a concentration of 200 μg/mL, the uptake of SPIO nanoparticles was sufficient and did not have any noticeable influence on the viability of NSCs. Therefore, we used 200 μg/mL for all experiments.

3.2. Differentiation of SPIO-labeled neural stem cells

Biosafety is a crucial requirement for nanotracers to label stem cells. To investigate whether SPIO is involved in the regulation of NSC differentiation, NSCs were labeled with SPIO at a concentration of 200 μg/mL. WB analysis showed that the levels of GFAP (the astrocyte marker) and TUJ1 (the neuronal marker) increased during NSC differentiation, while the expression of Nestin (NSC marker) significantly decreased. However, there was no significant difference in the expression of Nestin, GFAP, and TUJ1 between the SPIO-labeled NSCs (SPIO) group compared with the no SPIO-labeled NSCs (control) group (Figure 2A and B). The detection of GFAP expression at 1 day, 7 days, and 14 days after NSC treatment by IF staining showed that there was no significant difference in GFAP expression between the SPIO-labeled group and the control group (Figure 2C and D). Meanwhile, as shown in Figure 2E and F, there was no significant difference in TUJ1 expression between the SPIO-labeled group and the control group, indicating that SPIO did not influence the differentiation of NSCs.

3.3. Tracking the signal of SPIO nanoparticles in a tMCAO stroke model

To investigate the migration of transplanted NSCs labeled with SPIO, MRI was used to detect signals in the coronal plane of mouse brains that were detected by MRI. Signals were detected over the injection site at 7 days and the ischemic area at 14 days, 21 days, and 28 days in the SPIO-labeled NSCs group after tMCAO (Figure 3A). However, no signals of SPIO nanoparticles were detected in the control group. This suggests that NSCs labeled with SPIO nanoparticles could migrate from the injection site to the ischemic area. Besides, signals were also detected in the horizontal plane of mouse brains by MRI (Figure 3B).
On the other hand, PB staining can also be used for tracking transplanted NSCs in tMCAO mice. As shown in Figure 3C, SPIO-labeled NSCs were identified over the injection site and infarcted area at 28 days after tMCAO. These results suggest that SPIO labeling allows in vivo tracking of transplanted NSCs with MRI. The results also confirmed the migration of transplanted NSCs from the injection site to the infarcted area in tMCAO mouse brain.

3.4. Transplanted neural stem cells labeled with SPIO in tMCAO mice

To confirm whether the signals of SPIO nanoparticles detected by MRI and PB staining in the ischemic area are carried by NSCs, we examined the correlation between transplanted NSCs and SPIO nanoparticles. The synthesis of SPIO nanoparticles labeled with rhodamine B (Rhb) has been described in previous research. As shown in Figure 4A, the GFP+ NSCs labeling rate (green) was colocalized with Rhb-labeled SPIO (red) in vitro. Microinjection of SPIO-Rhb-labeled GFP+ NSCs was then performed in tMCAO mice. At 28 days after tMCAO, a fluorescence signal was detected in the peri-infarct area of the cortex in the ischemic hemisphere (Figure 4B). This result demonstrated that the SPIO-labeled NSCs had migrated to the ischemic area. The above experiments not only confirmed that the transplanted NSCs labeled with...
SPIO migrated to the infarcted side, but also verified that the signals of SPIO nanoparticles detected by MRI and PB are carried by NSCs.

4. Discussion

In recent years, stem cell therapy has become a very promising and advanced scientific research method\cite{26} that can be used for various diseases, such as stroke\cite{27}, Parkinson’s disease\cite{28}, amyotrophic lateral sclerosis\cite{29}, cancer\cite{30}, and heart disease\cite{31}. Among them, for stroke, stem cell therapy shows promising prospects as it offers potential neurorestorative benefits. The potential of NSCs for cell therapy is enormous. Transplanted NSCs have been used in pre-clinical and clinical trials to restore function\cite{32}. However, some relevant problems need to be addressed, especially pertaining the persistence and differentiation of transplanted cells into brain tissue.

With regard to this issue, we developed a method that allows us to observe the fate of transplanted cells in vivo, that is, to track stem cells and monitor stem cell homing, migration, and proliferation to evaluate the therapeutic effect.

In the present work, we developed a novel cell labeling strategy based on SPIO nanoparticles and explored its application for long-term MRI imaging tracking of transplanted NSCs. The SPIO nanoparticles showed a narrow size distribution, superparamagnetism, and low cytotoxicity. Our result demonstrated the tracking ability of SPIO nanoparticles. However, the biosafety of SPIO nanoparticles should be evaluated before further clinical application. In
Long-term in vivo MRI tracking of SPIO-labeled NSCs

Figure 3. Tracing of transplanted NSCs labeled with SPIO in tMCAO mice. (A) Representative images of MRI T2-star coronal plane showing the migration and distribution of transplanted NSCs. MRI was taken at 7, 14, 21, and 28 days after tMCAO surgery. n = 3 in each group. (B) Representative images of MRI T2-star horizontal plane showing the migration and distribution of transplanted NSCs. MRI was taken at 7, 14, 21, and 28 d after tMCAO surgery. n = 3 in each group. (C) Representative images of PB staining showing the distribution of iron deposits in the brain at 28 days after tMCAO surgery. Insets, individual images from PB staining. Red arrows point to SPIO signals. Scale bar = 100 μm.

MRI: Magnetic resonance imaging, NSCs: Neural stem cells, SPIO: Superparamagnetic iron oxide, tMCAO: transient middle cerebral artery occlusion.

Figure 4. Tracing transplanted NSCs labeled with SPIO in tMCAO mice. (A) Verification of NSCs labeling rate of synthetic rhodamine B (Rhd B)-SPIOs in vitro. Scale bar = 50 μm. (B) Immunofluorescence evaluation of peri-infarct cortex implanted with SPIO-labeled NSCs. DAPI (blue), nucleus; GFP (green), NSCs; SPIO-Rhd-B (red), which were coated with dextran and visualized with a rhodamine-conjugated dextran. Scale bar = 100 μm.

this study, we first identified the appropriate concentration of SPIO nanoparticles. The results showed that the amount of iron increased with the increase of SPIO concentration, demonstrating that NSCs internalized SPIO nanoparticles in a dose-dependent manner; thus, we chose an SPIO nanoparticle concentration of 200 μg/mL. This SPIO concentration had no significant effect on the proliferation and differentiation of NSCs, providing high biological security to track NSCs in the tMCAO mice model. Developing long-term tracking is a major problem in stem cell therapy. A marker should first effectively bind to the stem cell and the signal should not disappear within a short time\cite{33,34}. In this study, SPIO nanoparticles were used to label NSCs and were tracked by MRI at different time points, demonstrating that transplanted SPIO-labeled NSCs can migrate to infarcted areas. Taken together, the current work based on SPIO-based MRI imaging enables the visualization of the distribution, migration, and survival of transplanted NSCs in tMCAO mice, thereby facilitating a good understanding of the role of NSCs in ischemic stroke.

5. Conclusion

In this study, we demonstrated that SPIO nanoparticles could be used for tracking NSCs in a mice tMCAO stroke model by MRI. Our result showed that the uptake of SPIO nanoparticles by NSCs is concentration dependent, in which better magnetic properties were observed with higher concentrations of SPIO nanoparticles than lower concentrations. Moreover, our result showed that transplanted NSCs labeled with SPIO can migrate from the injection site to the infarcted area \textit{in vivo}, thus providing the salient point for stroke treatment with NSCs. We conclude that SPIO nanoparticles can be employed to track the migration of NSCs, thereby advancing stem cell-based therapy toward clinical application.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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\textbf{Ethics approval and consent to participate}

All animal experiments were performed in accordance with the standard guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the Medical School of Southeast University.

\textbf{Consent for publication}

Not applicable.

\textbf{Availability of data}

Data can be obtained from the corresponding author following request.

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