**EVALUATION OF A HIGH-YIELD TECHNIQUE FOR PANCREATIC
ISLET ISOLATION FROM DECEASED CANINE DONORS**

Master's Thesis

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is one of the most frequently diagnosed endocrinopathies in dogs with an increasing prevalence, challenging life-long management and frequent complications. Pancreatic islet transplantation is a noninvasive and potentially curative treatment for T1DM. We hypothesized that clinically acceptable islet yield and purity could be achieved by using deceased canine donors and standard centrifugation equipment.

Pancreata were procured from dogs euthanized for reasons unrelated to this study. Initial anatomic studies were performed to evaluate efficacy of pancreatic perfusion. Infusion into the accessory pancreatic duct resulted in perfusion of approximately 75% of the pancreas. Additional cannulation of the distal right limb of the pancreas allowed complete perfusion. Collagenase digestion was performed with a Ricordi chamber and temperature-controlled perfusion circuit. Islets were separated from the exocrine tissue with the use of a discontinuous density gradient and a standard laboratory centrifuge. After isolation, islet yield was calculated and viability was assessed with dual fluorescent staining techniques.

Islet isolation was completed in 6 dogs. Median (interquartile range) islet yield was 36,756 (28,527) islet equivalents (IEQ) per pancreas. A high degree of islet purity (percentage of endocrine tissue; 87.5% [10%]) and viability (87.4% [12.4%]) were achieved.

The islet yield achieved using this technique would require approximately 1 pancreas per 5 kg body weight of the recipient dog. Purity and viability of the isolated islets were comparable to those achieved in human islet transplantation program. According to initial results, clinically relevant islet yield and quality can be obtained from deceased canine donors with the use of standard laboratory equipment.

This thesis is dedicated to my family and especially my mother who supported me each step of the way; my boyfriend Chad for his unconditional love and support; Allison Kenzig for her help and late nights she spent working on this project; Dr. Gilor and Dr. Rajab for their invaluable suggestions; Dr. Hadley for his kind agreement to be on my committee; and my advisor Dr. Adin for his enthusiasm, expertise, and the best mentorship I could ever wished for.

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CHAPTER 1 INTRODUCTION

1.1 Diabetes as a Challenging Disease in Veterinary and Human Medicine

Diabetes mellitus is one of the most frequently diagnosed endocrinopathies in companion animals and prevalence of this disease continues to increase ¹. Type 1 diabetes mellitus (T1DM), previously termed insulin-dependent diabetes mellitus, is considered the most common type in dogs ². It is an immune-mediated disease with gradual destruction of beta cells, mirroring the pathogenesis of T1DM in human beings. The progression from normal, to glucose intolerant, to overt diabetes is generally slow, so that most islets (over 90%) are lost before clinical signs of diabetes occur. Similar to canine diabetes, the incidence rate of T1DM in people is rising, a trend that has been explained on the basis of increased contacts with adverse environmental factors acting on a background of complex genetic factors ³. The rate of progression to absolute insulin deficiency is quite variable in humans and has not been studied in dogs. The epidemiological factors of canine diabetes closely match those of human patients with late onset diabetes who are usually not obese and tend to be middle aged. Most affected dogs are over 7 years of age with insidious onset of clinical signs of several weeks to months duration ⁴.

Medical management of diabetes in dogs poses a number of financial and emotional challenges to the pet owner, and complications such as blindness caused by cataracts or retinal disease, atypical infections, change in body weight, life threatening hypoglycemia or

diabetic ketoacidosis are common ^{5,6}. Faced with the challenges of life-long medical management, up to 40% of owners elect to euthanize their dog on the day of diagnosis ⁷.

1.2 Medical Management

Insulin was discovered by Banting and Best in 1921 ⁸ who first treated a dog with canine islet extracts and then started treating human diabetics with bovine pancreatic extracts. The full amino acid sequence of human insulin was deduced in 1960 ⁹. Bovine and porcine pancreata were the source of all insulin formulations until the early 1980's ¹⁰. Recombinant human products largely replaced them by the late 1990's. Therapeutic insulin remains one of the most commonly used products of pharmaceutical biotechnology and insulin-based products command annual global sales in excess of \$4.5 billion ¹¹. Intermittent insulin injections delay the onset of clinical signs, but fail to approximate the moment to moment glycemic control that is accomplished by functioning pancreatic islets.

Innovation in insulin therapy is focused upon the development of engineered insulin molecules with altered pharmacokinetic properties, a range of both fast- and slow-acting insulin analogues and alternatives to the traditional subcutaneous injection delivery system ¹¹, including insulin pens, implanted or external insulin pumps ¹², jet injectors, transdermal insulin patches and inhalation devices ¹³.

Despite the technological advancements, all methods of exogenous insulin therapy fall short in mimicking the complex patterns of natural insulin secretion and tight glycemic control is difficult to achieve. The widespread effects of this failure of adequate glycemic control are hard to overstate- it is estimated that 1 in every 10 U.S. healthcare dollars is spent dealing with the complications associated with diabetes, with costs totaling \$174 billion in

2007¹⁴. Long-term diabetics inevitably suffer from serious complications such as nephropathy, neuropathy or retinopathy and half of T1DM patients experience chronic neurovascular complications due to microangiopathic lesions secondary to imperfect control of glycemia¹⁵. Attempts at strict glycemic control through frequent insulin injections increases the risk of hypoglycemia unawareness two to three folds¹⁶.

Despite the high prevalence of diabetes in dogs, the evolution of diabetes therapy in this species has been negligible after the development of intermediate-acting insulin formulations (NPH, Lente and PZI). Diabetic dogs today are largely treated the same way they were treated 50 years ago. While physicians can choose from more than 40 different types of insulin formulations when deciding how to best control a particular patient, veterinarians have a much more limited armamentarium.

Thus, although insulin therapy has proven effective in delaying complications and prolonging life, the metabolic control that is achieved through this method is inadequate and the failure of these therapies is manifested by the unacceptable rates of morbidity and mortality suffered by both human and canine diabetics.

1.3 Whole Pancreas Transplantation in Humans

Due to the inadequacies of insulin, many investigators have sought to develop whole pancreatic transplant as a potential cure for T1DM in human beings. With the development and application of anti-rejection agents (cyclosporine and azathioprine), performance of solid-organ transplantation dramatically increased in the time period from 1985 to 1995¹⁷. Whole pancreas transplantation can replace beta cell mass in selected human patients, restoring normal glucose homeostasis¹⁸.

Major drawbacks to whole pancreas transplantation include technical challenges of the surgical procedures required to reestablish pancreatic duct drainage and vascularization, the presence of unnecessary exocrine pancreatic tissue along with the desired pancreatic islets of Langerhans, hyperinsulinemia, highly variable functional life span of these grafts and need for massive and lifelong immunosuppressive therapy. High perioperative mortality and morbidity rates are related to the risk of thrombosis, bleeding, infection, anastomotic leak, and pancreatitis¹⁹. Due to these disadvantages and a desire to avoid immunosuppression until absolutely necessary, whole pancreas transplantation is rarely employed in diabetics as a sole therapy; rather, pancreas transplantation is delayed until development of end stage renal disease and dual kidney-pancreas transplant is required.

1.4 Whole Pancreas Transplantation in Dogs

A small number of experimental studies have used a canine model for pancreas transplantation to refine technical skills, or to evaluate the surgical risks and rejection pictures before application in clinical human pancreas transplantation programs²⁰⁻²². In pancreatectomized dogs receiving 3-drug immunosuppression (prednisolone, cyclosporine and mycophenolate mofetil), whole pancreas allograft transplantation caused normalization of serum glucose, but rejection rates approached 90% and only 50% of animals survived beyond 14 days²³. Surgical complications included duodenal stump leakage, wound infection, graft necrosis and graft vascular thrombosis, chyle leakage, intraabdominal abscess and pneumonia. Based on the technical challenges of surgery and the poor efficacy of immunosuppression in preventing rejection, application of whole pancreas transplantation is

not considered practical as a treatment for companion animals and has not been pursued in dogs with spontaneously occurring diabetes.

1.5 History of Pancreatic Islet Transplantation

Transplantation of endocrine cells was identified early on as a “low hanging fruit” for application of cell-based therapies. With diabetes arguably providing the largest market and need, much of the attention has focused on developing methods for successful transplantation of functioning pancreatic islets. Islet transplantation provides an attractive alternative to whole pancreas transplantation, allowing implantation of lower cell mass and minimizing the potential immune challenge (islets constitute only 1-2% of the total pancreas weight) and avoidance of technically challenging surgery, thereby minimizing morbidity to diabetic patients. Thus, pancreatic islet transplantation is considered a minimally invasive, curative, and physiologic treatment for T1DM in human diabetics ²⁴ and, as such, tremendous research efforts have been directed towards this strategy over the past four decades.

The concept of islet transplantation began as early as 1893, when investigators implanted fragments of ovine pancreas under the skin of a boy with ketoacidosis ²⁵. With the discovery of insulin and subsequent development of techniques for islet isolation in larger mammals, modern islet transplantation began to progress more rapidly and in 1974 Sutherland performed the first pancreatic islet transplant from a deceased human donor into a human patient with diabetes ^{26,27}. While this initial effort showed promise, insulin independence was not achieved and physicians soon recognized that large numbers of islets were required to produce equivalent effects to those seen after pancreas transplantation,

presumably due to the fact that cellular transplantation does not provide an immediate vascular supply and results in cell loss due to hypoxia and nutrient deprivation.

From this point onward, research in dog models was integral to progress in the human field. The inability to extract and purify sufficient numbers of islets was resolved by the introduction of an automated method for islet isolation in large mammals that replaced all previously tested procedures²⁸⁻³⁴. Investigators went on to use the dog model to show the importance of islet purity, demonstrating that pure islets from a single canine donor were superior to pancreatic fragments containing exocrine debris^{35,36}.

Despite these advances in technique, diabetic patients receiving islet transplants achieved only short-term insulin independence, most likely due to inadequate recipient immunosuppression and the presence of autoantibodies to pancreatic beta cells³⁷. The first successful series of islet allografts was reported in 1990 in patients that developed diabetes secondary to total pancreatectomy³⁸, while the results in T1DM slowly improved during the 1990s until 1999. Initial attempts at the use of cyclosporine showed improvement in allograft survival in outbred dogs, but poor long-term glycemic control³⁹. It appeared that long-term success was limited by immunosuppressive protocols that, ironically, depended upon diabetogenic drugs such as corticosteroids and calcineurin inhibitors⁴⁰.

A dramatic improvement was achieved with the Edmonton protocol in 2000²⁴ when seven consecutive human patients who received islet transplants achieved insulin independence and a marked decrease in the frequency of hypoglycemic episodes at 1 year post-transplantation. The major breakthrough of this protocol was elimination of corticosteroids, using instead a combination of sirolimus, tacrolimus, and daclizumab (an

anti-interleukin-2-receptor antibody) to prevent rejection. After this major success, islet transplant programs expanded significantly, with >750 human patients transplanted at multiple international institutions over the past 12 years ⁴¹. Over 80% of transplanted diabetic patients demonstrate persistent islet function at 5 years based on C-peptide production, and have a significantly improved quality of life ⁴². However, graft function decreases over time and only 15% to 50% of patients are insulin independent after 5 years. With each evolution of the Edmonton protocol, success rates for this novel procedure improve and reversal of diabetes by islet transplantation is now being performed at major medical centers around the world ^{43,44}.

1.6 History of Islet Transplantation in Dogs with Diabetes

As with many other novel therapeutic approaches, pancreatic islet transplant techniques that were originally evaluated and refined in dogs have had little application in dogs with naturally occurring disease. While canine diabetes mellitus has been described an ideal preclinical model for T1DM in humans ^{45,46}, the vast majority of islet transplant studies were actually carried out in healthy research animals that underwent pancreatectomy or chemically-induced diabetes, where success would be expected to be higher than in clinical patients with autoimmune induced beta cell destruction. Nonetheless, a vast amount of information has been assembled in over 40 studies involving canine islet isolation, preservation, or transplantation ^{28,31,32,36,47-86}. A small number of recent studies have even shown some promise in resolution of spontaneous diabetes in client-owned dogs ^{76,79}.

1.7 Islet Sources for Use in Human Diabetics

Currently, islet transplantation requires large “doses” of cells to achieve insulin independence in human diabetic recipients. Typically 2 to 3 complete donor pancreata are required to achieve the 6,000-10,000 islet equivalents/kg needed to achieve a successful outcome in a single recipient. This fact, in addition to the relatively high risks involved with partial pancreatectomy in living donors, have required the use of 2 to 3 deceased donor organs for each islet transplantation. Donor body mass index, pancreatic blood flow prior to harvesting (use of positive inotropes during resuscitation), blood glucose prior to harvesting, and the time of preservation in a hypothermic solution (cold ischemia time), are all known to affect outcome after islet transplantation ⁴¹. However, due to a shortage of human organ donors, there is little ability to select ideal organs for use in islet transplantation. In fact, with current disparity between the incidence of T1DM and available organs for cellular transplantation being approximately 1 human pancreas per 333 diabetics with T1DM, it appears that the use of xenograft pig islets are the most viable long-term approach to islet transplantation in human diabetics ⁸⁷. The use of pig donors has several advantages, including the known efficacy of porcine insulin in human diabetics, rapid growth of pigs to adult size and the social acceptability of using pig organs from animals that are already destined for human consumption. Unfortunately, pig islets are xenograft tissues and will require novel approaches to immunosuppression or immunoisolation, as described later. It would appear that the regenerative medicine would offer a practical solution to the problem of organ shortage, and many groups are working on differentiation of a stem-cell derived, insulin-producing cell line. However, there is significant difficulty in producing an

immortal cell line that maintains sensitivity to glucose and no success has been reached in this arena, to date ⁸⁸.

1.8 Islet Sources For Use In Canine Diabetics

In companion animals, there is no administrative support to maintain an organ-sharing network such as that used to coordinate organ donation in human beings. Thus, veterinarians will need to find a readily available source of islet donors that will allow procurement of islets in a manner that is ethically acceptable and humane. As in people, a number of issues must be considered in evaluating a potential donor. Ideally, donor animals should be screened through a rigorous pre-transplant protocol to rule out infectious, metabolic or neoplastic diseases that would affect the outcome of transplantation. Several investigators have explored the use of pig islet xenografts in canine recipients as a translational model for xenograft transplantation in human diabetics. If the issues of xenograft immunorecognition can be addressed, pig islets may provide another alternative to the use of canine donors. Live kidney donation is occurring at a limited number of companion animal transplantation centers ⁸⁹, but further study would be required to establish whether partial pancreatectomy from healthy donors would offer a safe and effective alternative for islet donation in dogs. If islet transplantation becomes successful and widespread treatment modality for diabetic dogs, shortages in cadaveric canine pancreas will be a reality and consequently living donation may be explored.

1.9 Islet Isolation Technique

The organ donor shortage has been an excellent motivator in refining islet isolation techniques and techniques have advanced rapidly since the first method of islet isolation was described using hand dissection under the microscope ⁹⁰. Initial steps in organ procurement are similar to those for other solid organs and involve rapid flushing of the pancreas via infusion of chilled organ preservation solution into the abdominal aorta or the celiac artery. After the pancreas is resected, it is placed in a dual layer of preservation solution and perfluorocarbon oxygen carriers while it is taken to the transplant center for further processing. The overall goal of islet isolation is to physically separate the endocrine components (Islets of Langerhans) from the exocrine pancreatic tissue. Larger mammals have higher connective tissue content in the pancreas, making it difficult to achieve isolation of the cellular components without injuring the cells in the process. Currently used technology is based on an automated method introduced by Ricordi in 1986 ³⁴. Digestion is achieved by a collagenase blend and composition of the enzymes is important for successful islet isolation. Currently, a standardized mixture of highly purified enzymes (Liberase) is considered the collagenase of choice ⁹¹. Once the islets are freed from the exocrine tissue, physical separation of the components is achieved by a continuous density-gradient purification of the islets using a COBE 2991 cell processor and multiple gradient layers of Ficoll ⁹². Because islet cells are lighter than acinar cells, they remain in a separate layer when centrifuged with continuous or discontinuous density gradients. The cell processing equipment used in human islet transplant centers allows rapid and effective purification of islets, but carries significant expense when compared to traditional laboratory centrifuges.

1.10 Methodology in Dogs

Canine pancreata are similar to human pancreata in size and connective structure and experimental studies have demonstrated that islet yield would ideally be performed in a similar manner to that described by human islet centers^{28,39,62,93,94}. However, the expense associated with cell processors and organ perfusion solutions would preclude their use at most veterinary referral centers. Previously published reports in research dogs used trituration of pancreatic tissue by passage through metal needles and maceration of tissue with a blade to achieve mechanical disruption³⁰. While these methods are less expensive and avoid the need for specialized equipment, they lead to excessive damage to islet cells and yield only limited numbers of non-sterile islets of variable purity that would not be sufficient for clinical use^{30,95}.

1.11 Sites of Transplantation

The optimal site for islet transplantation has not yet been defined, although many different sites have been proposed in experimental animal models including the liver, kidney subcapsule, spleen, pancreas, peritoneum, omentum, gastrointestinal wall, testis, thymus, bone marrow, anterior chamber of the eye, cerebral ventricles, and subcutaneous and intramuscular spaces⁹⁶. Intrahepatic islet implantation through the portal vein is currently the site of choice for clinical islet transplantation in people due to the easy access without significant morbidity⁹⁷ providing a high degree of vascularity, proximity to islet-specific nutrient factors, and more physiologic first-pass insulin delivery to the liver⁹⁸. Intrasplenic islet autotransplants have been associated with life-threatening complications such as splenic

infarct, rupture, and gastric perforation⁹⁹. In dogs, the majority of experimental studies have used intraportal or intrasplenic islet infusion^{30,52,53,74} although transplantation into the peritoneal cavity and omentum has also been reported^{63,100,101}.

1.12 Current Challenges in Islet Transplantation

Two main issues have to be resolved before pancreatic islet transplantation becomes a widespread reality in human and canine patients: donor availability and immunosuppression of the recipient or immunoisolation of the islets.

Despite early optimism, islet transplantation for T1DM has produced variable success with unpredictable duration of insulin independence and frequent requirement of multiple islet transfusions in human diabetics⁴⁴. One of the major limitations impeding islet transplantation from becoming a widespread clinical reality are the requirement for large numbers of islet per patient and paucity of high-yield protocols for pancreatic tissue purification and preservation¹⁰²⁻¹⁰⁴. The use of deceased pancreas donors has been associated with lower islet yield and potency, with only 50% of pancreas processing in human medicine achieving sufficient yield to convert to clinical transplantation¹⁰⁵.

Another major challenge impeding pancreatic transplantation is the need for life-long multidrug immunosuppressive protocols to prevent graft rejection. Conventional immunosuppressive drugs used in transplant recipients include cyclosporine, tacrolimus, azathioprine, and corticosteroids. Long-term use of these medications has been associated with a number of significant side effects including nephrotoxicity, hypertension, hyperlipidemia, microvascular disease, gastrointestinal problems, weight gain, skin changes, and increased risk of infection and malignancy^{106,107}. In addition, cyclosporine, tacrolimus

and corticosteroids have adverse effects on beta cell function and survival, with potential of actually inducing diabetes¹⁵. Therefore, islet transplantation is only performed in selected diabetic patients with unstable glycemic control and life-threatening hypoglycemic episodes or in patients with diabetic nephropathy requiring renal transplant. The corticosteroid-free regimen used in Edmonton protocol, consisting of sirolimus, tacrolimus, and daclizumab, proved to be effective in graft rejection with less side effects²⁴. Recent studies demonstrated poorer graft survival and decreased renal function in sirolimus-based protocols compared to mycophenolate mofetil¹⁰⁸.

Early studies using traditional cyclosporine immunosuppression in dogs showed prevention of islet transplant rejection if trough cyclosporine levels were maintained over 400-500 ng/ml^{109,110}. Long-term normoglycemia but also significant side effects were seen with cyclosporine levels of 600 to 1000 ng/ml measured by high-pressure liquid chromatography²³. Another study showed that in pancreatectomized mongrel dogs receiving 30 days of cyclosporine A, rapamycin, or both, neither drug alone showed significant increase in islet allograft survival. However, dogs treated with both drugs simultaneously demonstrated prolonged graft survival with no evidence of adverse effects¹¹¹.

Cryopreservation of canine islets was investigated for the purpose of storage and decreased immunogenicity. Cryopreserved islet allografts were shown to maintain normoglycemia if transplanted at higher volumes⁶⁰ however reduction of immunosuppressive medications was not achieved¹¹². It is important to note that none of the studies of traditional immunosuppression for canine islet transplantation were performed in animals with naturally occurring, autoimmune diabetes. Thus far, experience suggests that it is highly unlikely that traditional immunosuppression alone will provide an adequate long term solution for

transplantation in dogs with naturally occurring disease, as the side effects from these immunosuppressive drugs approach or exceed the morbidity of the primary disease^{89,113,114}.

An essential step in the exploration of clinical islet transplantation in dogs is the development of a protocol of islet isolation that is both practical and ethically acceptable. The goal of this study was to establish a simple and affordable canine pancreatic islet isolation method yielding sufficient islet mass and purity for clinical transplantation in a medium-sized dog (> 5000 islets/kg). We hypothesized that clinically acceptable islet yield and purity could be achieved using deceased canine donors and standard centrifugation equipment.

CHAPTER 2 MATERIALS AND METHODS

2.1 Donor Animals

Pancreatic tissues were obtained from animals euthanized for reasons unrelated to this study. Young (< 3 years of age) and apparently healthy dogs were selected, based on the lower incidence of pancreatic pathology in dogs less than 5 years ¹¹⁵. The study protocol and animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Ohio State University.

2.2 Anatomic Study

Initial anatomic studies were performed in two dogs to evaluate efficacy of pancreatic perfusion after cannulation of the accessory pancreatic duct. Pancreata were excised en-bloc with the associated descending duodenum. The accessory pancreatic duct was cannulated and the efficacy of pancreatic perfusion was characterized in each dog after injection of Evan's blue dye.

2.3 Pancreas Procurement

Immediately after confirmation of death, dogs were placed in dorsal recumbency and the abdomen was clipped and aseptically prepared. Pancreas procurement was

performed using sterile technique. A ventral midline laparotomy was performed and the pancreas was located by medial displacement of the duodenum. Beginning with the right limb, the pancreas was sharply dissected from the duodenum and stomach, being careful to avoid penetration of the viscera and contamination from intestinal microflora. In-situ vascular flushing of the donor organs or isolated pancreas was not performed. The retrieved pancreas was placed in a 500-mL sterile beaker with 100 to 150 mL of chilled (4°C) CMLR 1066 (Mediatech, Cellgro) supplemented with 2% fetal calf serum (FBS) to completely cover the tissue. The jar was deposited on ice inside an insulated transport cool-box and transported to the laboratory for immediate processing. The warm ischemia time (time from euthanasia until placement of the extracted pancreas on ice) was recorded for each dog. After arrival at the laboratory, the pancreas was weighed and subsequently placed on a sterile surface. The accessory pancreatic duct was identified and cannulated with a 22 gauge intravenous catheter. The catheter was then secured by a transfixation suture to prevent dislodgement. Cold enzyme solution was prepared containing 1 mg/mL of Collagenase P (Roche Diagnostics, Germany) and 50 µg/mL of DNase (Roche Diagnostics, Germany) in RPMI Medium 1640 (Gibco, Invitrogen, USA). The enzyme solution was administered at 2 to 4 mL/min using a syringe pump to maintain a consistent flow rate. Perfusion was continued to a maximum of 200 mL of fluid or until the entire pancreas was grossly distended (**Error! Reference source not found.**).

2.4 Pancreatic Digestion

The distended pancreas was cut into 15 - 20 pieces and placed in a modified Ricordi chamber (Error! Reference source not found.) along with 6 silicon nitride spheres to aid in disruption of the exocrine tissue and liberation of islets. Additional collagenase solution (identical to that used in initial pancreatic digestion) was added to fill the lower chamber of the apparatus. A filter (508- μ m mesh) was placed inside the Ricordi chamber to allow passage of dissociated pancreatic islets while maintaining connective pancreatic tissue in the chamber. The digestion was conducted in a closed, temperature-controlled perfusion circuit (Error! Reference source not found.) in which perfusion solution (Mediatech, Cellgro) was aspirated to fill the circuit by a pump set at 225 mL/min. Pump speed was decreased to 150 mL/min once the tubing of the entire circuit was primed with the solution. The heating coil was placed in a 37°C water bath to activate collagenase activity. The Ricordi chamber was manually shaken in a vertical direction approximately 60-90 times per minute.

Islet content in the solution was monitored by microscopic examination of Dithizone (diphenylthiocarbazone) stained samples obtained via the sampling stopcock at 5-minute intervals. Dithizone stain was used to differentiate the zinc-containing beta cells from the exocrine pancreatic tissue (Error! Reference source not found.). Digestion was continued until approximately 50% of islets were free of acinar tissue, based on guidelines used in our institutional human islet isolation protocol. The digestion was stopped by transferring the heat exchange coil from the warm water bath into an ice bath, and digestion time was

recorded for each dog. Cold Hanks' Balanced Salt Solution (HBSS, Gibco, Invitrogen, USA) that contained 10% of FBS was infused to inactivate the collagenase. The recovered islet cells and associated exocrine debris were collected into 250 mL collection tubes and washed twice with RPMI with 10% FBS at 250x g at 4°C. The resuspended pellet was filtered through a 450- μ m wire mesh and centrifuged for 2 min at 166x g at room temperature.

2.5 Islet Purification

The purification of the islet preparation was accomplished with a discontinuous density gradient and standard laboratory centrifuge (Thermo Scientific Sorvall Legend). Briefly, the pellets were combined and resuspended in two tubes, each containing 50 mL of a Biocoll gradient with a density of 1.100 g/cm³ (Biochrom, Berlin). This solution was carefully layered over with 50 mL of Biocoll (density 1.077 g/cm³) to maintain two layers. Finally, a third layer was added that consisted of RPMI and 10% FBS. The tubes were then centrifuged at 750x g at room temperature. The zone between the top two density gradients (RPMI and Biocoll 1.077) that contained purified islets was collected with a pipette, washed with RPMI solution, and pelleted by centrifugation at 166x g for 2 min at room temperature in a 45 mL conical tube.

2.6 Islet Quantification

After resuspension of the pellet, a 0.1 mL aliquot of the islet solution was removed to determine islet yield and purity. Dithizone stained samples were examined using an inverted phase contrast microscope (Fisher Scientific Microscope). An eyepiece reticle was used to determine the islet size and islets were counted and divided into six categories based on diameter (50-100, 101-150, 151-200, 201-250, 251-300, 301-350 μm). Islets smaller than 50 μm in diameter were not included in the manual count. Islet quantity is routinely expressed as the number of islet equivalents (IEQ) in order to compensate for variations in islet volume²⁹. A standard islet diameter of 150 μm was used in this study. The number of islets in each diameter class was converted to the standard number of IEQ using a multiplication factor. The purity of the preparation was determined qualitatively by comparing the relative quantity of endocrine tissue (islets) to unstained exocrine tissue as a percentile.

2.7 Viability Assessment

Freshly isolated islets were placed in 10 mL petri dishes at a concentration of 500 IEQ/dish in 10 mL of culture medium (RPMI) and cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The culture medium was supplemented with 10%FBS, penicillin G (100 U/mL), and streptomycin sulfate (100 $\mu\text{g}/\text{mL}$). After 24 h in culture, 100 islets were handpicked with 20 to 200- μL pipettes (Thermo scientific) and transferred to a 3 mL petri dish containing 0.5 mL of RPMI medium. Viability staining was performed as previously described¹¹⁶. A dual fluorescent staining technique was performed using

propidium iodide exclusion to identify viable cells (Error! Reference source not found.**A**).

Images were captured using an epifluorescent inverted microscope (Nikon Eclipse Ti) and were analyzed using a custom macro for NIH ImageJ^{116, 117} that identifies regions of interest and determines % islet viability (Error! Reference source not found.**B**).

CHAPTER 3 RESULTS

3.1 Pancreatic Donors and Anatomic Study

Pancreata were isolated from six young-adult mixed breed dogs immediately after euthanasia. Body weight ranged from 18 to 27 kg. The primary exocrine duct in the dog (accessory pancreatic duct) was found to be consistently located 7 to 8 cm from the pylorus. Evan's blue dye infused into the accessory pancreatic duct resulted in perfusion of approximately 75% of the pancreas, including the entire left limb and body of the pancreas, but failing to perfuse the apex of the right limb. Complete perfusion of the distal right limb of the pancreas was achieved by incising the pancreas transversely and cannulating the intra-parenchymal pancreatic duct approximately 5 cm from the apex of the gland (Error! Reference source not found.).

3.2 Pancreatic Digestion

Results of pancreatic islet isolation procedure are summarized in **Table 1**. Warm ischemia time (time from euthanasia to placement of the pancreas on ice) decreased with experience from 30 min in the first dog to 15 min in the final dog. Fifty percent islet dissociation from exocrine debris was detected in all dogs by manual examination of Dithizone-stained samples, and enzymatic digestion was stopped after 19 to 33 min.

3.3 Islet Purity and Quantification

Immediately after discontinuous gradient centrifugation, islet solutions from the pancreata were evaluated for quality and enumerated using a standard islet equivalent (IEQ) calculation. Most of the isolated islets were of high quality based on having spherical shape, excellent integrity, and well-rounded borders, with only a few single cells present (**Figure 5**). The total islet yield ranged from 18,945 to 70,180 IEQ with a median of 36,756 IEQ (interquartile range, IQR = 28,527 IEQ). The median islet number obtained per gram of pancreas was 608 IEQ/g (IQR = 807 IE/g). Based on Dithizone stain, conferring the characteristic red color to the islets and by means of a calibrated grid, islets were divided into 6 groups based on diameter (**Table 2**). Most islets were classified into the smaller groups (50-100 μm and 101-150 μm), with no islets exceeding 350 μm . The median (IQR) islet purity after isolation was 87.5% (10%).

3.4 Viability Assessment

Islet integrity and shape were preserved after 24 h culture. Viable islets will maintain a smooth border while necrotic (dark) center can be seen in the largest islets. Islet viability was evaluated by dual staining with background stain (Hoechst, blue fluorescence) and propidium iodide (red fluorescence) depicting unviable cells (**Error! Reference source not found.A**) and analyzed with epifluorescence microscopy. A custom macro for NIH ImageJ was used to identify region of interest and to calculate the percentage of dead cells present

in each islet (Error! Reference source not found.B). **Table 1** shows a median viability of the recovered islets 87.4% with an interquartile range of 12.4%.

Figure 1. Pancreatic cannulation.

Cannulation of the accessory pancreatic duct alone was insufficient to produce complete perfusion of the entire pancreas. Additional cannulation of the distal right limb of the pancreas was performed by incising the parenchyma and inserting a 22-gauge catheter into the pancreatic duct. Successful perfusion results in diffuse distention of the pancreas with collagenase solution and separation of the lobules of pancreatic tissue, as depicted in this image.

Figure 2. Perfusion circuit and the Ricordi chamber.

This schematic depicts the temperature-controlled perfusion circuit used to digest canine pancreata.

Figure 3. Dithizone-stained canine islets.

Dithizone-stained islet embedded within the exocrine pancreatic tissue (A) and free islets (B) on completion of the digestion process. Magnification, x100 (Fisher Scientific Inverted Microscope).

Figure 4. Islet viability staining.

Islet viability was quantified with a custom macro. Islet area was determined with a background DNA stain (Hoechst; blue). A second DNA stain (propidium iodide; red) is taken up only by cells with loss of membrane integrity, indicating cell death (A). Images were analyzed with epifluorescence microscopy (A). A custom macro for NIH ImageJ was then used to identify region of interest and to calculate the percentage of dead cells present in each islet (B). Magnification x40.

Figure 5. Pancreatic islet.

Image shows a high quality islet with spherical shape, excellent integrity, and well-rounded borders, with only a few single cells present. The islet was imaged at 20x using HMC.

Dog #	Pancreas weight (g)	Warm ischemia (min)	Digestion time (min)	IEQ	IEQ/g	Purity (%)	Viability at 24h (%)
1	37.9	30	19	23,044	608	80	NA
2	NA	25	25	50,965	NA	90	91.2
3	60	18	25	50,467	841	97	87.4
4	42	15	25	70,180	1,671	90	91.2
5	56	17	33	18,945	338	75	71.4
6	40	15	27	22,439	561	85	81.3
Median	42	17.5	25	36,756	608	87.5	87.4
IQR	19	10	2	28,527	807	10	12.4

Table 1. Results of pancreatic islet isolation in 6 dogs.

Abbreviations: IEQ, islet equivalent; IQR, interquartile ranges; NA, not assessed.

Islet diameter (µm)	Dog #1	Dog #2	Dog #3	Dog #4	Dog #5	Dog #6
50-100	95 (52)	185(49)	68(34)	116(46)	70(75)	33(56)
101-150	67(37)	113(30)	62(31)	93(37)	16(17)	14(24)
151-200	15(8)	67(18)	46(23)	26(10)	5(5)	8(14)
201-250	4(2)	9(2)	20(10)	13(5)	2(2)	3(5)
251-300	1(0.5)	1(0.3)	4(2)	0	0	1(2)
301-350	1(0.5)	0	0	2(0.8)	0	0

Table 2. Size variation of harvested islets.

Islets were divided into 6 classes based on their diameter. Percentage of islets falling into each size class for each dog is listed in parentheses. Most islets retrieved fall into the smaller categories.

CHAPTER 4 DISCUSSION

4.1 Summary

This study described isolation of viable pancreatic islets from deceased canine donors. Using a single canine cadaveric pancreas, it is possible to obtain high quality, viable islets in sufficient numbers for clinical transplantation thus overcoming current ethical difficulties of suitable donors. Furthermore, the described method of islet isolation uses a readily available fluid pump and laboratory centrifuge equipment, making the technique accessible to veterinary referral centers that do not have a cell separator available for use on animal patients.

4.2 Islet Characteristics

4.2.1 Islet Yield

Although islet yield in the current study is estimated to be sufficient for clinical transplantation in a 5 kg dog, multiple pancreas donors would be required for a larger transplant recipient. Inability to harvest enough islets from a single donor to reverse diabetes in a single recipient is a longstanding problem in human transplant medicine. Unfortunately, isolated islets removed from their blood supply face a fundamental problem with oxygen

delivery which results in up to 70% loss of functional islet mass due to ischemia, inflammation, apoptosis, and necrosis before revascularization can occur^{118, 119}. Recurrence of hyperglycemia after pancreatic islet autotransplant in dogs has been found to be strongly influenced by the number of islets implanted⁸² with 100% of dogs that received > 5000 islets/kg achieving normoglycemia. Studies using experimental heart-beating dogs and automated isolation methods (the Cobe 2991 cell separator) reported islet yield ranging from 3,481 to 6,340 IEQ/g of pancreas^{120,121}. Results of the current study showed a lower islet yield per gram of pancreatic tissue, which is expected with the use of deceased donors. Jung et al.¹²² showed that islet yield from living human donors is higher compared deceased donors. Islets are highly susceptible to prolonged ischemia due to their lack of enzymes necessary for ATP generation under anaerobic conditions; therefore cold ischemia of the cadaveric pancreas is detrimental to islet yield¹²³. In another study, the use of marginal pancreas donors (donors with advanced age, obesity, hyperglycemia, or other negative predictors for islet function) has been associated with lower islet yield and potency, with only 50% of pancreas processing in human medicine achieving sufficient yield to convert to clinical transplantation¹⁰⁵. On the basis of the successful use of deceased donors in the present study, we expect that veterinary institutions will be able to overcome any limitations on organ availability by identifying sources of canine tissues through organ donor programs (animals euthanized at the hospital for other reasons), research facilities or through the use of tissues obtained from animals euthanized at local animal shelters.

4.2.2 Islet Purity

Islet purity is defined as the percentage of islets compared to all tissue present in the islet preparation including islets, acinar, and ductal cells. Cell separation techniques are based on differences in cell density and diameter. Currently, the preferred technique for large-scale human islet purification uses continuous density gradient centrifugation and requires an automated cell processor. Although this advancement has increased processing efficiency¹²⁴, it is inaccessible for most veterinary hospitals because of the high cost of the equipment and disposables. A discontinuous Ficoll density gradient can be used to effectively separate small volumes of cells with the use of a standard laboratory centrifuge, but, before this study, no previous reports had effectively “scaled-up” this technique to reach clinically applicable doses of >5000 IEQ/kg body weight^{51,125}.

Our discontinuous gradient technique can be accomplished by a standard laboratory centrifuge that is easily accessible to laboratories and veterinary hospitals that are not associated with major human transplantation centers. In our study, we have achieved an islet purity of 87.5%, higher than in previously described canine studies that reported 30 to 80% purity with the use of different methods³⁰ and comparable to reports that used modern automated methodology¹²⁶. High islet purity is critical to decrease the level of immunosuppression required for islet allograft survival¹²⁷ and to limit the risk of hemodynamic instability and portal hypertension¹²⁸. Therefore, guidelines for islet quality control in people recommend the use of highly purified (> 80%) islet preparations for clinical transplantation purposes¹²⁹.

4.3 Islet Isolation Methodology

4.3.1 Digestive Enzyme Solution

Repeatability and success of islet isolation has been related to the type and purity of enzyme solutions that are used in pancreatic digestion ¹³⁰. In the present study, we elected to use a standard type P collagenase for pancreatic digestion, a less expensive form of isolation enzyme that is composed of a mixture of proteolytic enzymes of *Clostridium histolyticum*. Use of crude collagenase can contribute to the variability in islet yield and, based on previous reports, isolation recovery can vary up to fourfold with the use of different lots of collagenase ¹²⁰. Lakey et al. compared three types of collagenase with Liberase-CI (a purified enzyme blend specifically developed for canine islet isolation) and found significant improvement in islet recovery and function using this blend ⁴⁵. In the future, use of purified Liberase enzyme will be considered as a means to increase islet yield and purity with the present protocol.

4.3.2 Cold Preservation

In an effort to simplify donor organ harvest and to limit expense, we elected to avoid perfusing the donor with cold organ preservation solutions prior to organ harvest, using only surface cooling of the organ. In human organ procurement it is standard practice to perform regional in-situ cooling of the pancreas by cannulating the abdominal arteries to allow intravascular infusion of a hypothermic preservation solution in the transplant donor ¹³¹. University of Wisconsin solution (UWS) has been the standard preservation solution since 1989 ¹³², whereas recent reports describe effective alternative solutions ¹³³. The goal of

cold organ preservation is to suppress the rate of cell deterioration in the organ and to reduce metabolic activity of pancreatic tissue. A study using pig pancreata showed that infusion of preservation solution into the celiac and superior mesenteric arteries significantly improved islet yield when compared to surface cooling¹³⁴. Moreover, Van den Burg et al achieved higher islet purity and recovery (93% and 52%, respectively) using UWS for canine islet isolation compared with RPMI (15% purity and 41% recovery)¹³⁵. Although it is likely that the use of human organ perfusion solutions and performance of donor perfusion would improve islet yield when compared to our technique, the high cost and practicality of achieving donor perfusion must be weighed against the need for multiple pancreatic isolations.

4.4 Study Limitations

The animals used in the present study were derived from a population that was euthanized at an animal shelter because of behavior problems that prevented adoption. Although these animals were young (< 3 years) and appeared healthy on physical examination, we did not perform further screening to rule out the possibility of underlying metabolic, neoplastic, parasitic, or infectious disease, a step that would be absolutely necessary before institution of a clinical islet transplantation program. Similar guidelines have been developed for use in solid organ transplantation programs and will provide an excellent basis for initial screening of cellular transplant donors¹³⁶. Due to the need for harvest of the entire pancreas, it is not anticipated that live animal donor/adoption

programs will be a viable option for islet transplant programs as they are for kidney transplant programs in veterinary referral centers ⁸⁹.

Another consideration is the use of pentobarbital sodium as a euthanasia agent, a factor that was not specifically examined in this study. Although we have no control group to compare whether pentobarbital has a negative effect on islet quality, the results of our study suggest that the drug did not decrease islet viability or yield to an unacceptable level.

4.5 Future Directions

While the ultimate treatment of DM will be its prevention through gene therapy or, once established, induction of beta cell regeneration, these approaches still require major research breakthroughs. At this point, the most feasible and physiological strategy to replace diseased beta cell function is transplantation of pancreatic islet grafts ¹⁹. Introduction and acceptance of any technique into veterinary medicine must take financial and practical considerations into account. Through creation of an islet isolation protocol that allows for clinically acceptable islet quality from deceased canine donors, our group has overcome one of the major hurdles in making islet transplantation an ethically acceptable practice in client-owned dogs. Given the poor success of traditional immunosuppressive therapies in canine organ transplantation and the lack of access to modern monoclonal antibody therapies, it is likely that the future of canine islet transplantation lies in either tolerogenic therapies or in simple encapsulating strategies to provide immunoisolation without the need for cost-prohibitive, lifelong immunosuppressive drugs.

4.6 Conclusions

Successful isolation of highly pure and viable islets was achieved using canine cadaveric donors and accessible laboratory equipment. To our knowledge, this is the first report describing harvest of pancreatic islets from canine cadavers. The ready availability of the current technique to veterinary centers will overcome some of the most important practical difficulties in canine islet isolation, opening the door for preclinical testing of new isolation technologies, sites of implantation, immunoisolation, and immunotherapeutic strategies. Now that we have a method to obtain large quantities of purified islets, work in our laboratory is focused on islet encapsulation strategies that will allow the application of islet transplantation without the need for systemic immunosuppression, making islet transplantation a viable option for dogs with T1DM. Development of a clinical canine islet transplant program can have an enormous impact in veterinary medicine and change our approach in treatment of diabetic patients. Cooperation with human transplant specialists and utilizing their expertise and new developments would provide potential for direct benefits to dogs with diabetes and offer a simultaneous model for the preclinical testing of novel therapies prior to application in human diabetics.

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