Iron-Oxide Labeling and Outcome of Transplanted Mesenchymal Stem Cells in the Infarcted Myocardium

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Background—Cell labeling with superparamagnetic iron oxide (SPIO) nanoparticles enables noninvasive MRI and tracking of transplanted stem cells. We sought to determine whether mesenchymal stem cell (MSC) outcome is affected by SPIO labeling in a rat model of myocardial infarction.

Methods and Results—Rat MSCs were labeled with SPIO (ferumoxides; Endorem; Guerbet, Villepinte, France). By trypan-blue exclusion assay, almost 100% of the cells remained viable after labeling. Seven days after MI, rats were randomized to injections of $2 \times 10^6$ SPIO-labeled MSCs, $2 \times 10^6$ unlabeled MSCs, or saline. Labeled cells were visualized in the infarcted myocardium as large black spots by serial MRI studies throughout the 4-week follow-up. The presence of labeled cells was confirmed by iron staining and real-time polymerase chain reaction on postmortem specimens. At 4 weeks after transplantation, the site of cell injection was infiltrated by inflammatory cells. Costaining for iron and ED1 (resident macrophage marker) showed that the iron-positive cells were cardiac macrophages. By real-time polymerase chain reaction, the Y-chromosome-specific SRY DNA of MSCs from male donors was not detected in infarcted hearts of female recipients. Serial echocardiography studies at baseline and 4 weeks after cell transplantation showed that both unlabeled and labeled MSCs attenuated progressive left ventricular dilatation and dysfunction compared with controls.

Conclusions—At 4 weeks after transplantation of SPIO-labeled MSCs, the transplanted cells are not present in the scar and the enhanced MRI signals arise from cardiac macrophages that engulfed the SPIO nanoparticles. However, both labeled and unlabeled cells attenuate left ventricular dilatation and dysfunction after myocardial infarction.

Key Words: cells  ■ magnetic resonance imaging  ■ myocardial infarction  ■ remodeling  ■ transplantation

To develop effective stem cell-based therapies for myocardial repair, the location, distribution, and long-term viability of the cells must be determined in a noninvasive manner.¹ Magnetic resonance imaging (MRI) of cells labeled with magnetically visible contrast agents has the potential to fulfill this aim.² The value of MRI in monitoring and tracking stem cells injected into the heart has been established with various cell types, including mesenchymal stem cells (MSCs),³ myogenic precursors,⁴ and embryonic stem cells.⁵ The most sensitive existing markers for cell labeling using MRI are superparamagnetic iron oxide (SPIO) particles.⁶ They are nontoxic and biodegradable and do not affect proliferation and multilineage differentiation capacity in vitro.⁷⁻⁹ However, it is unclear how cell labeling with magnetic contrast material, and the use of transfection agents, influences the outcome and therapeutic capacity of stem cells. Thus, the aim of the present research was to determine whether the therapeutic capacity of MSCs is affected by iron-oxide labeling in a rat model of extensive myocardial infarction (MI).

Methods

Mesenchymal Stem Cell Culture and Labeling

Rat MSCs were isolated from bone marrow aspirate of Sprague-Dawley rats as previously described.¹⁰¹¹ MSCs were magnetically labeled with ferumoxide (Endorem; Guerbet, Villepinte, France), complexed to poly-L-lysine (catalogue No. P1524; Sigma, MW >388 000 and cell culture grade), as previously described.¹² Ferumoxide is an iron oxide nanoparticle solution provided with a total iron content of 11.2 mg/mL, whereas poly-L-lysine is used as a transfection agent that provides efficient labeling with significantly lower iron concentrations. Ferumoxide is approved for clinical use in liver imaging and is commercially available. Ferumoxide act by reducing the transverse relaxation time (T2) on T2-weighted MRI scans; therefore, labeled cells appear as areas of reduced signal intensity. Cells were incubated with the labeling medium containing 25 µg/mL iron and 0.375 µg/mL poly-L-lysine for 24 hours in a cell density of $3 \times 10^5$ cells/mL. After labeling, cells were washed 3 times.
with phosphate-buffered saline and harvested using 0.25% trypsin-EDTA (Sigma-Aldrich). The viability of the cells before transfer was assessed by trypan-blue exclusion. To confirm MSC engraftment in the scar after cell transfer, another group of cultured MSCs were labeled with 1% thymidine analogue 5-bromo-2'-deoxyuridine (BrdU; Zymed), as previously described.13

**Cell Transfer**

MI was induced as previously described.11,12 Sprague-Dawley male rats (approximately 250 g) were anesthetized with a combination of ketamine (50 mg/kg) and xylazine (10 mg/kg), intubated, and ventilated. The left coronary artery was permanently ligated with a 6–0 Prolene stitch. Seven days after MI, the chest was reopened and rats were randomized to receive 2×10^6 SPIO-labeled MSCs, unlabeled MSCs, or phosphate-buffered saline by direct injection into the scar area. These 3 groups underwent serial echocardiographic and MRI examinations. An additional group of intact rats (sham-MI) were injected with 2×10^6 unlabeled MSCs and underwent serial MRI scans. Finally, to detect the survival of the implanted cells by their specific DNA, MSCs were obtained from male Sprague-Dawley rats, expanded, and transplanted into female Sprague-Dawley recipient hearts 7 days after MI.

**Histology**

To assess iron oxide particle uptake by MSCs, a sample containing 1×10^5 cells was cultured in a chamber slide and then fixed with 70% ethanol and processed for iron staining (Sigma-Aldrich) and co-staining with eosin. After the last MRI study, the rats were killed. The hearts were sectioned into 3 to 4 transverse slices parallel to the atriовentricular ring and were fixed with 4% buffered formalin, embedded in paraffin, and sectioned with a microtome (5-μm thick). The lungs, liver, and kidneys were excised, fixed, and embedded in paraffin. Serial sections were stained with iron staining to detect iron particles and hematoxylin and eosin and immunolabeled with antibodies against ED1 (Serotec), a marker for tissue-resident macrophages, and ED2 (Serotec) for tissue resident macrophages. Slides with positive iron staining were co-stained with ED1 and ED2 antibodies. Biotinylated mouse anti-BrdU (Zymed) was used to localize the donor cells in the 2 rats injected with BrdU-labeled cells 24 hours after transplantation.13

**Real-Time Polymerase Chain Reaction Detection of SRY DNA**

Polymerase chain reaction analysis for the rat Y-chromosome-specific SRY gene was performed from scar and border zone cardiac tissue areas 4 weeks after cell transplantation. The DNA extraction was performed with a QIAamp Tissue Kit (Qiagen, Valencia, Calif) according to the manufacturer’s instructions.14 Primers for GAPDH and SRY gene segment were based on bioinformatics search at the National Center for Biotechnology Information (NCBI) and were designed to detect the specific DNA of normal myocardium. SRY sequence represents the signal intensity (arbitrary units) of normal myocardium, SISPIO represents the signal intensity of SPIO-labeled cell injection sites, and SD represents the SD of different background noise.

**Statistical Analyses**

Data are presented as means±SE. Because each animal was used as its own control, changes between baseline and 4 weeks in the control and treated groups were assessed with paired *t* tests. In addition, relative change (%) in baseline parameters was calculated as 

\[ \text{relative change} = \frac{\text{value at week 4} - \text{baseline}}{\text{baseline}} \times 100 \]

Analysis of variance was used to test differences in relative changes in LV variables among the 3 groups. Comparisons of the changes from baseline to 4 weeks in the control and treatment groups were made with repeated-measures 2-way analysis of variance. The analysis of variance model included the control versus treatments and baseline versus 4 weeks as factors and also included the interaction between the 2 factors. GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, Calif) was used for analysis.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the article as written.

**Results**

**Myocardial Infarction Operation and Mortality**

Overall, 66 rats were included in the study, 61 with MI and 5 with sham-MI. Among the rats with MI, 12 died within 24 hours of the surgical procedure and 3 died during cell injection (25% postoperative mortality). Seven rats were found to have no or only very small infarctions on echocardiography (Fractional shortening >45%) and were not included in the study. Thus, 39 rats with MI were included in the final analysis: (1) functional and morphometric evaluation by echo and cell tracking by MRI (*n* = 29); (2) short-term cell tracking by BrdU staining (*n* = 2); and (3) cell tracking by real-time PCR for the Sry gene (*n* = 8). Another group of 5 sham-MI rats were injected with SPIO-labeled cells and were included in the in vivo cell tracking by MRI.

**Efficacy and Safety of Cell Labeling With Iron Nanoparticles**

In a series of pilot experiments, we improved the distinction of the injected areas by decreasing the iron loading of cells.
(mainly by lowering the SPIO and transfection agent doses), but at the cost of lower signal-to-noise ratio at the site of injection. Iron staining of SPIO-labeled MSCs demonstrated highly efficient iron uptake manifested as numerous blue granules in the cytoplasm (Figure 1A–B). By trypan-blue exclusion assay, almost 100% of the cells remained viable after labeling with SPIO. Before cell transfer to animals, the magnetic properties of labeled cells were assured by MRI scans of agar phantoms prepared from cell suspensions. A phantom of 0.8×10^6 cells appeared gray, whereas a phantom of 1.8×10^6 cells appeared black (Figure 1C).

MRI-Guided Stem Cell Tracking in Animals With and Without Myocardial Infarction

We performed serial MRI exams to track the cells at 1, 2, and 4 weeks after cell injection into the anterior wall of the LV. Well-defined hypointensities (“black spots”) were observed at the region of cell injection in all the animals who received SPIO-labeled MSCs (Figure 2). In the sagittal section of the LV 1 week after treatment, the site of injection demonstrated wide hypointensities, which extended beyond the actual location of the labeled cells (“blooming effect”) and, in some of the animals, almost obliterated the whole thickness of the LV free wall. Control unlabeled MSCs or saline-injected hearts had no hypointensities on the tissue MRI appearance (Figure 2C). Four weeks after cell delivery, “black spots” could still be visualized by MRI (Figure 2). Retention of the magnetic signal throughout 4 weeks was similar in rats both with and without MI (percent change in contrast-to-noise ratio 79±17% and 62±8, respectively; P=0.38, Figure 2A–B).

Superparamagnetic Iron Oxide Nanoparticles Were Engulfed by Resident Macrophages

Proof of successful cell engraftment was demonstrated by numerous BrdU-labeled MSCs at the site of injection 24 hours after injection (Figure 3A). Four weeks after cell delivery, iron staining showed large clusters of positive cells at the site of injections at the scar and perinfarct zones (Figures 3B and 4). Higher-magnification photomicrographs demonstrated the intracytoplasmic localization of the iron particles with sparing of the nucleus and absence of extracel-
lular iron (Figure 3C). Examination of the remote organs, including the lungs, liver, and kidneys, did not reveal iron-positive cells in rats without MI, but did reveal a few positive cells in lung tissue of rats with MI (Figure 3D).

The site of MSC (labeled and unlabeled) injection was infiltrated with inflammatory cells with numerous macrophages (Figure 4). Costaining for iron and ED1 (a marker of resident macrophage) showed that most of the iron-positive cells were also stained positive for ED1 (Figure 5). These findings suggest that 4 weeks after injection, most of the transplanted labeled MSCs did not survive, and their iron content was engulfed by resident macrophages (Figure 5).

No labeled cells were found in the adjacent healthy myocardium. Iron-positive cells were also found in sections from sham-MI rats treated with SPIO cells, although clusters of cells tended to be smaller. Notably, scarce iron-positive cells could also be found in rats with MI injected with unlabeled cells, probably representing tissue macrophages loaded with hemosiderin from hemorrhages in the infarcted myocardium.17

Detection of Donor Cell DNA in the Scar by Real-Time Polymerase Chain Reaction
For identification of transplanted cells of male donor origin, real-time PCR analysis for the rat Y-chromosome-specific SRY gene was performed on DNA from infarcted hearts of 8 female recipients treated with labeled (n = 3) and unlabeled (n = 2) MSCs from donor males or with saline (control, n = 2). Four weeks after transplantation, no SRY sequences were detected in any of the female recipients treated with male MSCs (Figure 6). Thus, based on PCR and histological analysis, neither the labeled nor the unlabeled MSCs were present in the infarcted heart 4 weeks after injection.

Labeled and Unlabeled Cells Attenuated Left Ventricular Dilatation and Dysfunction After Myocardial Infarction
Transthoracic echocardiographic examination was performed in 3 groups of rats with MI at baseline (1 day before cell transplantation) and 4 weeks later. The typical course of scar thinning, LV dilatation and functional deterioration was observed in all groups (Tables 1 and 2). Both labeled and unlabeled cells attenuated LV dilatation and dysfunction (Tables 1 and 2; Figure 6). Compared with controls, the protective effect of both labeled and unlabeled cells was significant on LVESD, LVEDA, LVESA, and fractional shortening (repeated-measures analysis of variance, Table 1). The beneficial effect tended to be greater with unlabeled MSCs, but this difference was not significant compared with labeled cells (Table 2; Figure 5).

Discussion
The main new findings of the present study are: (1) At 4 weeks after transplantation of SPIO-labeled MSCs, the trans-
planted cells are not present in the infarcted myocardium and enhanced MRI signals arise from cardiac macrophages that engulfed the SPIO nanoparticles. This finding suggests that iron nanoparticles are not a reliable marker to monitor transplanted stem cell traffic and survival. (2) Despite cell loss, both labeled and unlabeled cell maintain their protective effect against progressive LV dilatation and dysfunction, probably attributable to paracrine effect or in situ modulation of healing response. Compared with SPIO-labeled MSCs, unlabeled MSCs tend to provide a slightly greater protective effect but this advantage was not statistically significant.

Effect of Iron Labeling on Protective Capacity of Stem Cells

SPIO is considered nontoxic and biodegradable and do not affect proliferation and multilineage differentiation capacity in vitro.7–9 However, chondrogenic differentiation of MSCs is inhibited after magnetic labeling with ferumoxides.22 High concentration of free intracellular iron may be toxic to cells. Arbab et al conducted a series of experiments to define the optimal protocols for using ferumoxides in combination with various transfection agents for cellular magnetic labeling.7,23,24 In their work, increasing the iron concentration in labeling solution from 50 to 125 μg/mL resulted in a 3-fold rise in MSCs intracellular iron concentration after 24 hours but was associated with almost 40% cell mortality compared with unlabeled control MSCs signifying the existence of a safety threshold for iron concentration above which iron toxicity appears.

In the present study, we chose a relatively low SPIO and transfection agent concentrations that were shown to enable clear visualization of MSCs without altering in vitro long-term viability, growth rate, and apoptotic indices.7 The same concentrations and labeling protocol were previously used in several trials in which SPIO labeling was applied to track MSCs.18,19,25,26 Nevertheless, in the present study, despite viability of almost 100% before transplantation, neither labeled nor unlabeled cells survived in the infarcted myocardium after 4 weeks. The cause of this observation that contradicts many previous reports is unclear. We may suggest several potential explanations. First, contrary to many previous studies, we injected the cells directly into the infarct and not into the border zone, which may be significant regarding cell loss attributable to leakage and inflammation. Second, Sprague-Dawley rat donors, unlike mouse donors from the
same inbred strain, are not syngeneic and can evoke significant immune response that kill the cells. Third, the cells were expanded for several weeks in culture dish, and therefore it is possible that incubation with xenogenic growth factors caused phenotypic changes in cultured cells that could have triggered an immune response after transplantation. Finally, it has been suggested that an immunological reaction to the histocompatibility antigen on the Y chromosome of the donor cells could account for the death or disappearance of the implanted cells. Despite the fact that the cause of cell

| TABLE 1. Comparison of LV Remodeling and Function in Unlabeled Cells, SPIO-Labeled Cells, and Saline-Treated Groups by 2-Dimensional Echocardiography Before (baseline) and 4 Weeks After Injection |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                  | Unlabeled MSCs (n=11) | SPIO-Labeled MSCs (n=10) | Saline (n=8) | P†               |
| AW d, cm                         |                  |                  |                |                  |
| Baseline                         | 0.15±0.01        | 0.14±0.01        | 0.13±0.01      |                  |
| 4 weeks                          | 0.10±0.01        | 0.10±0.01        | 0.09±0.003     | 0.92             |
| P                                | 0.005            | 0.008            | 0.005          |                  |
| AW s, cm                         |                  |                  |                |                  |
| Baseline                         | 0.17±0.01        | 0.16±0.01        | 0.14±0.01      |                  |
| 4 weeks                          | 0.12±0.01        | 0.11±0.01        | 0.10±0.004     | 0.70             |
| P                                | 0.002            | 0.003            | 0.001          |                  |
| LVEDD, cm                        |                  |                  |                |                  |
| Baseline                         | 0.78±0.03        | 0.75±0.03        | 0.78±0.02      |                  |
| 4 weeks                          | 0.98±0.02        | 0.98±0.03        | 1.03±0.02      | 0.22             |
| P                                | <0.001           | <0.001           | <0.001         |                  |
| LVESD, cm                        |                  |                  |                |                  |
| Baseline                         | 0.59±0.04        | 0.56±0.05        | 0.61±0.04      |                  |
| 4 weeks                          | 0.76±0.04        | 0.79±0.05        | 0.89±0.03      | 0.03             |
| P                                | <0.001           | <0.001           | <0.001         |                  |
| LVEDA, cm                        |                  |                  |                |                  |
| Baseline                         | 0.41±0.03        | 0.36±0.02        | 0.41±0.02      |                  |
| 4 weeks                          | 0.60±0.03        | 0.64±0.05        | 0.72±0.03      | 0.05             |
| P                                | <0.001           | <0.001           | <0.001         |                  |
| LVESA, cm                        |                  |                  |                |                  |
| Baseline                         | 0.23±0.03        | 0.20±0.02        | 0.24±0.04      |                  |
| 4 weeks                          | 0.36±0.04        | 0.41±0.05        | 0.50±0.03      | <0.01            |
| P                                | <0.001           | <0.001           | <0.001         |                  |
| FS, %                            |                  |                  |                |                  |
| Baseline                         | 25±2             | 26±4             | 23±3           |                  |
| 4 weeks                          | 23±3             | 20±3             | 14±2           | 0.01             |
| P                                | 0.145            | 0.005            | 0.006          |                  |
| FAC, %                           |                  |                  |                |                  |
| Baseline                         | 48±4             | 48±5             | 45±5           |                  |
| 4 weeks                          | 42±4             | 39±3             | 30±1           | 0.18             |
| P                                | 0.051            | 0.024            | 0.025          |                  |
| Heart rate, beats/min            |                  |                  |                |                  |
| Baseline                         | 288±18           | 292±19           | 293±19         |                  |
| 4 weeks                          | 246±19           | 247±13           | 259±9          | 0.89             |
| P                                | 0.012            | 0.156            | 0.131          |                  |

Values are means±SE.

*P values derived from paired comparisons between baseline and 4-week measurements.
†P values for the differences between groups over time (repeated-measure analysis of variance).
AW d indicates anterior wall diastolic thickness; AW s, anterior wall systolic thickness; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; LV EDA, LV end-diastolic area; LV ESA, LV end-systolic area; LV FS, LV fractional shortening—[(LVIDd−LVIDs)/LVIDd]×100; FAC, fractional area change—[(EDA−ESA)/ESA]×100.
cell survival or transdifferentiation. Our findings suggest that the benefit is likely a result of factors secreted by the MSCs (paracrine effect) or another type of interaction with the healing infarct. It is also possible that accumulation of activated resident macrophages at the site of injection contributed to infarct healing and repair.

Limitations
We are aware of several limitations in our work. First, the number of animals in each group was relatively small. It is possible that with larger numbers the differences between cell-treated groups have been more significant. Second, the echocardiography parameters used to assess heart function are still controversial in the small animal model. Using a different method such as small animal cardiac MRI to assess LV remodeling and function could support our findings.

Implications and Future Research
Persistent positive MRI signal from the infarcted tissue after transplantation of iron-labeled cells might arise from resident cardiac macrophages that engulfed the labeling iron nanoparticles. Our findings raise concern regarding the power of iron-labeling method in preclinical and clinical trials of cardiac cell therapy. In addition, it is possible that cell labeling could affect survival and therapeutic capacity. Further research is therefore needed to determine the reliability and safety of various labeling methods. New transfection agents and new mechanisms for generating contrast with MRI may help to overcome these challenges. With further research and development, the promise of molecular imaging using novel magnetic labels to track cells may be translated into clinical practice.

Acknowledgments
We thank Mrs Pat Benjamin (echocardiography) for excellent technical assistance and Mrs Vivienne York for skillful editing.

Source of Funding
This work was supported by Schlezak Foundation, Tel Aviv University.

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TABLE 2. Comparison of Percent Change in LV Remodeling and Function by 2-Dimensional Echocardiography 4 Weeks After Injection of Unlabeled Cells, SPIO-Labeled Cells, or Saline

|                      | Unlabeled MSCs (n=11) | SPIO-Labeled MSCs (n=10) | Saline (n=8) | p
|----------------------|-----------------------|--------------------------|--------------|---
| AW d                 | -46±13                | -45±12                   | -26±5        | 0.45
| AW s                 | -51±13                | -47±13                   | -28±4        | 0.36
| LVEDD                | 20±2†                 | 23±2†                    | 33±3         | 0.005
| LVESD                | 22±2§                 | 29±3§                    | 49±7         | <0.001
| LVEDA                | 32±3§                 | 42±3§                    | 76±10        | <0.001
| LVESA                | 37±5§                 | 50±3§                    | 140±28       | <0.001
| LV SF                | -12±7§                | -30±7                    | -37±8        | 0.052
| FAC                  | -16±6                 | -25±9                    | -23±14       | 0.75
| HR                   | -20±6                 | -23±11                   | 9±6          | 0.52

Values are mean percent change±SE observed during the 4-week interval between the 2 echo examinations.

*P values derived from one-way analysis of variance comparisons between the 3 groups.
†P value <0.05; ‡P value <0.01; and §P value <0.001 for the post hoc comparison with saline-treated group.

AW d indicates anterior wall diastolic thickness; AW s, anterior wall systolic thickness; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; LV EDa, LV end-diastolic area; LV ESVa, LV end-systolic area; LV FS, LV fractional shortening=[(LVIDd−LVIDs)/LVIDD]×100; FAC, fractional area change=[(EDA−ESA)/EDA]×100; HR, heart rate (beats per minute).

disappearance after 4 weeks remains unclear, it does not diminish the validity of our conclusions regarding the false-positive findings by MRI.

Of interest, despite massive cell loss, both labeled and unlabeled MSCs provide some therapeutic effect on cardiac remodeling and function after MI (Tables 1 and 2; Figure 7). This finding is in agreement with previous reports suggesting that the therapeutic effects of transplanted cells on LV remodeling and function might be independent of implanted cell survival or transdifferentiation. Our findings suggest that the benefit is likely a result of factors secreted by the MSCs (paracrine effect) or another type of interaction with the healing infarct. It is also possible that accumulation of activated resident macrophages at the site of injection contributed to infarct healing and repair.

Figure 7. Comparison of relative change in LV variables by 2-dimensional echocardiography. Compared with saline-treated rats, both unlabeled and labeled MSCs attenuated dilatation of the left ventricle (A, B) and there was a trend toward greater preservation of LV contractility (C, D). Probability value near each bar is derived from post hoc comparison with saline group.
Disclosure
None.

References
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doi: 10.1161/CIRCULATIONAHA.106.680231

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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