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Feasibility of islet magnetic resonance imaging using ferumoxytol in intraportal islet transplantation

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ABSTRACT

There is a clinical need for an alternative labeling agent for magnetic resonance imaging (MRI) in islet transplantation. We aimed to evaluate the feasibility of islet MRI using ferumoxytol, which is the only clinically-available ultrasmall superparamagnetic iron oxide. We compared islet function and viability of control islets and islets labeled with ferumoxytol and/or a heparin-protamine complex (HPF). Efficacy of ferumoxytol labeling was assessed in both *ex vivo* and *in vivo* models. Labeling for 48 h with HPF, but not up to 800 μg/mL ferumoxytol, deranged *ex vivo* islet viability and function. The $T2^*$ relaxation time was optimal when islets were labeled with 800 μg/mL of ferumoxytol for 48 h. Prussian blue stain, iron content assay, transmission electron microscopy (TEM) supported internalization of ferumoxytol particles. However, the labeling intensity in the *ex vivo* MRI of islets labeled with ferumoxytol was much weaker than that of islets labeled with ferucarbotran. In syngeneic intraportal islet transplantation, there was a correlation between the total area of visualized islets and the transplanted islet mass. In conclusion, islet MRI using ferumoxytol was feasible in terms of *in vitro* and *in vivo* efficacy and safety. However, the weak labeling efficacy is still a hurdle for the clinical application.

1. Introduction

Pancreatic islet transplantation offers a potential cure of type 1 diabetes mellitus. However, this procedure is still not ideal because a large proportion of transplanted islets are rapidly eliminated from the recipients due to immune reactions and non-specific inflammation [1,2]. Therefore, there is a strong clinical need for real-time assessment of functioning islet mass in the recipients of islet grafts, because the majority of islet injury in clinical islet transplantation takes place before changes in recipient glycemic levels become apparent. In this regard, magnetic resonance imaging (MRI) of superparamagnetic iron oxide (SPIO)-labeled islets is one of the most promising solutions, because it can track the transplanted islets with high resolution but without safety concerns [3,4]. Unfortunately, the clinical-grade SPIOs such as ferumoxides and ferucarbotran, which were used as contrast agents for liver diseases, were withdrawn from the market for economical reasons. Although alternative contrast agents are under development [5,6], their immediate application in clinical cell therapy is impossible. Therefore, an alternative clinically-available labeling agent for MRI

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is urgently required for use in the cell therapies, including islet transplantation.

Ferumoxytol, an ultrasmall superparamagnetic iron oxide (Feraheme®, AMAG Pharmaceuticals, Cambridge, MA, USA), was approved for clinical use as an iron supplement for anemia [7]. Several attempts have been made to use ferumoxytol as an alternative-labeling agent for labeling stem cells such as hematopoietic stem cells, bone marrow stromal cells, neural stem cells, and mesenchymal stem cells [8–10]. In these studies, several assisted labeling techniques were required because short-term labeling with ferumoxytol alone was not effective. To date, heparin and/or protamine [8] and long-term *in vivo* labeling of ferumoxytol prior to the isolation of therapeutic cells [9] have been reported to be effective. However, the validity of these strategies has not been tested in islet transplantation.

Pancreatic islets contain several kinds of cells, including insulin-secreting beta cells, glucagon-secreting alpha cells, and somatostatin-secreting delta cells. This affects the internalization efficacy of ferumoxytol because these cells have strong cell-to-cell interactions due to tight junctions between them, as well as significant extracellular collagen coverage on the islet surface. Therefore, the purpose of this study was to examine the feasibility of islet MRI with ferumoxytol using two labeling techniques: 1) a system using anionic heparin and cationic protamine to effectively internalize the ferumoxytol into the islet and 2) long-term (48 h) *ex vivo* labeling of islets with a high concentration of ferumoxytol. The latter was chosen instead of long-term *in vivo* labeling [9] because it is relevant to the *in vitro* culture of isolated islets before clinical islet transplantation. In this setting, it is possible to expose islets to high concentrations of ferumoxytol for a prolonged period without concern for systemic toxicity. We evaluated the optimal internalization of ferumoxytol into islets without any damage to viability and insulin secretion *in vitro* and *in vivo*. In addition, we further visualized the ferumoxytol-labeled islets through an *in vitro* phantom and explored the ability of the area of visualized islets to predict *in vivo* islet function in syngeneic intraportal islet transplantation.

2. Materials and methods

2.1. Animals

We used 12 to 16 week-old male C57BL/6 mice (Orient-Bio, Seongnam, Korea) for *ex vivo* assessment of labeling efficacy, islet viability, and function, and for syngeneic islet transplantation experiments. SD rats (Orient-Bio) were used to assess internalization of rhodamine isothiocyartate (RITC)-conjugated ferumoxytol into the islet cells. All animal procedures in this study were approved by the Institutional Animal Care and Use Committee of the Samsung Medical Center, Seoul, Republic of Korea.

2.2. Pancreatic islet isolation and ferumoxytol nanoparticle labeling

Islets were isolated using intraductal injection with collagenase P (Roche, Indianapolis, IN, USA) and purified using Biocoll (Biochrom, Berlin, Germany). Isolated islets were cultured while free-floating in 10 ml of Opti-MEM (Gibco, Grand Island, NY, USA) in a humidified CO2 incubator at 37 °C with a 5% CO2 atmosphere.

Isolated islets were labeled by culturing for 48 h in Opti-MEM (Gibco) supplemented with 0–1600 μg/mL of ferumoxytol (Feraheme®, AMAG Pharmaceuticals, Cambridge, MA, USA) with or without the heparin-protamine complex. The heparin–protamine–ferumoxytol complex was prepared by sequential addition of protamine sulfate (up to 60 μg/mL) and heparin sulfate (2 IU/mL) to each concentration of ferumoxytol in serum-free Opti-MEM (Gibco).

2.3. In vitro assessment of islet viability and function

To assess islet viability, acridine orange (0.67 μmol/L) and propidium iodide (75 μmol/L) staining were used to simultaneously observe living and nonviable islets. To determine the composition of islets, cultured islets were stained with dithizone (Sigma Chemicals, St. Louis, MO, USA). For glucose stimulatory insulin secretion assays, the islets were incubated for 1 h in Krebs–Ringer bicarbonate buffer (KRBB) supplemented with 0.2% bovine serum albumin (Sigma) and 3.3 mM glucose followed by re-incubation with basal (3.3 mM) and high (16.7 mM) glucose for an hour. The supernatant was collected and the amount of secreted insulin was measured using a mouse ultrasensitive insulin ELISA kit (ALPCO, Salem, NH, USA). Glucose-stimulated insulin release was expressed as the stimulation index, calculated as the ratio of stimulated (16.7 mM glucose) to basal (3.3 mM glucose) insulin release.

In addition, the viability of islets was also evaluated using alamar blue staining according to the manufacture's protocol (Invitogen, Grand Island, NY, USA). Briefly, the islets derived from C57/BL6 mice were cultured at a density of 10 islet equivalent (IE) per well in 96 well plates with RPMI 1640 containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. In the same way as the mouse islet, MIN-6 cells were cultured at a density of 1000 cell/well in 96 well plates with DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. After terminating the treatment of HPF in the cells, HPF solutions were discarded from wells, and the cells were washed with PBS. And then, alamar blue solution, which was diluted with the complete media, was directly added to the each well, and the plates were incubated at 37 °C for 3 h protecting from direct light. The fluorescence intensity of each well was measured at 570/585 nm (excitation/emission) using a GloMax-Multi Plus Detection System (Promega, WI, USA).

2.4. Ex vivo MR imaging of the labeled islet

Islets labeled with ferumoxytol were observed using a 7-T BrukerBioSpec MRI system (BIOSPEC 70/20 USR; Bruker-Biospin, Fallanden, Switzerland) with ParaVision 5.0 software (Bruker-Biospin). The islets treated with ferumoxytol appeared as hypointense spots on T2-weighted MR images. Coronal T2*-weighted MR images were acquired using the FLASH sequence with the following acquisition parameters: repetition time (TR) of 193.3 ms; echo time (TE) of 8 ms; field of view (FOV) of 31.2 mm × 31.2 mm; matrix size of 312 × 312; flip angle of 15.0°; number of signal averaging of 8; resolution of 100 um/pixel; slice thickness of 0.5 mm; and no interslice gaps. We observed the relationship between MR and optical imaging of islets for 5 to 7 sets in 1.5% agarose gel. Optical images of islets were obtained using a light microscope (Olympus CKX41SF, Olympus Co., Tokyo, Japan).

To determine the optimal labeling concentration, we compared the $T2^*$ relaxation time in *ex vivo* MRI of islets labeled with 100, 200, 400, 800, and 1600 μg/mL of ferumoxytol with Hank's Balanced Salt Solution without ferumoxytol, medium containing unlabeled islets, and medium containing 1600 μg/mL of ferumoxytol.

2.5. RITC-conjugation of ferumoxytol

Ferumoxytol was cross-linked by modification of a polysaccharide by epichlorohydrin. 1 ml of ferumoxytol (30 mg Fe/ml) was incubated with 1 ml of 4N NaOH for 15 min and then mixed with 1 ml of epichlorohydrin for 24 h at room temperature. After reaction, the cross-linked product was purified with dialysis using a 10,000 molecular weight cut-off (MWCO) membrane (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). Then, the purified cross-linked ferumoxytol was aminated with 2.5 ml of concentrated NH_4OH (30%, w/v) for 24 h at room temperature. The aminated ferumoxytol was again dialyzed with 0.1 M sodium phosphate buffer for purification and then reacted with NHS-Rhodamine (Thermo Scientific, Rockford, IL, USA), which was dissolved in DMSO for 1 h at room temperature. After reaction, the non-reacted NHS-Rhodamine was removed by dialysis. The final product was stored at 4 °C and protected from light until use.

2.6. Prussian blue staining

After 48 h of 800 μg/ml ferumoxytol or RITC-conjugated ferumoxytol labeling of pancreatic islets from SD-Rats, the islets were fixed with 4% paraformaldehyde for 15 min at room temperature and then washed twice with PBS. A mixture of equal volumes of hydrochloric acid (20% v/v, sigma) and potassium ferrocyanide (10% w/v, Sigma) was then added for 30 min. Images were observed with optical microscopy (TE 2000E, Nikon, Tokyo, Japan).

2.7. Confocal microscopy

To observe the existence of ferumoxytol in the islet, pancreatic islets from SD-Rats were labeled with RITC-conjugated ferumoxytol at a concentration of 800 μg/ml for 48 h. Unreacted ferumoxytol was removed from cells and images were obtained using confocal laser scanning microscopy (FV-1000 spectral; Olympus, Tokyo, Japan).

2.8. Quantification of iron content in labeled islets

Islets labeled with different concentrations of ferumoxytol were washed three times with PBS. The iron content of the islets was determined using a total iron assay kit (BioVision Research, Mountain View, CA, USA). Average iron content per cell was calculated by dividing the total mean value by the number of cells in each sample.

2.9. Transmission electron microscopy of labeled islets

Ferumoxytol-labeled and unlabeled islets were harvested and fixed for 24 h with a 4% paraformaldehyde solution containing 2% glutaraldehyde. The samples were washed with cacodylate buffer and post-fixed in 1% osmium tetroxide (OsO_4) solution containing 0.05% potassium ferrocyanide ($K_4Fe(CN)_6$) for 1 h. Post-fixed samples were dehydrated using graded ethanol buffer impregnated with