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A Novel Protocol for Culturing Adult Porcine Islets for Transplantation in Type 1 Diabetic Patients

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This study shows that lengthening the culture period for pre-transplantation adult porcine islets from 7 days to 30 days significantly lessens the chance of immunorejection after xenotransplantation for type 1 diabetes. Immunofluorescence staining was chosen to analyze antigen expression, islet viability, and insulin production after culture periods of 7, 15, and 30 days. Because an inexpensive humidity chamber to provided 95% humidity was not available, an inexpensive humidity chamber was engineered from materials purchased at Target and found in the laboratory. Additionally, silane gold-coated slides were optimized for immunofluorescence staining. Immunocytochemistry staining showed that after 30 days, there was a significant reduction in Gal^+ cells and in $CD45^+$, Class I, and Class II antigens ($p < 0.05$), all of which are associated with immunorejection of porcine islets. Additionally, there was a significant increase in insulin production over a 30-day culture period ($p < 0.05$), as well as a significant reduction in mononuclear cells that are responsible for immunorejection. Furthermore, viability staining showed that a culture period of 30 days provides a rehabilitative environment for islets that experience stress after extraction ($p < 0.05$). Finally, RT-PCR was done to validate results. RT-PCR showed no significant change in gene expression of insulin over a 30-day culture period ($p > 0.05$). As a result of our work, our research site is now running quality control trials to determine efficacy of incubating all pre-transplantation adult porcine islets for 30 days. Suarez-Pinzon W, Clark B, Godishala P. A novel protocol for culturing adult porcine islets for transplantation in Type I diabetic patients. *Minnesota Academy of Science Journal of Student Research* 2015; **3:**1-11.

Keywords: Type 1 diabetes, adult porcine islet cells, xenotransplantation, xenoresponse, islet culture period

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Abbreviations: ANOVA, Analysis of variance; DAPI, 4',6-diamidino-2-phenylindole; dH_2O, distilled water; DNA, Deoxyribonucleic acid; FITC, Fluorescein isothiocyanate; Gal, Galα-[1,3]Gal; PBS, Phosphate buffered saline, PIM, Prodo islet media, RNA, Ribonucleic acid; RT-PCR, Reverse transcription polymerase chain reaction; SLA-DQB, Swine leukocyte common antigen DQB allele; SLA-DRB, Swine leukocyte common antigen DRB allele; ΔCT, Normalized gene levels to cyclophillin; APIC, Adult porcine islet cells; APMNC, Adult porcine mononuclear cells, NOD, Non obese diabetic; TRITC, Tetramethylrhodamine

INTRODUCTION

Type 1 diabetes is an autoimmune disease that results in destruction of pancreatic islets that produce insulin[1]. Without insulin-producing islets, blood glucose levels rise, resulting in serious health problems for those who suffer from type 1 diabetes. Although type 1 diabetes can be managed by daily insulin injections, there is no permanent cure for this chronic disease. The purpose of our study was to improve a procedure for culturing islet cells that has the potential for use as a one-time treatment for type

1 diabetes. We were inspired to undertake this study because we both have close family members who suffer from diabetes.

In 1966, the University of Minnesota pioneered pancreas transplantation for chronic patients afflicted with type 1diabetes. Initially, the entire pancreas was transplanted from a deceased donor; however, scientists later discovered that type 1 diabetes affects insulin-producing β-cells in pancreatic islets[2]. Subsequently, islet cell therapy quickly developed.

Because there is a limited supply of suitable deceased pancreatic organ donors to supply the demand for human islets, scientists turned to swine for an alternative source of islets. Pig-to-human islet transplantation (xenotransplantation) has advantages over human-to-human islet (allogeneic) transplantation. Although slightly different in shape, porcine islets are closely related biologically to human islets[3]. Since adult porcine islets can be prepared from healthy pigs that are raised locally, donor pancreata are not subjected to long transportation times that can result in tissue deterioration. Furthermore, between 75,000 and 650,000 islets can be isolated and cultured from a single healthy porcine pancreas[4,5].

While xenotransplantation shows great promise as a possible therapy for type 1 diabetes, before xenotransplantation can be viable, four problems must be addressed. First, mononuclear cells, which are leukocytes that remain in islet cells after extraction, must be eliminated since they produce an autoimmune response to insulin producing β-cells[1]. Secondly, expression of the complex carbohydrate called Galα-[1,3]Gal (Gal), which is found on the endothelial surface of porcine mononuclear cells and islet cells, must be suppressed[6]. Expression of Gal during xenotransplantation results in rapid immunorejection of the islets[7]. In order to prevent immunorejection, scientists have bioengineered Gal-free pigs for xenotransplantation; however, these pigs cost up to three times more and are not as healthy as Gal^+ pigs[8]. For xenotransplantation to be economically feasible, a way to repress Gal expression without the need to genetically modify pigs must be found. The third problem that must be addressed is the presence of $CD45^+$, Class I, and Class II antigens that are naturally expressed on the surface of porcine mononuclear cells but are foreign to humans. These antigens are a problem following transplantation because they trigger a xenoresponse that destroys islets[9-12]. Fourth, islets experience stress (necrotic tendencies) during islet isolation when the pancreas is shaken to isolate porcine islets[4]. The shaking process results in islets succumbing to a comatose-like state, rendering them ineffective. Currently, in order to eliminate Gal^+ cells as well as $CD45^+$, Class I, and Class II antigens, blood is flushed out of the pancreas during porcine pancreatic procurement; however, this flush does not eliminate mononuclear cells in islets; therefore, the antigens are still expressed on the surfaces of the mononuclear cells in the islets[4].

After looking closely at studies that outlined these problems, it occurred to us that the solution might be to extend the standard seven-day islet culture period. We hypothesized that a longer culture period would reduce the numbers of mononuclear cells, Gal^+ cells and $CD45^+$, Class I, and Class II antigens, thus reducing the problem of immunorejection while providing a rehabilitative environment for comatose-like islets. We proposed culturing for 30 days based on the length of our summer research period, and selected 15 days because it was a midpoint. We also used seven days as a baseline. In undertaking this study, our specific goals were:

- To determine if a culture period longer than the standard seven days would reduce Gal^+ cells and expression of $CD45^+$, Class I, and Class II antigens as well as numbers of mononuclear cells in porcine islets while providing a rehabilitative environment for comatose-like islets;
- To determine if islets remain viable during the longer culture period;
- To engineer an inexpensive humidity chamber for use during immunofluorescence staining that would maintain a 95% humidity environment without condensation;

- To optimize slides for immunofluorescence staining; and
- To validate results by determining gene expression of the insulin, Class I, and Class II genes using RT-PCR.

MATERIALS AND METHODS

All protocols were conducted in a BSL2 environmental safety level laboratory.

Islet Culturing: The following islet culturing protocol was designed by the authors to replicate an *in vivo* environment. From six porcine donors sacrificed at the University of MN for other studies, 5,000 whole porcine islets were incubated at 37 °C + 5% CO_2 for 7, 15, and 30 days in PIM(S) islet media C GMP standard (PRODO Laboratories Inc. Irvine, CA) with heat-inactivated 10% porcine serum (Grand Island, NY), which are both ideal for islet rehabilitation and growth[1]. A culturing temperature of 37 °C was chosen based on a study by Mueller *et al.*[13] that found culturing islets at 37 °C is advantageous over culturing at 22 °C. The islets were then distributed into two aliquots of 2,500 islets each: one for cellular characterization and one for mononuclear characterization. Islets were fed by replacing 50% of the PIM(S) islet culture media with fresh culture media every 48 hours.

Islet Disassociation: To prepare islets for cellular characterization, an islet disassociation protocol outline by Suarez-Pinzon *et al.*[1] was used to separate mononuclear cells from islet cells, with the following modifications[1]. Intracellular connections within whole islets were disrupted by re-suspending the islets in enzyme-free phosphate buffer saline (PBS) based cell disassociation buffer (Invitrogen Grand Island, NY). Because the standard disassociation procedure that calls for disruption with 14, 16, 18, 20, and 22 gauge needles proved to be harmful to porcine islets, disruption was done using repetitive needling with 18, 20, and 22 gauge plastic needle heads. Mononuclear cells were separated from islet cells into two fractions by filtration using a 20-micron filter. (Dissociation does not totally eliminate mononuclear cells in islets; therefore, antigens are still expressed on the surfaces of the remaining mononuclear.) Viability staining was conducted twice per culture period on disassociated islet cells stained with acridine orange (Sigma-Aldrich, St. Louis, MO) and propidium iodine (Sigma-Aldrich, St. Louis, MO). The total number of cells per mL in the islet cell fraction and the mononuclear cell fraction were counted using a Becton hemocytometer (San Jose, CA).

Engineering a Humidity Chamber: A humidity chamber was needed for immunofluorescence staining; however, a working humidity chamber was not available, so it was necessary to engineer an inexpensive humidity chamber from readily available materials that would provide a perfectly level 95% humidity environments without condensation. Paper towels were laid into an 18 x 30 cm plastic cupboard box (Target, MN). To infuse humidity into the chamber, distilled water (dH_2O) was generously poured over the paper towels until total saturation. Four Corning Costar six-well incubation plates (Ithaca, NY) with lids were laid parallel to the long sides of the cupboard box in a 2 x 2 plate arrangement. Caps were taken from 24 75-cm suspension flasks (Starstedt, Germany) and placed into a 2 x 3 formation on each well plate, leaving space for placement of 12 slides for immunofluorescence staining. More paper towels were cut and wedged in between the well plates and the inside edge of the box to prevent movements of the well plates. Supplementary dH_2O was added to fully drench the paper towels.

Optimizing Silane Gold-coat Slides for Immunofluorescence Staining: Since there are no commercially available inexpensive slides for immunofluorescence staining that are ideal for adult porcine islet cells, it was necessary to optimize silane gold-coated slides (LabScientific, Irvine, NY) for use in porcine islet immunofluorescence staining. This was done by etching three 5-mm diameter circles (smears sites) into 145 silane gold-coat slides, using a Diamond Collection diamond tipped pen (Amazon). To distinguish hydrophobic environments around each smear site, a Pentel gel wax pen (Amazon) was run around each smear site.

Immunofluorescence Staining: At the cellular level, immunofluorescence staining was chosen to characterize porcine cells based on studies by Suarez-Pinzon *et al.*[9], Bornstein *et al.*[14], and Kiliminik *et al.*[15] that suggested immunofluorescence staining best enables visualization of antibody binding of all staining methods available. Islet-cell and mononuclear-cell fractions were fixed with 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA). Fixed islet-cell and mononuclear-cell fractions were seeded onto the optimized silane gold-coated slides in three smears of 2,500 cells per smear per slide. Three smears per slide were done to compare the control to two antibody stains on one three-well slide.

Choosing Primary and Secondary Antibodies: Primary antibodies were chosen as follows. Anti-Galα-(1,3) (Gal) (Sigma-Aldrich, St. Louis, MO) was chosen to visualize Gal^+ cells based on work by Kilimnik *et al.*[15]. Porcine anti-insulin (Abcam, England, UK) was selected to determine numbers of islet cells that stained positive for insulin based on work by Shizuru *et al.*[16]. Anti-porcine CD45 (Novas, St. Louis, MO) was selected to visualize presence of CD45 on mononuclear cells based on a study by Bas-Bernadet & Blancho[2], and anti-swine leukocyte antigen Class I antibody (Serotec, Hercules, CA) was chosen to visualize cells that stained positive for Class I antibodies based on work by Chen *et al.*[11] that showed Class I causes immunorejection by activating a T-cell response. Furthermore the anti-swine leukocyte Class II antigen (Sigma-Aldrich, St. Louis, MO) was chosen to visualize cells that stained positive for Class II atigens based on a second study by Chen *et al.*[12] that found Class II also causes immunorejection by activating a T-cell response.

Primary antibodies were added to each smear site on the optimized silane gold-coated slides with Fluoroshield mounting medium with 4',6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO) to stain nuclei. In order to visualize the primary antibodies under a fluorescent microscope, the following secondary fluorescein antibodies were chosen for their compatibility with the primary antibodies and for their fluorescence properties: goat anti-rabbit TRITC (Vector Labs, Burlingame, CA) was used to visualize Gal, while rabbit anti-mouse FITC (Santa Cruz Biotechnology, Santa Cruz, CA) was used to visualize insulin and CD45. Secondary antibodies for Class I and Class II were commercially conjugated with FITC (Thermo Scientific,Waltham, MA). Porcine islet cells were incubated with the primary and secondary antibodies.

Immunocytochemistry Staining: Slides from islet-cell and mononuclear cell fractions were sealed using prolong gold antifade reagent (Carlsbad, CA) and kept at 4 °C overnight prior to quantification of cells under a LEICA fluorescent microscope (Solms, Germany). Six different fields per section at 20x magnification were observed by counting a minimum of a hundred cells per microscopic field. Nuclear counterstaining was observed to determine numbers of cells in each field. Percent of positive cells was quantified as a percent of total cells. Frequencies of cells that stained positive for Gal, insulin, and $CD45^+$, Class I, and Class II antigens were compared after 7, 15, and for 30 days of culturing.

Analysis: Frequencies of cells that stained positive as percent of the total were reported in triplicates as the mean ± standard error of the mean. The mean of the triplicate was calculated, and the standard error of the mean was calculated from the standard deviation of the triplicates. Significance was determined by a one-way ANOVA, followed by a Tukey-Kramer post-hoc test to compare the three time points in a pair-wise manner. Significance was set at $p \leq 0.05$.

Validating Results: The following RT-PCR procedure was run to validate results. RNA was extracted from two samples of 2,500 undissociated islets taken at 7, 15, and 30 days, using a RNeasy mini kit (Hilden, Germany). In order to assess the RNA concentration, a Nanodrop spectrophotometer (Hercules, CA) was used. RNA quality was determined using a 2100 expert Eukaryote Total RNA Nano Kit (Santa Clara, CA). Then, 2 µg of RNA were converted to cDNA with a Quantitect reverse transcription kit (Hilden, Germany).

The following genes were chosen: insulin, SLA-DQB, and SLA-DRB. Insulin was chosen based on a

study by Mueller *et al*.[13] that affirmed the importance of the insulin gene. SLA-DQB and SLA-DRB were chosen because DQ and DR alleles cause immunorejection by activating a T-cell response[11,12]. Insulin, SLA-DQB, and SLA-DRB primers were selected to complement the genes. Perfecta SYBR green fastmix (Quanta Biosciences, Gaithersburg, MD) was added to the cDNA along with 100 nM of the gene-specific primer sets (Quanta Biosciences, Gaithersburg, MD) and a 6 ng sample of adult porcine islets. Then, qRT-PCR was performed using an ABI 7500 instrument (Pittsburgh, PA). Each gene marker was run in a triplicate. Cyclophillin, an internal control/reference gene (housekeeping gene), previously synthesized in the lab from swine, served as the internal control, and RNAse-free water (Sigma-Aldrich, St. Louis, MO) served as the negative control. This internal control gene was chosen based on a study by Schmid *et al*.[17] that showed cyclophillin to be an optimal housekeeping gene because of its consistency. Results were recorded as the mean gene expression (C_t) value, normalized to the cyclophillin mean (ΔC_t).

Analysis: Analysis was performed by determining a semi-quantitative score of the marker gene expression with respect to the cyclophillin reference for each individual pig donor ($n = 6$). Significance in gene expression during culture for 7, 15, and 30 days was compared using a one-way ANOVA. Significance was set at $p \leq 0.05$.

RESULTS

Humidity chamber: The design for the humidity chamber provided a uniform 95% humidity environment that showed no condensation on the chamber walls during each incubation period. Figure 1 shows the average number of deceased islets and mononuclear cells out of an average cell total of 85 at each culture period. The mortality rate for islets and mononuclear cells was less than 5% for all culture periods, which indicates the effectiveness of the humidity chamber.

Islet Cell and Mononuclear Cell Count: Figure 2 shows total numbers of islets and mononuclear cells after each incubation period. There was no significant decrease in the total numbers of islet among the three culturing periods (7, 15, and 30 days) ($p = 0.181$); however, there was a significant decrease in total numbers of mononuclear cells after 30 days of culturing compared to a 7 day culture period ($p = 0.049$).

Cell Viability: Figure 3 shows total numbers of viable cells in the islet cell fraction and in the mononuclear-cell fraction. Change in total numbers of viable islets among the three culturing periods was not significant ($p > 0.05$); however, there was a significant decrease in the number of viable mononuclear cells from day 7 to day 30 ($p < 0.05$).

Immunocytochemistry Staining: Figure 4 shows that while numbers of Gal^+ cells in the islet cell fraction significantly increased from the 7-day culture period to the 15 day culture period ($p > 0.05$), numbers of Gal^+ cells in the islet fraction significantly decreased from the 15 day culture period to the 30-day culture period. Gal^+ cells in the mononuclear cell fraction significantly decreased after the 15 day and 30 day culture periods compared to the 7-day culture period ($p < 0.05$).

Figure 5 shows a significant increase in insulin production in the islet cell fraction after the 30 day culture period compared to the 7 day culture period ($p < 0.05$). (Insulin production in the mononuclear cell fraction was negligible, so was not reported.)

Figure 6 shows there was a significant decrease in presence of CD45 on surfaces of mononuclear cells in the mononuclear cell fraction after a 30-day culture period compared to the 7 day culture period ($p < 0.05$). (Presence of CD45 was negligible in the islet cell fraction, so was not reported.)

Figure 7 shows a significant decrease in presence of Class I antigens on surfaces of mononuclear cells remaining in the islet cell fraction and on mononuclear cells in the mononuclear cell fraction after a 30-day culture period compared to the 7 day culture period ($p < 0.05$).

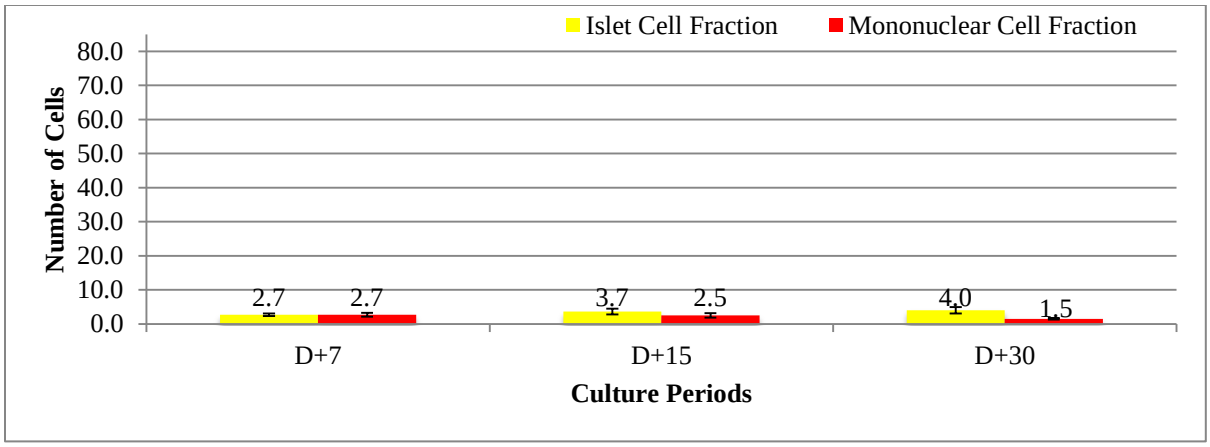

Figure 1. Average number of deceased islet and mononuclear cells during testing of the humidity chamber. Total average numbers of deceased cells plotted against culture periods. Bars show standard error of mean for deceased cells. (D+7 = seven-day culture period) (D+15 = 15 day culture period) (D+30 = 30 day culture period)

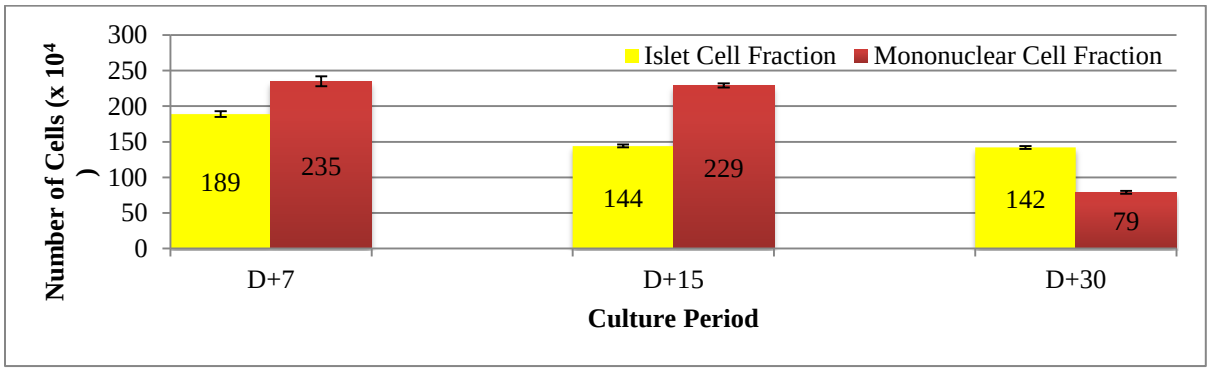

Figure 2. Total numbers of islets calculated and mononuclear cells after each incubation period. ANOVA, followed by Tukey post hoc test was done to find significance among culture period. Standard error of the mean was calculated from the values above, and error bars express the mean ± SEM (standard error of the mean). Significance was set at $p < 0.05$ ($n = 6$)

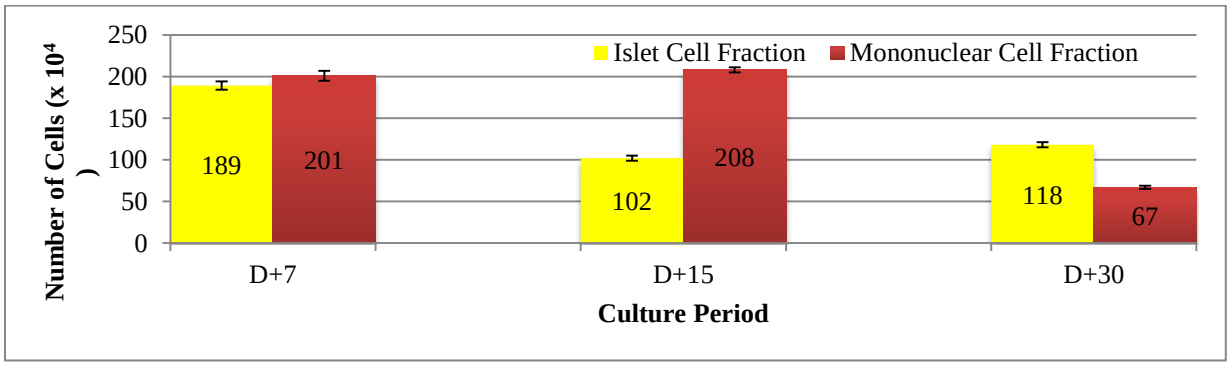

Figure 3. Total numbers of viable cells in the islet cell fraction and in the mononuclear cell fraction**.** The bars show the mean ± SEM of all six pigs calculated from triplicate measurements for each pig. One-way ANOVA followed by a Tukey-Kramer post-hoc test was done to compare the three culture periods in a pairwise manner, with significance set at $p \leq 0.05$. ($n = 4$ for D+7) ($n = 6$ for D+15 and D+30)

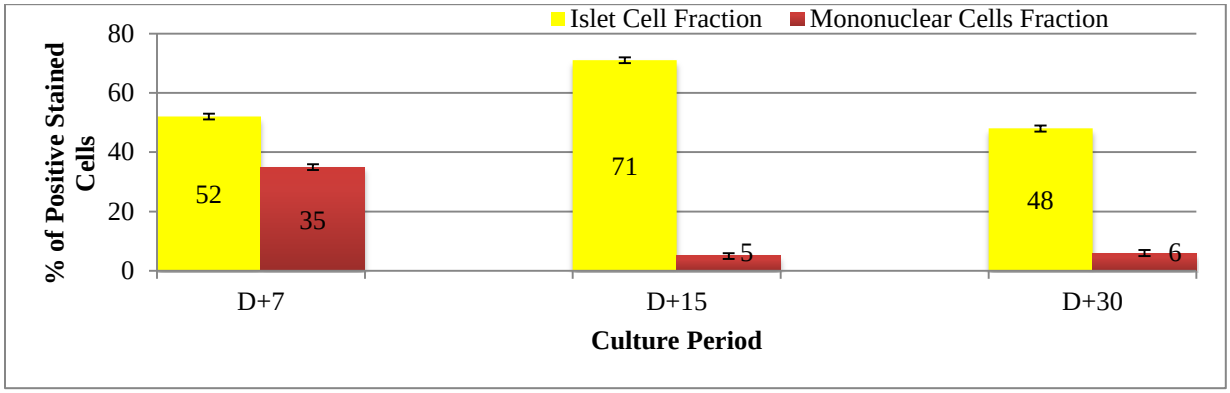

Figure 4. Average stain results for Gal$^+$ cells in the islet cell fraction and in the mononuclear cells fraction. ANOVA, followed by Tukey-Kramer post hoc test was done to find significance among time points, with significance was set at $p \leq 0.05$. Error bars represent the mean of each culture period ± SEM calculated from four measurements for each pig. Significance was set at $p < 0.05$. (n = 4 for D+7) (n = 6 for D+15 and D+30).

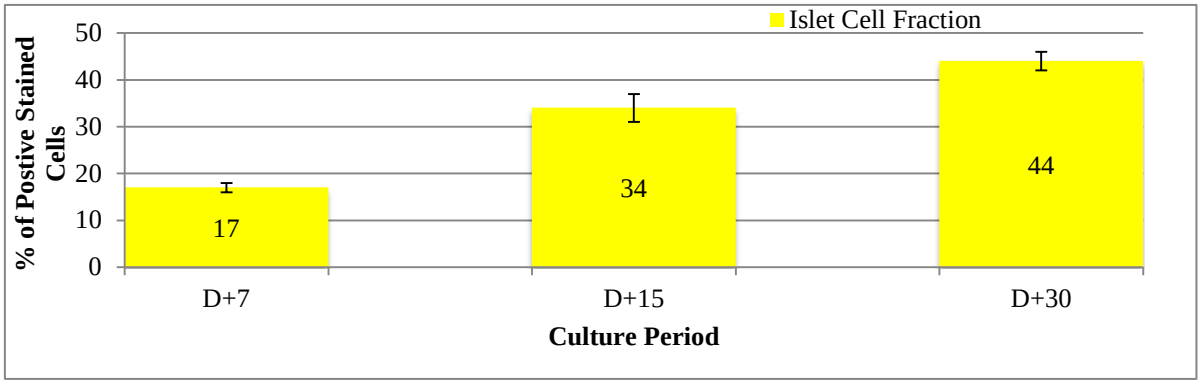

Figure 5. Average stain results for insulin production in the islet cell fraction. ANOVA, followed by Tukey-Kramer post hoc test was done to find significance among time points, with significance was set at $p \leq 0.05$. Error bars represent the mean of each culture period ± SEM calculated from four measurements for each pig. Significance was set at $p < 0.05$. (n = 4 for D+7) (n = 6 for D+15 and D+30)

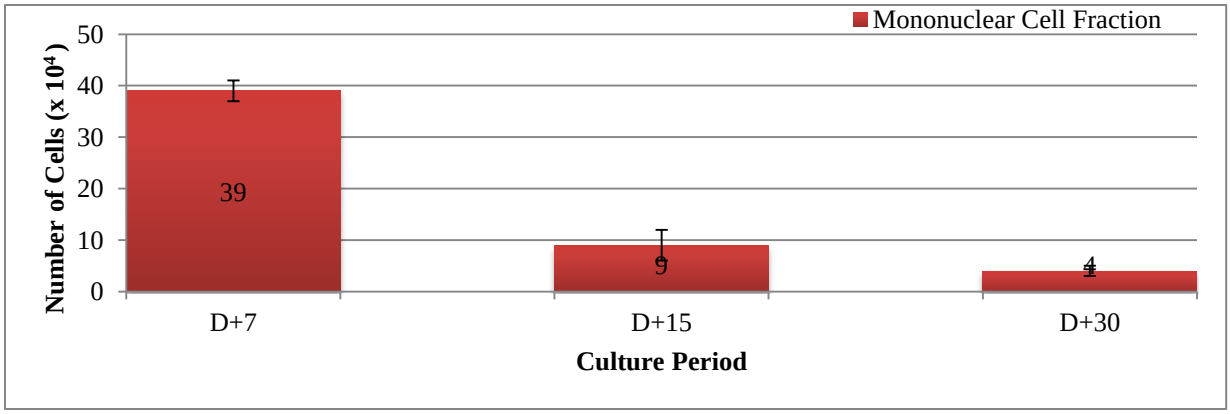

Figure 6. Average stain results for presence of CD45 in the mononuclear cell fraction. ANOVA, followed by Tukey-Kramer post hoc test was done to find significance among time points, with significance was set at $p \leq 0.05$.

Error bars represent the mean of each culture period ± SEM calculated from four measurements for each pig. Significance was set at $p < 0.05$. (n = 4 for D+7) (n = 6 for D+15 and D+30).

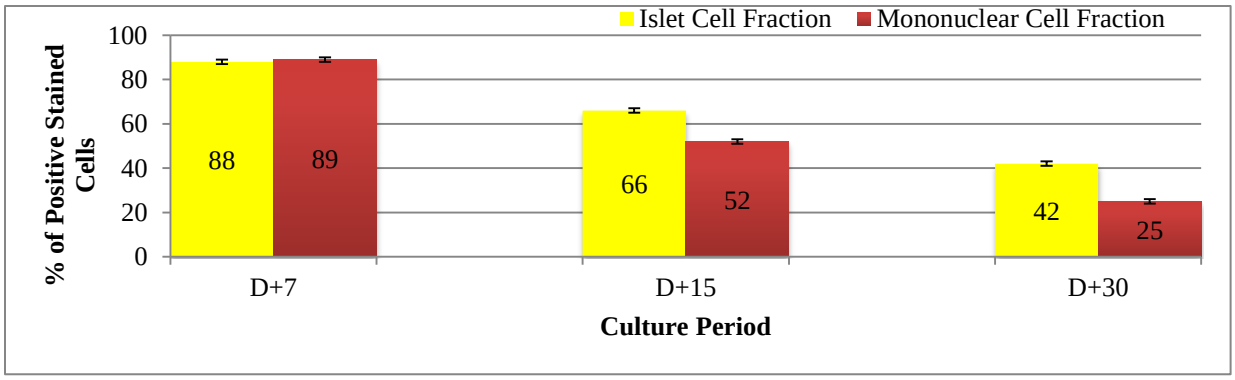

Figure 7. Average stain results for presence of Class I antigens on mononuclear cells remaining in the islet cell fraction and on mononuclear cells in the mononuclear cell fraction. ANOVA, followed by Tukey-Kramer post hoc test was done to find significance among time points, with significance was set at $p \leq 0.05$. Error bars represent the mean of each culture period ± SEM calculated from four measurements for each pig. Significance was set at $p < 0.05$ (n = 4 for D+7) (n = 6 for D+15 and D+30).

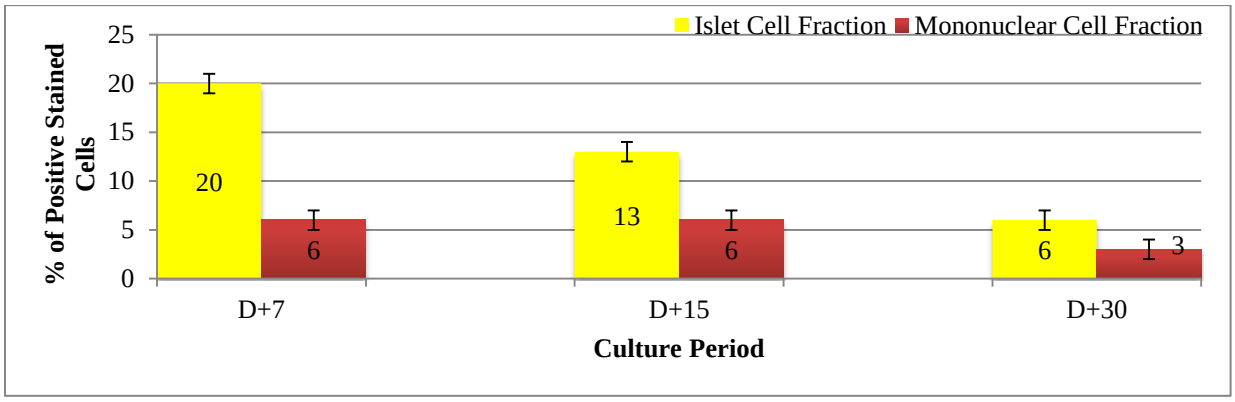

Figure 8. Average stain results for Class II antigen expression by mononuclear cells remaining in the islet cell fraction and by mononuclear cells in the mononuclear cell fraction. ANOVA, followed by Tukey-Kramer post hoc test was done to find significance among culture periods, with significance was set at $p \leq 0.05$. Error bars represent the mean of each time point ± SEM calculated from four measurements for each pig. Significance was set at $p < 0.05$. (n = 4 for D+7) (n = 6 for D+15 and D+30).

Figure 8 shows a significant decrease in presence of Class II antigens on surfaces of mononuclear cells remaining in the islet cell fraction and on mononuclear cells in the mononuclear cell fraction after a 30-day culture period compared to the 7 day culture period ($p < 0.05$).

Validating Results: Figure 9 shows ΔCT gene expression values normalized to cyclophillin of insulin, SLA-DQB (gene for the class I antigen), and SLA-DRB (gene for the class II antigen) for the 7, 15, and 30-day culture. There was no significant change in insulin gene expression ($p > 0.05$), no significant change in SLA-DQB gene expression ($p > 0.05$), and no significant change in SLA-DRB gene expression ($p > 0.05$).

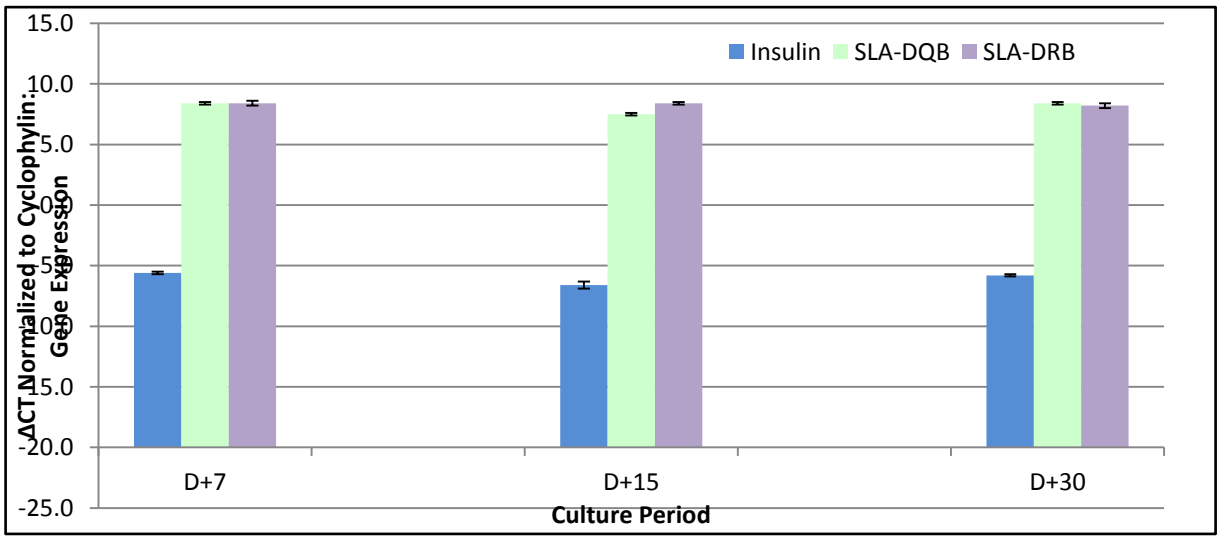

Figure 9. Average ΔCT gene expression values normalized to cyclophillin of insulin, SLA-DQB (gene for the class I antigen), and SLA-DRB (gene for the class II antigen). ANOVA was done to find significance among culture period. Standard error of the mean was calculated from the values, and error bars express the mean ± SEM (standard error of the mean). Significance was set at $p < 0.05$ ($n = 6$).

DISCUSSION

After successfully engineering an inexpensive humidity chamber that provided a uniform 95% humidity environment and optimizing slides for immunofluorescence staining, we successfully improved a protocol for culturing porcine islets for xenotransplantation by lengthening the culture period from 7 to 30 days. The 30 day culture period has not been tried prior to this study.

Results supported our hypothesis. Cell counts done on adult porcine islets at 7 days, 15 days, and 30 days showed that at 30 days there were significantly fewer mononuclear cells. Staining for cell viability also showed that a culture period of 30 days provided a rehabilitative environment for comatose islets. Furthermore, immunofluorescence staining showed that after 30 days of culturing there was a significant reduction in Gal^+ cells as well as in $CD45^+$, Class I, and Class II antigen expression, all of which are associated with immunorejection of porcine islets. Additionally, results were validated by RT-PCR that showed gene expression for Class I and Class II antigens continued; therefore, the significant decrease in these antigens was due to the longer culture period. Moreover, RT-PCR showed that insulin production did not change over the course of the culture period. This suggests that a 30-day culture period will result in less chance of immunorejection after xenotransplantation.

Due to the length of the summer, we were unable to culture the islets for a period longer than 30 days, given the time required for planning and analysis. Future work should include extending the culture period beyond 30 days to determine if islets remain viable and if the longer culture period further reduces mononuclear cell counts, Gal^+ cells, and expression of $CD45^+$, Class I, and Class II antigens that cause immunorejection after xenotransplantation.

As a result of our work, our research site is now running quality control tests on the efficacy of incubating all pre-transplantation adult porcine islets for 30 days. Following quality control tests, a 30-day pre-transplantation pre-clinical trial will be run using a NOD mouse model to determine length of time that mice are independent of exogenous insulin as a result of a longer culture time for islet xenotransplantation with no immunosuppressive drugs.

In conclusion, we went against the established concept that islets would not remain viable in culture longer than seven days. Our study shows that untrained young people are able to generate fresh ideas and design procedures that contribute to important research because we are not afraid to think outside the box.

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