Externally Applied Static Magnetic Field Enhances Cardiac Retention and Functional Benefit of Magnetically Iron-Labeled Adipose-Derived Stem Cells in Infarcted Hearts

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ABSTRACT

Although adipose-derived stem cells (ASCs) hold the promise of effective therapy for myocardial infarction, low cardiac retention of implanted ASCs has hindered their therapeutic efficiency. We investigated whether an externally applied static magnetic field (SMF) enhances cardiac localization of "magnetic" cells and promotes heart function recovery when ASCs are preloaded with superparamagnetic iron oxide (SPIO) nanoparticles. The influence of SMF (0.1 Tesla) on the biological activities of SPIO-labeled ASCs (SPIOASCs) was investigated first. Fifty-six female rats with myocardial infarction underwent intramyocardial injection of cell culture medium (CCM) or male SPIOASCs with or without the subcutaneous implantable magnet (CCM-magnet or SPIOASC-magnet). Four weeks later, endothelial differentiation, angiogenic cytokine secretion, angiogenesis, cardiomyocyte apoptosis, cell retention, and cardiac performance were examined.

INTRODUCTION

Adipose tissue comprises a heterogeneous cell population including endothelial cells and mesenchymal stem cells. The latter are called adipose-derived stem cells (ASCs) [1, 2]. Adipose tissue has several advantages over other tissues as a resource for mesenchymal stem cells: the abundance in most of population, easily and repetitive harvest with a minimally invasive procedure, and rapid proliferation [3]. ASCs have been demonstrated to be able to differentiate into endothelial cells and smooth muscle cells [4, 5] and to secrete proangiogenic and antiapoptotic growth factors [6, 7]. The transplantation of ASCs improves cardiac function following myocardial infarction.
infarction (MI), primarily by induced neovascularization and prevention of cardiomyocyte apoptosis [8–11]. Although stem cell transplantation is a promising therapy for MI, low cardiac cell retention and engraftment are major obstacles to achieving a significant functional benefit. At 6 days after cell transplantation, the percentages of the delivered cells trapped in the lung are approximately 26%, 47%, and 43% for intramyocardial, intracoronary, and intravenous delivery, respectively [12]. The corresponding cardiac retention rates are 11%, 2.6%, and 3.2%, respectively [12]. Intra-aortic delivery accompanies the cell distribution in heart, kidney, lung, liver, and spleen [13]. After intravenous delivery, many cells are observed in the lungs, with only a few implanted cells presented in the hearts [13]. Although intramyocardial delivery produces the greatest cell retention in the hearts in comparison with other approaches, approximately 50% of cells escape from the heart to other organs, such as the lungs, within 1 hour of cell delivery [14]. Evidently, most implanted cells are trapped and colonized, none specifically in untargeted extracardiac organs. Most studies only demonstrated that ejection fraction (EF) was mildly or moderately increased by the transplantation of stem cells [8–11].

Externally applied static magnetic field (SMF) has been used to direct drugs, cytokines, and other molecules chemically bound to magnetic particles into targeted organisms for maximization of therapeutic effects and minimization of systemic side effects [15]. The SMF around the tumor significantly enhanced the delivery of magnetic particle-conjugated anticancer drugs into the targeted solid tumor after intravascular administration [16, 17]. Externally applied SMF increased the uptake of biocompatible magnetic nanoparticle-labeled monocyties into tumor by threefold after intravenous injection [18]. The main mechanism was predominantly related to the fact that the magnetic targeting approach induced extravasation of magnetic particle-combined agents through the vascular wall and led to localization and retention in the targeted site [17]. Superparamagnetic iron oxide (SPIO) nanoparticles coated with dextrin (Feridex, Bayer HealthCare Pharmaceuticals, Whipppany, NJ, http://www.bayer.com/) are nontoxic and biodegradable and have been approved by the U.S. Food and Drug Administration for clinical practice as magnetic resonance contrast agents in humans [19, 20]. SPIOs are efficiently internalized by a wide variety of cells, including ASCs. The “magnetic” iron-labeled ASCs were thus obtained. The labeling of ASCs with SPIOs is safe without affecting the viability, proliferation, differentiation potential, antigenic phenotype, and cell factor secretion of ASCs [21, 22].

In this study, ASCs were preloaded with SPIOs and an SMF was generated above the heart by subcutaneous insertion of a magnet over the chest cavity to attract these magnetic cells to the targeted area. We first examined the magnetic safety and magnetophoretic attraction of the SPIO-labeled ASCs (SPIOASCs) in vitro, with the same magnet as that subcutaneously implanted in vivo. We further investigate whether externally applied SMF enhances the cardiac retention and functional benefit of magnetic SPIOASCs in infarcted hearts.

**Materials and Methods**

All animals received care by humans, and the study was approved by the Institutional Review Board and Animal Care Committee of Huazhong University of Science and Technology and National Research Council of Canada.

Preparation of ASCs

ASCs were isolated from 12-month-old male transgenic rats expressing green fluorescent protein (GFP). Subcutaneous adipose tissue was obtained from the abdominal and inguinal regions of the rats, minced and digested with collagenase I (2 mg/ml, Worthington Biochemical Corp., Lakewood, NJ, http://www.worthington-biochem.com) at 37°C for 20–30 minutes. Collagenase activity was neutralized by adding DMEM-F12 (HyClone, GE Healthcare, Logan, UT, http://www.gelifesciences.com) containing 15% fetal bovine serum (FBS, HyClone, GE Healthcare). The digested adipose tissue was filtered twice with a 100-μm and then with a 25-μm nylon membrane to eliminate the undigested fragments. Cellular suspension was centrifuged at 1,000 g for 10 minutes. Cell pellets were resuspended in cell-culture medium (CCM) and cultivated for 24 hours at 37°C in 5% CO₂.

The GFP-positive ASCs were incubated for 2 days in a CCM containing 50 μg/ml SPIO nanoparticles (Feridex) and 6 μg/ml protamine sulfate. On the day of cell transplantation, the SPIOASCs were trypsinized and the detached cells were then centrifuged. The supernatant was removed, and FBS-free medium was added to the cell pellet.

Flow Cytometry Analysis

Adherent ASCs were resuspended with 0.25% trypsin. Then the suspended ASCs were fixed for 10 minutes in 1% paraformaldehyde. The ASCs were then washed twice with phosphate-buffered saline (PBS) and incubated with primary antibodies at room temperature for 30 minutes. The antibodies used were fluorescein isothiocyanate-conjugated anti-rat CD11b, CD34, CD45, CD59, CD29, and CD90.1 (Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermoscientiﬁc.com). Flow cytometric analysis was performed on a fluorescence-activated cell sorter (BD Biosciences, San Jose, CA, https://www.bdbiosciences.com).

In Vitro SPIOASCs Under the Exposure of SMF

The SPIOASCs were resuspended in one column of a 24-well plate, which was superimposed on the top of the same magnet as that subsequently used in vivo. The magnetic field intensity was approximately 0.1 Tesla on the inner wall of plate by measurement with a handheld Gauss meter. The ASCs and SPIOASCs cultured without SMF were used as control. One week later, the cells from three cell groups (ASCs, SPIOASCs, SPIOASCs-magnet) were collected for assessment of growth, proliferation, cytokine secretion, and DNA integrity.

To investigate the ability of a magnet to capture SPIOASCs in vitro, the magnetic SPIOASCs were suspended in a cell-cultured dish. A circular magnet was directly applied to the outside dish bottom wall. After 2 days of culture, cell condensation was assessed visually under a phase-contrast microscope.

MTT Assay

The viability of ASCs was measured by the (3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) (MTT) assay (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Briefly, a 5-mg/ml MTT salt solution was added to cells to give a final concentration of 2.5 mg/ml MTT. Cells were then incubated at 37°C for 1 hour. The final formazan product was dissolved in dimethyl sulfoxide and absorbance was measured at 570 nm. The amount of formazan was directly proportional to the number of live cells.
Cell Proliferation Assay

Proliferation of ASCs was assessed by cell counting kit assays. A cell counting kit reagent (Sigma-Aldrich) was added to the well and incubated for 2 hours. Absorbance value was measured at 450 nm using a microplate reader.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA from the ASCs was extracted using the TRIzol Reagent (Thermo Fisher Scientific Life Sciences) protocol. One microgram of RNA was reversely transcribed using SuperScript III reverse transcriptase (RT; Thermo Fisher Scientific Life Sciences). cDNA was used as a template for polymerase chain reaction (PCR) amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for RNA extraction and RT-PCR assay. The following primers were used: for vascular endothelial growth factor (VEGF), were upstream primer 5'-TCTTCCCTGGA-GACCTGAGA-3' and downstream primer 5'-ACGGAAATCTCG-CAAAACTGCTC-3'; for hepatocyte growth factor (HGF), upstream primer 5'-ACACAAAAACAGTAGGGTGGGAC-3' and downstream primer 5'-TTGGATGCGGACATCCACGACTCAGG-3'; for insulin-like growth factor-1 (IGF-1), upstream primer 5'-CATGTCGTTCTCACATCTCTTCTAC-3' and downstream primer 5'-TCTTCCCTGGA-GACCTGAGA-3'; and for GAPDH, upstream primer 5'-ATCTGACATGCCGCCTGGAGAAACC-3' and downstream primer 5'-CAGGGTTTTCTTACTCCCTGGAGGCC-3'.

Single Cell Gel Electrophoresis (Comet Assay)

ASCs at the cell density 2 × 10⁴ cells/ml were spread into the agarose-covered surface of a precoated slide. After the agarose has gelled, the sides were submerged in a covered dish containing alkaline lysis solution overnight at 4°C. After three rinses, the electrophoresis was conducted in the slides submerged in a chamber for 25 minutes at a voltage of 0.6 V/cm. Then, the slides were rinsed with distilled water and stained in 2.5 m g/ml propidium iodide for 20 minutes. The stained slides were analyzed under a fluorescence microscope. Normal ASCs treated with 2.5 mM H₂O₂ at room temperature for 10 minutes were used as positive control.

Animal Model and Experimental Protocol

Female rats (n = 56) with an average body weight of 200 g were anesthetized through inhalation of 1.5%–2% isoflurane in oxygen, followed by intubation and mechanical ventilation with a rodent ventilator at a rate of 60–70 breaths/min and tidal volume of 2–3 ml. A left anterior thoracotomy was made through the fourth intercostal space. The left anterior descending artery (LAD) was permanently occluded at about 2–3 mm from its origin using a 7-0 silk suture. Coronary ligation generated an infarct demonstrated by macroscopic blanching (supplemental online Fig. 1A). Cardiac cine magnetic resonance imaging (MRI) was performed before and after surgery with double inversion recovery fast spin echo black-blood sequence. The postinfarct hearts displayed the depressed shortening of left ventricular (LV) anterio-lateral wall (supplemental online Fig. 2A). Absolute reduction of approximately 15% in the shortening fraction and 26% in the LV ejection fraction (LVEF) indicated the efficiency of the surgical procedure to induce MI (supplemental online Fig. 2B and 2C). Moreover, in the magnet groups, post-surgery MRI was impossible because of the subcutaneous implantation of magnet.

Immediately after LAD occlusion, the rats were randomly divided into four groups (CCM, n = 8; CCM-magnet, n = 8; SPIOASCs, n = 20; and SPIOASCs-magnet, n = 20). In the rats receiving ASC implantation, four injections of approximately 1.5 × 10⁶ GFP-positive SPIOASCs with total volume of 150 μl were made into the infarct border using a 30-gauge needle (supplemental online Fig. 1B). The CCM-magnet treated rats and SPIOASCs-magnet-treated rats underwent the placement of a circular magnet above the heart for 60 seconds (supplemental online Fig. 1C) and the subsequent subcutaneous insertion of the disc magnet over the chest cavity immediately after suture closure of the costal margin (supplemental online Fig. 1D). There was a 1.6 ± 0.2-mm distance from the disc magnet to the surface of infarcted myocardium. The disc magnet produced the approximately 0.1-Tesla SMF on the surface of LV anterio-lateral wall. CCM control rats and CCM-magnet-treated rats underwent four injections of 150 μl CCM in the same regions. Rats (n = 6 for each cell-injected group) were euthanized and hearts were cryo-sectioned 1 week after surgery to assess short-term cell localization and retention. The numbers of GFP-positive cells were counted in at least 3 high-power fields (HPFs) in infarcted regions of each section under a fluorescence microscope. The histological analysis indicated that the preinfarct region contained the cellular cluster of GFP-positive SPIOASCs and cells containing blue-stained particles (supplemental online Fig. 1E and 1F).

Hematoxylin-Eosin and Prussian Blue Staining

Heart tissue sections from rats (n = 6 for each cell-injected group at 1 week after surgery) were immersed in Prussian blue reagent (4% potassium ferrocyanide/12% HCl, 50:50 vol/vol) for 40 minutes under agitation. The tissue sections were then washed once in PBS and twice in deionized water. The tissue sections were counterstained through standard hematoxylin-eosin procedure. Then, the slides were mounted by antifade mounting media. The sections were examined under a light microscope.

Cardiac Cine MRI

Rats (n = 8 for each CCM-injected group, n = 14 for each cell-injected group at 4 weeks after surgery) were endotracheally incubated, mechanically ventilated with oxygen comprising 1.5%–2% isoflurane using a ventilator, and positioned prone in a cradle. Standard limb leads were constructed from electrodes placed on both forepaws and the left hind paw. The cradle was inserted into an in-house manufactured quadrature MR coil. The coil was positioned in the center of the horizontal bore of a 7-Tesla Bruker magnet interfaced to Bruker Biospec console (Bruker, Karlsruhe, Germany, https://www.bruker.com/).

Cardiac cine imaging was performed using a gradient-echo sequence with a repetition time of 9.2 milliseconds, echo time of 3.5 milliseconds, field of view of 8 × 8 cm², and matrix size of 256 × 256. The cine images were acquired from 5 consecutive slices along the short cardiac axis with a slice thickness of 2.0 mm and no gap between the slices.

Real-Time PCR

Male SryASCs were intramyocardially injected into the myocardium of female rats, enabling detection of Sry gene located on the Y chromosome as an index of engraftment. Quantitative real-time PCR was performed 4 weeks after cell injection (n = 6
for each cell-injected group at 4 weeks after surgery). The DNA of the hearts \( n = 6 \) for each cell-injected group at 4 weeks after surgery \( n = 6 \) for each cell-injected group at 4 weeks after surgery for each rat group at 4 weeks after surgery) were transversely cryosectioned into 8-μm-thick slices from apex to the base. Tissue sections were fixed with 4% paraformaldehyde for 10 minutes at room temperature, washed three times with PBS containing 0.3% Triton X-100, and blocked with 2% goat serum and 1% bovine serum albumin for 30 minutes at room temperature. Slides were then incubated with primary antibodies against von Willebrand factor (vWF), VEGF, HGF, and IGF-1 for 1 hour at 37°C. After 3 washes with PBS, slides were incubated with Alexa Fluor 594-conjugated rabbit anti-goat IgG (Thermo Fisher Scientific Life Sciences) for 45 minutes at 37°C. After 3 more washes with PBS, the slides were stained for nuclei with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Capillaries were counted in at least 3 random fields in infracted regions of each section under a fluorescence microscope. The capillary density was expressed as counts/mm².

**Staining Analysis of Apoptosis**
The cytopreserved tissue sections \( n = 8 \) for each rat group at 4 weeks after surgery\( n = 6 \) for each cell-injected group at 4 weeks after surgery\( n = 6 \) for each cell-injected group at 4 weeks after surgery) were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 20 minutes at room temperature. Then tissue sections were stained by terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) with In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN, https://usdiagnostics.roche.com) according to the manufacturer’s instructions. After TUNEL, the sections were counterstained for nuclei with DAPI. The TUNEL- or DAPI-positive nuclei were counted in 3 random fields in the border zone of each section under a fluorescence microscope. The percentage of apoptotic cells was calculated as the ratio of TUNEL-positive nuclei to total DAPI-positive nuclei.

**Image Processing and Data Analysis**
Cine MRIs were analyzed by using the image processing software Marevisi (Institute for Biodiagnostics, Winnipeg, Canada). LV endocardial and epicardial contours were drawn semiautomatically on serial short-axis slices of both end-diastolic and end-systolic images. The volume of the LV chamber was calculated using a simple numerical integration algorithm. LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV) in each short-axis slice were determined and added to obtain the maximum volume of blood within the LV over a cardiac cycle. Stroke volume (SV) was calculated as the value of LVEDV minus LVESV. EF was calculated as the ratio of stroke volume to LVEDV.

**Statistical Analysis**
All data are expressed as means ± SD. Statistical significance was evaluated with unpaired Student’s t test for comparisons between two means, with analysis of variance for more than two means. Data were considered significant when the p-value was < .05. All statistical analyses were performed with SPSS software, version 12.0 (IBM, Inc., Chicago, IL, http://www.ibm.com).

**Results**

**Characterization of Cultured ASCs**
The cultured ASCs contained a significant number of mesenchymal stem cells, identified by the expression of CD29 (82.07% ± 8.62%), CD59 (83.43% ± 8.45%), and CD90.1 (94.29% ± 5.65%), a very small percentage of endothelial cells, identified by the expression of CD34 (4.56% ± 1.07%), but no hematopoietic lineages, identified by a negative expression of CD11b (0.07% ± 0.04%) and CD45 (0.06% ± 0.02%)(Fig. 1A). These results indicated that the cultured ASCs contained a large population of mesenchymal stem cells and a small percentage of endothelial cells.

Cultured ASCs showed a fibroblast-like morphology (Fig. 1B). ASCs derived from GFP transgenic rats expressed green fluorescence (Fig. 1C). Prussian blue staining confirmed particle uptake by ASCs (Fig. 1D).

**Effect of SMF on Cell Growth, Paracrine Cytokines, and DNA Integrity**
At 1 week after SMF exposure, cell viability evaluated by MTT assay did not differ among ASCs (100% ± 1.6%), SPIOASCs (97.3% ± 2.5%), and SPIOASCs-magnet (96.8% ± 2.5%) suplemental online Fig. 3A). Cell proliferation percentage reflected by direct cell counting did not differ among ASCs (100% ± 2.2%), SPIOASCs (100.3% ± 3.7%), and SPIOASCs-magnet (99.2% ± 3.6%) (supplemental online Fig. 3B).

Figure 2A showed the mRNA bands of angiogenic cytokines expressed in three cell groups. The relative band intensity (VEGF/GAPDH) did not differ among the ASCs (3.55 ± 0.48 arbitrary units [A.U.]), SPIOASCs (3.58 ± 0.27), and SPIOASCs-magnet (3.50 ± 0.36) (Fig. 2B). Moreover, the relative band intensity (IGF-1/GAPDH) did not differ among the ASCs (0.86 ± 0.08 A.U.), SPIOASCs (0.87 ± 0.07), and SPIOASCs-magnet (0.85 ± 0.08) (Fig. 2C).

Figure 2D showed the comet assay results in three cell groups. DNA damage with comet tail was not observed in ASCs, SPIOASCs, and SPIOASCs-magnet by visual estimation. The percentage of cells with integral DNA without comet tail did not differ among the ASCs (98.8% ± 0.41%), SPIOASCs (98.7% ± 0.33%), and SPIOASCs-magnet (98.6% ± 0.29%) (Fig. 2E). These indicated that the SMF (0.1 Tesla) did not adversely affect viability, proliferation, cytokine secretion, and DNA integrity of SPIOASCs.

**Proangiogenic Effect of Implanted ASCs**
The implanted ASCs expressed vWF, indicating that some injected cells differentiated into endothelial cells (Fig. 3A). The
GFP-positive ASCs directly incorporated into newly formed blood vessels (Fig. 3B). The implanted ASCs expressed the angiogenic cytokines, such as VEGF, HGF, and IGF-1 (Fig. 3C–3E). These indicated that ASC transplantation promoted neovascularization by differentiation into endothelial cells, incorporation into vasculature, and secretion of multiple angiogenic cytokines.

Externally Applied SMF Captures Magnetic SPIOASCs In Vitro

The SPIOASCs were resuspended in a cell-cultured dish and a circular magnet was positioned behind the dish (supplemental online Fig. 4A). The magnet was the same as that subsequently used in vivo. After two days of cultivation, the ‘magnetic’ SPIOASCs were obviously attracted toward magnet and accumulated focally on the adjacent inner wall (supplemental online Fig. 4B). In contrast, less SPIOASC accumulation was observed on the remote area (supplemental online Fig. 4C).

Externally Applied SMF Enhances Cardiac Retention of Implanted SPIOASCs

One week after cell transplantation, more cells containing blue-stained particles were distributed in the SPIOASC-magnet-treated rats than in the SPIOASC-treated rats (Fig. 4A). SPIOs released upon the death of transplanted cells have been engulfed in macrophages [23]. Prussian blue staining might reflect the location of SPIOs in the myocardium, but not necessarily the number of engrafted SPIOASCs. Likewise, more GFP-positive cells were evident in the SPIOASC-magnet-treated rats than in the SPIOASC-treated rats (Fig. 4B). The number of SPIOASCs, quantified as GFP-positive cells per HPF, was significantly greater in the SPIOASC-magnet-treated rats (64.7 ± 8.01 cells per HPF) than in the SPIOASC-treated rats (34.7 ± 5.31) (Fig. 4C).

Figure 4D shows the reproducibility of the Y-chromosome quantification by real-time PCR. The correlation between the number of copies of male cell DNA and number of cycles for detecting threshold was excellent ($r^2 = .992; p < .0001$). The number of alive male ASCs transplanted into female heart was significantly greater in the SPIOASC-magnet-treated rats (213.96 ± 6.28 cells per milligram of heart tissue or $3.62 \times 10^5$ cells per heart) than in the SPIOASC-treated rats (116.88 ± 7.22 or 1.98 ± 0.12) at 4 weeks after cell transplantation (Fig. 4E and 4F). These indicated that externally applied SMF enhanced myocardial retention of the magnetic SPIOASCs implanted using intramyocardial injection.

Externally Applied SMF Enhances ASC-Induced Angiogenesis and Inhibition of Apoptosis

Capillaries were identified according to positive vWF staining in the peri-infarct zone 4 weeks after cell transplantation (Fig. 5A). Capillary density was substantially higher in the SPIOASC-magnet-treated rats (374.49 ± 64.54 vessels/mm²) than in the SPIOASC-treated rats (285.82 ± 55.33), in the CCM-magnet-treated rats (195.71 ± 74.87), and in the CCM
Figure 2. Angiogenic cytokines secretion and DNA integrity of ASCs under the exposure of 0.1-Tesla static magnetic field. (A): Typical example of reverse-transcriptase polymerase chain reaction analysis of VEGF and IGF-1 expression in ASCs, SPIO ASCs, and SPIO ASCs-magnet. (B and C): Expression of two cytokines from three groups of ASCs were indistinguishable (n = 3 experiments for each cell group). (D): DNA damages with comet tail were not observed at three cell groups. (E): The percentage of cells with integral DNA was identical among three cell groups (n = 3 experiments for each cell group). Scale bar = 50 μm. Abbreviations: ASC, adipose-derived stem cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF-1, insulin-like growth factor-1; SPIO ASC, superparamagnetic iron oxide-labeled adipose-derived stem cell; VEGF, vascular endothelial growth factor.
control rats (197.67 ± 60.70) (Fig. 5B). This indicated that externally applied SMF significantly strengthened SPIOASC-induced angiogenesis.

Apoptotic cardiomyocytes were stained red with TUNEL reagent in the peri-infarct zone 4 weeks after cell transplantation (Fig. 6A). TUNEL-positive cardiomyocytes in the CCM-magnet-treated
rats (2.13% ± 0.35%) and the CCM control rats (2.07% ± 0.20%) were indistinguishable. Apoptotic cardiomyocytes was significantly lower in the SPIO ASC-magnet-treated rats (0.86% ± 0.23%) than in the SPIOASC-treated rats (1.46% ± 0.46%) (Fig. 6B). These findings suggested that externally applied SMF did not induce cardiomyocyte apoptosis but enhanced SPIOASC-induced inhibition of ischemic cardiomyocyte apoptosis.

**Externally Applied SMF Enhances Functional Benefit of SPIOASC Transplantation**

Figure 7A showed three stacks of representative cine MR images acquired at 4 weeks after cell transplantation. The SPIOASC-magnet-treated rats displayed a smaller LV slice volume in comparison with the SPIOASC-treated rats, the CCM-magnet-treated rats, and the CCM control rats.

There were no significant differences in LVEDV, LVESV, and LVEF between the CCM-magnet-treated rats and the CCM control rats (476.77 ± 72.49 versus 489.56 ± 62.61 mm³ for LVEDV, 329.43 ± 76.31 versus 330.06 ± 57.03 mm³ for LVESV, 33.42% ± 3.92% versus 32.87% ± 4.34% for LVEF). This suggested that externally applied SMF did not adversely affect cardiac contractile function.

The LVEDV and LVESV were significantly lower in the SPIOASC-magnet-treated rats than in the SPIOASC-treated rats (353.26 ± 43.88 versus 421.62 ± 57.01 mm³ for LVEDV, 140.34 ± 36.80 versus 213.02 ± 47.52 mm³ for LVESV) (Fig. 7B and 7C). LVEF was substantially greater in the SPIOASC-magnet-treated rats (58.87% ± 5.87%) than in the SPIOASC-treated rats (49.73% ± 6.85%) (Fig. 7D). This suggested that improved cell retention by externally applied SMF indeed translated into superior heart function.

Figure 3. Continued from previous page.
Figure 4. Effect of the externally applied static magnetic field on cell retention and engraftment. (A, B): One week after cell implantation, more GFP-positive SPIOASCs and cells containing blue-stained particles were detected in the SPIOASC-magnet-treated rats ($n = 6$) than in the SPIOASC-treated rats ($n = 6$). Regions 1 and 2 selected in left column are amplified in two right columns, respectively. (C): At 1 week after cell transplantation, the SPIOASC-magnet-treated rats ($n = 6$) exhibited an approximately 1.86-fold greater cell numbers per high-power field than the SPIOASC-treated rats ($n = 6$). (D): The reproducibility of the standard curve obtained by real-time polymerase chain reaction. A serial 10-fold dilution of DNA was tested 6 times in separate experiments. Each circle corresponds to the result of one dilution in one assay. The solid line corresponds to the regression analysis. (E, F): At 4 weeks after cell transplantation, the SPIOASC-magnet treated rats ($n = 6$) showed an approximately 1.82-fold greater cell numbers per milligram of heart tissue (E) and per heart (F) than the SPIOASC-treated rats ($n = 6$). Scale bar = 20 μm. *, $p < .05$ versus SPIOASCs. Abbreviation: SPIOASC, superparamagnetic iron oxide-labeled adipose-derived stem cell.
Figure 5. Immunofluorescence analysis of capillary density in the peri-infarct zone 4 weeks after cell transplantation. (A): Cardiac sections from four rat groups were stained with DAPI (blue) and antibody to endothelial cell marker vWF (red). (B): Capillary density was greatest in the SPIOADSC-magnet-treated rats \(n = 8\) compared with the SPIOASC-treated rats \(n = 8\), the CCM-magnet rats \(n = 8\), and the CCM control rats \(n = 8\). Scale bar = 50 \(\mu\)m. \#\#, \(p < .05\) versus the CCM; \(\Delta\), \(p < .05\) versus the CCM or CCM-magnet; \(*\), \(p < .05\) versus the SPIOASCs, CCM-magnet, or CCM. Abbreviations: CCM, cell culture medium; DAPI, 4′,6-diamidino-2-phenylindole; SPIOASC, superparamagnetic iron oxide-labeled adipose-derived stem cell; vWF, von Willebrand factor.
Figure 6. TUNEL staining of apoptotic cardiomyocytes in the peri-infarct zone 4 weeks after cell transplantation. (A): Cardiac sections from four rat groups were stained with DAPI (blue) and with TUNEL reagent for apoptosis (red). (B): TUNEL-positive cardiomyocytes were fewest in the SPIOASC-magnet-treated rats (n = 8) compared with the SPIOASC-treated rats (n = 8), the CCM-magnet rats (n = 8), and the CCM control rats (n = 8). Scale bar = 20 μm. #, p > .05 versus the CCM; ∆, p < .05 versus the CCM or CCM-magnet; *, p < .05 versus the SPIOASCs, CCM-magnet, or CCM.

Abbreviations: CCM, cell culture medium; DAPI, 4',6-diamidino-2-phenylindole; SPIOASC, superparamagnetic iron oxide-labeled adipose-derived stem cell; TUNEL, terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling.
The safety of SMF on ASCs is the prerequisite for the feasibility of magnetic targeting technique. The subcutaneously implanted magnet produced an approximately 0.1-Tesla SMF on the LV anterior wall. The SPIOASCs under the 0.1 Tesla of SMF had viability and proliferative ability similar to those of the normal SPIOASCs. Exposure to 0.1-Tesla SMF for 1 week did not alter the expression of VEGF and IGF-1 of the ASCs. Comet assay demonstrated that no DNA single-strand break was observed in the SPIOASCs regardless of whether the SMF was present. Those findings agree with those of previous investigations. Victora et al. found that the number of hematopoietic stem cells was not reduced by the 3- to 5-month exposure of magnetic field (180–200 gauss) and that proliferative capacity was likewise unimpaired [24]. Schäfer et al. found that the exposure to 0.6-Tesla magnetic field for 24 hours had no influence on the migration capacity, viability, proliferation rate, and chondrogenic differentiation of ASCs.

**DISCUSSION**

The safety of SMF on ASCs is the prerequisite for the feasibility of magnetic targeting technique. The subcutaneously implanted magnet produced an approximately 0.1-Tesla SMF on the LV anterior wall. The SPIOASCs under the 0.1 Tesla of SMF had viability and proliferative ability similar to those of the normal SPIOASCs. Exposure to 0.1-Tesla SMF for 1 week did not alter the expression of VEGF and IGF-1 of the ASCs. Comet assay demonstrated that no DNA single-strand break was observed in the SPIOASCs regardless of whether the SMF was present. Those findings agree with those of previous investigations. Victora et al. found that the number of hematopoietic stem cells was not reduced by the 3- to 5-month exposure of magnetic field (180–200 gauss) and that proliferative capacity was likewise unimpaired [24]. Schäfer et al. found that the exposure to 0.6-Tesla magnetic field for 24 hours had no influence on the migration capacity, viability, proliferation rate, and chondrogenic differentiation of ASCs.

**Figure 7.** Magnetic resonance imaging (MRI) analysis of cardiac function. (A): Typical short-axis cine MRIs from end-diastole to end-systole during the whole cardiac cycle. The SPIOASC-magnet-treated rats (n = 14) displayed a smaller left ventricular (LV) slice volume in comparison with the SPIOASC-treated rats (n = 14), the CCM-magnet-treated rats (n = 8), and the CCM control rats (n = 8). (B, C): LV end-diastolic (B) and endsystolic (C) volumes were lowest in the SPIOASC-magnet-treated rats (n = 14) compared with the SPIOASC-treated rats (n = 14), the CCM-magnet rats (n = 8), and the CCM control rats (n = 8). (D): LV ejection fraction was highest in the SPIOASC-magnet-treated rats (n = 14) compared with the SPIOASC-treated rats (n = 14), the CCM-magnet rats (n = 8), and the CCM control rats (n = 8). #, p < .05 versus the CCM; Δ, p < .05 versus the CCM or CCM-magnet; *, p < .05 versus the SPIOASCs, CCM-magnet, or CCM. Abbreviations: CCM, cell culture medium; SPIOASC, superparamagnetic iron oxide-labeled adipose-derived stem cell.
capacity of SPIO-labeled or unlabeled mesenchymal stem cells [25]. Reddig et al. reported that exposure of human peripheral blood mononuclear cells to 7-Tesla SMF did not induce DNA double-strand breaks [26].

The magnetic field has enhanced the delivery of magnetic particle-conjugated anticancer drugs to the targeted tumor organ in both preclinical and clinical studies [15–17]. A magnet positioned near the subcutaneous tumor increased the retention percentage of intravenously delivered SPIO-labeled monocytes from 4.9% ± 3.5% to 16.9% ± 4.2% [18]. This study found that locally applied SMF (0.1 Tesla, 7 days) enhanced cardiac retention of magnetic SPIOASCs by approximately 1.82-fold at 4 weeks after intramyocardial injection. A subcutaneously implanted magnet (0.1 Tesla, 24 hours) over the chest cavity increased cardiac retention of intraventricular arterial infusion [35]. More importantly, the superior result in greater improvement in LVEF relative to intracoronary arterial infusion [34]. At a same cell dose, direct intramyocardial injection and intravenous delivery of the umbilical cord blood cells demonstrated with intramyocardial, intracoronary arterial, vascularization and inhibits ischemic cardiomyocyte apoptosis, through endothelial differentiation, incorporation into vessels, and paracrine secretion. An externally applied SMF enhanced myocardial retention of intramyocardially injected magnetic SPIOASCs and promoted cardiac function recovery after MI.

CONCLUSION

A 0.1-Tesla SMF did not adversely affect the viability, proliferation, angiogenic cytokine secretion, or DNA integrity of the SPIOASCs. ASC implantation promotes myocardial neovascularization and inhibits ischemic cardiomyocyte apoptosis through endothelial differentiation, incorporation into vessels, and paracrine secretion. An externally applied SMF enhanced myocardial retention of intramyocardially injected magnetic SPIOASCs and promoted cardiac function recovery after MI.

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AUTHOR CONTRIBUTIONS

J.W.: financial support, manuscript writing, final approval of manuscript; B.X. and D.Z.: collection and/or assembly of data, data analysis and interpretation; J.D.: administrative support, data analysis and interpretation; H.-Y.L.: administrative support, collection and/or assembly of data; D.H.F. and R.C.A.: provision of study material or patients, conception and design; G.T.: conception and design, financial support, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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