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Noninvasive Fluorine-19 Magnetic Resonance Relaxometry Measurement of the Partial Pressure of Oxygen in Acellular Perfluorochemical-loaded Alginate Microcapsules Implanted in the Peritoneal Cavity of Nonhuman Primates

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Abstract

Background.—We have utilized a noninvasive technique for measuring the partial pressure of oxygen (pO₂) in alginate microcapsules implanted intraperitoneally (i.p.) in healthy non-human primates (NHPs). Average pO₂ is important for determining if a transplant site and capsules with

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Authorship

S.A.S. and M.L.G. are co-first authors; S.A.S. directed the PFCE encapsulation; M.L.G. directed all aspects of the NHP experiments, performed implant, biopsy, gross pathology, and associated data analysis; B.L.W. constructed the surface coil used in experiments, generated the calibration curve, performed MRI imaging of NHPs, analyzed the MRI data, and co-drafted the article; S.A.E. advised on MRI imaging, analyzed the MRI data, and co-drafted the article; G.F.B. generated the PFCE capsules for all experiments and contributed to the manuscript; J.J.J. and L.A.M. provided expert animal care, carried out experiments, and collected data, AS and SR developed the flow cytometry panels used in this study and acquired and analyzed the phenotyping data presented in this manuscript, M.G. designed the ¹⁹F MRI experiments, reviewed and analyzed data; A.S. contributed to the design of the PFCE capsules, consulted on oxygen measurement and contributed to drafting the manuscript; K.K.P. assisted in designing the ¹⁹F MRI experiments and served as consultant on oxygen measurement; B.J.H. directed the flow cytometric analysis and microscopic evaluation of capsules, contributed to overall experimental design, and reviewed the manuscript; C.J.W. provided expertise in encapsulation and reviewed the manuscript. All authors read the journal's authorship agreement, and all authors read and approved the final version of the article.

Conflicts of interest: The authors declare no conflicts of interest.

certain passive diffusion characteristics can support the islet viability, metabolic activity, and dose necessary to reverse diabetes.

Methods.—Perfluoro-15-crown-5-ether (PFCE) alginate capsules were infused i.p. into three healthy NHPs. Peritoneal pO₂ levels were measured on days 0 and 7 using ¹⁹F-MR relaxometry (¹⁹F-MRR) and a fiber optic probe. ¹⁹F-MR imaging (¹⁹F-MRI) was used to determine the locations of capsules within the peritoneal space on Days 0 and 7. Gross and histologic evaluations of the capsules were used to assess their biocompatibility postmortem.

Results.—At Day 0 immediately after infusion of capsules equilibrated to room air, capsules were concentrated near the infusion site and the pO₂ measurement using MRR was 147 ± 9 mmHg. On day 7 after capsules were dispersed throughout the peritoneal cavity, the pO₂ level was 61 ± 11 mmHg. Measurements using the fiber optic oxygen sensor were 132 ± 7.5 mmHg (Day 0) and 89 ± 6.1 mmHg (Day 7). PFCE capsules retrieved on Day 7 were intact and free-floating without host cell attachment, although the numbers of peritoneal CD20⁺ B cells, CD4⁺ and CD8⁺ T cells, and CD14⁺ macrophages increased consistent with a mild foreign body reaction.

Conclusions.—The peritoneal pO₂ of normal NHPs is relatively low and we predict would decrease further when encapsulated islets are transplanted i.p.

Introduction

Our goal was to measure, noninvasively, the partial pressure of oxygen (pO₂) in alginate microcapsules implanted intraperitoneally (i.p.) in healthy non-human primates (NHPs) using fluorine-19 magnetic resonance relaxometry (¹⁹F-MRR), and to identify their location within the peritoneal space by fluorine-19 magnetic resonance imaging (¹⁹F-MRI). Since encapsulated islets typically are transplanted in the peritoneal cavity, we used a large animal model to characterize the pO₂ in microcapsules at implant and following exposure to normal peritoneal pO₂ levels, a critical parameter that is currently not well defined.

In diabetic mice, we have found using ¹⁹F-MRR that the average capsular pO₂ decreases in a dose-dependent manner following transplantation of encapsulated murine insulinomas.¹⁻³ We hypothesized that a similar reduction in the average capsular pO₂ could occur following implantation of encapsulated islets in healthy large animals and during the clinical use of encapsulated islets in diabetic patients. Suboptimal pO₂ in microcapsules may be sufficient to allow a fraction of encapsulated islets to survive, but inadequate to support the function of the islet graft over time,⁴ and may provoke detrimental hypoxia-induced pro-inflammatory changes in host immune cells.⁵⁻⁸ Microencapsulated islets (both allografts and xenografts) lose function over time when transplanted in immunosuppressed diabetic murine and NHP recipients^{1,4,9-12} in the absence of pericapsular overgrowth.⁴ Hypoxia of 60 mmHg oxygen tension and below has been shown to have an immediate effect on insulin secretion from rat islets, which is reversible upon re-oxygenation.¹³ These observations led to our operating assumption that relatively low average capsular pO₂ might be a major contributing factor in the gradual loss of islet function.

We pursued an imaging-based approach considering the principles of the ‘3Rs’ (Replacement, Reduction, and Refinement) in animal studies. Our intention was to utilize a

noninvasive technique capable of serially evaluating the condition of islet grafts without multiple survival surgeries (refinement) or euthanasia of multiple animals at several time points (reduction). The replacement of serial survival surgeries or timed euthanasia with percutaneous catheter placement and noninvasive MR techniques has the potential to give quantitative data regarding the i.p. environment of transplanted islets and to determine the pO₂ level at which islet function may decline or provoke immune mediated changes. The ¹⁹F method presented here allows such valuable data to be obtained without confounding issues, such as surgical interventions that could cause wound-induced inflammation.

Materials and Methods

Animals

All procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee and conducted in compliance with the Animal Welfare Act. All animals were purpose-bred, purchased from institutionally approved commercial vendors, and housed and cared for according to the standards detailed in the Guide for Care and Use of Laboratory Animals. Three healthy adult female rhesus macaques (*Macaca Mulatta*), average weight 8.1 ± 1.8 kg, with a naïve abdomen were used for this study (Table 1). They were housed in same sex pairs, except when there was demonstrated social incompatibility, in which case they were housed in direct visual, olfactory, and auditory contact with conspecifics. Their diet consisted of biscuits (2055 Teklad Global 25% Protein Primate Diet or 7195 Teklad Hi-Fiber Primate Diet, Envigo, Madison, WI, USA) based on body weight along with free access to water. The diet was liberally enriched with fresh fruits, vegetables, grains, beans, nuts and a multivitamin supplement. The NHPs participated in an environmental enrichment program that included social play, toys, music, and regularly scheduled access to large exercise and swimming areas. To facilitate interaction with caregivers, all animals were trained using a positive reinforcement paradigm for routine study procedures.¹⁴ NHPs were observed daily for general appearance, gait, alertness, temperament, social interaction, urination, defecation and body condition as part of routine health monitoring.

Placement of intraperitoneal catheter

The test animals were fasted overnight. Under general anesthesia, each animal was positioned flat in the supine position, the periumbilical area was aseptically prepared, and 1% lidocaine was administered at the intended needle insertion site. An MR safe catheter (16G, 5 cm) was inserted at a 45 degree angle distal to the umbilicus, into the lower left quadrant of the abdomen. When penetration of the peritoneal wall was detected, the guide needle was withdrawn. The catheter lumen was protected with a cap while not in use and temporarily secured to the skin with sterile dressing (Tegaderm, 3M Company, Maplewood, MN, USA). This catheter was used to deliver the capsules, to sample peritoneal fluid for various assays, and to provide an introducer for fiber optic probe insertion.

Production of alginate microcapsules

Perfluoro-15-crown-5-ether (PFCE) emulsion was prepared following a protocol adapted from Joseph, et al.¹⁵ Briefly, an aqueous dispersion of egg yolk lecithin (Sigma-Aldrich, St.

Louis, MO, USA) was made in Tyrode's solution (Sigma-Aldrich) by sonication in an ice bath. PFCE (Exflur Research Corporation, Round Rock, TX, USA) was added, and the sample was sonicated. The resulting emulsion was filtered through a 0.8 μm membrane filter. PFCE-loaded alginate capsules (average diameter 1000 μm , Figure 1A) were prepared by mixing the PFCE at a 1:20 ratio (5%) with 2.0% low viscosity, high mannuronic acid (LVM) alginate (FMC Biopolymer, NovaMatrix, Sandvika, Norway). Using an electrostatic droplet generator (Nisco Engineering Inc., Zurich, Switzerland) with a 0.9 μm encapsulation nozzle (Nisco), the alginate/PFCE mixture was aspirated into a 50 mM strontium chloride (SrCl_2) solution containing 4.6% mannitol (Sigma-Aldrich) and 25 mM HEPES buffer (Corning Mediatech, Tewksbury, MA, USA). The PFCE capsules were washed in Hanks' Balanced Salt Solution (HBSS) and assessed by MR using a 7 T system. A single, narrow ^{19}F peak indicated high purity of the preparation (Figure 1B). We expected these Sr-gelled LVM alginate capsules to be highly biocompatible and durable, similar to our barium-gelled LVM alginate capsules.¹⁶

Establishment of oxygen calibration curve

An application specific calibration curve was necessary to calculate the pO_2 . This calibration is based upon the linear relationship between the spin-lattice relaxation rate constant (R_1) of the fluorine resonances from PFCE and the pO_2 . PFCE capsules (three independent preparations) were used to generate a ^{19}F -MRR oxygen calibration curve (Figure 1C) by adapting a previously-described technique.¹⁷⁻¹⁹ Briefly, the capsule samples were suspended in buffered salt solution and maintained at 37°C during the calibration procedure. Each sample was allowed to equilibrate to a series of oxygen partial pressures ($\text{pO}_2 = 0, 38, 76, 114, \text{ and } 160 \text{ mmHg}$) by sparging the sample with calibrated gas mixtures for at least 30 minutes for each pO_2 , at least 2.5 hr total sparging time. "Each calibration sample was centered on the MR coil and then positioned at the isocenter of the MR system, with the same configuration used for subsequent studies with NHPs. The R_1 for each sample at each pO_2 was measured as described,¹⁷⁻¹⁹ and a linear calibration with two fitting constants (A and B) was generated using a weighted least-squares analysis ($R_1 = A \cdot \text{pO}_2 + B$).

Administration of PFCE alginate capsules

Before implantation, the capsules were tested for sterility (Marshfield Labs, Marshfield, WI, USA). Sterile capsules (200 μL) were loaded into syringes with a warm physiologic carrier solution (Plasmalyte, Baxter, Deerfield, Illinois, USA). The total volume of the suspension ranged from 290 μL to 450 μL and was slowly infused over a period of 3-10 minutes *via* the i.p. catheter (Table 2).

Introduction of a fiber optic probe through the lumen of the i.p. catheter

A fiber optic probe with an oxygen microsensor tip (diameter of approximately 230 μm ; Microx 4, PreSens, Regensburg, Germany) was passed through the 16G i.p. catheter to extend just past the catheter tip. The probe was allowed to dwell in the peritoneal space throughout the MR procedure and then was removed. At Day 7, the microsensor was placed at the identical location to Day 0, in order to make a direct comparison between the environment of the capsules at implant, in medium equilibrated to room air, and the peritoneal environment one week later.

MR imaging and spectroscopy

Animal care and monitoring: NHPs were anesthetized with a combination of inhalation isoflurane (0.5-3%) mixed with low flow 100% oxygen (0.4L/min), as is standard during isoflurane anesthesia, to counteract an acute hypoxic ventilatory response. Isoflurane produces a ^{19}F signal that does not interfere with the PFCE signal.² The NHPs were placed into a custom MR cradle with warm padding underneath and warm MR-safe water packs surrounding the animal. Warm supplemental fluids were administered at 60-100 mL/hr, adjusted for hydration status during the entire imaging session. The imaging session duration averaged 155 min. (range 117–200 min.). Animal monitoring was performed using an MR-conditional patient monitor (Invivo 3150; Koninklijke Philips N.V., Amsterdam, Netherlands). End tidal carbon dioxide (ETCO₂), peripheral capillary oxygen saturation (SpO₂), heart rate, blood pressure, and temperature were continuously monitored and recorded every 10 – 15 minutes (Table 2). NHP core body temperature ranged from 96.4–101.3°F (Table 2). NHP subjects were positioned on top of the custom-built ^{19}F - ^1H dual resonance surface coil so that the abdominal cavity was centered over the sensitive region.

General MR procedures: The target area of the animal was positioned at the iso-center of the MR system (4 T, 67 cm bore Oxford Instruments magnet with Varian Inova console) using a motorized bed, and the coil was tuned and matched for standard ^1H -MR. A series of MR images were acquired from the peritoneal space using a localizer sequence to verify proper coil function and animal position. A small number of ^1H -MR images were acquired using an adiabatic spin-echo multi-slice (A-SEM) sequence to obtain reference images for determining the location of the capsules within the abdomen. With the animal remaining in position, the coil was then re-tuned and matched for ^{19}F -MR and the magnetic field was shimmed for the single PFCE resonance. The R_1 of the fluorine signal was measured in triplicate and then used to determine the average capsular pO₂. Finally, a series of ^{19}F -MR images were acquired to determine the location of capsules within the abdomen.

Scans performed on the day of capsule injection (Day 0) optimized clinical practices to support imaging. For example, we evaluated the respiratory motion artifact, the surface coil performance, and techniques to maintain a precise temperature of the NHP subjects during the scanning procedure.

MR acquisition details: The general acquisition protocols used for these studies has been described previously¹⁸ and adapted. Briefly, ^{19}F and ^1H spectra and images were acquired using a custom-built double-loop quadrature surface coil tuned to the appropriate resonance frequency as needed (160.2 MHz for ^{19}F , and 170.3 MHz for ^1H). An inversion-recovery pulse sequence with adiabatic excitation and inversion pulses (to ensure uniform flip angles) was used to measure R_1 . The sequence was repeated with a series of optimized delay times, and the observed magnetization-recovery curve was fit using non-linear regression to obtain R_1 for each measurement. *In vivo* measurements of pO₂ were obtained from the bulk R_1 of the detected PFCE in the abdomen of each subject, and the corresponding pO₂ was calculated using the calibration previously described.

Peritoneal lavage

On days 0 and 7, peritoneal lavage was performed to evaluate numbers/phenotypes of mononuclear cells in the fluid and degree of host cell adherence to the capsules. Following MR, warm physiologic Plasmalyte (10-15ml/kg, based on pediatric approach²⁰) was infused and allowed to dwell for at least 5 min. Lavage fluid (approximately 10mL) was recovered by gentle syringe aspiration. The gross appearance of capsules recovered on Day 7 was compared with capsules collected on Day 0 by phase contrast microscopy.

Euthanasia and gross evaluation of PFCE alginate capsule distribution

Following lavage on Day 7, the animals were euthanized under anesthesia with Beuthanasia-D (390 mg pentobarbital plus 50 mg phenytoin), 1 mL per 4.5 kg (~86 mg/kg pentobarbital). Immediately following euthanasia, a necropsy was performed to grossly evaluate the capsule distribution for comparison with MR imaging.

Cell viability staining and imaging

Fluorescence microscopy: Capsules explanted from the NHPs on Days 0 and 7 were stained with acridine orange (AO) and propidium iodide (PI) for viability analysis of adherent host cells. Briefly, AO and PI were prepared by mixing 1 mL of AO (A6014, Sigma-Aldrich, 1mM stock) and 2 mL of PI (P4170, Sigma-Aldrich, 0.5 mg/mL stock) with 47 mL of PBS. AO permeates both live and dead cells and stains all nucleated cells to generate green fluorescence. PI enters dead cells with compromised membranes and generates red fluorescence. For cells stained with both AO and PI, all live nucleated cells fluoresce green and all dead nucleated cells fluoresce red. For viability determination, 20 μ L of cell sample and 20 μ L of AO/PI staining solution were combined. Then 10 μ L of the stained sample was added to a hemocytometer, the total live (green) and dead cells (red) were counted, and the percent viability was calculated. These cells were used for subsequent phenotyping.

Immunophenotyping by flow cytometry

Multicolor flow cytometric analysis was performed on host cells from peritoneal fluid samples. Staining for surface markers was performed using α -CD3 APCCy7, α -CD4 FITC, α -CD8PEy7, CD11b PerCP, α -CD14 APC, CD20 PE, α -HLA-DR BV421 and α -CD45 BV500 antibodies (BD Biosciences, CA, USA). Cells (1.0×10^6) were stained for 25 min at 4°C in the dark with a cocktail of antibodies followed by fixation (eBiosciences) and wash. Appropriate fluorescence-minus-one (FMO) and/or isotype controls were used. Stained cells were acquired on a 3-laser instrument (Canto II, BD Bioscience), and data were acquired with FACSDiva 6.1.3 (BD Bioscience). The relative percentages and the absolute count of each subpopulation were determined using FlowJo 10.1 software (TreeStar San Diego CA, USA).

Histologic analysis of explanted PFCE alginate capsules

Capsules collected on days 0 and 7 were transferred to neutral buffered formalin, paraffin-embedded, sectioned (4 μ m sections), and stained by hematoxylin and eosin (H&E), as

described.²¹ Detailed evaluation of host cellular responses or fibrosis was performed by a board-certified cytopathologist/anatomic pathologist.

Statistical analysis

Peritoneal pO₂ measurements are expressed as mean $\pm$ 1 standard error of the mean (SEM). This preliminary study of three NHPs was not designed or powered to detect statistically significant differences between groups. With that caveat, Prism (Graph-Pad Software, San Diego, CA) was used to perform a nonparametric Wilcoxon test on the data.

Results

Measurement of peritoneal pO₂ and capsule location by noninvasive ¹⁹F-MR

Our aims were to determine noninvasively the average pO₂ in PFCE capsules dwelling in the peritoneal cavity of NHPs and to identify the location of the capsules within the peritoneal space.

Immediately after PFCE capsule infusion, the pO₂ levels measured in capsules by MRR were 165, 137, and 141 mmHg for the three NHPs imaged (147 ± 9 mmHg) (Figure 2A, Table 3). These intracapsular pO₂ measurements were in relative agreement with expected pO₂ since capsules and the infusion solution had been equilibrated with atmospheric air (pO₂~160 mmHg), prior to infusion. Day 0 ¹⁹F-MRI scans showed a large mass of capsules concentrated near the infusion site (representative NHP, Figure 3, left panels).

On Day 7, the intracapsular pO₂ levels by MRR were 66, 76, and 41 mmHg, for the three NHPs, respectively (61 ± 11 mmHg) (Figure 2A, Table 3). After 7 days, ¹H and ¹⁹F MR images demonstrated that the capsules were widely distributed throughout the abdomen (Figure 3, right panels). However, some capsules were concentrated in local pockets (indicated by high signal intensities at various locations in the ¹⁹F-MR images, Figure 3, right panels), suggesting that the capsules had pooled. The locations of PFCE capsules within the peritoneal space were determined by differential coloring of sequential MRI “slices”. In the axial orientation, coloring the cranial, abdominal, and caudal slices blue, green, and red, respectively, showed that the capsules had moved away from the infusion site and were dispersed throughout the abdominal space by day 7 (Figure 4, left panel, axial view). Coloring the ventral and dorsal slices green and red, respectively, revealed that many of the capsules had aggregated in the lower part of the pelvis (Figure 4, right panel, coronal view, red pixels, dorsal region).

The i.p. distribution of PFCE capsules visualized by ¹⁹F-MRI was verified by findings at gross necropsy of the NHPs on Day 7. Capsules were distributed throughout the abdomen (Figure 5A), with some aggregation noted around the bladder and uterus. Although the capsules were generally mobile, in all three animals a substantial portion of them were found trapped (but rarely adhered within) in the peritoneal folds composing the greater omentum (Figure 5B). In addition, a large number of capsules had accumulated in the caudal region of the peritoneal cavity (Figure 5C).

Parallel measurement of peritoneal pO₂ by fiber optic probe

In parallel with ¹⁹F MRR, pO₂ was measured using a fiber optic probe inserted in the peritoneal cavity *via* the catheter, a more widely used method than MRR. This probe with an oxygen microsensor was capable of measuring pO₂ at a specific point (~ 1 mm), not globally, and a degree of error was likely introduced when some atmospheric oxygen was introduced into the peritoneal space during insertion. This approach could not unequivocally place the tip in direct proximity to the capsules since they were freely moving. However, imaging and necropsy observations demonstrated the non-adherent capsules were dispersed throughout the sub-peritoneal space and peritoneal cavity, specifically on the surface of all peritoneal organs and peritoneal wall with pockets of aggregation in peritoneal recesses. The fiber optic probe was positioned in the same area as the capsules, between the peritoneal wall and the surface of the omentum/bowel, to sample the general environment to which the capsules were exposed. The average i.p. measurement using the fiber optic probe over the period of MRR acquisition on Day 0 was 132 ± 23 mmHg (immediately after implantation of capsules) and decreased to 78 ± 13 mmHg by Day 7 (Figure 2B).

Microscopic assessment of biocompatibility of cell-free PFCE capsules in NHPs

Evaluation of capsules from all three NHPs by phase microscopy showed that the capsules were intact and the morphology was unchanged after one week *in vivo*. In order to examine host reactions to PFCE capsules, we assessed the presence of viable host cells on the surfaces of the capsule on Days 0 and 7 histologically and by viability staining. On Days 0 and 7, freshly explanted, non-fixed capsules showed no cellular attachment or fibrosis (representative animal, Day 0, Figures 6A and 6B; Day 7, Figures 6C and 6D). Because insignificant numbers of host cells were present in the peritoneal lavage on Day 0, no assessment of cell viability could be performed at that time. However, low numbers of host cells (1.7 X 10⁶ to 5.2 X 10⁶) were present in the peritoneal fluid surrounding the capsules at necropsy on Day 7 (Table 4 and Figure 6D, arrows), and their viability was more than 96% (Table 4).

The absolute numbers of CD45⁺ hematopoietic cells and the total number of leukocytes were uniformly low, (correlating with our observation of low numbers of host cells in the peritoneal fluid), although there were substantially more total cells in one of the animals compared to the other two (Table 5). The peritoneal fluid of all NHPs contained minimal numbers of CD4⁺ and CD8⁺ T cells, CD20⁺ B cells, and CD14⁺ monocytes/macrophages (Table 5 and Figure 7A). Of the cells that were present, CD11a (lymphocyte function-associated antigen-1, LFA1, a cell adhesion/homing receptor important for cell activation and migration) was expressed by less than 40% of the CD20⁺ B cells, 80 –100% of the CD8⁺ T cells, and close to 100% of the CD4⁺ T cells and CD14⁺ macrophages (Figure 7B).^{22,23} The CD14⁺ macrophages also expressed relatively high levels of HLA-DR (MHC Class II), which is constitutive on these antigen presenting cells and can also be upregulated in response to immune stimulation (Figure 7C).²⁴

Representative aliquots of capsules from each NHP were analyzed histologically. Small numbers of free peritoneal cells (resident macrophages, mesothelial cells, and a few lymphocytes) were observed surrounding but not attached to the capsules (Figure 8A, free-

floating capsules). The few capsules that had become attached to the omentum were surrounded by host macrophages, eosinophils, neutrophils, and some lymphocytes (Figure 8B). These host cells were representative of a benign foreign body reaction, and not an acute inflammatory response.

Discussion

Intraportal pancreatic islet transplantation is a successful treatment modality for type 1 diabetes, but widespread clinical application has been hampered by the limited availability of donor islets and decline of graft function over time.^{25,26} Loss of graft function is related to auto- and allograft immune reactivity and to the direct toxicity of some immunosuppressive therapies.²⁷⁻³⁰ One strategy used to avoid or minimize immunosuppression is to encapsulate islets using micro- or macro-devices.^{31,32} Encapsulated islets transplanted i.p. in dogs, NHPs, and in a few human patients have temporarily restored glycemic control but without long-lasting success.^{33,34} Factors contributing to the loss of graft function include damaging conditions intrinsic to the i.p. site, such as hypoxia or immunoreactivity.³⁵⁻³⁷ Our studies in NHPs transplanted i.p. with encapsulated porcine islet xenografts showed that fibrosis, capsule breakage, and host immune responses were not responsible for the loss of graft function, but that hypoxia could have an important role.⁴ In the current study, we evaluated ¹⁹F-MRR as a means to assess capsular pO₂ in the peritoneal cavity in a large animal model.

¹⁹F-MRR has several advantages over a fiber optic or electrolytic probe for measuring pO₂ in NHPs and in human patients: (1) MRR is noninvasive and therefore reduces trauma and the possibility of infection in the subject, (2) MRR facilitates serial measurements to monitor the graft over time; (3) While a probe allows a limited measure of pO₂ intrinsic to the region just adjacent to the tip, MRR allows pO₂ to be mapped in specific anatomical areas of the peritoneum, where capsules may be more or less crowded; and (4) MRR does not introduce atmospheric air into the peritoneal site, avoiding the error that could be associated with introducing a probe into the peritoneum.

The usefulness of ¹⁹F compounds for clinical MR imaging has been known for decades.^{38,39} Because oxygen is necessary for cellular function and metabolism, ¹⁹F-MRR was utilized with PFCs as oxygen concentration markers, to noninvasively measure pO₂ in vivo. A major advantage of ¹⁹F is that fluorine is absent from biological tissue, eliminating background signals that could interfere with interpretation of the data.⁴⁰ ¹⁹F-MR has been used to evaluate the metabolic activity and bioenergetics of encapsulated cells in vitro and also to measure peritoneal pO₂ in mice *in vivo*, using cell-free PFCE capsules.^{2,19} While ¹⁹F-MR is an established technique for noninvasively measuring peritoneal pO₂ in vitro and in vivo in murine models,^{2,3,19,41,42} to our knowledge no other investigators have attempted to measure pO₂ in NHPs by ¹⁹F-MRR. One objective of our study was to determine whether the PFCE capsules were biocompatible in vivo in NHPs and whether they induced an inflammatory response. We found that free-floating capsules did not become covered with host cells or fibrosis after 7 days in vivo, and that very few host cells (T cells, B cells, and macrophages/monocytes) were present in the peritoneal lavage at necropsy. The few

capsules that became attached to the omentum represented a benign foreign body reaction and not an inflammatory reaction.

On Day 0, immediately after infusion, the PFCE capsules were still localized near the infusion site. Since these capsules had been equilibrated with atmospheric oxygen, we were not surprised to find a relatively high level of oxygen in this area (147 ± 9 mmHg). By Day 7, the pO_2 of capsules implanted i.p. was relatively low (61 ± 11 mmHg). Given the small number of animals in this study ($n=3$), the data on Days 0 and 7 were not significantly different (nonparametric Wilcoxon test). Nevertheless, we believe that the differences are still biologically meaningful and can be used to appropriately power future studies that would reach statistical significance if similar data is observed.

In native pancreatic rodent islets, the mean pO_2 is 40 mmHg.⁴³ Using cell-free microcapsules implanted i.p., we measured a pO_2 of 60 mmHg. This level may be sufficient for islet function, but when encapsulated islets are present, the pO_2 level within capsules will decrease further due to the oxygen consumption of the highly metabolic cells,^{2,3,44,45} having the potential to negatively influence functional transplant outcomes.^{13,46}

It should be noted that the pO_2 was measured during a period of hyper- oxygenation, when the animals were being ventilated with 100% oxygen while under anesthesia during the MR procedure, suggesting that the pO_2 may be substantially lower under normal respiration.^{47,48} The administration of inspired oxygen has been shown to increase the pO_2 in tissues^{49,50} and is beneficial to outcomes in certain applications, for example in reduction of surgical site infections.⁵¹ However, our i.p. capsules are not vascularized and are mobile within the peritoneum. There are many factors that influence the degree of oxygen available to the capsules, including proximity to vessels, density of cells within the capsules, and the presence of fluid in the peritoneum. Therefore, the increase in intracapsular pO_2 is likely to be less than in the blood and tissues and may change over time in the peritoneal site. To address this problem, it will be important to focus future studies on translational oxygen delivery methods and implantation techniques that will sustain pO_2 levels sufficiently for long-term function of encapsulated islets.

The finding by ^{19}F -MRR imaging (with verification by gross necropsy) that a large number of capsules had accumulated in the caudal region of the peritoneal cavity by day 7 post-implantation is relevant for the consideration of an optimal transplant site for microcapsules in bipedal recipients. In both NHPs and human patients, we expect that the sedimentation of encapsulated islets would be detrimental to the long-term function of the grafts, because of reduced oxygenation in the aggregated capsules. A potential solution to the problem of microcapsule aggregation could involve the use of a bioengineered construct that supports more uniform dispersal and prevents aggregation of cells. One example of the success of such an approach is the use of a plasma/thrombin clot to support adhesion and engraftment.⁵²

We believe that by establishing protocols to quantitate the potential need for enhanced oxygen in macro-, micro- and also non-encapsulated islet transplantation, by optimizing the islet dose, and by assessing transplant sites (such as an omental pouch or the bursa

omentalis) that may lessen capsule pooling/aggregation and local hypoxia, it will be possible to develop solutions to the problem of inadequate oxygenation of islet grafts. Thus, islet transplantation may become significantly more successful in patients with type 1 diabetes.

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Abbreviations

AO	acridine orange
EtCO₂	end tidal carbon dioxide
H&E	hematoxylin and eosin
i.p.	intraperitoneal
LVM	low viscosity high mannuronic acid
MRI	magnetic resonance imaging
MRR	magnetic resonance relaxometry
NHP	non-human primate
NOD	nonobese diabetic
pO₂	partial pressure of oxygen
PFCE	Perfluoro-15-crown-5-ether
PI	propidium iodide
SEM	standard error of the mean
SpO₂	peripheral capillary oxygen saturation
STZ	streptozotocin

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Figure 1.

A, ¹⁹F PFCE microcapsules. PFCE (5%) was incorporated into 2% LVM alginate and Sr-gelled capsules (1000 μm diameter average, red scale bar) were produced as described. B, ¹⁹F-NMR spectrum of PFCE capsules. ¹⁹F was acquired with a 7 T system. A single, narrow ¹⁹F peak was indicative of the high purity of the preparation. C, Oxygen calibration curve. Three independent samples of capsules were prepared. Each sample was allowed to equilibrate to a series of oxygen partial pressures (pO₂ = 0, 38, 76, 114, and 160 mmHg) by sparging the sample with calibrated gas mixtures at least 30 minutes for each pO₂. The spin-lattice relaxation rate constant (R₁) for each sample at each pO₂ was measured, and the resulting calibration with two fitting constants (A and B) was generated using a weighted least-squares analysis (R₁ = A · pO₂ + B). The slope, A, was found to be 0.00246 ± 0.0004 mmHg⁻¹ · s⁻¹ and the intercept, B, was found to be 0.3397 ± 0.009 s⁻¹ (R² = 0.9963). These data represent the average of three separate experiments and a linear regression fit of the entire data-set. The standard error of the mean (SEM) at each point was <1% of the average value for R₁ (pO₂ 0, mean R₁ = 0.348274, SEM = 0.000822, n=3; pO₂ 38, mean R₁ = 0.427474, SEM = 0.003654, n=3; pO₂ 76, mean R₁ = 0.521029, SEM = 0.00198, n=3; pO₂ 114, mean R₁ = 0.609969, SEM = 0.005241; pO₂ 152, mean R₁ = 0.722372, SEM = 0.000967, n=3).

Figure 2.

A, Day 0 and Day 7 Peritoneal pO₂ Measurements by Fluorine-19 Magnetic Resonance Relaxometry (MRR). Capsule pO₂ levels in three NHPs (indicated by circle, square, and triangle symbols) were assessed by ¹⁹F-MRR in three replicate measures on days 0 and 7 post-infusion of PFCE capsules. With the anesthetized animals positioned on a custom-built ¹⁹F-¹H dual-resonance surface coil, ¹H-MRI was performed to obtain reference images for determining the location of the capsules within the abdomen. With the animal remaining in position, the coil was then re-tuned for ¹⁹F-MR. R₁ of the fluorine signal was measured in triplicate. The R₁ values were subsequently used to determine the peritoneal pO₂ using an oxygen calibration curve. B, Day 0 and Day 7 Parallel Peritoneal DO Measurements by Fiber Optic Probe (FO Probe). Using the i.p. catheter, a fiber optic oxygen microsensor measured local pO₂ during MR pO₂ acquisition for comparison. The probe measured pO₂ at a specific point (~ 1 mm), not globally.

Figure 3.

Representative ^1H - and ^{19}F -MRI Slices of Abdomen on Days 0 and 7. At each time point, preliminary scan was done using a custom-built ^{19}F - ^1H dual resonance surface coil with standard proton MRI to verify proper coil function and image acquisition, and then ^{19}F MRI scans were performed. Representative slices (axial and coronal) are shown on day 0 (left panels) and on day 7 (right panels). The images are digitally contrast-optimized for visual presentation.

Figure 4.

Fused ^1H - ^{19}F MR Images Demonstrating Capsule Distribution on Day 7. With a 4 T MR system, ^1H and ^{19}F images of the NHPs were acquired using a double-loop quadrature surface coil tuned to the appropriate resonance frequency as needed (160.2 MHz for ^{19}F , and 170.3 MHz for ^1H). For analysis, the ^1H MR (black and white) images and ^{19}F MR (colored) images were fused. Representative ^{19}F MR slices from different areas of the peritoneum were labeled with different colors. For the axial slices: red = caudal; blue = mid-abdomen; green = cranial. In the coronal slices: red = dorsal; green = ventral.

Figure 5.

Gross Necropsy Showing Intraperitoneal Distribution of Capsules on Day 7. Left panel: View of many clean, intact PFCE alginate capsules distributed throughout the peritoneal cavity of a representative, supine NHP. Center panel: Omentum from a representative NHP showing many PFCE alginate capsules within in the folds composing the greater omentum. The majority of the capsules were not adherent to the omentum and could be easily moved by touching with a blunt instrument. Right panel: PFCE capsules that had aggregated in the lower portion of the abdomen (caudal).

Figure 6.

Microscopic Assessment of PFCE Beads from Representative NHP on Days 0 and 7 Post-infusion. PFCE alginate capsules explanted from the NHPs by lavage after the MR procedure on days 0 and 7 were assessed by phase contrast microscopy and also AO/PI stained and examined for the presence of viable (green) or dead (red) host cells by fluorescence microscopy. A, Unstained capsules assessed by phase microscopy on day 0, magnification 10X, B, Unstained capsules assessed by phase microscopy on day 7, magnification 10X, C, Capsules stained with AO/PI and assessed by fluorescent microscopy on day 0 (10X), D, Capsules stained by AO/PI and assessed by fluorescent microscopy on day 7 (10X), Arrows indicate clusters of host cells floating in the lavage media.

Figure 7.

Peritoneal Cell Populations in NHPs on Day 7 after Administration of PFCE Capsules.

Hatched bars = NHP #1; Solid black bars = NHP #2, Gray bars = NHP #3. A, Numbers of leukocytes in peritoneal lavage. B, Percent of CD20⁺ B cells, CD4⁺ and CD8⁺ T cells, and CD14⁺ macrophages expressing CD11a (LFA1). C, Percent of CD20⁺ B cells, CD4⁺ and CD8⁺ T cells, and CD14⁺ macrophages expressing HLA-DR.

Figure 8.

Histologic Analysis of Explanted PFCE Alginate Capsules. Capsules collected during peritoneal lavage on days 0 and 7 were paraffin-embedded, sectioned, and stained by H and E. Detailed evaluation of host cellular responses or fibrosis was performed by a pathologist at the Emory University Hospital. A, H&E stain of capsules free-floating in the peritoneal fluid. B, H&E stain of capsules adherent to the omentum.

Table 1.

NHP Study Groups

Animal ID	Species	Sex	Body weight (kg) pre	Body weight (kg) Day 4	Study Duration (days)
11GP1	Rhesus macaque	Female	6.22	6.36	7
11GP5	Rhesus macaque	Female	9.89	9.6	7
11GP3	Rhesus macaque	Female	8.11	8.09	7

Table 2.

Study Participant Management during Imaging Procedures on Days 0 and 7

Animal ID	Body Temp (°F) Day 0	S_pO₂ level (%) Day 0	ETCO₂ (mmHg) d 0	Body Temp (°F) Day 7	S_pO₂ level (%) Day 7
11GP1	96.1 - 98.2	95 - 98	38 - 47	96.4 - 101.1	96 - 100
11GP3	99.0 - 100.5	96 - 100	42 - 48	97.8 - 101.3	93 - 100
11GP5	96.9 - 100.1	98 - 100	33 - 48	96.5 - 100.3	99 - 100

Table 3.

pO₂ measurements by ¹⁹F MRR on days 0 and 7 in three NHPs

Animal ID	Day 0			Day 7		
	Mean	SEM	(n)	Mean	SEM	(n)
11GP1	165	4	3	66	1	3
11GP5	137	2	3	76	1	3
11GP3	141	2	3	41	3	3

Table 4.

Numbers of host cells, viability, lavage fluid volume, and capsule volume in samples from three NHPs necropsied on day 7 post-infusion.

Animal ID	Total Cell # (x10 ⁶)	Viability (%)	Fluid Vol. (mL)	Capsule Vol. (mL)	Total Vol. (mL)
11GP1	5.2	96	4.15	3.75	7.9
11GP5	1.7	99	6.2	3.6	9.8
11GP3	4.5	100	5.35	7.6	12.95

Table 5.

Phenotypes and absolute numbers of host leukocytes in the peritoneal fluid

Immune Subsets	11GP1 Absolute # cells	11GP5 Absolute # cells	11GP3 Absolute # cells
CD45 ⁺	1061	358	4644
CD3 ⁺	290	86	1268
CD4 ⁺	95	43	414
CD8 ⁺	192	41	840
CD20 ⁺	79	21	348
CD14 ⁺	232	31	1016
CD11a ⁺	782	273	3422