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Early Barriers to Neonatal Porcine Islet Engraftment in a Dual Transplant Model

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Abstract

Porcine islet xenografts have the potential to provide an inexhaustible source of islets for β cell replacement. Proof-of-concept has been established in nonhuman primates. However, significant barriers to xenoislet transplantation remain, including, the poorly understood instant blood mediated inflammatory reaction (IBMIR), and a thorough understanding of early xeno-specific immune responses. A paucity of data exist comparing xeno-specific immune responses to allo-islet responses in primates. We recently developed a dual islet transplant model, which enables direct histological comparison of early engraftment immunobiology. In this study, we investigate early immune responses to neonatal porcine islet (NPI) xenografts compared to rhesus islet allografts at 1 hour, 24 hours, and 7 days. Within the first 24 hours after intraportal infusion, we identified greater apoptosis (caspase 3 activity and TUNEL positive cells) of NPIs when compared to alloislets (AIs). Macrophage infiltration was significantly greater at 24 hours compared with 1 hour in both NPI (WT) and AIs. At 7 days, IgM and macrophages were highly specific for NPIs (GTKO) compared to AIs. These findings demonstrate an augmented macrophage and antibody response towards xenografts compared to allografts. These data may inform future immune or genetic manipulations required to improve xenoislet engraftment.

Introduction

Intraportal islet allotransplantation has been established as an alternative to solid organ pancreas transplantation.¹,² However, neither approach has achieved widespread application relative to the total number of patients with type 1 diabetes.³ The overall lack
of pancreata available for either form of transplantation (alloislet or solid organ) is a major impediment to clinical utilization, but it is particularly so in islet transplantation, as many recipients require multiple pancreata to provide sufficient islets to achieve insulin independence. Islet graft efficiency is also considerably lessened by the instant blood mediated inflammatory reaction (IBMIR), which leads to early islet destruction. Current understanding suggests that an innate inflammatory process, consisting of complement, coagulation, antibodies, macrophages, and neutrophils, drives this process upon intraportal infusion. The mechanistic processes driving IBMIR in vivo remains incompletely defined. Early responses to islet grafts have a major impact for long-term function, and thus remain a significant obstacle to achieving functional outcomes similar to whole organ pancreas transplantation. Even so, overcoming IBMIR in islet allotransplantation will fail to meaningfully impact the roadblock of limited donor tissue, and will at best approach parity with whole organ pancreas transplantation.

Porcine islet xenotransplantation may provide an alternative, limitless source of islets for β cell replacement therapy. Xenotransplantation has seen a recent resurgence in interest due to breakthroughs in genetic manipulation, which may facilitate targeted reductions in tissue immunogenicity. Many aspects of islet xenotransplantation present it as unique for early phase clinical testing. However, a high bar has been set for safety for early xenotransplant trials, and this requires a concerted effort to understand mechanisms specific to xenoislet engraftment and rejection using preclinical models. Thus far, preclinical models have demonstrated that islet xenotransplantation can provide sufficient glucose homeostasis without the need for insulin. The current long-term graft survival data also reveal the finite longevity of these islet xenografts, presenting opportunities for significant improvement before clinical efforts are undertaken. As with islet allotransplantation, xenogeneic IBMIR must be overcome for the functional engraftment and survival of porcine islets. A thorough understanding of early xenospecific immune responses is imperative when considering clinically relevant immunosuppression to facilitate xenoislet engraftment and long-term function. A rigorous method to interrogate xenoislet engraftment biology would rapidly advance the field towards the goal of clinical application.

We recently described a novel dual islet transplant model in which the early inflammatory components governing engraftment are compared within a single recipient. Xenotransplantation is a species-specific endeavor, making preclinical (e.g. pig to primate) graft survival models laborious and resource-intensive. The dual transplant model takes advantage of hepatic vascular anatomy to examine differences in immunobiology within a single recipient, providing an effective internal control for each animal, thereby reducing the number of animals required to delineate significant differences. Here we employ the dual transplant model to delineate early xeno-specific immune responses compared to alloislets.

**Materials and Methods**

**Neonatal porcine islet and Rhesus islet procurement, isolation, and culture**

Neonatal piglets, either α1,3-galactosyl transferase knockout (GTKO) or hemizygous (phenotypic wild-type), were obtained from Fios, Inc. (Rochester, MN, USA) and bred at the...
University of Georgia Department of Animal and Dairy Science (Athens, GA, USA). The extended dual islet transplants were performed with GTKO piglets generously provided by Dr. David Sachs at the Harvard Transplantation Biology Research Center. Piglets were transported to our institution and then underwent terminal pancreatectomy 3–6 days after birth followed by immediate collagenase digestion and islet isolation via a previously described modified Korbutt technique. (28) NPIs were maintained in culture using NPI culture media (Corning, Corning, NY, USA) supplemented with penicillin/streptomycin (Corning, Corning, NY, USA) for 6–8 days and were quantified in islet equivalents (IEQs) on day of transplantation. Rhesus islets were isolated from terminal rhesus donors by Dr. Norma Kenyon at the University of Miami as previously described. (29) Rhesus islets were cultured overnight and quantified the day of transplantation, with islet equivalents (IEQs).

On the day of transplantation, islets were assessed for quantity using dithizone (Sigma-Aldrich, St. Louis, MO, USA), for viability by fluorescein diacetate (Sigma-Aldrich, St. Louis, MO, USA) and propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) live-dead counterstaining, for bacterial contamination by Gram stain and culture with endotoxin testing, and for in vitro function by static incubation assay to obtain a glucose stimulation index (GSI). (28)

**Dual islet transplantation**

Rhesus macaques (*Macaca mulatta*) weighing 3–10 kg were selected as NPI xenograft recipients (Table 1). All procedures and care of animals was performed in accordance with the Guide for the Care and Use of Laboratory Animals (30) and approved by the Emory University and/or Duke University Institutional Animal Care and Use Committees. Individual islet infusions were prepared using 20mL CMRL 1066 without phenol red (Corning, Corning, NY, USA), 100 units of heparin sodium, and 1.5 mg/kg of etanercept (Enbrel Immunex Corp, Thousand Oaks, CA). Two islet preparations were made for each transplant recipient, one for each islet phenotype, and balanced to provide comparable IEQ infusions to each hemiliver, with an exception of recipient WT/AI1 due to poor rhesus islet yield. This graft was included in the analysis, as this model is focused on histological impact of the islets rather than on insulin independence. Also, NPIs require in vivo maturation and if used clinically, the islet mass required to achieve insulin independence will be larger for xenogenic islets than for allogenic islets. Thus, differences in islet mass are a clinically relevant variable to be understood. Transplantation was performed as previously described. (25) The side of the infusion was randomized so that differences segregating with lateralization were controlled. Six animals underwent dual transplant of rhesus alloislets and wild type neonatal porcine islets (WT NPIs) without immunosuppression with endpoints of 1 hour (n=3) and 24 hours (n=3) (Table 1A).

**Extended dual islet transplantation**

Rhesus macaques were rendered diabetic through streptozocin induction (STZ, 1250 mg/m² IV; Sigma, St Louis, MO, USA) after baseline IV glucose tolerance testing (IVGTT) data were obtained. Post-diabetes induction IVGTT was performed to confirm significant reduction and poor responsiveness of rhesus C-peptide levels (C-peptide ELISA assay; Abcam, Cambridge, MA, USA) to dextrose bolus. Animals were monitored with twice daily glucose checks and administration of NPH and glargine insulin based on sliding scale to
maintain appropriate blood glucose levels. Three diabetic recipients underwent rhesus islet and GTKO xenoislet dual transplantation (Table 1B) and given a robust costimulation blockade based regimen using CTLA-4Ig, anti-CD154, and anti-LFA1 therapy used in our initial GTKO islet xenotransplantation series, (21) with an experimental endpoint of 7 days after transplant.

**Tissue collection and immunohistochemistry**

Livers were recovered from euthanized islet recipients at 1 and 24 hours post-infusion time-points for detailed immunohistochemical analysis. The explanted liver was divided into right and left lobes with 1.0cm of central tissue excluded from analysis in each hemiliver to preclude crossover contamination between islet phenotypes. Hemilivers were divided into 5 sections each and preserved in 10% neutral buffered formalin for histological evaluation, as described. (27) Tissue samples were embedded in paraffin and underwent subsequent sectioning and hematoxylin and eosin (H&E) staining for morphologic observation. Tissue slides also underwent independent staining for standard hematoxylin and eosin, as well as galactose-α1,3-galactose (Gal, Enzo Life Sciences, Farmingdale, NY, USA) for one and 24 hour assessments, porcine specific CD44 (PORC24A IgG2a, Washington State College of Veterinary Medicine, Pullman, WA, USA) for 7 day post infusion assessments, insulin (Sigma-Aldrich, St. Louis, MO, USA), C3d (Abcam, Cambridge, MA, USA), C4d (American Research Products, Waltham, MA, USA), IgG (Sigma-Aldrich, St. Louis, MO, USA), IgM (KPL, Gaithersburg, MD, USA), neutrophil (neutrophil elastase, Dako, Carpinteria, CA, USA), IFN-gamma (Abcam, Cambridge, MA, USA), macrophage (CD68, Dako, Carpinteria, CA, USA), natural killer (NK) cell (CD3-CD8+, CD3, Abcam, Cambridge, MA, USA; CD8, Abcam, Cambridge, MA, USA), Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, Roche Diagnostics GmbH, Mannheim, Germany), cleaved caspase 3 (Cell Signaling Technology, Danvers, MA, USA) and platelets (CD61, Dako, Carpinteria, CA, USA). The signal was visualized using 3,3 diaminobenzidine peroxidation and counterstained with hematoxylin. Whole slide digital images were captured using the Aperio ScanScope XT (Leica Biosystems, Vista, CA, USA) slide scanner system with 20× objective magnification. Quantitative immunohistochemical analysis was performed using Aperio Imagescope (Leica Biosystems, Vista, CA, USA) digital pathology software. (27) Individual slides were scanned and islets were manually selected and analyzed using an optimized positive pixel algorithm to obtain a percent pixel positivity of the individual stain per islet. The sum of individual islets within a hemiliver was compiled to compute a percent positive staining representative of each hemiliver, termed the positivity index. Data for a specific stain were excluded from an animal if the total number of islets found in a hemiliver was less than five.

**Statistical analysis**

Pixel positivity index was calculated within each hemiliver and paired against the contralateral hemiliver positivity for each animal. As positivity was measured in a percentage format, a paired t-test with logarithmic transformation was performed to analyze stain differences between islet preparations at the study time points of 1 and 24 hours and 7 days. An unpaired t-test was performed to measure differences in positivity from 1 to 24 hours for rhesus alloislet and WT NPI experiments. Statistical analysis was performed using
GraphPad Prism (GraphPad Software Inc., LaJolla, CA, USA) version 6 statistical software. A $p \leq 0.05$ was determined to be statistically significant.

**Results**

*Islet segregation was achieved by the dual islet transplant model*

Immunohistochemical staining of galactose-alpha-1,3-galactose was analyzed to determine adequate segregation of alloislets (AI) and WT NPIs in the dual islet transplant model (1 hour and 24 hours). At both 1 hour ($p<0.01$) and 24 hours ($p<0.01$) staining between islet phenotypes was significantly different (Figure S1A). Similarly, Immunohistochemical staining of CD44 was analyzed to determine adequate segregation of GTKO NPIs and AI by hemiliver in the extended dual islet transplant model (7 days). Staining between islet phenotypes was also significantly different ($p<0.02$, Figure S1B).

*Wild type neonatal porcine islets demonstrate enhanced susceptibility to apoptosis and cell death compared to alloislets*

We investigated the induction of apoptosis in WT NPIs and the AIs at both 1 and 24 hours. In Figure 1A, we demonstrate significantly greater caspase 3 activity at 1 hour in the WT NPIs compared to the AIs. Consistent with these data, we observed a trend toward significance at 24 hours with increased caspase 3 activity in the WT NPIs compared to the AIs. To make sure this increase in caspase 3 activity is not a phenomenon intrinsic to WT NPIs, we performed similar immunohistologic staining on WT NPIs prior to transplantation and demonstrated minimal caspase 3 activity in WT NPIs cultured ex vivo (Figure 1D). In addition, we evaluated differences in TUNEL staining, as a late marker of apoptosis and cell death. In Figure 1B, we demonstrate significantly greater TUNEL-positive cells at 24 hours in the WT NPI compared to the AIs ($p=0.03$).

*Early immune responses to wild type neonatal porcine islets compared to alloislets at 1 and 24 hours*

Detailed Immunohistochemical analysis was undertaken to investigate potential early differences in the immune response to xenoislets compared to alloislets. In Figure 2A, macrophage infiltration was not significantly different between WT NPIs and AIs at either 1 or 24 hours. However, significantly greater macrophage infiltration at 24 hours was seen in both WT NPI and AIs. In Figure 2B, there is significant reduction in NK cell staining in the AIs at 24 hours compared to AIs at 1 hour. NK cell staining in the WT NPIs is not significantly different at 1 and 24 hours. Similarly, there is no significant difference between WT NPI and AIs at 1 and 24 hours. In Figure 2C, there is a significant reduction in IFN-gamma staining in the WT NPIs at 24 hours compared to 1 hour. In addition, IFN-gamma staining tended to be higher in the WT NPIs at 1 hour compared AIs ($p=0.06$). Lastly, there was no significant difference in staining for insulin, C4d, IgM, IgG, neutrophils, CD3, and platelets at both 1 hour and 24 hours (Figure S2).
Evaluation of acquired immune responses to GTKO NPIs in the presence of rigorous costimulation/adhesion blockade compared to alloislets

Immunohistochemical staining was performed in 3 diabetic animals on postoperative day 7 in GTKO NPIs and AIs to identify specific immune responses capable of overcoming a potent immunosuppressive regimen consisting of CTLA-4Ig, anti-CD154, and anti-LFA1 therapy, a regimen that has been shown to facilitate long term xenograft acceptance. (19, 20)

C4d staining remained higher in AIs compared to GTKO NPIs (p=0.06, Figure 3). IgM responses remained consistently higher in GTKO NPIs compared to AIs across all 3 recipients (p=0.06, Figure 4C). Cellular infiltration of macrophages were highly specific for GTKO NPIs compared to AIs (p=0.02, Figure 4D). NK cell staining was not significantly different between GTKO NPIs and AIs. Staining of platelets, neutrophils, IgG, and complement C1q and C3d staining did not reveal significant differences between GTKO NPIs and AIs (data not shown).

Discussion

This series of experiments applies the dual transplant model to compare early engraftment immunobiology between xenogeneic and allogeneic islets within the first week after intraportal infusion. We find that xenogeneic islets evoke an augmented antibody and macrophage response compared to alloislets, and further demonstrate that the immune response to islet xenografts remains significantly more robust even when using GTKO islets under the cover of immunosuppression that is known to foster long term islet survival. These data were obtained under a rigorous, internally controlled environment, which indicates that current regimens known to permit eventual engraftment incompletely manage the early immune response to islet xenografts. This highlights additional opportunity for genetic manipulation or early maneuvers designed to better control these early events. Further, this reveals a significant gap in our understanding of xenoislet immunobiology with significant implications for xeno-specific immunosuppression and future genetic modifications.

Our histologic assessments at 1 and 24 hours after infusion of AIs and WT NPIs demonstrated a mostly nonspecific milieu of antibody, complement, and coagulation. These findings are likely a part of the inflammatory nature of the islet preparations in general as a significant amount of debris and islet stress is produced during isolation and infusion. Our introductory description of the dual transplant model also supports this, as no major differences existed between GTKO and WT NPIs in the first 24 hours after transplantation. (27)

Macrophage infiltration was nonspecific to either WT NPIs or AIs, yet there was a uniform increase over 24 hours, which is similar to our previous findings comparing WT NPIs and GTKO NPIs. (27) Although macrophage infiltration was common to both sources of islet, we observed a higher level of caspase-3 activation and TUNEL positive cells in WT NPIs, indicative of increased apoptosis early in the xenogeneic process. Macrophage infiltration in the first 24 hours may thus be more related to the removal of necrotic material and debris without specificity for an islet phenotype at this stage.
Our initial series of GTKO islet transplants demonstrated increased engraftment and long term function over WT NPIs. These results were facilitated by the use of an experimental drug regimen using anti-CD154 and anti-LFA1. Although results in preclinical long-term function of porcine islet xenografts by various groups have been promising, it is important to note that these studies with experimental agents remain outside the realm of clinical application. We sought to compare islet allografts and xenografts at one week after transplantation based on this regimen due to our prior experience with successful GTKO engraftment.

NPIs typically requires a two-week timeframe to reach physiologic maturity. Thus, a direct comparison between xenogeneic and allogeneic islets with regards to achievement of glucose homeostasis was not appropriate to this study. The delayed responsiveness of NPIs is supported by in vitro culture data and although these findings may not affect our observations of the immunobiology in this model, there are implications for future preclinical studies wherein the relevance of NHP to porcine glucose physiology may require critical examination when considering porcine to human transplantation.

Complement activation has been an extensively studied phenomenon in xenotransplantation, leading to the development of complement regulatory transgenic modifications of donors. Interestingly, our experiments demonstrated increased C4d deposition on AIs compared to GTKO NPIs, although there were no differences in C1q, C3d, or IgG staining between AIs and GTKO NPIs. Thus, we are not able to point towards a classical or alternative pathway for the activation of C4 complement. Increased IgM deposition in GTKO NPIs also contradicts a classical activation pathway cause for this finding. As complement activation is a significant component of both allo- and xenoislet IBMIR, the increased presence of C4d in AIs may indicate a relatively greater allospecific complement activation process.

Costimulation blockade appears to prevent donor specific antibody production, with evidence suggesting inhibition of germinal center responses. The dual transplantation of islet xenografts and allografts with costimulation blockade generated a xenospecific IgM response, which was not prevented by our experimental regimen. Indeed, non-Gal natural antibodies exist, and our findings may point to the need for immunosuppressive interventions addressing the initial immune activation and additional genetic modifications of porcine islets to minimize natural IgM binding to non-Gal xenoantigens. Screening for these antibodies and selecting recipients that are naturally low in these antibodies might also be warranted.

The infiltration of macrophages was significantly higher in GTKO NPIs than in AIs, however an exact mechanism for macrophage responses to xenografts has yet to be elucidated. Early macrophage infiltration was indiscriminate between AIs and WT NPIs, and thus may be the result of direct activation and infiltration along with islet graft production of macrophage specific factors such as MCP-1, RANTES, with macrophage TLR activation and upregulation of chemokine receptors. It should be noted, however, that most of these findings have been described primarily in rodent models; thus, there is a need to further define the xenoreactivity of macrophages in a pre-clinical setting. The lack of...
functional interaction between porcine CD47 and human SIRP1α interactions may propagate further macrophage-mediated xenograft phagocytosis.\(^{50, 51}\) In fact, transgenic pigs expressing human CD47 are currently being investigated in a hematopoietic stem cell xenotransplant model in an effort to induce chimerism and xeno-tolerance. Tena et al. have demonstrated a protective effect of hCD47 expressing on porcine HSC engraftment, likely through inhibition of early macrophage phagocytosis.\(^{52, 53}\) These overall findings point towards the inefficacy of a robust costimulation based immunosuppressive regiment to inhibit innate IgM, and macrophage xenospecific responses. Currently available experimental immunomodulation has largely been based on adaptive immune responses; although pertinent for allotransplantation, the xenotransplantation of islets will likely require major consideration of innate immune responses in development of drugs designed to prolong engraftment and function.\(^{54, 55}\)

The dual transplant model provides a unique, internally controlled \textit{in vivo} environment in which islet phenotype specific responses can be effectively compared. This study comparing AIs and NPIs clearly illustrates our inability to contain innate immune responses with advanced biologics developed for adaptive alloimmune responses, necessitating transgenic modification or specific immunosuppression to improve islet xenograft outcomes. Further implementation of this model towards evaluating xenoislet genetic and pharmacological manipulations will reveal beneficial interventions prior to investment in longer-term studies.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

We would like to thank Dr. Norma Kenyon and Dora Berman-Weinberg at the University of Miami for their help in providing rhesus islets, and Dr. David Sachs and Scott Aron at the Harvard Medical School Transplantation Biology Research Center for their contribution of GTKO piglets. We would also like to thank William Davis and the Washington State University College of Veterinary Medicine for generously providing us with the PORC24A mAb. This study was funded by NIH grant # AI090956.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>NHPs</td>
<td>Non-human primates</td>
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<tr>
<td>IBMIR</td>
<td>Instant blood mediated inflammatory reaction</td>
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<tr>
<td>GTKO</td>
<td>α1,3-galactosyltransferase knockout</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>NPIs</td>
<td>Neonatal porcine islets</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural Killer cells</td>
</tr>
<tr>
<td>IEQs</td>
<td>Islet equivalents</td>
</tr>
<tr>
<td>IVGTT</td>
<td>IV glucose tolerance test</td>
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References


Figure 1.
(A) Immunohistochemical staining of caspase-3 was analyzed at 1 hour and 24 hours in WT NPI and AIs. (B) Representative immunohistochemistry image of caspase 3 staining of WT NPI at 1hr. (C) Representative immunohistochemistry image of caspase 3 staining of WT NPI cultured ex vivo on Day 4 after islet isolation. (D) TUNEL positive cells were analyzed at 1 hour and 24 hours in WT NPI and AIs. Representative immunohistochemistry image of TUNEL staining of WT NPI (E) and AIs (F) at 1hr.
Figure 2.
(A) Macrophage staining of CD68 demonstrated increasing infiltration of islets over 24 hours which was indifferent to phenotype (both phenotypes from 1 to 24 hours, p=0.01). (B) NK cell staining (CD3-, CD8+) of WT NPI and AIs at 1 and 24 hours. (C) IFN-gamma staining of WT NPI and AIs at 1 and 24 hours. (D) Representative immunohistochemistry image of IFN-gamma staining of WT NPI at 1hr. (E) Representative immunohistochemistry of IFN-gamma staining of AIs at 1hr.
Figure 3.
C4d deposition greater in AIs 7 days post infusion with costimulation based regimen. (A)
An increased staining of C4d in alloislets over GTKO NPIs was observed approaching statistical significance (p=0.06). Shown are representative immunohistochemistry images of C4d staining of AI (B) and GTKO NPIs (C) on post-transplant day 7.
Figure 4.
GTKO NPIs incur greater IgM and cellular responses than AIs 7 days after portal infusion despite intensive immunosuppression. Representative immunohistochemistry image of IgM staining of AI (A) and WT NPI (B) on post-transplant day 7. Representative immunohistochemistry image of CD68 (macrophage) staining of AI (C) and WT NPI (D) on post-transplant day 7. (E) IgM staining was higher in all GTKO NPI grafts compared to AIs (p=0.06). (F) Macrophage infiltration and (G) NK cell infiltration were both significantly higher in GTKO NPIs than AIs (p=0.02 and p=0.05 respectively).
Table 1

Dual islet transplant rhesus macaque recipients.

<table>
<thead>
<tr>
<th>Recipient ID</th>
<th>Weight</th>
<th>Endpoint</th>
<th>Left lobe</th>
<th>IEq/kg</th>
<th>GSI</th>
<th>Right Lobe</th>
<th>IEq/kg</th>
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<td>WT/AI1</td>
<td>3.14 kg</td>
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<td>Allo</td>
<td>4,745</td>
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<td>WT/AI2</td>
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<td>1 hour</td>
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<td>22.61</td>
<td>Allo</td>
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<td>Allo</td>
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<td>1.5</td>
<td>WT NPI</td>
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<td>WT/AI4</td>
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<th>Recipient ID</th>
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<th>Left lobe</th>
<th>IEq/kg</th>
<th>GSI</th>
<th>Right Lobe</th>
<th>IEq/kg</th>
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<td>GTKO/AI1</td>
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<td>7 days</td>
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<td>Allo</td>
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<tr>
<td>GTKO/AI3</td>
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<td>7 days</td>
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<td>1.39</td>
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A. WT NPI and AI recipients transplanted for histological assessment at 1 (n=3) and 24 (n=3) hours after infusion.

B. Recipients of GTKO NPIs and AIs (n=3) assessed at one week post infusion.

GSI – glucose stimulation index