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Research Article

Accelerated Porcine islet Maturation Through Efficient Isolation and Culture Methods: Neonatal and Juvenile Aged Pigs

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Abstract

Successful porcine islet isolation is crucial for making xenotransplantation as a treatment for type 1 diabetes. Having a protocol that efficiently produces maximal amount of intact islets, while maintaining their quality and functionality, would allow xenotransplantation to be applicable for clinical use. In our previous protocol, islets were isolated and cultured for 7-9 days to mature. We revised our protocol's isolation methods and culture media, decreasing the length of culture to 3 days. Pancreas tissue was mechanically and enzymatically digested, and cultured till 7 days. Quality control tests were performed on days 3 and 7, such as islet yield, purity, viability, function, and cellular content. Neonatal islet yield was 1.48±0.21x104IE/g of pancreas on day 3 and decreased to 8.02±1.60x103IE/g on day 7. Juvenile islet yield was 3.43±0.53x103IE/g on day 3 and 4.11±1.06x103IE/g on day 7. Neonatal and juvenile islets were both functional by day 3 with an SI of 1.66±0.11 and SI of 1.86±0.14, respectively. Insulin-positive cells decreased from 627.0±54.9IE/g on day 3 to 466.8±36.8IE/g on day 7 for neonatal islets, while juvenile islets' insulin-positive cells increased from 370.4±127.5IE/g on day 3 to 514.3±136.9IE/g on day 7. The results suggest that this method of isolating porcine islets is able to accelerate their maturation process by only requiring 3 days of culture, improving efficiency and accessibility for transplantation.

Keywords: Neonatal Porcine Islets, Juvenile Porcine Islets, Islet Isolation, Islet Culture, Type 1 Diabetes Mellitus

Introduction

Diabetes mellitus type 1 (T1D) is an autoimmune disorder characterized by the gradual destruction of insulin producing β-cells in the Islets of Langerhans (11095106). The function of pancreatic β-cells is to store and release the peptide hormone insulin, which primarily regulates the metabolism of carbohydrate, protein, and fat in response to changes in plasma glucose concentration (22974359). The loss of β-cells can impair insulin secretion, which results in dysregulation of blood glucose, hyperglycemia, and ultimately, T1D (9720788). Untreated T1D can cause irreversible damage to abdominal organs, diabetic coma, and death. Lifelong exogenous administration of insulin is currently being used as a treatment to manage T1D. Even though it has improved quality of life, daily treatment using intensive insulin therapy can lead to a threefold increase in the rate of severe hypoglycemia, which can be associated with coma and/or seizure. In addition, this practice

is inconvenient to manage and can cause daily stress on a patient. Allotransplantation of insulin-secreting islets by way of the Edmonton protocol can restore euglycemia, result in long-term insulin independence, and has been shown to be a promising cure for T1D (1738936, 12499894, 10911004, 12086945). Still, this treatment is hampered by challenges, and a major one is the scarcity of cadaveric donors. Donor selection is very particular because donor age, body mass index, and health affect the quality of islets isolated (19110649). To universalize islet transplantation as a treatment, a need for more donors and maximal islet production must be recognized.

In response to the high demand for islets, xenotransplantation of porcine islets has been strongly studied and shown as a viable alternative for islet transplantation. Pigs are appropriate donors in that they are comparable to human physiology and pancreatic molecular structure. Their supply is unlimited, and their husbandry is unchallenging. On the contrary, xenogeneic porcine islets can consequently introduce different species' pathogens and have a risk of rejection (16719044). However, methods are being tested to potentially overcome these issues, such as encapsulation of porcine islets in biocompatible immunoisolation material prior to xenotransplantation (25990050). Several studies have shown that encapsulating islets lengthens their survival (11473027, 12518896, 16491083, 16501570). Moreover, pig donor age affects the islet yield and functionality, and the optimal ages that have shown a prolonged T1D reversal in non-human primates or humans are neonatal and adult pigs (25091986). However, neonatal pancreases are limiting due to month-long maturation processes to develop β-cells and function sufficiently *in vivo* (25676501, 12005100). Adult pig pancreases are more probable to vary from one another. Additionally, adult pancreases result in substantial loss during mechanical and enzymatic digestion and in *in vitro* culture considering the weight of the pancreas (23394130, 17214707), which is why our protocol focuses on neonatal pigs. In addition, we applied our protocol to juvenile pigs because their islets have the potential to restore and sustain euglycemia in diabetic athymic nude mice in our previous study (25793438).

The need for a reproducible, accessible, and cost-effective method of porcine islet isolation and culture is critical for universal utilization in clinical transplantations. Our standard protocol was able to address those needs (23394130), but reducing culture time can be even more advantageous. The standard protocol cultured young porcine islets for up to 7-9 days while the revised protocol, adjusting isolation methods and culture media, was able to shorten the culture time to 3-4 days. This protocol produces viable, mature islets in a shorter time while also maintaining a significant amount. Our revised protocol aims to be reproducible and expedite the use of porcine islets in xenotransplantations for clinical use to treat patients with T1D.

Materials and Methods

Pancreas Procurement and Digestion Process

Donor pancreases were obtained from neonatal and juvenile (3-6 & 9-12 day-old, respectively), pre-weaned Yorkshire pigs (S&S Farms, Ramona, CA) of either sex. All animal procedures including but not limited to monitoring, surgery, and euthanasia were done with approval by the University of California, Irvine Institutional Animal Care and Use Committee (IACUC). Pancreases were removed using a rapid surgical procurement (≤10 mins) and placed in cold (4°C) HBSS (Gibco, cat#24020117) till digestion. After cold ischemia (<2 hrs), using aseptic sterile technique from this point on, pancreases were transferred out of the HBSS into a 50mL conical each and weighed. Each pancreas was mechanically digested using 2 curved blunt/blunt 18cm Metzenbaum scissors (14019-18, Fine Science Tools Inc., Foster City, CA) to cut till the tissue fragments were a majority in 1mm3 in size, while 3-4 washes of HBSS were performed in between to get rid of blood, connective tissue, fat, lymph nodes, etc. When all fragments were in 1-2mm3 in size, all pancreases were combined into one, mixed, and split into 50mL conical tubes (~2g tissue/tube). Tissue was washed with HBSS and centrifuged at 1000rpm for 1min, and supernatant was vacuumed. To enzymatically digest the pancreas further from acinar tissue, enzyme solution (8.33mg/g tissue, dissolved for >30 mins at 2-8°C in HBSS, Sigma-Aldrich, cat#C8051) was added till ending volume of 25mL of tissue with solution. Tubes were parafilmed and placed in a water bath at 37°C at 100rpm for 15 minutes to activate the enzyme. Tubes were swirled every 3 minutes during water bath to break the pellet. After the 15 minutes, tubes were hand shook vigorously side to side for 30 seconds. Enzyme activity was stopped immediately after by filtering the islet tissue clusters through a 500μm metal mesh and washing the mesh with HBSS supplemented with 1% porcine serum (Gibco, cat#26250084). To make sure enzyme was inactivated, islet tissue clusters were centrifuged and fully washed twice more with neutralization solution. Islet tissue clusters were then transferred to a new culture media we created called UCI media: Ham's F-12 medium (Corning Inc., cat#10-080) supplemented with HEPES (Sigma-Aldrich, cat#H3375), L-glutathione (Sigma-Aldrich, cat#G4251), ITS+3 (Sigma-Aldrich, cat#I2771), nicotinamide (Sigma-Aldrich, cat#N5535), gentamycin sulfate (Corning Inc., cat#30-005-CR), trolox (Sigma-Aldrich, cat#238813), heparin (Sagent Pharmaceuticals, cat#400-10), pefabloc (Santa Cruz Biotechnology, cat#sc-202041B), L-glutamine (Alfa Aesar, cat#56-85-9), medium 199 (Corning Inc., cat#50-051-PB), calcium chloride dihydrate (Fisher Scientific, cat#C79-3), DNase (Sigma-Aldrich, cat#D4263), antibiotic/antimycotic solution (Corning Inc., cat#30-004-CI), and 10% porcine serum. Islets were cultured in a T-225 untreated suspension flask (Corning Inc., Corning, NY) at 37°C and 5% CO2 to mature into islets. Islet clusters density in the flask was approximately 2g of digested tissue in 30mL of UCI media.

Culture of Islets

Islets were cultured in a humidified incubator at 37°C, 5% CO2 (Thermo Forma Series II 3120 Water Jacketed CO2 Incubators, Carlsbad, CA) for up to 7 days, and media change was done to remove debris and exocrine cells every 48 hrs after day 1. Islets had a 100% media change on day 1 and day 3, and a 33% media change on day 5. Quality control of islets were performed on day 3, 4, 7, and 8.

Islet Yield & Islet Purity

Islet count (IC) and islet equivalence (IE) was obtained by collecting an aliquot of approximately 20-30 IE, staining them with 1mL dithizone (DTZ, MP Biomedicals, cat#150999) for 5 minutes, and counting them at 25x on a standard stereomicroscope (Max Erb, Santa Ynez, CA) with a 10x eye piece graticule (2075782, 23394130). Multiplying IC & IE by dilution factor and dividing by gram of digested tissue to get IC/g and IE/g. Islet purity was also determined through DTZ staining by the equation: (area of islet with positive DTZ staining/total area of islet) x 100 = % purity. Determine % purity of 5 islets in the DTZ aliquot and calculate the average.

Islet Viability

An aliquot of 100 IE islets were stained with calcein AM (Invitrogen, cat#C1430) and propidium iodide (Invitrogen, cat#P3566) (23304445). Viability-stained islets were analyzed through fluorescence with a Microplate reader (Tecan Infinite F200; Tecan, Mannedorf, Switzerland). Viability percentage is calculated by the equation: live/(live+dead) x 100.

Islet Function

A n=3 of 100IE islets per sample were incubated at 37°C and 5% CO2 for 1 hour in each media in the corresponding order: low glucose (2.8 mM; L1), high glucose (28 mM; H), high glucose plus 3-isobutyl-1-methylxanthine (28mM + 0.1mM IBMX; H+), and again in low glucose (2.8 mM; L2) (11579292, 25793438). Supernatant was collected and stored at -20°C for analysis. Insulin concentration released during incubation were measured by using a porcine insulin enzyme-linked immunosorbent assay (Porcine Insulin ELISA, Mercodia, cat#10-1200-01). Absorbance was measured using a Microplate reader (Tecan Infinite F200 and Magellan V7) and expressed as (ug/L)/IE. Stimulation index (SI) was calculated as the ratio of insulin concentration secreted in high glucose over the insulin concentration secreted in the first low glucose.

Flow Cytometry Assay

Islets were washed twice with DPBS (cat#14190250, Thermofisher Scientific) and dissociated with Accutase (cat# AT104-500, Innovative Cell Technologies) for 15 minutes in a 37°C, 100 rpm shaking water bath to obtain a single cell suspension (15943621). The cell suspension was filtered through a 40µm filter (VMR, Visalia, CA) to remove any debris and un-dissociated islet clusters. Cell samples were stained with 7-AAD (7-aminoactinomycin D; cat#A1310, Invitrogen) for 30 minutes on ice to detect live and dead cells. The cells were fixed with 4% paraformaldehyde for 10 minutes on ice and permeabilized with Intracellular Staining Permeabilization Wash Buffer (cat#421002, BioLegend) for 15 minutes on ice. Cells were blocked with Protein Block (cat#ab64226, Abcam) to reduce nonspecific binding for 30 minutes on ice followed by staining with fluorescently conjugated antibodies for intracellular markers in Intracellular Staining Permeabilization Wash Buffer (cat#421002, BioLegend) with 0.5% bovine serum albumin (BSA; cat#BAL62-0500, Equitech-Bio, Inc.) for 30 minutes on ice. FITC conjugated anti-amylase (Anti-amylase-FITC; cat#ab21156, Abcam) was used as a marker for acinar cells, PE conjugated anti-insulin (Anti-insulin- PE; cat#8508, CST, Danvers, MA) was used as a marker for β-cells, and APC conjugated anti-glucagon (Anti-glucagon- APC; cat#NBP2-21803AF647, Novus Biological) was used as a marker for α-cells. Cell populations was then quantified using the NovoCyte 3000VYB Flow Cytometer (ACEA Biosciences, Inc., San Diego, CA). An unstained, single-stained, fluorescence minus one, and matching isotype control were used as controls. Cell populations were then analyzed using FlowJo software (FlowJo, Ashland, OR).

Immunohistochemical Evaluations of Islets

Pancreas tissue and islets collected on day 3 and day 7 were fixed in neutral buffer formalin (Cat # 22-026-354, Fischer Scientific) for at least 48 hours. The fixed samples were prepared for paraffin processing through serial dehydration in increasing concentrations of ethanol solutions using a Leica TP1020 tissue processor (Leica Microsystems, Buffalo Grove, IL). After preparation, the tissues were embedded in paraffin wax (Sigma Aldrich) using a Leica EG 1150C tissue embedder (Leica Microsystems). The tissue blocks were sectioned into 5µm sections using a Leica RM 2255 microtome (Leica Microsystems) and incubated at 65°C for one hour before staining. Mouse and Rabbit Specific HRP/DAB (ABC) Detection Kit (cat#ab64264, Abcam) was utilized to detect insulin-positive cells according to manufacturer protocol and slides were counterstained with hematoxylin.

Statistical Analysis

All data are expressed as mean ± standard error of mean (SEM). An unpaired t-test or a one-way ANOVA followed by a post-hoc Tukey's HSD test was performed to determine statistical significance. P-values <0.05 were considered to be statistically significant and $p<0.01$ were considered to be highly statistically significant.

Results

Young Porcine Islet Yield

To determine if culture time had a substantial difference in IC/g and IE/g, counts were evaluated at days 3 and 7 of culture. All counts were represented per gram of pancreas tissue digested since pancreases procured had different weights, therefore, normalizing the data for better comparison. Neonatal pig pancreases ranged in 2-6g of tissue; juvenile pig pancreases ranged in 4-6g of tissue. At day 3, neonatal tissue resulted in 1.11 ± 0.16x104 IC/g (n=12) and 1.48 ± 0.21x104 IE/g (n=11). At day 7, yield decreased significantly ($p<0.05$) and resulted in 6.07 ± 0.48x103 IC/g (n=11) and 8.02 ± 1.60x103 IE/g (n=11) (Table I). There was no apparent difference in size distribution between day 3 and day 7 islets. Juvenile pancreases, on the other hand, yielded less islets than neonatal. At day 3, juvenile tissue resulted in 2.89 ± 0.91x103 IC/g (n=8) and 3.43 ± 0.53x103 IE/g (n=7). At day 7, yield was similar to day 3, increased insignificantly (p=NS) and resulted in 3.22 ± 0.87x103 IC/g (n=8) and 4.11 ± 1.06x103 IE/g (n=8). Again, no apparent difference in size distribution between day 3 and day 7 islets.

Characterization of Islets

Purity and viability were collected to analyze the quality of the islets at their respective time of culture. In both age groups, purity and viability remained at reasonable levels throughout culture. Purity of the neonatal islets were not significantly (p=NS) different between day 3 (n=12) and day 7 (n=10) (83 ± 2% and 85 ± 2%, respectively), and increased by day 7 (Table 1, Figure 1A & 1C). Day 3 (n=8) and day 7 (n=8) purity (81 ± 3% and 77 ± 4%, respectively) of the juvenile islets was also not significantly (p=NS) different between each other, but decreased by day 7 (Figure 1B & 1D). Neonatal islets maintained a viability over 90% over 7 days of culture and both time points were not significantly (p=NS) different from each other, 93 ± 1% on day 3 and 92 ± 1% on day 7 (Figure 2A). Viability at or above 90% was also seen in juvenile islets, and both time points were not significantly (p=NS) different from each other, 90 ± 3% on day 3 and 94 ± 2% on day 7 (Figure 2B), but did increase over the culture period.

Functionality of Islets *in vitro*

To determine if islets were responding accurately to different glucose concentrations, glucose stimulated insulin release assays were performed on days 4 and 8. Insulin concentration (μg/L) was represented per IE to normalize all data for comparison. By day 4 neonatal islets had an SI of 1.66 ± 0.11 (L1: 2.16 ± 0.57x10-3μg/L/IE, H: 2.65 ± 0.41x10-3μg/L/IE) (Figure 4C), and they responded accurately following the trend of the different glucose concentrations (Figure 4A). By day 8 they were more responsive with an SI of 2.50 ± 0.31 (L1: 1.72 ± 0.32x10-3μg/L/IE, H: 3.30 ± 0.53x10-3μg/L/IE) (Figure 4C), and also followed the trend of different glucose concentrations (Figure 4A). Juvenile islets had an SI of 1.86 ± 0.14 (L1: 1.57 ± 0.50x10-3μg/L/IE, H: 3.16 ± 1.08x10-3μg/L/IE) (Figure 4D) by day 3, and were responsive to the different glucose concentrations (Figure 4B). They were greatly more responsive (Figure 4D) by day 7 with an SI of 3.04 ± 0.47 (L1: 4.29 ± 1.08x10-3μg/L/IE, H: 8.97 ± 1.94x10-3μg/L/IE) (Figure 4B).

Flow Cytometry Analysis

To confirm the viability of islets, dissociated single-cell suspension of islets was evaluated on day 3 and day 7 by flow cytometry after staining. Neonatal islets had significantly higher viable cells on day 3 compared to day 7. 93.8 ± 0.8% of neonatal islets were viable on day 3 and 86.3 ± 2.1% on day 7 ($p<0.01$) (Figure 4A). Juvenile islets had a viability of 90.2 ± 1.4% on day 3 and 90.51 ± 2.0% on day 7 (Figure 4B). No significant changes in viability of juvenile islets were found on day 3 and day 7, which was consistent to the viability results obtained from calcein AM and PI staining. Flow cytometry was also used to quantify the major cellular composition (insulin, amylase, and glucagon) of neonatal and juvenile porcine islets. The percentage of insulin positive cells in neonatal islets significantly increased from day 3 to day 7. Insulin positive cells in neonatal increased from 4.2 ± 0.7% on day 3 to 9.1 ± 1.6% on day 7 ($p<0.01$) (Figure 5A). Similarly, the percentage of insulin positive cells in juvenile islets was higher on day 7 but not statistically significant when compared to day 3. The percentage of insulin positive cells in juvenile islets were 5.9 ± 1.4% on day 3 and 10.1 ± 1.7% on day 7 (Figure 5D). The percentage of amylase positive cells in both neonatal and juvenile islets decreased from 0.97 ± 0.3% and 3.6 ± 1.9% on day 3 to 0.33 ± 0.1% and 2.1 ± 0.6% on day 7, respectively; however, the differences were not statistically significant (Figure 5B & 5E). The percentage of glucagon positive cells increased from day 3 to day 7 in both neonatal and juvenile islets. The percentage of glucagon positive cells in neonatal islets was 4.3 ± 0.7% on day 3 and 8.5 ± 2.5% on day 7 (p=NS) (Figure 5C). The percentage of glucagon positive cells in juvenile islets was 7.8 ± 1.2% on day 7, which was significantly higher compared to day 3, 4.7 ± 0.8% ($p<0.05$) (Figure 5F).

Viable Insulin Positive IE per gram of Pancreas

The numbers of viable insulin positive IE per gram of pancreas were calculated as described to compare the numbers of viable insulin positive cells obtained from the pancreas. The numbers of insulin-positive IE per gram of pancreas significantly decreased from 627.0 ± 54.9 IE/g on day 3 to 466.8 ± 36.8 IE/g on day 7 for neonatal islets ($p<0.05$) (Figure 6A). For juvenile islets, the numbers of insulin-positive IE per gram of pancreas were higher on day 7, 514.3 ± 136.9 IE/g, compared to day 3, 370.4 ± 127.5 IE/g, but were not statistically significant (p=NS) (Figure 6B).

Immunohistochemical Analysis of Islets

To confirm our flow cytometry data, results from immunohistochemical analysis of neonatal islets revealed higher amount of insulin positive cells per islet on day 7 comparing to day 3 (Figure 6A & 6B). Similarly, juvenile islets showed a higher amount of insulin positive cells per islet on day 7 than on day 3 (Figure 6C & 6D).

Discussion

Young porcine islet xenotransplantation is seen to be a promising treatment for patients affected by T1D. Still, challenges occur, such as prolonged β-cell maturation and low islet yields, that impedes the regular utilization of porcine donors in the clinical setting. Our revised protocol provides a possible resolution to these challenges by creating an efficient isolation and culture method that results in an accelerated islet maturation process *in vitro*, while still maintaining maximal islet yields. Our previous protocol produced considerable quantity of islets of both neonatal and juvenile pigs with a culture time of 7-9 days. By modifying isolation methods and culture media, the revised protocol minimized culture time to 3-4 days.

Many studies have reported achieving optimal and responsive neonatal islets from their own methods of isolation and culture that require as long as 12 days and as little as 5 days to mature (25676501, 26377964). In this study, neonatal pigs resulted in significant yields of pure and viable islets by day 3 of culture, also improving our previous 7-day protocol. Remarkably, these islets have shown that they are mature and able to function *in vitro* with a short culture time. These islets did result in a moderate, but insignificant, increase in function with more time in culture. However, a prolonged culture resulted in a significant loss of 50% in the

quantity of islets. At day 3, using our revised protocol neonatal pigs would theoretically yield ~30,000-89,000 IE per pancreas. At day 7, they would theoretically yield ~16,000-48,000 IE per pancreas. Thus, there is a tradeoff, shortening the culture time will produce a substantial amount of islets that function or prolong culture and produce a good amount of islets that function greatly. Having said that, studies have reported a dose as small as 1500 IE to 2000 IE per mouse to achieve normoglycemia in nonhuman primates (25793438, 8621802). Moreover, humans would demand much greater doses, which has not been determined yet, like reported in Elliott's study which required a dose of 15,000 IE/kg bodyweight (17381690). Therefore, producing a maximal amount of islets per pancreas is essential for xenotransplantation. For that reason, it would be beneficial to stop islet culture at day 3, allowing islets to be more readily available and decreasing the number of total donors required for transplant.

In contrast, producing juvenile islets for xenotransplantation has not been as explored, and in our previous studies we established a protocol where islets had to culture for 7 days for complete maturation (23394130, 25793438). In our revised protocol, juvenile pigs are able to achieve quality islets that function well on day 4 as well, but producing only fair islet yields. Even though there is a significant increase in islet response on day 8, a 63% increase in function, islets are still able to function by day 3. There is no noticeable difference in islet yield between day 3 and day 7, making culturing for 3 days more sensible.

In this study, we have shown that the viability of islets remained similar on both day 3 and day 7 of culture, except neonatal islet viability markedly decreased on day 7. Both neonatal and juvenile islets showed higher populations of viable β-cells and α-cells after prolonged culture. Likewise, immunohistochemical analysis confirmed that both neonatal and juvenile islets had higher amounts of insulin-positive cells on day 7 when compared to day 3. Although longer culture time allowed the development of endocrine cells without affecting their viability, there was a significant decrease in the numbers of viable insulin positive IE in neonatal islets on day 7 compared to day 3. The numbers of viable insulin positive IE in juvenile islets on day 7 was higher than day 3; however, the increase was insignificant, suggesting that the longer culture time may not justify its benefits. Prolonged culture has been suggested to allow exocrine content to decrease (133470). We have shown in this study that the insignificant decrease in exocrine content from day 3 to day 7, as indicated by the percentage of amylase positive cells, combined with the low exocrine content on day 3 further showed that the benefit of prolonged culture may not be effective.

Conclusions & Future Directions

Our improved protocol has shown that it is able to efficiently obtain a consistent quantity of functional neonatal and juvenile porcine islets through a shortened culture time and production of high islet yield per pancreas. These islets contain both β-cells and α-cells with low exocrine component, making them a possible source of islets for xenotransplantation. Not many studies have reported on isolation of juvenile-aged pigs, opening up for an opportunity to test ways to increase yields in this specific age range. By shortening culture time to reduce islet loss, without a significant increase in endocrine cells or islet function, we optimize islet isolation and transplantation protocols for future clinical trials. Going forward, examining how islets produced from this protocol will function *in vivo* to restore and maintain euglycemia will be the next step. Furthermore, characterizing the *in vivo* development of neonatal and juvenile porcine islets after xenotransplantation would support our efforts to carry out clinical trials in non-human primates and T1D patients.

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