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·Original Article·

Magnetic resonance evaluation of human mesenchymal stem cells in corpus cavernosa of rats and rabbits

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Abstract

Aim: To investigate whether the biological process of superparamagnetic iron oxide (SPIO)-labeled human mesenchymal stem cells (hMSCs) may be monitored non-invasively by using *in vivo* magnetic resonance (MR) imaging with conventional 1.5-T system examinations in corpus cavernosa of rats and rabbits. **Methods:** The labeling efficiency and viability of SPIO-labeled hMSCs were examined with Prussian blue and Tripan blue, respectively. After SPIO-labeled hMSCs were transplanted to the corpus cavernosa of rats and rabbits, serial T2-weighted MR images were taken and histological examinations were carried out over a 4-week period. **Results:** hMSCs loaded with SPIO compared to unlabeled cells had a similar viability. For SPIO-labeled hMSCs more than 1×10^5 concentration *in vitro*, MR images showed a decrease in signal intensity. MR signal intensity at the areas of SPIO-labeled hMSCs in the rat and rabbit corpus cavernosa decreased and was confined locally. After injection of SPIO-labeled hMSCs into the corpus cavernosum, MR imaging demonstrated that hMSCs could be seen for at least 12 weeks after injection. The presence of iron was confirmed with Prussian blue staining in histological sections. **Conclusion:** SPIO-labeled hMSCs in corpus cavernosa of rats and rabbits can be evaluated non-invasively by molecular MR imaging. Our findings suggest that MR imaging has the ability to test the long-term therapeutic potential of hMSCs in animals in the setting of erectile dysfunction. *(Asian J Androl 2007 May; 9: 361–367)*

Keywords: human mesenchymal stem cells; magnetic resonance; stem cells; penis; cell labeling; corpus cavernosa

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1 Introduction

The main cause of erectile dysfunction is the damage of penile cavernous smooth muscle cells and sinus endothelial cells by various metabolic conditions or mechanical manipulations. Because the integrity of cavernous endothelial cells and smooth muscle cells is critical in maintaining and regulating erectile function, accelerating intact cavernous smooth muscle cells and sinus endothelial cells repair can be a novel treatment for erectile dysfunction. Several drugs have been investigated *in vitro* and *in vivo* in animal and/or human studies, and showed relaxant activity in cavernous smooth muscle and an increase in the percent of smooth muscle [1, 2].

Because several populations of bone marrow-derived cells have the potential to differentiate into endothelial-like cells, they may be good candidates for tissue repair. Bone marrow contains several types of stem cells: hematopoietic stem cells, mesenchymal stem cells (MSCs), endothelial stem/progenitor cells (EPCs), and multipotent adult progenitor cells (MAPCs). MSCs fulfill all criteria of true stem cells, that is, self-renewal, multilineage differentiation, and *in vivo* reconstitution of tissue [3]. Human (h)MSCs are a population of rapidly self-renewing adult stem cells with varying differentiation potentials. The relative ease of isolating MSCs from bone marrow and the great plasticity of the cells make them ideal tools for an autologous or allogeneic cell therapy.

A reliable *in vivo* imaging method to localize transplanted cells and monitor their restorative effects will enable a systematic investigation of cell therapy. However, most studies of stem cell transplantation have been carried out using immunohistological staining, which does not provide an opportunity to follow the migration of transplanted cells *in vivo* in the same host. Molecular imaging aims to visualize targeted cells in living organisms. Through molecular imaging with nanoparticles, the biological process of transplanted stem cells can be monitored non-invasively as materials can be evaluated without sacrifice and repeated evaluation is possible [4, 5]. Among several molecular imaging techniques, magnetic resonance (MR) imaging provides a high resolution and sensitivity for transplanted cells [6].

Superparamagnetic iron oxide (SPIO) nanoparticles are being used for intracellular magnetic labeling of stem cells to monitor cell trafficking by MR imaging as part of cellular-based repair, replacement and treatment strategies. Because of the small crystal size (approximately 7–10 nm in diameter), SPIO particles exhibit magnetic moments that are unaffected by lattice orientation and align in an applied magnetic field, creating extremely large microscopic field gradients around the particles that dephase the neighboring proton magnetic moments, thereby reducing the T_2 relaxation time and facilitating the detection of labeled cells. Furthermore, SPIO particles have shown no adverse effects on the viability and proliferation of labeled cells [7, 8]. This study was performed to investigate whether the biological process of reticuloendothelial system-specific iron oxide nanocrystal SPIO-labeled hMSCs in the corpus cavernosum of rats and rabbits can be evaluated non-invasively by using molecular MR imaging.

2 Materials and methods

2.1 Preparation of hMSCs

hMSCs were isolated and culture-expanded according to the method described by Pittenger *et al*. [9]. Briefly, 10 to 20 mL of bone marrow aspirate was obtained under sterile conditions by puncture of the posterior iliac crest of bone marrow transplantation donors after receiving informed consent. Mononuclear cells were isolated from the bone marrow using Ficoll-Hypaque (1.077 g/cm^3, Sigma, St Louis, MO, USA) density centrifugation (400 × *g* for 25 min). The interface mononuclear cells were collected and washed twice with phosphate buffered saline (PBS). The cells were re-suspended, counted, and plated at 2×10^5/cm^2. The cells were cultured in hMSC medium composed of Dulbecco's modified Eagle's medium with low glucose (DMEM-LG; GibcoBRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; GibcoBRL, Grand Island, NY, USA) and 1% antibiotic-antimycotic solution (GibcoBRL). Cells were plated into 75-cm^2 flasks (Falcon, Franklin Lakes, NJ, USA) and the cultures were incubated at 37ºC in 5% CO_2 in air and 95% relative humidity. The medium was replaced after 72 h, and every 3 to 4 days thereafter. When the cultures reached approximately 90% of confluence, hMSCs were detached with 0.05% trypsin-EDTA solution (GibcoBRL, Grand Island, NY, USA) and replated into passage culture at a density of 1×10^6 cells per each 175-cm^2 flask. In either case, the hMSCs were confirmed to be negative for hematopoietic markers by flow cytometry and capable of differentiating into osteocytes, chondrocytes, and adipocytes *in vitro*.

2.2 Labeling of cells with iron oxide particles

A liposome transfection agent, GenePORTER (GTS, San Diego, CA, USA) was used as the transfection agent. A stock solution of GenePORTER was added to the culture medium at a dilution of 1:250 and mixed with SPIO (Feridex; AMI, Cambridge, MA, USA) for 60 min at room temperature on a rotating shaker. These cultures containing the SPIO-GenePORTER were added to the hMSCs such that the final concentration of SPIO was 25 μg/mL and the final dilution of GenePORTER was 1:500. The cell cultures were kept for 4 h at 37ºC, 5% CO_2 incubator with rotation.

Labeled cells were harvested for transplantation by gentle trypsinization. To remove excess iron oxide particles, trypsinized cells were washed in PBS and concentrated by centrifugation, resuspended in PBS and kept on ice. The labeling efficiency of the SPIO-labeled hMSCs was examined with Prussian blue staining and electron microscopic image (H-7600; Hitachi, Tokyo, Japan). The labeling viability was done with Tripan blue staining. For Prussian blue staining, which indicates the presence of iron, the cells were fixed with methyl alcohol, washed, incubated for 30 min with 2% potassium ferrocyanide (i.e., Perl reagent) in 6% hydrochloric acid, washed again, and counterstained with nuclear fast red.

2.3 In vitro MR imaging

We performed *in vitro* MR imaging of SPIO-labeled hMSCs compared with the control. SPIO-labeled hMSCs were labeled as described above, and a cell suspension at variable concentrations of (at concentrations of 1×10^2, 1×10^3, 1×10^4, 1×10^5, and 2×10^5 hMSCs/1 000 mL) was suspended in 2.0% agarose gel. It was compared with distilled water and agarose gel without SPIO-labeled hMSCs. All MR examinations were performed on a 1.5-T scanner (Sonata; Siemens, Erlangen, Germany) by using a surface coil. The imaging parameters for T2-weighted images acquired were: TR, 600 msec; TE, 6.18 msec; flip angle, 30°; and acquisition time 4 min and 37 sec; field of view, 100 × 100 mm; matrix size, 512 × 512 pixels. To check the sensitivity of the MR imaging, we performed *in vitro* imaging of SPIO-labeled hMSCs with variable concentration.

2.4 In vivo MR Imaging

All procedures were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (2001) and were approved by the Institutional Animal Care and Use Committee of our hospital. Eight-week-old 250 g Sprague-Dawley rats (n = 20) and 13–14-week-old 3 kg New Zealand White male rabbits (n =10) were purchased. Rats and rabbits were anesthetized with ketimine hydrochloride (50 mg/kg) and xylazine (5 mg/kg) i.p., respectively. Penile skin incision was made. 1×10^6 SPIO-labeled hMSCs were transplanted into the rat and rabbit cavernosa using a 500 micrometer syringe with 26G needle. The incised penile skin was sutured.

The imaging parameters for T2-weighted images were as described above. MR examinations were performed before and after transplantation of SPIO-labeled hMSCs. Follow-up serial T2-weighted gradient-echo MR imaging was performed within one hour and until 4 weeks in the rabbits and 12 weeks in the rats. Intraperitoneal Cypol (cyclosporine; Chong Kun Dang, Seoul, Korea) with 10 mg/kg injection was carried out everyday.

2.5 Histological examination

At the end of the *in vivo* MR imaging experiments (4 weeks for rabbits and 12 weeks for rats), animals were killed under deep anesthesia. The penises were removed and rinsed with PBS solution, cryoprotected in 30% sucrose, embedded in OCT and frozen on a bed of crushed dry ice. Twenty micrometer-thick cryosections were made and alternate sections were stained with H&E or Prussian blue.

3 Results

3.1 Cell labeling and histological analysis

hMSCs were separated and SPIO were transferred to hMSCs *in vitro* using GenePORTER. Prussian blue staining of labeled human mesenchymal stem cells revealed abundant uptake of the SPIO-GenePORTER complex in the cytoplasm. However, no stainable iron was detected in the nonlabeled human mesenchymal stem cells. Intracellular transfer of SPIO-GenePORTER complex was confirmed by electron microscopic image (Figure 1).

3.2 Viability

SPIO-labeled hMSCs were cultured for 6 days and the viability of the labeled cells measured by using Tripan blue staining. hMSCs loaded with SPIO compared to unlabeled cells had similar viability. The viabilities of hMSCs with and without SPIO-labeling were 98% and

95%, respectively (not shown).

3.3 *In vitro MR imaging*

In vitro MR imaging for SPIO-labeled hMSCs compared with the control are shown in Figure 2A. Overall, a dramatically decreased signal intensity was observed in the SPIO-labeled hMSCs compared with that of distilled water and agarose gel without SPIO-labeled hMSCs. To check the sensitivity of the MR imaging, we performed *in vitro* MR imaging with a variable concentration of SPIO-labeled hMSCs. MR imaging showed a clear hypo-intense signal at all concentrations greater than 1×10^5 hMSCs/1 000 mL (Figure 2B).

3.4 *In vivo MR imaging*

After the injection of SPIO-labeled hMSCs into the corpus cavernosum of rabbits, their presence was evident by distinct regional signal intensity loss induced by the susceptibility effects of iron oxide particles (Figure 2). The distribution of the signal intensity loss was located in the corpus cavernosum.

On follow-up serial T2-weighted gradient-echo MR imaging, signal intensity loss faded but persisted for 4 weeks after SPIO-labeled hMSCs injection. After injection of SPIO-labeled hMSCs into the corpus cavernosum of rats, T2-weighted gradient-echo MR imaging clearly demonstrated signal intensity loss induced by the injection of labeled cells (Figure 3). The distribution of the signal intensity loss was located in the corpus cavernosum. The signal intensity decrease faded with successive examinations but was observed 12 weeks after SPIO-labeled hMSCs injection.

3.5 *Histological examination*

The presence of iron oxide was confirmed 4 weeks after transplantation to the rabbit corpus cavernosum. The presence of iron oxide was also confirmed with Prussian blue staining 12 weeks after SPIO-labeled hMSCs

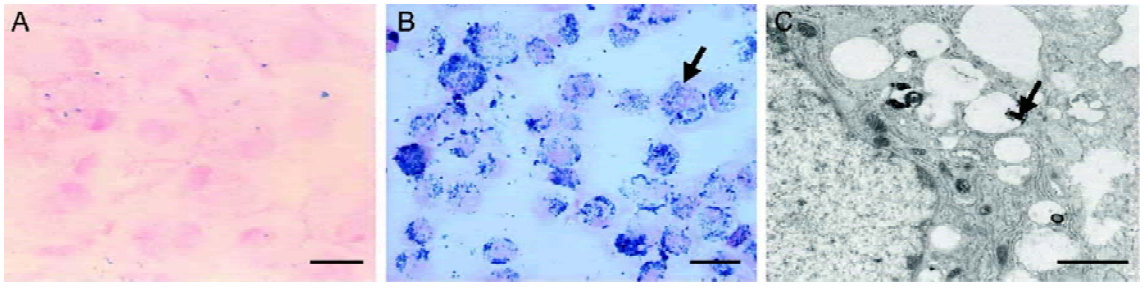

Figure 1. (A): Prussian blue-stained non-labeled human mesenchymal stem cells (hMSCs). Original magnification × 400. Scale bar = 20 μm. (B): Prussian blue-stained labeled hMSCs. Original magnification × 400. Scale bar = 20 μm. Note the abundant iron particles (blue dots) in the cytoplasm of the cells (arrow). Scale bar = 20 μm. (C): Electron microscopic image of hMSCs. Arrows indicate intracellular iron particles. Scale bar = 50 nm.

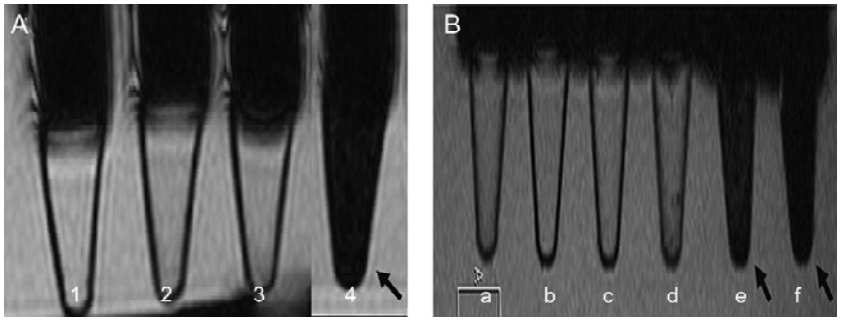

Figure 2. (A): *In vitro* magnetic resonanc (MR) imaging. 1: human mesenchymal stem cells (hMSCs). 2: Distilled water. 3: Agarose gel. 4: Superparamagnetic iron oxide (SPIO)-labeled hMSCs using GenePORTER. (B): *In vitro* MR image of various magnetically labeled hMSC concentrations (hMSCs/1 000 mL). a: Agarose gel; b: 1×10^2; c: 1×10^3; d: 1×10^4; e: 1×10^5; f: 2×10^5. Arrows show the decrease of MR signal intensity.

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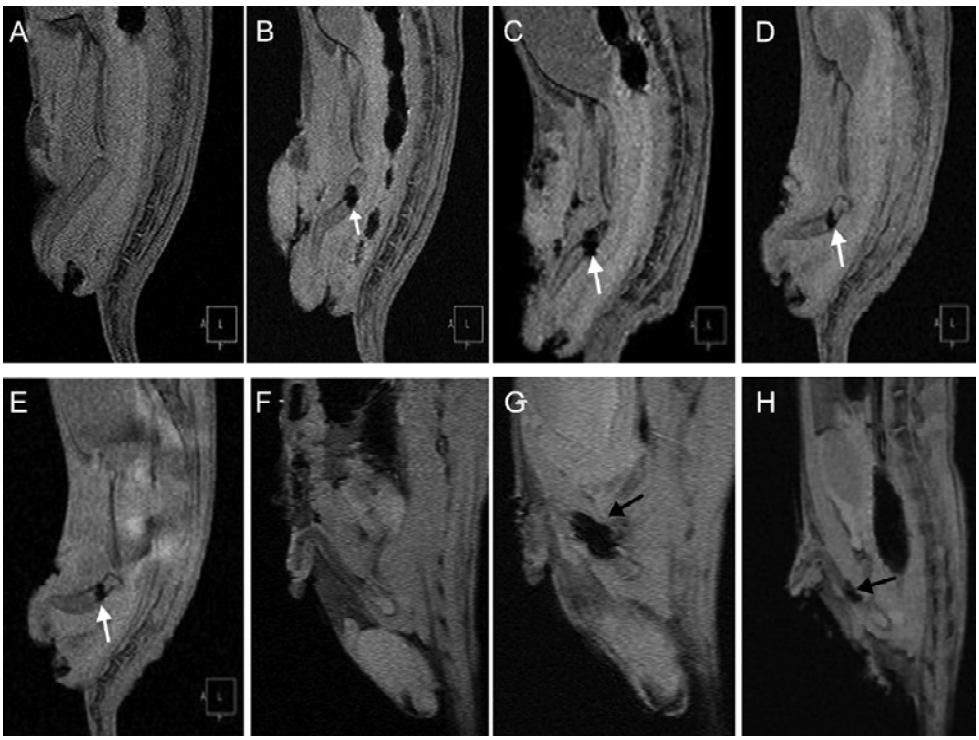

Figure 3. *In vivo* magnetic resonanc (MR) imaging of magnetically labeled human mesenchymal stem cells (hMSCs). (A): Before transplantation in rabbit. (B): Immediately after transplantation in rabbit. (C): At 1 week after transplantation in rabbit. (D): At 2 weeks after transplantation in rabbit. (E): At 4 weeks after transplantation in rabbit. (F): Before transplantation in rat. (G): Immediately after transplantation in rat. (H): At 12 weeks after transplantation in rat. Arrow shows the decrease of MR signal intensity. The areas of decreased MR signal intensity in the penile cavernosum were confined locally.

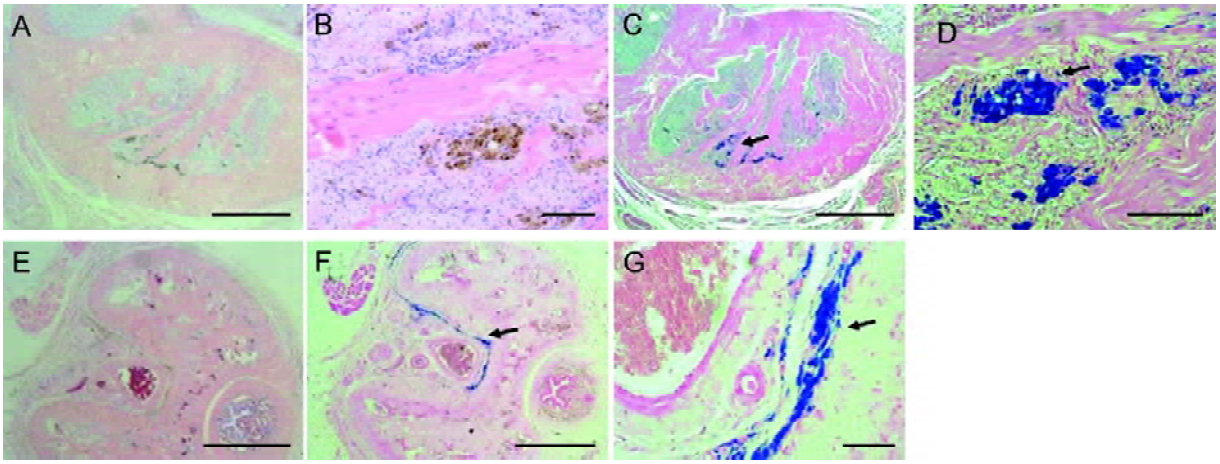

Figure 4. Histological findings of magnetically labeled human mesenchymal stem cells (hMSCs) in rabbit corpus cavernosum at 4 weeks after transplantation (A–D) and in rat corpus cavernosum at 12 weeks after transplantation (E–G). (A): H&E staining. Original magnification × 12.5. Scale bar = 50 μm. (B): H&E staining. Original magnification × 100. Scale bar = 20 μm. (C): Prussian blue staining. Original magnification × 12.5. Scale bar = 50 μm. (D): Prussian blue staining. Original magnification ×100. Scale bar = 20 μm. (E): H&E staining. Original magnification × 12.5. Scale bar = 50 μm. (F): Prussian blue staining. Original magnification × 12.5. Scale bar = 50 μm. (G): Prussian blue staining. Original magnification × 100. Scale bar = 20 μm. Intracytoplasmic SPIO particles (blue dots) are clearly visible with Prussian blue staining (arrows).

transplantation to the rat corpus cavernosum (Figure 4).

4 Discussion

There is increasing interest in using MR imaging to monitor the *in vivo* behavior of stem cells. Such cell trafficking studies would be a valuable tool for development and evaluation of cell-based repair, replacement or treatment strategies. Cells labeled with iron oxide particles exhibit much higher stability *in vivo* and reveal stronger contrast [8, 10]. Iron oxide-labeled cells appear as hypo-intense areas in tissues associated with the decreased signal intensity on iron sensitive T2-weighted and T2-weighted gradient echo images. The major advantage of using iron oxide particles for labeling cells is that it is an FDA-approved MR contrast agent, and therefore, quality control, sterility, and stability have all been well documented. Several modifications of dextran-coated iron oxide nanoparticles have been used for cell labeling in the past. The use of transfection agents significantly improves the internalization of iron oxide particles without altering cell physiology and therefore is preferable for long-term MR imaging monitoring of labeled cells.

Iron in its cationic states (i.e., Fe^{2+} and Fe^{3+}) is essential for the normal cycle and growth of cells. Increases in intracellular unbound iron result in oxidative stress and injury to the cells by causing the formation of reactive oxygen species, which may also lead to cell death [11, 12]. The introduction of SPIO-nonviral transfection agent complex into cells may increase the formation of reactive oxygen species and hydroxyl-free radicals. This phenomenon, in turn, may alter cell metabolism or increase the rate of apoptosis or cell death [11, 12]. However, SPIO particles that are used for cellular labeling have not demonstrated any adverse effects in cell viability and differentiation [13]. In this study, intracellular endosomal magnetic labeling of hMSCs with SPIO combined with the appropriate dilution of the nonviral transfection agent, GenePORTER, caused no toxic effect on stem cell viability (data not shown).

Labeling stem cells with iron oxide particles enhances the cell-to-background contrast and makes them visible in MR images. In *in vitro* study, the decrease of MR signal intensity was found only in SPIO-labeled hMSCs using GenePORTER but not in other control groups, including distilled water, agarose gel and hMSCs only. The intensity and area of the negative (hypo-intense) signal caused by SPIO particles depend on the concentration of iron oxide particles per cell and the image acquisition parameters. In the present study, *in vitro* experiments were carried out to estimate the minimum concentration of iron oxide particles that would generate optimal contrast for *in vivo* studies. Through the minimal concentration of 1×10^5 SPIO-labeled hMSCs that could be detected on a magnetic field, if the cells form a cluster of 1×10^5 or more it will be possible to detect them for *in vivo* experiments. However, we decided to use a concentration of 1×10^6 SPIO-labeled hMSCs for *in vivo* studies to compensate for signal loss caused by cell division and phagocytosis.

In this study, we confirmed the ability of *in vivo* MR imaging to detect labeled stem cells in normal rabbit and rat corpus cavernosa. Transplanted SPIO-labeled hMSCs were observed *in vivo* up to 12 weeks after injection. No migration/relocation of transplanted cells was noted in the corpus cavernosum after 12 weeks of transplantation. It is interesting to note that, with serial imaging, hypo-intense MR imaging decreases over time because of the presence of SPIO-labeled hMSCs. This could possibly result from the dilution of iron (cell division) and/or metabolism of iron oxide particles. However, we observed that even after 12 weeks of injection, the majority of the cells remain at or near the injection site. In the present study, validation of intracytoplasmic iron in labeled cells by using simple Prussian blue staining was shown.

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