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Ferumoxytol can be used for quantitative Magnetic Particle Imaging of Transplanted Stem Cells

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Abstract

Purpose: To evaluate, if clinically translatable ferumoxytol nanoparticles can be used for *in vivo* detection and quantification of stem cell transplants with magnetic particle imaging (MPI).

Procedures: Mesenchymal stem cells (MSCs) were labeled with ferumoxytol or ferucarbotran and underwent MPI, magnetic resonance imaging (MRI), prussian blue staining and inductively coupled plasma (ICP) spectrometry. Unlabeled, ferumoxytol, and ferucarbotran-labeled MSCs were implanted in calvarial defects of eight mice and underwent MPI, MRI and histopathology. The iron concentration calculated according to the MPI signal intensity and T2 relaxation times of the three different groups were compared using an analysis of variance (ANOVA) with Bonferroni correction, and a $p < 0.05$.

Results: Compared to unlabeled controls, ferumoxytol and ferucarbotran labeled MSC showed significantly increased iron content, MPI signal and MRI signal. The ferumoxytol MPI signal was approximately 4× weaker compared to ferucarbotran at equimolar concentrations ($p = 0.0003$) and approximately 1.5 times weaker for labeled cells when using optimized labeling protocols ($p = 0.002$). *In vivo*, the MPI signal of ferumoxytol-labeled MSC decreased significantly between day 1 and day 14 ($p = 0.0124$). This was confirmed by histopathology where we observed a decrease in Prussian blue stain of MSCs at the transplant site. The MRI signal of the same transplants did not change significantly during this observation period ($p = 0.93$).

Conclusion: Ferumoxytol nanoparticles can be used for *in vivo* detection of stem cell transplants with MPI and provide quantitative information not attainable with MRI.

Summary Statement:

Ferumoxytol nanoparticle-labeled stem cells can be detected and tracked longitudinally *in vivo* with magnetic particle imaging (MPI).

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Disclosures of Conflicts of Interest:

Prachi Pandit holds equity interest in Magnetic Insight Inc.

Keywords

Stem cell; MRI; MPI; Molecular imaging

Introduction:

Complex bone injuries due to osteoarthritis, trauma or surgery are debilitating for individuals and require significant medical intervention [1–4]. More than two million bone grafts are transplanted each year, representing the second most commonly transplanted materials after blood products and causing healthcare costs in the order of \$21 billion every year [5]. Considering escalating demands, limited availability and limited efficacy of bone allografts, new options for bone repair are urgently needed. Stem cell transplants are attractive alternatives because they are immediately available in potentially unlimited quantities, less expensive and represent “live” tissue with potentially better engraftment outcomes [1–4].

However, successful development of stem cell mediated bone repair is hampered by limited survival and engraftment of locally administered therapeutic cells. Reported engraftment outcomes of locally engrafting stem cells are quite modest [6–8]. Important factors that may cause death of the transplanted cells are a locally hostile environment or graft rejection [9–11]. A non-invasive imaging test, which could accurately monitor and quantify transplanted stem cells over time would substantially advance stem cell based bone regeneration approaches.

Magnetic Particle Imaging (MPI) is an emerging molecular imaging technique that can non-invasively detect and quantify iron oxide-labeled cells *in vivo* [12–13]. MPI images detect iron without non-specific background signal and provide exquisite sensitivity and specificity [14]. Though promising, stem cell imaging with MPI has been performed with experimental iron oxide nanoparticles thus far (e.g. ferucarbotran). To enable clinical translation, it would be preferable to label and track therapeutic cells with a clinically applicable iron label. Ferumoxytol is currently FDA approved as an iron supplement for the treatment of anemia in patients with renal insufficiency. Several institutions in North America are using ferumoxytol “off label” for vascular imaging and cancer imaging purposes [15]. Therefore, ferumoxytol is immediately clinically available for imaging applications in patients through an “off label” use. While initially not detectable with MPI, improvements of the MPI technology recently enabled detection of ferumoxytol nanoparticles *in vitro* [16]. It is not known, if MPI is sensitive enough to detect ferumoxytol labeled stem cells *in vivo*. Therefore, the goal of this study was to evaluate, if ferumoxytol nanoparticles can be used for *in vivo* detection and quantification of stem cell transplants with MPI.

Materials and methods:

Iron nanoparticles

Ferumoxytol (Feraheme™, AMAG Pharmaceuticals) is an iron oxide nanoparticle compound composed of an iron oxide core and a carboxymethyl-dextran coat [17].

Ferumoxytol is approved by the FDA for the treatment of iron deficiency anemia [18]. Ferumoxytol nanoparticles have a hydrodynamic diameter of 20–30 nm, an r_1 relaxivity of $15 \text{ mM}^{-1}\text{s}^{-1}$ and an r_2 relaxivity of $89 \text{ mM}^{-1}\text{s}^{-1}$ at 1.5 Tesla and 37 degree Celsius [19]. Ferumoxytol can be internalized into stem cells using optimized labeling procedures [20–21].

Ferucarbotran (VivoTrax™, Magnetic Insight Inc, Alameda, CA) is an iron oxide nanoparticle compound composed of an iron oxide core and a carboxydextran coat [22–23]. Ferucarbotran nanoparticles have a mean hydrodynamic diameter of 62 nm, an r_1 relaxivity of $7.2 \pm 0.1 \text{ mM}^{-1}\text{s}^{-1}$ and an r_2 relaxivity of $82.0 \pm 6.2 \text{ mM}^{-1}\text{s}^{-1}$ at 1.5 Tesla and 37 degree Celsius [24]. Ferucarbotran has been used extensively for clinical MRI [23, 25], but has been withdrawn from the US clinical market and recently reformulated for pre-clinical MRI and MPI applications [16].

Stem cell labeling

To obtain murine mesenchymal stem cells (MSCs), a bone marrow aspiration was performed on eight 6–8-week-old female C57BL/6J (Jackson Laboratory) mice. Mice were euthanized by CO₂ inhalation and both femurs and tibias were isolated. Upon removal of the epiphyses, the bone marrow was flushed with DMEM/F12 with Glutamax. The cells were collected after a round of centrifugation and re-suspended in full culture medium supplemented with 10% FBS, 1% pen-strep, and 50 pg/mL of human fibroblastic growth factor. The cell dispersion was plated in a T75 flask and maintained at 37°C with 5% CO₂ for 5–7 days. The medium was replaced every three days or when the cells reached confluence. Non-adherent hematopoietic stem cells, red blood cells, and white blood cells were eliminated during changes of culture medium, leaving the adherent MSCs. 24 hours before labeling, cells were detached and re-plated at a confluency of 5 million cells per flask. Then, cells were labeled with either ferumoxytol nanoparticles (Feraheme™, AMAG Pharmaceuticals, Waltham, MA, 100 µg/mL media) using Lipofectin® (Life Technologies), or labeled with ferucarbotran (Magnetic Insight, Inc., Alameda, CA, 50 µg/mL media) as previously described [20].

Cellular iron uptake

25, 50, 100, 200, and 500×10^3 cells – of both ferumoxytol- and ferucarbotran-labeled MSCs were washed three times with phosphate-buffered saline (PBS) (Gibco, Grand Island, NY), mixed with 30 µl of the polyethylene glycol scaffold and moved to 3 mm NMR tubes for further *in vitro* assessments. Iron uptake of labeled cells was visualized via Prussian blue staining with the Accustain Iron Stain Kit (Sigma-Aldrich, St. Louis, MO) using a pararosaniline counter stain. Iron uptake was further quantified by inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 5300 DV, Perkin-Elmer, Waltham, MA).

Animal model preparation

Animal experiments were approved by the Stanford Administrative Panel on Laboratory Animal Care (APLAC protocol 27357). All animals were treated as established by the National Research Council's Guide for the Care and Use of Laboratory Animals. All experiments were performed under isoflurane anesthesia, and buprenorphine was given for

pain control after surgeries. One million murine bone marrow-derived MSCs were suspended in scaffolds (50 μ L) consisting of polyethylene glycol conjugated to dimethacrylate. Ammonium persulfate and tetramethylethylenediamine (TMEDA) were used to catalyze the polymerization of acrylamide, forming a polyacrylamide gel. A circular window (diameter: 4 mm) was created in the parietal skull bone of eight female C57BL/6J mice under 1.5% isoflurane inhalation anesthesia. The MSC-seeded scaffolds were implanted into these windows. Three mice received transplants of ferumoxytol-labeled MSCs, three mice received ferucarbotran-labeled MSCs, and two mice receive unlabeled MSCs transplants. All mice underwent MRI and MPI on days 1 and 14 under isoflurane anesthesia (Figure 1).

Magnetic particle imaging

Magnetic particle imaging was performed with the Momentum MPI scanner (Magnetic Insight Inc, Alameda, CA), a projection Field Free Line (FFL) scanner, operating with a magnetic field gradient strength of 6×6 T/m. Samples translated along the z-axis of the scanner using a single-axis translation stage, with field-free line along y-axis and excitation field (45kHz, 20mT peak amplitude) along z-axis. Images were reconstructed using x-space MPI reconstruction Algorithm [26].

For nanoparticle characterization, tracer stock solutions were serially diluted with deionized water and 1 μ L point source phantoms were prepared with tracer concentrations of 100%, 50%, 20%, 10%, 5%, and 2%. For ferumoxytol (stock concentration of 30 μ g/ μ L Fe) this ranged from 30 μ g to 0.6 μ g of Fe in PBS, while for ferucarbotran (VivoTrax™ stock concentration of 5.5 μ g/ μ L Fe) this ranged from 5.5 μ g to 0.11 μ g of Fe in PBS. Samples were prepared and imaged in triplicate for both tracers using 2D coronal projection images with a field of view (FOV) of 6 cm $\times$ 10 cm and acquisition time of 10 seconds per projection.

In vitro samples were prepared in duplicate as described earlier and 2D coronal projection images with a FOV of 6 cm $\times$ 10 cm and acquisition time of 10 seconds per projection were acquired for both tracers for each of the duplicate test tubes, containing 25, 50, 100, 200, and 500×10^3 labeled cells in 30 μ L of the agarose scaffold.

For the *in vivo* experiments, 2D coronal projection images with a FOV of 6 cm $\times$ 10 cm were first acquired for localization. This was followed by 3D tomographic images with 55 projections with total imaging time of 40 mins, including reconstruction. A FOV of 6 cm $\times$ 6 cm was imaged to include the mouse head with the implant, as well as a set of three fiducials with known tracer concentration (10%, 5%, 5% in 1 μ L volume) for quantification purposes.

Data analysis was performed with VivoQuant (inviCRO, Boston, MA). For tracer characterization, regions of interest (ROIs) were defined in the MPI images and a linear regression model from the MPI signal at each concentration was used to assess the signal linearity. Tracer resolution was estimated using the measured full-width, half-maximum (FWHM) generated by a single point source of signal. A calibration curve for iron content to MPI sum signal of the whole ROI was established for both tracers, and subsequently used to

quantify the iron content in the *in vitro* cell samples. For *in vivo* quantification, volumetric ROIs were defined over the cell scaffold as well as the fiducials. Iron content and number of implanted cells in the scaffolds were evaluated from a regression model based on the MPI sum signal from the three fiducials with known iron content.

Magnetic resonance imaging

All mice with stem cell implants were scanned with a 7T MR scanner (Discovery MR901; collaboration between Agilent [Santa Clara, Calif] and Bruker) using a 2 cm inner diameter mouse head RF coil (Bruker Biospin, Billerica, MA). T2 relaxation times were measured using multi-echo spin echo (MESE) sequences with a repetition time of 2000 ms and multiple echo times (TE) of 6, 13, 20, 27, 34, and 41 ms. All images were obtained using a field of view (FOV) of 25 × 25 mm, a 256 × 256 matrix, 0.5 mm slice thickness, and two acquisitions. T2 relaxation times were calculated by using Paravision software (Bruker Biospin, Billerica, MA). After the last MRI scans, mice were sacrificed, and defects were explanted, fixed, paraffin embedded, and cut into 7 μm slices for histological processing. For iron nanoparticle staining, DAB-Prussian blue with the Accustain Iron Stain kit (Sigma-Aldrich, St. Louis, MO) and a Pararosaniline solution counterstain was performed. The number of labeled cells were quantified under the microscope in five random high power fields (40×) and compared between day 1 and 14.

Statistical analysis

T2 relaxation time, ICP results, and iron concentration calculated according to the MPI signal of the three different groups were compared using an analysis of variance (ANOVA) with Bonferroni correction, and a $p < 0.05$.

Results:

Magnetic particle imaging of ferumoxytol nanoparticles

First, we evaluated the sensitivity of MPI to detect ferumoxytol nanoparticles in solution by evaluating MPI images of increasing concentrations of ferumoxytol and ferucarbotran. Ferumoxytol showed a blooming effect with an MPI signal that expanded beyond the test tube, over an area of 5 mm³. Ferumoxytol showed a linear relationship between ferumoxytol concentration and MPI signal (Figure 2, $R^2 > 0.99$). The minimum detectable ferumoxytol concentration was 1% (0.3 μg/μL Fe) in a 1 μL volume. By comparison, ferucarbotran showed approximately four times stronger MPI signal at equivalent concentrations (Figure 2b) and a narrower signal spread compared to ferumoxytol (Figure 2). Using the FWHM measurement from the point source signal, the resolution for ferumoxytol is ~5 mm, compared ~1 mm for ferucarbotran.

In vitro imaging of ferumoxytol labeled MSCs

Magnetic particle imaging—Next, we evaluated the sensitivity of MPI to detect ferumoxytol labeled MSC. The calibration curve from figure 2c was used to calculate the total iron content in each cell sample. The MPI signal, and correspondingly the total iron content in ferumoxytol labeled MSC (2.11 ± 0.12 μg of Fe in 500K cells) was significantly higher compared to unlabeled cells (0.0005 ± 0.002 μg of Fe in 500K cells) and varied

linearly with the number of labeled cells ($R^2 > 0.98$). When using optimized labeling protocol for either tracers, the MPI signal of ferumoxytol labeled cells was approximately 1.5 times weaker compared to ferucarbotran labeled cells ($p=0.002$) (Figure 3b).

The iron uptake per cell was calculated to be 4.656 ± 0.46 pg/cell for ferumoxytol-labeled MSC (100 $\mu\text{g/mL}$), 6.267 ± 0.61 pg/cell for ferucarbotran-labeled MSC (50 $\mu\text{g/mL}$), and 0.001 ± 0.00 pg/cell, $p=0.0001$ for unlabeled cells.

Histopathology and ICP

Ferumoxytol and ferucarbotran were efficiently internalized into MSCs, as confirmed by Prussian blue staining and ICP results (Figure 4). Prussian blue stains demonstrated marked iron uptake within MSCs labeled with ferumoxytol and ferucarbotran (Figure 4a). The quantitative iron uptake, as determined by ICP-OES, for MSCs labeled with 100 $\mu\text{g/mL}$ ferumoxytol and 50 $\mu\text{g/mL}$ ferucarbotran and was $4.01 \text{ pg/cell} \pm 0.18$ and $4.58 \text{ pg/cell} \pm 0.50$ respectively which did not show a significant difference ($p=0.14$).

In vivo imaging of labeled-MSC implants

Magnetic particle imaging—Finally, we investigated the ability of MPI to detect and quantify transplanted stem cells *in vivo* at day 1 and day 14 after transplantation of ferumoxytol-labeled, ferucarbotran-labeled and unlabeled MSC in calvarial defects. As seen in figure 5, Ferumoxytol-labeled MSC implants showed significantly higher MPI signal and correspondingly higher calculated Fe content compared to unlabeled MSC implants ($p=0.0005$) and significantly lower calculated Fe content compared to ferucarbotran-labeled MSC implants ($p=0.0018$). The MPI signal decreased significantly between day 1 and 14 for both ferumoxytol- and ferucarbotran-labeled MSC ($p=0.0124$, $p=0.0001$) This corresponded to a decreased DAB-Prussian blue stain in the transplant site on histology (figure 5 e–f).

Magnetic Resonance imaging—T2 relaxation times of ferucarbotran-labeled MSCs (4.16 ± 0.40 ms) and ferumoxytol-labeled MSCs (4.58 ± 0.04 ms) were significantly shorter than those of unlabeled MSCs (26.3 ± 0.5 ms). The mean T2 relaxation time did not change significantly between day 1 and day 14 for both ferumoxytol and ferucarbotran-labeled MSC ($p=0.93$ and $p=0.23$ respectively).

Discussion:

Our results show that Ferumoxytol nanoparticles can be used for *in vivo* detection of stem cell transplants. MPI provided information about decreasing ferumoxytol quantities at the transplant site, which was not attainable with MRI.

MPI has several advantages compared to MRI: MPI directly detects the electronic magnetization of iron oxide nanoparticles by applying a time-varying homogeneous excitation field that causes magnetization of the iron oxides to instantaneously flip and thus induce a signal in the receive coil [27]. The MPI signal is 10^8 times larger than nuclear magnetization of protons seen in MRI, making MPI a sensitive imaging modality for imaging iron. It should be noted that being a new and evolving modality, the detection sensitivity of MPI is yet to be fully realized. MPI directly detects iron oxide nanoparticles,

without false positive background signal [28]. Additionally, the nanoparticle signal on MPI increases linearly with increasing nanoparticle concentration, thereby allowing for accurate iron quantification [13]. Although there are no clinical scanners to date, the technology is in principle clinically translatable [29].

Thus far, iron oxide nanoparticle labeled cells have been tracked with MRI [30]. MRI also allows for quantification of decreasing T2 relaxation times over time [11, 31]. However, changes in T2- and T2* relaxation times are not linearly related to iron concentration or MSC cell number [32], because they are dependent on additional variables, such as intra- or extracellular iron oxide compartmentalization [33], distribution volume and proton content of the underlying tissue [34].

While we found a linear relationship between MPI signal and iron concentration at a given time point, caution has to be taken with regards to extrapolations of this observation towards changes in cell number over time. The iron content at the transplant site could change due to proliferation of labeled cells, dilution in progenies, iron metabolization, and potential cellular iron elimination. However, assuming differences in cell engraftment and iron metabolization, it would be possible to establish standards for MPI signal over time for successful and unsuccessful cell transplants. Deviations from a reference standard could then be used to diagnose complications of the procedure.

Technical refinements of the sensitivity and image quality of the MPI technique are ongoing including the design of optimized nanoparticles [35–36] and pulse sequences with improved sensitivity, such as pulsed excitation waveforms [37–38]. Hardware improvements such as incorporating dual-channel, orthogonal drive fields, along with advanced image processing tools will further improve image quality by achieving isotropic resolution [39–40] and reducing background image haze [41–42]. Ultimately, clinical translation of MPI will depend on clinical hardware and clinically applicable nanoparticles. We used Lipofectin as a transfection agent to label the cells with ferumoxytol, which is not clinically translatable, however there are alternate options of transfection agent free labeling techniques such as *in vivo* labeling [43] or using the advantage of protein corona around nanoparticles to facilitates cell labeling [21]. Ferumoxytol would immediately cover one of these fundamental needs for clinical translation.

Conclusion:

In conclusion, MPI is a new and emerging imaging modality, which can detect iron oxide nanoparticles with high specificity. Our data showed that clinically applicable ferumoxytol nanoparticles can be readily detected with MPI, allowing accurate *in vivo* detection and quantification of ferumoxytol labeled stem cells. This discovery is an important basis for clinical translation of MPI technologies.

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Figure 1: Principle of iron oxide nanoparticle detection with MPI and MRI.

a) nanoparticle-labeled therapeutic cells in calvarial defects of a mouse. **b)** MRI used for anatomical resolution and indirect quantification of iron-labeled stem cells. **d)** MPI used for direct quantification of iron-labeled stem cells.

Figure 2: MPI properties of ferumoxytol and ferucarbotran.

a) MPI images of increasing concentrations of ferumoxytol and ferucarbotran in 1 μ L of PBS ($=1 \mu\text{m}^3$). Ferumoxytol showed a blooming effect with an MPI signal that expanded beyond the test tube, over an area of 5 mm^3 . By comparison, ferucarbotran showed less blooming effect with a signal spread over an area of 1 mm^3 . **b)** Linear correlation between peak MPI signal and Fe concentration for both ferumoxytol and ferucarbotran. The slope for ferucarbotran is higher than ferumoxytol, indicating higher sensitivity of the MPI technique to detect ferucarbotran compared to ferumoxytol. For equivalent iron concentrations, ferucarbotran displays a higher peak signal intensity as compared to ferumoxytol. **c)** Calibration curve for total iron content to MPI sum signal for both tracers.

Figure 3: Magnetic particle images of ferumoxytol and ferucarbotran labeled MSCs.

a) MPI images of increasing numbers of ferumoxytol and ferucarbotran labeled MSCs in 30 µl of the agarose scaffold. **b)** Corresponding sum MPI signal for increasing concentrations of ferumoxytol and ferucarbotran labeled MSC. **c)** Corresponding total iron content (microgram per cell sample) of ferumoxytol and ferucarbotran labeled MSC, as determined by mass spectrometry.

Figure 4: Cellular iron uptake of ferumoxytol and ferucarbotran.

a) Ferumoxytol and ferucarbotran were internalized into MSCs as confirmed by Prussian blue staining. **b)** Cellular iron uptake in unlabeled, ferumoxytol, and ferucarbotran labeled MSCs as determined by ICP and MPI. There was no significant difference between iron measures with ICP and MPI ($p = 0.14$).

Figure 5: *In vivo* MRI, MPI, and corresponding quantifications of implanted labeled MSCs into calvarial defects.

a) Coronal fast spin echo (FSE; TE/TR = 42ms/ 3000ms) MR images at day 1 and 14 after implantation of unlabeled, ferumoxytol or ferucarbotran labeled MSC in calvarial defects of experimental mice. **b)** Corresponding MPI images of the calvarial defects at different time points after implantation of therapeutic cells. **c)** Corresponding T2 relaxation times of the calvarial defects at different time points after implantation of unlabeled, ferumoxytol or ferucarbotran labeled MSC, as measured using multi-echo spin echo (MESE) sequence (TR=2000 ms, TE = 6, 13, 20, 27, 34, and 41 ms) Data are displayed as means and standard deviations of three animals per experimental group. **d)** Corresponding calculated Fe content of the calvarial defects at different time points after implantation of unlabeled, ferumoxytol or ferucarbotran labeled MSC, as measured using MPI signals. **e-f)** Corresponding DAB-Prussian blue stain and quantification of transplanted MSCs seeded in the scaffold at day 1 and day 14. * indicates significant differences between data obtained at day 1 and day 14 ($p < 0.05$).