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Optimization of *In Vitro* Cultures of Neonatal Porcine Islets Pre-transplantation

by

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ABSTRACT

Islet transplantation is an attractive method to achieve blood glucose homeostasis. However, β-cell function declines over time. Therefore, it is necessary to explore strategies to enhance the β-cell mass and function. Also, because there is a severe shortage of human cadaver tissue, alternative sources of insulin secreting tissue need to be examined. Neonatal porcine islet (NPI) tissue has emerged as an attractive alternative source of β-cells. The aim of this thesis was to optimize the culturing conditions of NPIs pre-transplantation so that the available tissue can be used as efficiently and economically as possible.

The results from this study indicate that the treatment of NPI cultures with z-VAD-FMK, a pan caspase inhibitor and general protease inhibitor significantly enhances β-cell survival. Additionally, the optimum length of culturing NPIs pre-transplantation appears to be 3-5 days. Since widespread cell death stimulates immunogenic response, this treatment also has the potential benefit of reducing immunosuppression needs in the recipient.

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LIST OF ABBREVIATIONS

ABC	Avidin-biotin complex
Apaf-1	Apoptotic peptidase activating factor 1
BSA	Bovin serum albumin
CK	Cytokeratin
DAB	3,3-diaminobenzidinetetrahydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
ER	Endoplasmic reticulum
ESC	Embryonic stem cells
EGF	Epidermal growth factor
FACS	Fluorescence activated cell sorter
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
Gal-α-gal	Galactose-α(1,3)-galactose
HBSS	Hank's Balanced Salt Solution
HLA	Human leukocyte antigen
HNF-6	Hepatocyte nuclear factor 6
IBMIR	Instant blood-mediated inflammatory reaction
IBMX	Isobutylmethylxanthine
IEQ	Islet equivalent
JNK	c-Jun N-terminal kinase
MHC	Major histocompatibility complex

Ngn3	Neurogenin 3
NPI	Neonatal porcine islet
PBS	Phosphate buffered saline
Pdx-1	Pancreatic duodenal homeobox gene-1
PERV	Porcine endogenous retrovirus
Ptf-1	Pancreas transcription factor 1
SEM	Standard error of the mean
SI	Stimulation Index
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TGFβ	Transforming growth factor beta
TNFR	Tumor necrosis factor receptor
TUNEL	TdT-mediated dUTP nick-end labeling
VEGF	Vascular endothelial growth factor
XIAP	x-linked inhibitor of apoptosis

CHAPTER ONE

General Introduction

1.1 Diabetes Mellitus

Diabetes is a chronic disease that occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. Hyperglycemia, or raised blood sugar, is a common effect of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves and blood vessels. There are two major forms of diabetes mellitus, namely type 1 diabetes mellitus (T1DM) and Type 2 diabetes mellitus (T2DM). T1DM is a condition in which pancreatic β-cell destruction usually leads to absolute insulin deficiency (Devendra D, Liu E and Eisenbarth GS, 2004). On the other hand, T2DM results from the resistance to the actions of insulin, particularly in liver, and peripheral muscle and fat tissue leading to increased insulin secretion by β-cells. Ultimately, β-cells are unable to secrete sufficient insulin to maintain normoglycemia, leading to T2DM (Lomberk G and Urrutia R, 2009).

Diabetes is so widespread that it has become a global epidemic. According to the Canadian Diabetes Association, an estimated 246 million people worldwide are affected by diabetes. With a further 7 million people developing diabetes each year, that number is expected to hit 380 million by 2025 (The Canadian Diabetes Association). In 2005, an estimated 1.1 million people died from diabetes. The World Health Organization (WHO) projects that deaths due to diabetes will

increase by more than 50% in the next 10 years without urgent action (Setacci C et al., 2009).

The full impact of the disease is much larger, because although people may live for years with diabetes, their cause of death is often recorded as heart disease or kidney failure. Diabetes leads to a number of life-threatening complications, including heart disease (Nathan DM et al., 2005; Weissman A. J. et al., 2005), hypertension (De La SA and Ruilope LM., 2000), nephropathy (Soldatos G and Cooper ME, 2008) and neuropathy (Donaghue KC and Silink M., 1999). Diabetes is now the most frequent cause of end-stage renal failure and blindness. Life expectancy for people with T1DM may be shortened by as much as 15 years. Life expectancy for people with T2DM may be shortened by 5 to 10 years (The Canadian Diabetes Association). The financial burden of diabetes and its complication is enormous. By 2010, it's estimated that diabetes will cost the Canadian healthcare system $15.6 billion a year and that number will rise to $19.2 billion by 2020 (The Canadian Diabetes Association).

1.1.1 Type 1 Diabetes Mellitus (T1DM)

T1DM (previously known as insulin-dependent or juvenile diabetes) accounts for 10% of all cases of diabetes, however its incidence continues to increase worldwide and it has serious implications. T1DM in itself is of two types: type 1A results from a cell-mediated autoimmune attack on β-cells, whereas type 1B, also referred to as idiopathic diabetes, is far less frequent and has no known cause (Imagawa A, Hanafusa T, Miyagawa J et al., 2000; Redondo

MJ, Fain PR, Eisenbarth GS, 2001). The susceptibility to T1DM is largely inherited, residing predominantly in the HLA genotypes DR and DQ and a host of other genetic loci termed insulin-dependent diabetes mellitus susceptibility genes (Redondo MJ, Fain PR, and Eisenbarth GS, 2001). These susceptibility genes are thought to be important regulators of the immune response. The major histocompatibility complex (MHC) is reported to account for approximately 40% of the familial aggregation of T1DM (Noble JA, Valdes AM, Cook M, et al., 1996; Lambert AP, Gillespie KM, Thomson G, et., al 2004).

The next step requires exposure to some environmental triggers such as viruses, environmental toxins, or foods that alter immune function, leading to the initiation of β-cell destruction (Lammi N, Karvonen M and Tuomilehto J, 2005; Robles DT and Eisenbarth GS, 2001; Helgason T and Jonasson MR, 1981). The abnormal activation of the T-cell-mediated immune system in susceptible individuals leads to an inflammatory response within islets as well as to a humoral response with the production of antibodies to β-cell antigens. The presence of these antibodies can precede the clinical onset of T1DM by years or even decades. The continuing destruction of β-cells leads to progressive loss of insulin-secretory response with, in order, loss of first phase insulin response to an intravenous glucose tolerance test (Bingley PJ, 1996), then to clinical diabetes when insulin secretion falls below a critical amount, and finally in most cases, a state of absolute insulin deficiency and patent hyperglycemia.

The main target of T1DM care is to avoid hyperglycemia in order to prevent long-term microvascular (retinopathy, nephropathy, and neuropathy) and

macrovascular complications (cardiovascular, cerebrovascular, and peripheral vascular disease). Some newly diagnosed patients with T1DM who still produce endogenous insulin and maintain euglycemia are still recommended to start intensive insulin therapy since it appears to promote cell preservation (Linn T, Ortac K, Laube H, et al., 1996; The Diabetes Control and Complication Trial Research Group, 1993).

In general, treatment for T1DM includes taking insulin shots or using an insulin pump, making wise food choices, and exercising regularly. The never-ceasing challenge faced by diabetics has lead to strong efforts to find ways and means for achieving better control of blood glucose levels. As a result, various new areas for better blood glucose control and amelioration of a diabetic's quality of life are being explored. For example, Exubera, an inhaled powder form of recombinant human insulin is now available for the treatment of adult patients with T1DM (White NH, Quattrin T, St Aubin LB, et al., 2008). Also, avenues in immunotherapy to prevent autoimmune depletion of β-cells are being investigated (Sytwu HK et al., 2003) in addition to islet transplantation for achieving euglycemia without insulin injections, which also carries the added benefit of improving glucose control.

1.1.2 Type 2 Diabetes Mellitus (T2DM)

T2DM, formerly known as adult-onset or non-insulin-dependent diabetes, is the most common form of diabetes accounting for approximately 90% of the diabetes cases. In T2DM, either the body does not produce enough insulin or

there is diminished insulin responsiveness in peripheral tissues. According to the United Kingdom Prospective Diabetes Study (UKPDS) data, by the time the diagnosis of T2DM is made, the individual has already lost 50% of their β-cell function (1998). Research has demonstrated that risk factors include a family history of diabetes, as well as being overweight and inactive (United Kingdom Prospective Diabetes Study, 1995).

Current T2DM treatment focuses on primary metabolic defects, namely insulin resistance and pancreatic islet dysfunction. During early loss of β-cell function, corresponding to phase 1 of diabetes, the treatment often involves monotherapy with diet, metformin, sulfonylureas etc. However, with progressive β-cell failure, patients may need combination therapy to control their blood glucose and eventually may need insulin to maintain glucose homeostasis (Das SK and Chakrabarti R, 2005).

1.2. A Historical Perspective

1.2.1 The Pancreas

The pancreas was first identified by Herophilus (335-280 BC), a Greek anatomist and surgeon. A few hundred years later, Ruphos, another Greek anatomist, gave the pancreas its name (Tsuchiya R and Fujisawa N, 1997). The term "pancreas" is derived from the Greek *pan*, "all", and *kreas*, "flesh", probably referring to the organ's homogeneous appearance. Since 1642, Johann Georg Wurstung had established the role of pancreas as a secretory gland. However, it was not until 1887 when Etienne Lancereaux noted that glycosuria occurred upon

pancreatitis that the role of pancreas in glucose regulation started being known. In 1889, Oscar Minkowski in collaboration with Joseph Von Mering removed the pancreas from a healthy dog to test its assumed role in digestion. Several days after the dog's pancreas was removed, they found that there was sugar in the dog's urine and the relationship between the pancreas and diabetes was established (Luft R, 1989).

1.2.2 The Islets of Langerhans

Islets of Langerhans were first described in 1869 when Dr. Paul Langerhans, a medical student in Berlin, was studying the structure of the pancreas under a microscope when he identified some previously un-noticed tissue clumps scattered throughout the bulk of the pancreas (Langerhans PHM, 1937). Later in 1893, one year after the successful transplant of pancreatic fragments to reverse diabetes in dog (Minkowski, 1892), Laguesse, who had been studying the islet cluster of cells in human pancreas, associated them with those discovered by Langerhans and named them islets of Langerhans in honor of Langerhans (Laguesse E., 1893). In 1901, Eugene Opie clearly established the link between the islets of Langerhans and diabetes by showing a pathological connection between diabetes and damage to the cells Langerhans had discovered (Opie E, 1901).

Islets of Langerhans are scattered throughout the pancreas and comprise approximately 2-3% of total pancreatic mass. Although the islets only represent a small part of pancreatic mass, they receive a large portion of pancreatic blood

flow. The estimation of blood flow to the islets in rabbit pancreas by administration of microspheres trapped in capillaries showed that 11-23% of the total pancreatic blood flow went directly to the islets (Lifson N et al., 1980).

In humans, each pancreatic islet is comprised of roughly 2000 cells, which include α, β, δ and PP cells. β-Cells, the most abundant cell type, synthesize and release insulin in response to glucose and comprise 60-80% of the total islet mass (Soria B et al., 2000). The α cells, which comprise 10-65% of the pancreas (Soria B et al., 2000), synthesize and secrete glucagon primarily in response to hypoglycemia. During hypoglycemia, glucagon secretion provides primary counter-regulatory hormonal response by traveling via the hepatic portal vein to the liver where it stimulates glycogenolysis and glucose production by the liver. Glucagon secretion is inhibited by somatostatin and hyperglycemia. The δ cells (2-8%) synthesize and secrete somatostatin, a response enhanced by high glucose levels (Soria B et al., 2000). Since somatostatin inhibits both insulin and glucagon secretion, it is often advocated as the regulator of neighboring α cells and β cells. The PP cells (1-2%) synthesize and secrete pancreatic polypeptide, primarily in response to hypoglycemia and secretin (Soria B et al., 2000). However, the function of this hormone in humans remains unclear.

1.2.3 The β-cell

The β-cell was first described by M.A. Lane in 1905 (Lane M., 1907). Later, immunofluorescent studies by Lacy and Davis in 1959 confirmed that these β-cells were indeed the insulin producing endocrine cells (Lacy PE and Davis J.,

1959). The β-cell is distinctive in its ability to sense changes in circulating blood glucose concentrations and its response to hyperglycemia by insulin release. Also, β-cells respond to amino acids, β-adrenergic stimulation, and other hormones (Torres N, Noriega L, and Tovar AR., 2009). Its insulin secretion is inhibited by epinephrine, somatostatin, and prostaglandin E2. The expression of a number of key transporters and enzymes allows for the release of insulin within minutes.

1.2.4 Insulin

The discovery of insulin by Frederick Banting, Charles Best, James Collip and J.J.R. MacLeod in 1921 marks one of those incredible moments in medical history that lead to the beginning of a new phase in the study and treatment of diabetes. The most significant effect of diabetes before discovery of insulin was patients falling into a hyperglycemia induced coma. Before this discovery, the treatment of diabetic patients often consisted of starvation or under nutrition, which may have worked for type 2 diabetics but had no effect on type 1 diabetic patients. With luck, and through careful management of carbohydrate intake, pioneers such as Dr. Fred Allen and Dr. Elliott P. Joslin were able to help some diabetics prolong their life by as long as a year or two (Bliss M, 2007).

During this time, Frederick Banting began his research on diabetes under supervision of MacLeod, a professor in the Department of Physiology at the University of Toronto along with a student assistant, Charles Best. On July 27, 1921 Banting and Best injected the pancreatic extract isolated from ligated canine pancreata (resulting in partial atrophy) into a diabetic dog. Within hours, the dog

recovered from the diabetic coma and its blood glucose levels had returned to normal (Banting FG and Best CH., 1922). Banting and Best repeated their experiments and enthusiastically reported their findings to MacLeod, who himself had been away on his summer holidays during this time. Now James Collip, a chemist was asked to join this research in order to purify the extract obtained by Banting and Best. Collip was responsible for making breakthroughs in the process of developing the extract. The process involved removing harmful toxins and impurities, which detracted from the beneficial properties of the extract.

Finally, the first patient, Leonard Thompson, was given the pancreatic extract on January 11, 1922. After being given this extract, he improved dramatically and became the first successfully treated human patient (Bliss M, 2007). The demand for insulin after this spectacular success soon surpassed the capabilities of the research group. During this same time, Lilly was becoming interested in producing insulin on a mass scale. Thus, in May 1922, an agreement was reached between the University of Toronto and Eli Lilly and Company in which the latter would produce the pancreatic extract at cost in return for the company's exclusive right for the sale of insulin in North America. As a result, in July 1922, Lilly started production of Isletin- the name of insulin at the time (Bliss M, 2007).

In 1923, Banting and Macleod were awarded the Nobel Prize in Physiology for the discovery of insulin. However, Banting split his half with his research assistant, Charles Best, and Macleod then split his half with his colleague, James Collip. Although Abel and coworkers first crystallized insulin in

1925, the exact sequence of amino acids comprising the insulin molecule, the so-called primary structure, was determined by British molecular biologist Frederick Sanger in 1959 (Sanger F, 1959). It was the first protein the structure of which was completely determined. For this he was awarded the Nobel Prize in Chemistry in 1958. Later, in 1967, Dorothy Crowfoot Hodgkin determined the spatial conformation of the molecule, by means of X-ray diffraction studies and was awarded a Nobel Prize as well. In 1982, genetically engineered human insulin was introduced and is now the standard method of treatment of diabetic patients (Dingermann T, 2008).

1.3 Islet Transplantation

Discovery of insulin in 1922 by Banting and Best (Banting FG and Best CH., 1922) lead to the saving of millions of patients with T1DM associated with life-threatening consequences. In spite of the fact that death from the acute complications of diabetic ketoacidosis is now rare, insulin replacement, even with the most modern approaches of intensive treatment is unable to control transient variations in blood glucose. Chronic hyperglycemia and peripheral hyperinsulinemia are believed to accelerate diabetic microangiopathy (Despres JP et al., 1996). Thus, the extreme efficiency of the biological system for tying insulin secretion to insulin need is difficult to achieve with insulin replacement.

On the other hand, transplantation of insulin producing tissue as a therapy for T1DM has many advantages because the recipient achieves glucose homeostasis without fluctuations associated with taking exogenous insulin. The

concept of islet transplantation is not new- investigators as early as Charles Pybus (1882-1975) attempted to graft pancreatic tissue to cure diabetes. Watson Williams performed the first clinical attempt to transplant islets by implanting fragments of sheep pancreas in subcutaneous tissue of a young boy dying from diabetic ketoacidosis. However, there was only a temporary improvement before the boy died a few days later (Williams, PW, 1894).

After the discovery of insulin in 1922, interest in this area lay dormant because a cure of diabetes seemed to be at hand. However, as late micro and macrovascular complications of T1DM were being uncovered and related to poor levels of glycemic control associated with patients taking insulin, interest in pancreatic endocrine cell replacement therapy was renewed. The first major development in islet isolation occurred through the use of collagenase solution to isolate islets from finely diced guinea pig pancreas with the pioneer work of Moskalewski (Moskalewski S, 1965).

Later, Lacy and Kostianovsky introduced ductal distension of the pancreas as a refinement of the procedure and applied this technique for isolation of rat islets (Lacy PE and Kostianovsky M, 1967). Several research groups soon reported the first transplantation experiments. Kelly and associates performed first successful transplantation of islets in humans for the treatment of T1DM; this transplant was whole organ pancreatic transplantation (Kelly WD et al., 1967). In 1972, Ballinger and Lacy reported the first successful islet transplantation in rats with chemically induced diabetes (Ballinger WF and Lacy PE, 1972). Subsequent studies showed that transplanted islets could reverse diabetes in both rodents and

non-human primates (Kemp CB et al., 1973; Scharp DW et al., 1975). This was followed by the first human islet allograft resulting in insulin independence (Scharp DW et al., 1990).

Despite improvements in isolation techniques and immunosuppressive regimens, only about 10% of islet recipients in the late 1990s achieved normoglycemia (Robertson RP, 2004). Interest in the field of islet transplantation increased tremendously when an intensive study by the diabetes control and complications trial (DCCT) in 1993 reported limitations associated with taking exogenous insulin. DCCT was a major clinical study conducted from 1983 to 1993, which compared the effects of standard control of blood glucose versus intensive control on the complications of diabetes.

Intensive control meant keeping hemoglobin A1C levels as close as possible to the normal value of 6 percent or less. The study showed that intensive control via exogenous insulin slows the onset and progression of the eye, kidney, and nerve damage caused by diabetes. However, the most significant side effect of intensive treatment was an increase in the risk for hypoglycemia, including episodes severe enough to require assistance from another person (The Diabetes Control and Complication Trial Research Group, 1993). This study clearly established a stronger rationale for clinical islet transplantation that has the potential benefit of achieving blood glucose homeostasis without risks of hypoglycemia, especially in patients with “brittle” diabetes.

Finally in 2000, a research group from the University of Alberta in Edmonton published a report describing that they had managed to normalize

blood glucose for greater than one year by islet transplantation without exogenous insulin in seven consecutive patients using a steroid-free protocol (Shapiro AM et al., 2000). This protocol, famously known as the Edmonton Protocol, was adapted by islet transplant centers around the world and greatly increased islet transplant success. The results lead to the establishment of multi-centre clinical trials with success rates varying between centers due to site-specific modifications and improvements in the experimental protocol (Shapiro AM and Ricordi C., 2004; Hering BJ et al., 2005). The success rates were so high that since then, more than 550 islet transplants have been performed (Shapiro et al., 2005).

Although early results were very promising, a five year follow up study of patients transplanted at Edmonton found that only about 10% patients remained insulin independent at five years with about 80% retaining some level of graft function as demonstrated by C-peptide levels (Ryan EA et al., 2005). Thus, islet transplant therapy has still to overcome many limitations before it can be considered a definitive treatment of diabetes. Nonetheless, pancreatic islet transplantation offers renewed hope to many patients, especially those with a high incidence of hypoglycemic episodes.

The major limitations that preclude the widespread application of islet transplantation include, first and foremost, an inadequate supply of donor tissue for transplantation and, second, problems of immune rejection. The transplant procedure can trigger a blood-mediated inflammatory reaction (IBMIR) instantaneously, leading to leukocyte infiltration and graft damage (Bennet W et al., 2000). Not only that, the primary pathology of autoimmune destruction of

pancreatic β-cells can lead to selective destruction of transplanted β-cells as well (Stegall MD et al., 1996). Furthermore, the immunosuppressive regimen leads to complications in a number of patients who develop mouth ulcers, edema, pneumonia, weight loss (Ryan EA et al., 2005). Moreover these immunosuppressive agents are nephrotoxic (Ryan EA et al., 2005). Therefore, there is a need to develop strategies and immunosuppressive regimens with minimal side effects.

Many patients require islet transplants from two or more pancreases to achieve a functional mass of 12,000 islet equivalents per kilogram body weight (Shapiro AM et al., 2000). Thus, there is a demand for an abundant supply of transplantable tissue, which may be met by four alternative sources, namely, the derivation of islet tissue from stem cells (adult and embryonic), induced β-cell regeneration, transdifferentiation of non-endocrine tissue, and xenogeneic tissue.

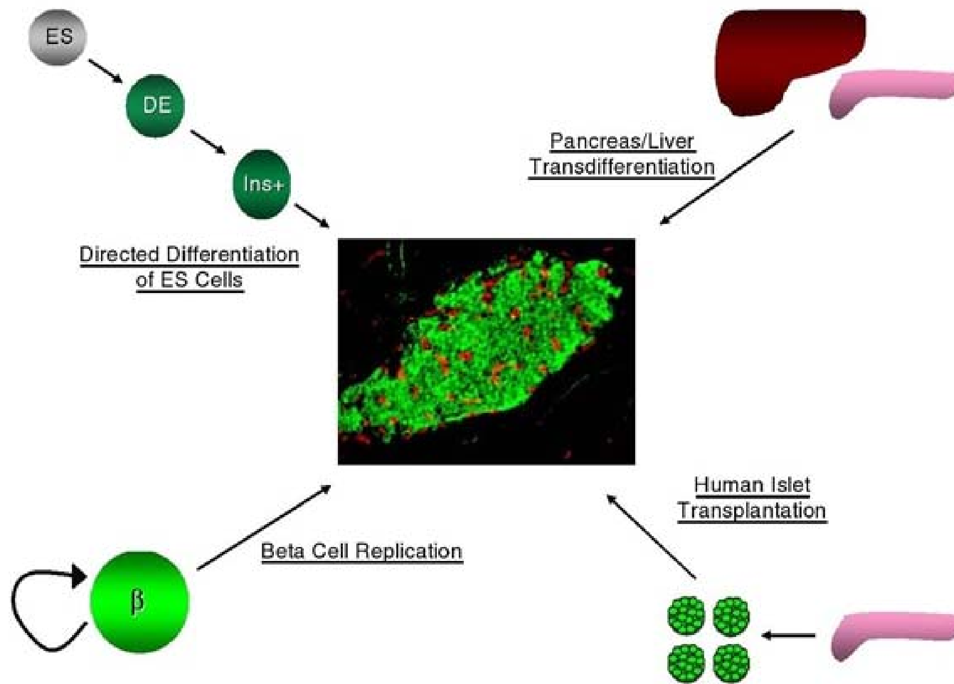

Figure 1.1 The possible alternative sources of new β-cells (Claiborn KC and Stoffers DA, 2008).

1.4 Alternative Sources of Islet Tissue

There is potential for liver and nonendocrine pancreatic cells to be reprogrammed by gene transfer or growth factor treatments to produce and release insulin in response to glucose. Recent evidence also suggests that adult β-cells are mitotically active and that replication of these cells can occur in response to increasing insulin demands. A well-known example of this is that β-cells of infants born to diabetic mothers (hyperglycemic environment) undergo hypertrophy, hyperplasia, and also demonstrate increased insulin content. A complete understanding of the factors and signals that control these processes could allow this innate ability to be harnessed in patients with diabetes. Finally, cadaveric human islets and xenogeneic islets represent a promising source of therapeutic beta cells through transplantation (Claiborn KC and Stoffers DA, 2008).

1.4.1 Stem Cells, β-Cell Regeneration, and Transdifferentiation

In recent years, there has been an increasing interest in the possible use of cell implants derived from stem cells to treat a variety of diseases. Use of epithelial skin grafts and hematopoietic cell transplants are already well-established clinical procedures, thus showing the utility of stem cell therapies. Possibly, inadequate supply of islet tissue could be overcome by the induction of embryonic or adult stem cells to differentiate into islets. One advantage to this approach is the possibility of propagating an unlimited number of cells that already possess the ability to become fully functioning endocrine tissue.

Furthermore, the potential use of adult stem cells offers the advantage of an autologous model where a patient's own cells can be used for transplantation, thereby circumventing the problem of immune rejection. Similarly, embryonic stem cells (ESCs) in an undifferentiated state are undefined immunologically and programmable by the recipient's own immune system as "self" tissue. Human ESCs are derived from the blastocyst, which is a group of 32 cells. Because of ESCs' special properties of renewable growth and selective differentiation, deriving insulin-secreting cells from this source is an exciting prospect for generating an unlimited supply of specialized beta cells for transplantation. The first report of successful generation of insulin-producing cells from mouse ESCs was published in 2000 (Soria et al., 2000). When transplanted into diabetic mice, these cells were able to restore normoglycemia.

There is increasing evidence that adult stem cells are located in the pancreatic duct. Chemical ablation of islet β–cell mass by selective cytotoxic drugs such as streptozotocin (STZ) is followed by a partial restoration of β-cell mass (Wang RN et al., 1996). It has been suggested that adult mouse islets contain progenitor cells that can replace β-cells destroyed by STZ (Fernandes A et al., 1997). Another pancreas regeneration model consists of surgical removal of part of the gland. Subtotal (90%) pancreatectomy has been shown to result in proliferation of the remaining ducts. These in turn form new ductules, acini and islets (Bonner-Weir S et al., 1993; Bonner-Weir S et al., 2000). Also, it has been shown that pancreas regeneration occurs following ligation of a part of the exocrine ductal system. In the ligated part, tissue remodeling occurs where the

predominantly acinar tissue is converted to ductal complexes. Although islets do not seem to be injured by this treatment, an important increase in the number of islet β-cells occurs between 4 and 7 days after ligation (Wang et al., 1995). New β-cells are seen in and around duct cells of the ductal complexes. A number of groups have since reported isolation and differentiation of stem cells derived from adult pancreatic ductal structures (Cornelius et al., 1997; Bonner-Weir et al., 2000; Ramiya et al., 2000) that express endocrine hormones. These observations suggest existence of adult pancreatic stem cells in ductal tissue, which contribute towards neogenesis of islets.

Recently, Gershengorn et al's experiments have added a new perspective to islet cell proliferation and differentiation mechanisms. It was shown that human islet cells may even undergo epithelial-mesenchymal transition into fibroblast cells, which can, after a period of substantial expansion, re-differentiate into aggregates of epithelial cells with a low level expression of islet endocrine genes (Gershengorn et al., 2004). These findings imply that in addition to proliferation of the β-cells, which is known to be minimal in human islets, the islet cell mass could expand through transient dedifferentiation and proliferation phase.

Currently the studies with human pancreatic cells are hampered by the fact that it is very difficult to obtain 100% pure cell populations-endocrine, exocrine or ductal. Also, one of the main drawbacks to the use of stem cells in islet transplantation is that techniques to identify and subsequently differentiate stem cells to an islet-endocrine phenotype are lacking. Although protocols result in enrichment of cells, many techniques still result in mixed cell populations.

1.4.2 Xenografts

Xenotransplantation is the transplantation of living cells, tissues or organs from one species to another such as from pigs to humans. Such cells, tissues or organs are called xenografts or xenotransplants. If successfully applied, the use of xenogeneic islets would provide relatively unlimited donor tissue. However, various questions remain undetermined concerning islet xenografts including a) the most appropriate xenogeneic donor for future implants into human diabetics, b) the most effective method for prevention of xenograft rejection, and c) the ability of xenografts to normalize glucose homeostasis.

A number of animal models have been considered in an attempt to find suitable sources for xenogeneic islets for transplantation. Non-human primates are an obvious potential source of islets for xenografting to human patients. Functional islets have been isolated from subhuman primates and xenografted into immunosuppressed rodents, with reversal of streptozotocin induced diabetes for up to one month (Chabot J, et al., 1987 and Weber C, et al., 1986). However, there are several problems associated with the use of primates. The supply of non-human primate donors is extremely limited because they have relatively few animals in each generation. Also, there are significant ethical issues surrounding their use. Amongst other species, some of the most promising sources of discordant xenogeneic islets seem to be bovine (Marchetti et al., 1995) and porcine islets (Korbutt GS et al., 1997; Groth CG et al., 1994).

Amongst many investigators, pigs are being regarded as the ideal donor source of xenogeneic islets for clinical use. Not only does porcine pancreas share anatomical and physiological similarities to human pancreas, the large supply, short gestation period, and large litter size make the pig a great functional model. Moreover, porcine insulin was the standard therapy for diabetes for many years before introduction of recombinant insulin. Porcine insulin differs from human insulin at only one amino acid (Home PD et al., 1982). Also, there have been successful results in transplants of pig islets to various mouse models of diabetes and islet transplantation including successful induction of tolerance (Wang G. et al., 2005) and immunosuppressive strategies (Adams AB et al., 2005; Bucher P et al., 2005; Mirenda V et al., 2005; Rayat GR and Gill RG, 2005; and Safley SA et al., 2005).

Porcine islet isolation yield and function are affected by factors such as age, diet, strain, and type of collagenase used. Adult pig islets are difficult to isolate and fragment easily in culture (Ricordi C, Finke EH, and Lacy PE., 1986). Fetal and neonatal islets or islet progenitors are more resilient than adult islets in terms of surviving early post transplant trauma perhaps due to the fact that they have a higher potential to proliferate and differentiate (Hayek A., 2004) than adult pancreas (Davalli A et al., 1995; Trivedi N et al., 2001). Because of these factors, fetal and neonatal pigs are being considered as better potential donors of islets than adult pigs for clinical transplantation. Moreover, *in vitro* insulin secretion of isolated islets from younger pigs is greater than those of older pigs (Jay T, Heald K, and Downing R., 1997). However, the advantages of using islets from older

pigs versus younger animals include faster normalization of blood glucose levels after transplantation (Hori H et al., 2001). It should be noted that the percentage volume density of β-cell in pancreas does not change as the pancreas matures (Jay TR et al., 1999). β cells do not cluster together into small islets until 10-13 days after birth (Alumets J, Hakanson R and Sundler F., 1983).

Thus, islets from younger animals are referred to as islet-like cell clusters since majority of these cells are epithelial precursor cells with few amounts of endocrine cells. Postnatal maturation of pancreatic islets makes neonatal pigs a more attractive source of β cells than fetal pancreas. Unlike immature fetal pig β cells, neonatal pig β-cells can respond to *in vitro* glucose stimulation (Korsgren O et al., 1991; Kuo C et al., 1993; Ohgawara H et al., 1991).

In 1996, Korbutt et al. developed a simple, reliable procedure for the large-scale isolation of islet cells from 1-3 day old neonatal pigs (Korbutt et al., 1996). The cellular composition of neonatal islet cell aggregates was defined and growth potential and viability of islets was assessed both *in vitro* and *in vivo*. Results showed that these islets survived well in tissue culture, during which time the total β cell mass increased 150%. These islets were able to normalize hyperglycemia in 100% of diabetic mice receiving 2000 islet aggregates. When grafts were recovered at 14-weeks post-transplantation, there was a 20-30-fold increase in cellular insulin in the host relative to the time of transplantation. Following this initial report, subsequent experiments have shown that these relatively immature islets are an ideal source of islets for transplantation (Yoon K et al., 1999; Korbutt G et al., 1997; Nielsen T et al., 2003). Moreover, the

surprising increase in β cell mass in a hyperglycemic environment has been attributed to both proliferation of ductal cells and their transdifferentiation into β cells (as shown by both CK-7 and insulin positive staining) as well as active proliferation of β cells (Trivedi N et al., 2001).

Neonatal pig islets were recently transplanted into the hepatic portal circulation of rhesus macaque monkeys (Cardona K et al., 2006). Immunosuppression was carried out via use of anti-CD25 and anti-CD 154 followed by long-term administration of sirolimus and belatacept. Insulin independence was achieved in all seven immunosuppressed animals by 60 days post-transplantation suggesting proliferation and differentiation of neonatal islets following engraftment.

Finally, neonatal pig islets were microencapsulated in alginate-polyornithine-alginate and transplanted into the intraperitoneal spaces of eight adult male cynomolgus monkeys rendered diabetic with streptozotocin. All animals received a second transplant and there was no immunosuppression administered. This approach resulted in the decrease of exogenous insulin requirement by a mean of 43% with equally good blood glucose control in the transplanted animals compared with control group (Elliott RB et al., 2005). Thus, the encapsulation of islets presents a potentially promising method to eliminate the requirement of long-term immunosuppression for islet xenografts. Therefore, results indicate that the pig islet xenografts in this non-human primate diabetic model can indeed provide a "cure" of the diabetes.

Although pig donors present a great source of islets, a large islet mass is required because a comparatively small islet yield is insufficient to cure an adult human of T1DM. Thus, having a more optimized system of the variables from isolation to post isolation culturing before transplantation could help immensely in the overall efficiency of the system.

1.5 Development of the Pancreas

Development of the islets of Langerhans within the pancreas is initiated from a pool of undifferentiated precursor cells associated with the pancreatic ductal epithelium (Weir GC and Bonner-Weir S, 1990; Peters et al., 2000). Thus, all the epithelial cell types of the pancreas, from both the exocrine and endocrine tissue originate from a common pool of endodermal progenitor cells during embryogenesis (Slack, 1995; Percival and Slack, 1999). This common pool of progenitor cells for liver and ventral pancreas becomes determined by a gradient of morphogen fibroblast growth factor (Deutsch et al., 2001).

Starting at embryonic day 9 (E9) in rodents, first the dorsal pancreatic bud and later the ventral bud is formed from the embryonic foregut and eventually fuse (Slack, 1995). Each structure communicates with the foregut through a duct. The ventral pancreatic bud becomes the head and unciate process, and comes from the hepatic diverticulum. Then, the differential rotation and fusion of the ventral and dorsal pancreatic buds results in the formation of defined pancreas. As the duodenum rotates to the right, it carries with it the ventral pancreatic bud and common bile duct. Once it reaches its destination, the ventral pancreatic bud fuses

with the larger dorsal pancreatic bud. The fusion of the main ducts of the ventral and dorsal pancreatic buds form the duct of Wirsung, the main pancreatic duct.

The endodermal cells in the ducts organize into ductal structures that become surrounded by abundant mesenchymal tissue. Immature acinar structures as well as endocrine cell groups bud off from the fetal ducts. Mesenchymally derived FGF-10 (Bhushan et al., 2001), and epidermal growth factor (EGF) (Cras-Meneur et al., 2001) promote the proliferation of undifferentiated progenitor cells.

Recent studies have found some major genes that encode for transcription factors regulating cell differentiation in the embryonic pancreas.

The first and major pancreatic gene is Pdx-1, which belongs to the family of homeobox- containing genes and transactivates the insulin gene (Ohlsson et al., 1993). Pancreas differentiation begins in epithelial cells expressing Hnf6, Hlxb9, Hnf3β, and Pdx-1. Hnf6 is required for induction of Hnf3β and Ngn3. Hnf3β is an important regulator of the Pdx1 gene (Wu et al., 1997). In the early pancreatic buds, all epithelial cells express Pdx-1. Later in development, Pdx-1 expression is downregulated in most cells, but a high level of expression is retained in β-cells and a small subset of δ-cells. In adult human pancreas, Pdx-1 is still expressed in ductal cells but shows a different pattern of activation and DNA binding compared with β-cells (Heimberg et al., 2000). Another major pancreatic gene is p48/Ptf-1, which is initially expressed in all pancreatic epithelial cells but later becomes restricted to exocrine cells (Krapp et al., 1996). In the case of both Pdx-1 and p48, the targeted ablation of the gene leads to pancreatic loss of development,

which indicates the importance of both factors for pancreatic cell differentiation (Jonsson et al., 1994).

Differentiation of cells of the pancreas proceeds through two different pathways, in accord with the dual endocrine and exocrine functions of the pancreas. Lateral inhibition seems to be involved in the specification of cells towards the endocrine or exocrine fate. Similar to neural system development, this specification involves expression of the Delta-Notch system. The differentiating cell expresses the Delta ligand while ligation of Notch, the receptor for Delta on a neighboring progenitor cell lead to the activation of the HES genes that encode for basic helix-loop-helix transcription factors. HES proteins repress pro-endocrine genes preventing endocrine cell differentiation but promote maintenance of a pool of undifferentiated progenitor cells. Ngn-3, a basic helix-loop-helix protein is thought to represent the major pro-endocrine gene that is directly antagonized by the Delta-Notch system (Gradwohl et al., 2000; Jensen et al., 2000; Schwitzgebel et al., 2000). Based on this model, it has been proposed that the direct progenitors of islet cell are Pdx-1, Ngn-3 positive while p48 and Hes-1 negative cells (Jensen et al., 2000).

By the fourth or fifth month of human embryonic development, insulin and glucagon can be detected in the fetal circulation. Just before birth, the endocrine cells cluster together into islets and thereafter undergo maturation, attaining full glucose responsiveness two-three weeks after birth.

Secretions by the pancreas are regulated both by neural and hormonal stimuli. The endocrine cells of pancreas are mainly organized into compact

collections of cell clusters called islets of Langerhans. In contrast to the endocrine pancreas, the exocrine pancreas produces digestive enzymes and an alkaline fluid, and secretes them into the small intestine through a system of exocrine ducts in response to the small intestine hormones secretin and cholecystokinin. Digestive enzymes including trypsin, chymotrypsin, pancreatic amylase, and pancreatic lipase are produced and secreted by acinar cells of the exocrine pancreas. Specific cells that line the pancreatic ducts, called centroacinar cells, secrete a bicarbonate- and salt-rich solution into the small intestine.

1.6 Physiology and Pathophysiology of Insulin and its Secretion

The gene encoding insulin is located on chromosome 11 and is expressed only in β-cells (Edlund T, Walker MD, Barr PJ, Rutter WJ, 1985). The insulin protein contains 51 amino acids and weighs 5808 daltons. Insulin is assembled into a hexamer and has threefold symmetry with the zinc ions holding it together. Transcription of the insulin gene leads to preproinsulin, which is the biosynthetic precursor of insulin. Preproinsulin has a short half-life and therefore is found only in trace amounts in β-cells. Proinsulin is progressively processed from the rough endoplasmic reticulum to the golgi apparatus ending in the mature secretory granule (Robbins DC, Tager HS, and Rubenstein AH, 1984). Most of the enzymatic processing of proinsulin to insulin occurs in secretory granules. The result of post-translational modification is an equimolar production of insulin with alpha and beta chains linked by disulfide bonds and a connecting peptide (C-peptide) (Hou JC, Min L, and Pessin JE, 2009).

The β-cell releases insulin in response to circulating blood glucose. The membrane potential of β-cells is controlled by potassium channels (K_{ATP} channel) and in the absence of glucose it is at -70 to -80 mV. Glucose binds glucose transporter (GLUT) in β-cell membrane and is moved into the cell by facilitated diffusion. Studies have shown that the expression of GLUT varies with developmental stage and also within species. With regards to age, for instance, compared to adult β-cell, fetal β-cell express significantly less GLUT2 (Hathout E et al., 1997). This reduced GLUT2 expression in fetal β-cell has been suggested to hamper the glucose uptake resulting in an immature insulin secretory response (Rorsman P et al., 1989).

Once glucose enters the cell, it is metabolized and ATP generation leads to closure of the K_{ATP} channels leading to depolarization of membrane to about -55 mV (Ashcroft FM, Harrison DE, and Ashcroft SJH, 1984). These channels are referred to as K_{ATP} channels since they are regulated by metabolites (Misler S et al., 1986) and inhibited by ATP (Cook DL and Hales CN, 1984). The depolarization caused by K_{ATP} channel closure in turn activates voltage gated calcium channels in the membrane. This leads to entry of extracellular calcium into cells and this rise in calcium in cytosol causes phospholipase C activation. The cleavage of membrane phospholipid phosphatidyl inositol 4,5-bisphosphate into inositol 1,4,5-triphosphate and diacylglycerol occurs as a result. The subsequent binding of 1,4,5-triphosphate to membrane receptors of endoplastic reticulum leads to calcium release from endoplastic reticulum via 1,4,5-triphosphate gated channels facilitating even higher calcium concentration.

Elevated levels of intracellular calcium trigger exocytosis of insulin granules, which has been previously synthesized and stored in the secretory vesicle.

If the glucose concentrations are high (above 7mM), the membrane potential oscillates between an active and silent phase and leads to firing of calcium action potentials (Atwater I, Carroll P, and Li MX, 1989). The higher the glucose concentration, the more time the β-cell spends in the "active phase" consisting of continuous firing of calcium action potentials. Thus, stimulated insulin release from β-cells is dependent on calcium entry into cells and is K_{ATP} channel dependent (Grodsky GM and Bennett LL, 1966). Basal insulin release induced by lower concentrations/absence of glucose, on the other hand, is not dependent on calcium entry and thus is K_{ATP} channel independent pathway (Straub S and Sharp G., 2002).

Thus, in the pancreatic β-cell, K_{ATP} channels play a critical role in coupling membrane excitability to glucose-stimulated insulin secretion, thereby maintaining blood glucose within a narrow physiologic range. Mutations that result in "overactive" K_{ATP} channels decrease membrane excitability and impair glucose sensing by the β-cell as demonstrated in mice constitutively expressing ATP-insensitive β-cell K_{ATP} channels, which develop profound neonatal diabetes (Koster et al, 2000). Genetic studies have now identified K_{ATP} channel mutations as the most common cause of neonatal diabetes mellitus in humans (Gloyn et al, 2004; Hamilton-Shield, 2007; Polak and Cave, 2007).

Insulin secretion by β-cells is phasic and exhibits cyclic oscillations (Rosario LM, Atwater I, and Scott AM, 1986). In the non-fed state in a normal

man, significant pulses of insulin release occur every 10-15 min (Lang DA et al., 1979). The first phase is transient and results in “burst” of insulin followed by second sustained phase lasting for remainder of the glucose stimulation period (Grodsky GM., 1972).

It has been hypothesized that pulsatile rather than continuous release of insulin to target tissues may have some benefit in terms of receptor regulation and thus may be a more effective method of hormone delivery (Matthews DR et al., 1983). Once released from the islet, insulin travels through the hepatic portal and systemic circulation to reach the liver, muscle and fat cells where it exerts its primary metabolic actions by decreasing glucose production by the liver and increasing glucose uptake by muscle and fat cells.

1.7 Culturing Neonatal Porcine Islets Pre-transplantation

After isolation from a donor pancreas, islets can either be transplanted immediately or may be cultured for some time. Culturing islets before transplantation has many advantages over immediate transplantation. Culturing allows for testing of *in vitro* and *in vivo* function before human transplantation and better matching of donor/recipient pairs. Maintaining islets in culture also allows time for possible immunologic or genetic modifications of islets. There are many clinical benefits of culturing islets pre-transplantation since it allows for selection of viable functionally tested islets and eliminates the discarding of islets because of low purity or yield (Gaber AO and Fraga D, 2004).

Short-term culture is associated with a loss of exocrine tissue and an increase in the purity of islet preparations, with increase in metabolic efficiency (Lupi R et al., 2004). Additionally, short-term culture reduces immunogenicity of islets by allowing removal of apoptotic and necrotic islets that not only affect performance of the islet preparation, but also are harmful to the implantation and engraftment process since they induce inflammatory responses to the islet infusion (Stein E et al., 1994; Lacy PE, Davie JM and Finke EH, 1979). Endothelial cells that are thought to play role in graft rejection are also reduced upon culturing (Rose ML., 1998).

In adult pigs, a recent study by Rijkelijkhuizen JK, et al. (2006) demonstrated that after 1 day of culture islet recovery was only 21% and grafts of these islets cured 12 of 17 mice. However, after 7 to 14 days of culture, the recovery had decreased to 11% but these islets reversed hyperglycemia in all mice (13/13), which then showed shorter time to normoglycemia and more tightly regulated blood glucose. This indicates that culturing is a valuable tool to improve graft quality and homogeneity.

Islet culture can be affected by the residual presence of proteases from the digestion phase of islet isolation (Brandhorst H et al., 2003). These enzymes lead to destruction of the islet's collagen matrix causing fragmentation and death. The addition of protease inhibitors to culture media may help counter these detrimental effects.

The first few days after isolation and engraftment are critical to islet survival and function. Islet isolation exposes the islets to a variety of cellular

stresses and disrupts the cell-matrix relationships, events known to be associated with apoptosis. Also, the rich blood supply of the islets has been lost upon collagenase digestion and islets have become dependent on oxygen and nutrients from periphery. Hypoxia experienced during isolation procedure and collagenase digestion has a profound effect upon the survival of islets and has powerful inhibitory effect upon insulin secretion. The islet cultures are thus subject to apoptotic and necrotic cell death from hypoxia and stress experienced during islet isolation (Andersson A., 1978; Brandhorst D et al., 1997; Ilieva A et al., 1999; Rosenberg L et al., 1999).

The various traumas to the islets mentioned above enhance apoptosis of the β cells through stress signaling pathways. It is believed that the most important death signalling pathway activated is c-Jun NH_2-terminal kinase (JNK1), a mitogen-activated protein kinase. Thus, inhibiting the JNK pathway should enhance the function of the grafted islets. This hypothesis has been confirmed. Noguchi et al. (2007) demonstrated that intraportal injection of JNK inhibitory peptide during islet transplantation in diabetic mice prevented the early loss of islet graft failure and improved the islet transplant outcome. Treatment of human islets in culture with a cell-permeable peptide inhibitor of JNK1 reduced apoptosis and inhibited the adverse effect of cytokines *in vitro*. When the treated islets were transplanted into diabetic immunodefficient mice, graft outcome was improved (Fornoni A et al., 2008).

Additionally, several studies aiming to inhibit cell death by blocking the conserved pathways leading to apoptosis have showed improved graft function. It

has been shown that islets transfected with genes preventing apoptosis prior to transplantation lead to protection of islets and greatly improve graft survival (Rabinovitch A et al., 1999). For example, the treatment of islets with broad-spectrum caspase selective inhibitor EP1013 potently enhances marginal-mass islet graft function post-transplant as confirmed by significantly higher graft insulin content and function using murine syngeneic islets and human islets transplanted into immunodefficient mice (Emamaullee JA et al., 2008). Similar studies using caspase inhibitor z-VAD-FMK (Emamaullee JA et al., 2007; Montolio M et al., 2005), and X-linked inhibitor of apoptosis protein (Emamaullee JA et al., 2005) led to prevention of early post-transplantation apoptosis and reduction in islet mass sufficient to achieve normoglycemia and enhanced graft survival post-transplantation. Also, selective caspase inhibitors, for example caspase-3 inhibitor (Z-DEVD-FMK) have been shown to prevent apoptosis in isolated human islet cells leading to improved islet yield and grafted islet function *in vivo* (Nakano M et al., 2004).

1.8. Mechanisms of Cell Death

There are three major types of cell death, namely apoptotic, necrotic, and autophagic cell death. Of these cell death types, caspases have been primarily linked with apoptotic cell death. Caspases, key mediators of apoptosis, are a structurally related family of cysteine proteases that cleave their substrate at aspartic acid residues either to cause cell death or to activate cytokines as part of an immune response (Callus BA and Vaux DL, 2007).

Apoptosis of beta cells is a feature of both T1DM and T2DM as well as loss of islets after transplantation. Loss of beta cells after islet transplantation is due to many factors including the stress associated with islet isolation, primary graft non-function and allogeneic graft rejection. Irrespective of the exact mediators, highly conserved intracellular pathways of apoptosis are triggered. There are two major pathways leading to apoptosis of mammalian cells, the 'extrinsic' (death receptor-induced) and 'intrinsic' (Bcl-2-regulated or mitochondrial) pathways. The extrinsic pathway is mediated by cell death receptors such as Fas or TNFR, which by a series of well described signaling steps lead to caspase activation and apoptosis. The intrinsic pathway is often activated by cell stress, such as growth factor withdrawal, application of chemotherapeutic drugs, and exposure to the cytotoxic granule constituents perforin and granzymes.

The intrinsic pathway is regulated by the balance between pro- and anti-apoptotic members of the Bcl-2 protein family. It reaches the point of no return when the outer mitochondrial membrane is disrupted causing an increased mitochondrial membrane permeability and subsequent release of cytochrome c leads to an initiation of the apoptosis cascade via activation of the apoptosis kinase Apaf-1. The activation of caspase 9 activates further downstream caspases such as caspases 3, 6, and 7 and this "caspase cascade" ultimately results in apoptosis of the cell (Thomas HE et al., 2009).

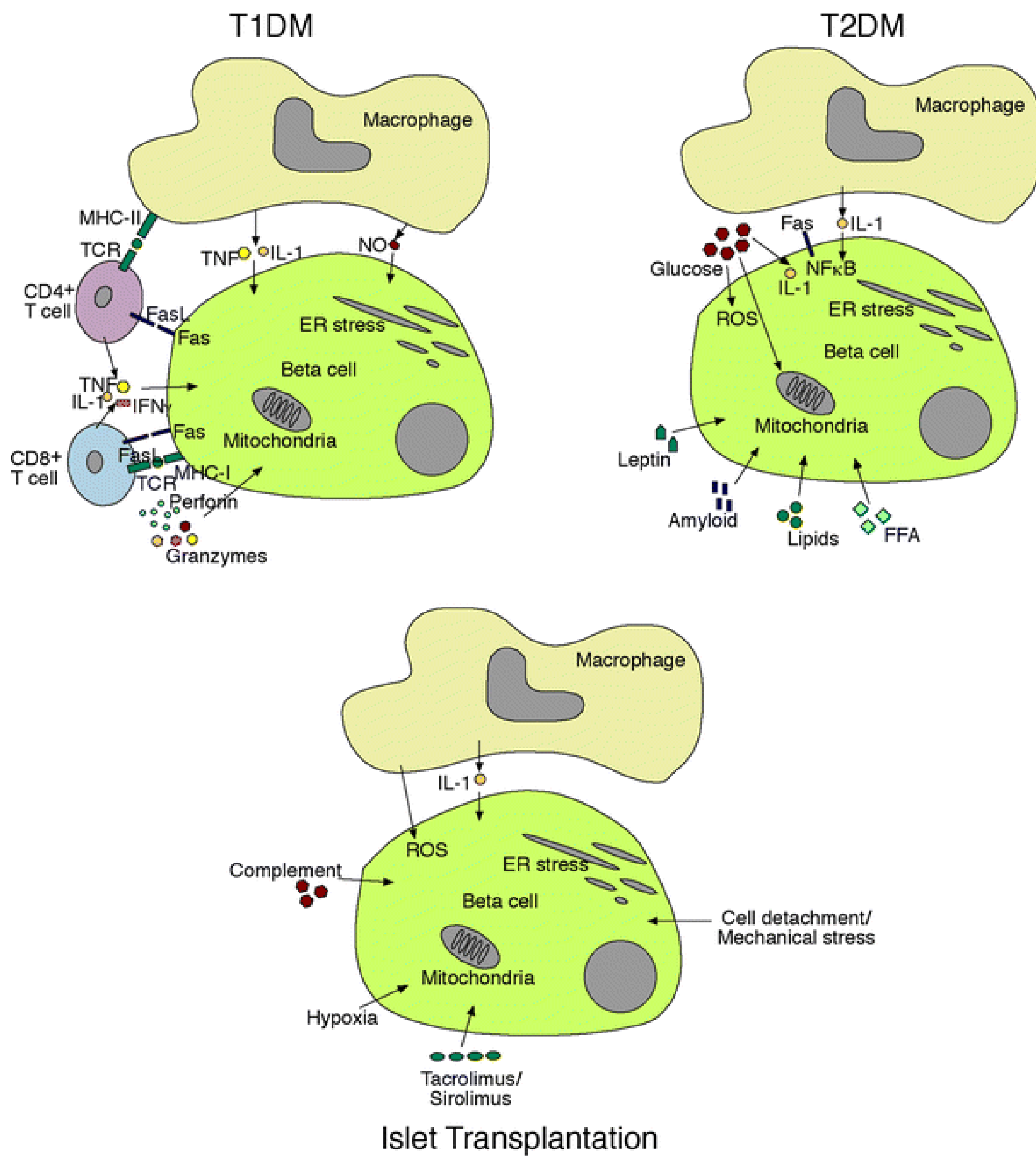

Figure 1.2. Beta cell apoptosis in T1DM, T2DM, and islet transplantation. The different cellular and molecular mediators of beta cell apoptosis are shown for T1DM, T2DM and primary non-function of islets after transplantation. After initial graft loss, transplanted islets become susceptible to the cellular and molecular mechanisms of apoptosis outlined for T1DM and T2DM (Thomas HE et al., 2009).

Altogether, as shown in Figure 1.2, apoptosis plays an important role in the development of T2DM, T1DM (Kurrer MO et al., 1997), its rodent model of nonobese diabetic (NOD) mice (O'Brien BA et al., 1997), and finally, in islet cell death post isolation (Cattan P et al., 2001; Paraskevas S et al., 2000). These findings suggest that the yield and the function of isolated islets could be improved by protecting islet cells from apoptotic cell death during the process of islet isolation and transplantation.

Several options to prevent apoptosis exist, including transfecting isolated islets with antiapoptotic proteins such as Bcl-2 (Contreras JL et al., 2001). An attractive alternative is to block apoptotic pathways through the pretreatment of islets with synthetic caspase inhibitors. As mentioned earlier, one such agent is N-benzyloxycabonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-FMK), a cell-permeable irreversible inhibitor of caspases whose permeability is facilitated by the presence of the benzyl-oxycarbonyl and OMe groups. z-VAD-FMK is a potent inhibitor of apoptosis induced by a wide range of stimuli (Cohen GM, 1997).

1.9 Thesis Objectives

The objective of this study is to assess the effects of different lengths of culture periods and the effect of caspase and protease inhibitors on the *in vitro* and *in vivo* function and viability of neonatal porcine islet cells. The overall aim of this study is to assess the optimum culturing conditions of neonatal porcine islets pre-transplantation with the intent of reducing time required to reach

normoglycemia in diabetic recipients along with making the procedure more cost-efficient.

More specific aims are:

1) Preparations from 1, 3 and 7 day of cultures will be assessed and compared for insulin and DNA content, glucose stimulated insulin secretion using standard static incubation assay, and cell composition by staining for insulin, glucagon and CK-7 positive cells. The data will be used to estimate the appropriate length of culturing neonatal porcine islets.
2) After isolation, neonatal porcine islets will be exposed to z-VAD-FMK, a pan-caspase inhibitor and cultured in modified Ham's F10 medium containing no BSA and supplemented with human serum albumin and protease inhibitors. After 1, 3 and 7 days of culture, preparations will be assessed for insulin and DNA content, glucose stimulated insulin secretion using standard static incubation assay, and cell composition by staining for insulin, glucagon and CK-7 positive cells. The data will be analyzed for evaluation of the hypothesis that the use of caspase inhibitors will reduce apoptotic cell death by initiation of caspase pathways, thereby improving graft outcomes.

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CHAPTER TWO

In Vitro Optimization of Neonatal Porcine Islet Cultures Pre-Transplantation

2.1 Introduction

The success of the Edmonton Protocol in 2000 has renewed the interest and belief in pancreatic islet transplantation as a viable strategy to treat patients with type 1 diabetes, especially "brittle" diabetes. For islet allo-transplantation, an average of 10,000 human IEQ/kg recipient body weight is required to achieve insulin independence (Shapiro AM, 2000), which usually can only be obtained from two donors, of which there already exists an extreme shortage.

This shortage is a significant impediment to the widespread application of clinical islet transplantation (Shapiro AM, 2006). Therefore, to circumvent this supply problem, porcine islet xenotransplantation currently represents the most appropriate solution. Although islets can be harvested from any developmental stage of the pig, neonatal porcine islet cells are more responsive to glucose than fetal β cells (Asplund K, 1973) and only neonatal and adult pig islets are shown to correct diabetes in non-human primate recipients (Rajotte RV, 2008).

Korbutt et al. in 1996 for the first time established not only the ability to reproducibly isolate porcine neonatal islet cells, but also the ability of these islets to correct diabetes in nude mice. Tissue from one neonatal pancreas was shown to yield approximately 50,000 islet cell aggregates (Korbutt GS et al., 1996). In the past, studies using porcine islet cell xenotransplantation reported a maximum islet graft survival of 2 months despite the use of strong immunosuppressive regimens

(Soderlund J et al, 1999; Komoda H et al., 2005; Sun Y et al., 1996; Kirchhof N et al., 2004).

However, in 2006, significant progress was made toward clinical trials of pig islet transplantation when two major studies reported that wild-type porcine islets reversed experimentally induced diabetes for up to 6 months in cynomolgus macaques (Hering BJ et al., 2006) and almost 9 months in rhesus monkeys (Cardona K, Korbutt GS, et al., 2006). For the first study, 25,000 IEQ/kg from two-day cultures of adult pigs were transplanted in recipient diabetic cynomolgus macaques. In second study, approximately 6.2 x 10^6 β-cells/kg (50,000 IEQ/kg) of neonatal porcine islets were cultured and transplanted on day 7 post-isolation. Diabetes had been established in all eight recipients by total pancreatectomy. Although the multi-drug immunosuppressive regimen used for these studies was more intensive than most clinicians and human patients would find acceptable, these studies demonstrated consistent, prevalent long-term insulin independence for the first time in pig-to-primate models.

Nevertheless, graft failure remains a significant problem leading to loss of insulin independence in recipients. Despite the early success of Edmonton Protocol, recent long-term follow-up studies indicate marked reductions in graft function, with only 15% of recipients maintaining normoglycemia without exogenous insulin after 5 years (Ryan EA et al., 2005).

Beta cells are lost during isolation as well due to damaging factors such as exposure to drugs and hypoxic environment in the donor before islet isolation, enzymatic and mechanical stress. After isolation, beta cells are also lost during

transplantation. Later, the loss of beta cells leading to deteriorating graft function over time can be attributed to β-cell autoimmunity, toxic immunosuppressive drugs, tissue factor release, and cell stresses such as hypoxia and inadequate revascularization of the graft. (Emamaullee JA and Shapiro AM, 2007). Additives during islet isolation or prior to transplantation may combat some of the adverse effect of both islet isolation and primary non-function. For example, the protease inhibitor α1-antitrypsin inhibits thrombin, and promotes early graft survival (Lewis EC et al., 2005). The loss of transplanted tissue can be attributed to apoptosis and necrosis especially since experimental studies in both human recipients and non-human primates have reported detection of circulating mRNA of genes for granzyme B, perforin and Fas-ligand in correlation with subsequent decrease or loss in islet graft function (Rood PP, 2007; Han D et al., 2004; 2002).

In mammalian cells, two distinct apoptotic pathways have been defined, the 'extrinsic' and 'intrinsic' (Bcl-2-regulated mitochondrial pathways) (Marsden VS and Strasser A et al., 2003). These pathways ultimately converge by activating common downstream effector cysteine proteases (caspases) that cleave a large number of intra-cellular proteins thereby causing cell death.

The extrinsic pathway is mediated by cell death receptors such as Fas or TNFR, which leads to caspase activation and apoptosis. The intrinsic pathway is often activated by cell stress, such as growth factor withdrawal, application of chemotherapeutic drugs, and exposure to the cytotoxic granule constituents perforin and granzymes. The intrinsic pathway is regulated by the balance between pro- and anti-apoptotic members of the Bcl-2 protein family. It reaches

the point of no return when the outer mitochondrial membrane is disrupted, leading to release of cytochrome *c* and downstream caspase activation (Figure 2.1). Upregulation of Fas expression on mouse or human islets makes them susceptible to *in vitro* killing by soluble FasL or anti-Fas Ab (Thomas HE, Darwiche R et al., 1999). This death is a caspase dependent process that can be inhibited with the caspase inhibitor z-VAD-fmk.

One of the major breakthroughs in islet transplantation was the use of islet-friendly immunosuppressive drugs that include a glucocorticoid-free regimen consisting of sirolimus, tacrolimus, and daclizumab (Shapiro AM et al., 2000). However, these drugs are toxic to islets, and it has been shown that incubation of islets with tacrolimus and sirolimus *in vitro*, either alone or in combination, causes significant inhibition of islet insulin secretory function and apoptosis. This is associated with an increase in the mitochondrial release of the pro-apoptotic factor Smac. The inhibitor of apoptosis, XIAP (Figure 2.1), was shown to reduce the toxic effects of immunosuppressive drugs on islets (Hui H et al., 2005), suggesting that blocking caspases may be a useful strategy to improve islet survival post-transplantation.

Therefore, it is possible that the use of agents that inhibit cell death will promote islet cell survival in early post-transplant period enhancing long-term graft outcome. As mentioned earlier, z-VAD-FMK, a broad-spectrum caspase inhibitor is one such agent that is well known to inhibit apoptosis (Hui H et al., 2005; Montolio M et al., 2005; Emamaullee JA et al., 2005, 2007, 2008).

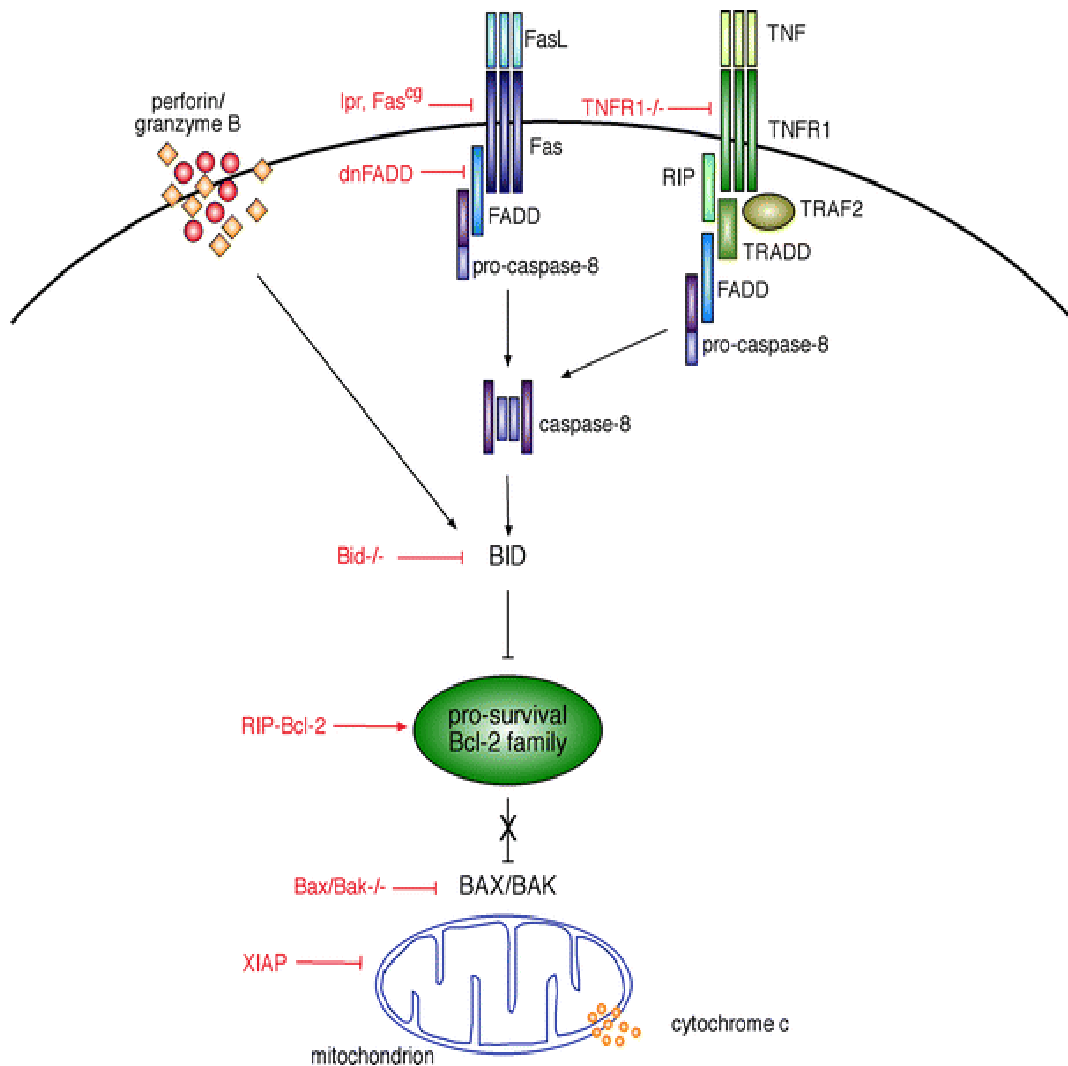

Figure 2.1. Pathways of apoptosis in β cells. *In vitro*, the perforin/granzyme, Fas/FasL and TNF pathways appear to be dependent on the pro-apoptotic molecule Bid in beta cells. Different parts of this pathway have been targeted in beta cells (indicated in *red*) (Thomas HE et al., 2009).

Additionally, protease inhibitors have been reported to provide improved islet yield (Wee YM et al., 2008). This study was undertaken as a means to use these strategies to improve neonatal porcine islet graft outcome.

2.2. Materials and Methods

2.2.1 Neonatal pig pancreatic islet isolation and culture

Isolation of islets was performed using male Duroc neonatal pigs (Swine Research and Techology Centre, University of Alberta) from ages of 1-2 days old (body weight range of 1.4-1.9 kg) as previously described (Korbutt GS et al., 1996). After anaesthetizing with Halothane, piglets were subjected to laparotomy and complete exsanguination. The pancreas was dissected from surrounding tissue and placed in cooled HBSS supplemented with 0.25% w/v BSA. Then, it was minced into fragments of about 1-2 mm^3, transferred to sterile tubes containing HBSS (supplemented as above) with 1.0 mg/ml collagenase XI (Sigma-Aldrich, Oakville, ON, Canada), and filtered through a 500 μm nylon screen. The digest was washed four times in HBSS and cultured in four plates per pancreas in Hams F10 medium (Invitrogen, Burlington, ON, Canada) supplemented with 10 mM glucose, 10 mM nicotinamide (BDH Biochemical, Poole, Dorset, UK), 2 mM L-glutamine, 0.5% w/v BSA (fraction V, Sigma), 50 μm IBMX (ICN Biomedicals, Montreal, QC, Canada), 100 U/ml penicillin and 100 μg/ml streptomycin (Cambrex Bio Science, East Rutherford, NJ, USA). The islets were cultured for the duration of 7 days at 37° C in humidified 5% CO_2/95% air, with media changes and samples drawn for assessment of insulin, DNA content and

immunohistochemistry at three time points of: after 1 day of culture, 3 days of culture, and 7 days of culture.

2.2.2 Caspase inhibitor exposure and inhibition of proteases

In order to accomplish the inhibition of caspases and active proteases *in vitro*, islets were exposed post isolation to general caspases inhibitor, Z-VAD-fmk (R& D Systems, Minneapolis, MN, USA) dissolved in DMSO as stipulated by the manufacturer. After washes with HBSS, the islets were incubated for 90 minutes in 24 ml of Hams F10 media containing no BSA and supplemented with 0.5% human serum albumin and 11.1 mM Z-VAD-FMK caspases inhibitor per pancreas. After exposure, they were washed two times in Hams F10 media (no BSA) containing 0.5% human serum albumin (Talecris Biotherapeutics Limited, Mississauga, ON, Canada). The islets were then cultured in one plate per pancreas in Hams F10 medium (no BSA) supplemented as above along with 0.5% human serum albumin (Talecris Biotherapeutics Limited, Mississauga, ON, Canada) and containing 1:500 protease inhibitor cocktail (Sigma, St louis, MO, USA) prepared per manufacturer's instructions.

At each time point of 1 day, 3 days and 7 days of culture, islets were washed with Hams F10 media containing 0.5% human serum albumin and samples were drawn for assessment of insulin, DNA content and immunohistochemistry. The islets were then cultured in Hams F10 medium supplemented as above along with 1:500 protease inhibitor and 0.5% human serum albumin until the final duration of 7 days.

2.2.3 Cellular Insulin and DNA content analysis

In order to assess the cellular insulin content, two samples each were measured for hormone content after extraction in 2mmol/liter acetic acid containing 0.25% BSA. The samples were sonicated, centrifuged (800 g, 10 minutes, 4° C) and supernatants were collected and stored at -20° C until the time of radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA, USA). DNA content was analyzed using PicoGreen, a fluorescent nucleic acid stain for double-stranded DNA according to the manufacturer's instructions (Invitrogen). Duplicate aliquot samples of islets for measuring DNA content were washed in citrate buffer (150 mM NaCl, 15 mmol/l citrate, 3 mM EDTA, pH 7.4) and stored as a dry pellet at -20° C. Cell pellets were resuspended in lysis buffer (10 mM Tris, 1 mmol/l EDTA, 0.5% Triton-X-100, 4° C at pH 7.5), sonicated and then analyzed (Korbutt GS et al., 1996).

2.2.4 Glucose-stimulated insulin secretion

In order to assess the function of the islets, the insulin secretion response of the islets to an *in vitro* glucose challenge was analyzed using a static incubation assay (Korbutt GS et al., 1996). Islets were washed three times with Hams F10 (0.5% BSA and 2.8 mmol/l glucose) and samples were drawn to analyze cellular insulin content. Islets were then incubated in 24 well plates with 1.5 ml of Hams F10 (0.5% BSA) containing either 2.8 or 20 mmol/l glucose for 120 minutes at 37° C. Then, supernatants were collected and insulin release was measured by radioimmunoassay. Glucose stimulated insulin release by islets was calculated by

dividing the amount of insulin released into the supernatant by the cellular insulin content of the islets (percent secretion). Stimulation indices were calculated by dividing the percentage of insulin released at 20 mmol/l glucose challenge by that of insulin released at 2.8 mmol/l glucose challenge. Insulin release per β-cell was calculated by dividing the amount of insulin released into the culture medium by the number of β cells. Based on the determination of total islet cellular DNA content and the percentage of insulin-positive cells, the following equation was used to calculate the total number of β cells (Korbutt GS et al., 1996):

$$\frac{\text{Total DNA content}}{7.1\ \rho\text{g DNA/cell}} \times \frac{\text{\% insulin positive cells}}{100} = \text{Number of } \beta \text{ cells}$$

2.2.5 Immunohistochemistry

To determine the percentage of insulin-positive β cells, the cellular composition of the islets at each time point was analyzed by collecting samples from each time point in a calcium-free HBSS media supplemented with 1mM EGTA and 0.5% BSA. Islets were then mechanically dissociated using siliconized glass pipettes at 37° C for 3 minutes before addition of 1.0 mg/ml trypsin and 0.4 mg/ml DNAse (Boehringer Mannheim, Laval, QC, Canada) and continued mechanical dissociation for another 4 minutes. Cells were then washed and resuspended in PBS and allowed to adhere to Histobond microscope slides before fixation in Bouin's fixative and storage in 70% ethanol at 4° C until analysis.

For CK-7 staining, the microwave antigen retrieval method was performed consisting of microwaving rehydrated slides in sodium citrate (10mM, pH 6.0) and allowing the slides to cool completely before quenching. Endogenous

peroxidases were quenched with 10% v/v hydrogen peroxide in methanol to reduce background staining. The slides were blocked for 30 minutes using 20% v/v normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Primary antibodies (Dako, Mississauga, ON, Canada) used were 1:1000 guinea pig anti-porcine insulin, 1: 5000 mouse anti-porcine glucagon (Dako, Mississauga, ON, Canada), and 1:50 mouse anti-human CK-7 (Dako, Mississauga, ON, Canada). The antibodies were added for 30 minutes at room temperature in a humidified chamber followed by 2 washes in PBS before addition of the secondary antibodies. Secondary antibodies used were 1:200 biotinylated goat anti-guinea pig and 1:200 biotinylated goat anti-mouse for 30 minutes. The slides were then incubated with avidin-biotin complex (ABC, Vector Laboratories, Inc., Burlingame, CA, USA) for 40 minutes before addition of diaminobenzidine (DAB) chromagen (Signet Laboratories).

A minimum of 500 single cells from each sample were counted and the percentage of cells positive for insulin (β-cells), glucagon (α-cells) and CK-7 (ductal cells) was determined. The total number of cells was determined using the total DNA content of the islets and the percentage of insulin, glucagon and CK-7 positive cells.

2.2.6 Statistical Analysis

Results are presented as mean ± SEM of the four independent experiments performed with islets from at least 3 pancreases each. Statistical comparisons were conducted using the *unpaired t-test* (two-tailed) with STATA

software. A p-value of less than 0.05 was considered to be a significant difference.

2.3. Results

2.3.1 Effect of culture duration of 1 day, 3 days and 7 days on insulin and DNA content in case of control and caspase inhibitor and protease inhibitor treated neonatal porcine islets (NPIs).

It is well known that during islet transplantation, a considerable amount of transplanted cells are lost due to apoptosis during isolation, post-isolation culture, and the early engraftment period. Therefore, it was hypothesized that using caspase inhibitors to block apoptotic cell death would prevent cell death in NPI cultures compared to controls. Furthermore, one plate per pancreas was used in case of experimental group in order to determine if this provides similar results as controls.

The control experiments were cultured in standard Hams F10 medium (as mentioned before) in four plates per pancreas. The second set of experiments included incubation of neonatal porcine islets in presence of caspase inhibitor z-VAD-FMK, a pan caspase inhibitor for a period of 90 minutes followed by 7 days culture in media supplemented with protease inhibitor in one plate per pancreas. In order to discern the optimum *in vitro* culture time of islets pre-transplantation, samples were drawn from each time point of 1 day, 3 days and 7 days and analyzed for insulin and DNA content. As shown in Table 2.1, there was a significant difference in DNA content in control versus experimental group.

However, there was not a significant difference in insulin content between the two groups (Table 2.5).

Overall, in addition to a decrease in insulin content levels of NPIs with increasing culture time in both groups, a decline also occurred in DNA content (Table 2.1). The treatment of islets with combination therapy of caspase inhibitor and protease inhibitor was unable to avert all of cell death. From the comprehensive values of insulin and DNA content of neonatal porcine islet cell cultures from duration of day 1, 3 days, and 7 days, it can be seen that the general trend is a decline in both insulin and DNA content of neonatal porcine islet cell cultures.

However for within each group of two independent experiments, there was not a significant decrease in insulin content. On the other hand, as shown in Figure 2.1, caspase and protease inhibitor treatment led to significantly better cell survival as depicted by a marked decrease in loss of DNA content ($p<0.05$, Table 2.5). Within the group, compared to controls, for caspase inhibitor and protease inhibitor treated NPIs cultured in one plate per pancreas, DNA loss is significantly lower (65.2%) compared to control NPIs (77.4%) cultured in four plates per pancreas.

On the other hand, as shown in Figure 2.3, there was no significant difference in the ratio of insulin to DNA content between control and experimental groups from day 1 to 3 days or 7 days of culture durations (Table 2.5).

Table 2.1. Cellular insulin and DNA content in control and caspase inhibitor treated neonatal porcine pancreatic islets after 1 day, 3 days and 7 days of culture.

Total Insulin and DNA Content (μg) per pancreas			
Treatment	Time in days	Insulin Mean ± SEM	DNA Mean ± SEM
Control			
	1	62.7 ± 3.7	1505.7 ± 85.2
	3	19.8 ± 1.4	340.3 ± 33.4
	7	9.0 ± 1.1	170.0 ± 26.0
Caspase and Protease Inhibitor			
	1	38.7 ± 4.9	642.5 ± 106.4
	3	12.6 ± 0.8	223.7 ± 29.5
	7	6.8 ± 0.4	115.8 ± 20.6

Values are expressed as mean ± SEM from two independent experiments (n=6). The experimental group consisted of a caspase inhibitor, z-VAD-FMK exposure followed by culture in one plate per pancreas in Hams F10 medium supplemented with general protease inhibitor and human serum albumin (no BSA) while the control group was cultured in four plates per pancreas in standard Hams F10 medium.

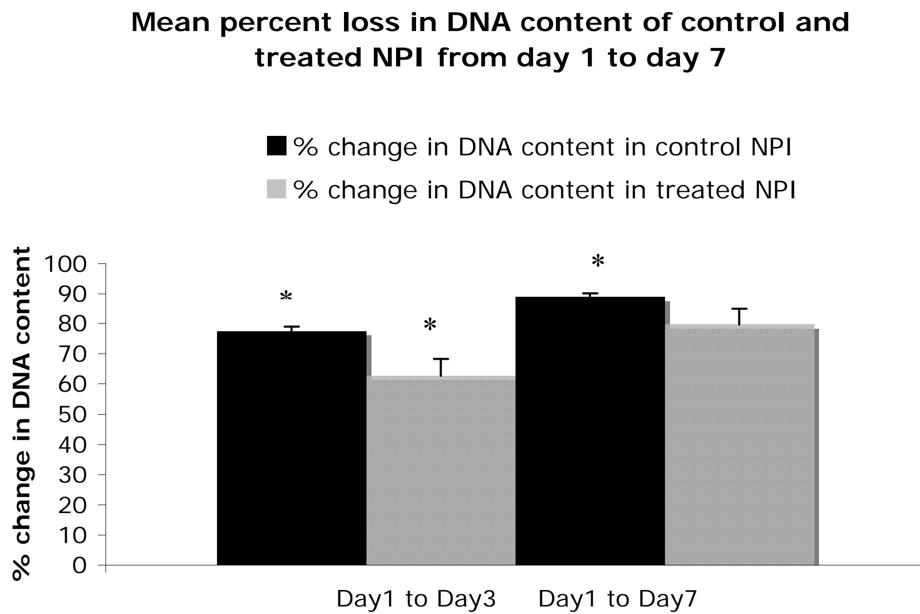

Figure 2.2. Mean percent loss in DNA content of two independent experiments. NPIs were cultured for 7 days under control conditions in one set of experiments; the second set of experiments included a caspase and protease inhibitor treatment (n=6). Statistical significance of difference was calculated by *unpaired t-test* (two-tailed); ∗ denotes p< 0.05 vs. control. Caspase and protease inhibitor treatment led to a better cell survival of NPIs cultured in one plate per pancreas compared to control NPIs cultured in four plates per pancreas in standard Hams F10 medium.

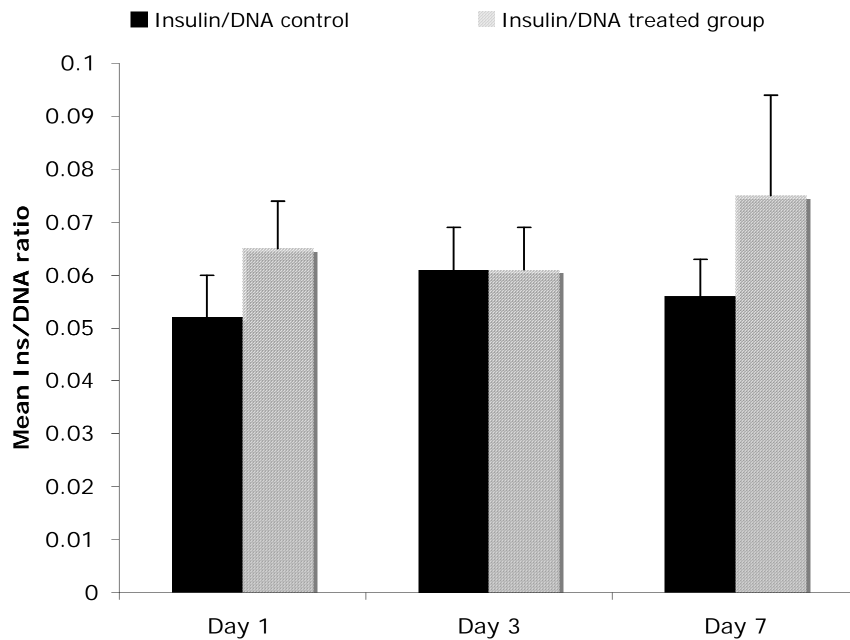

Figure 2.3. There was no significant difference between the insulin: DNA of NPI control and treated cultures. The experimental group consisted of a caspase inhibitor, z-VAD-FMK exposure followed by culture in one plate per pancreas in Hams F10 medium supplemented with general protease inhibitor and human serum albumin (no BSA) while the control group was cultured in four plates per pancreas in standard Hams F10 medium. Values are expressed as mean ± SEM from two independent experiments with an n=6 each.

2.3.2 Islet responsiveness to a glucose challenge increases with a longer culture duration in case of both control as well as caspase and protease inhibitor treated NPIs.

The viability of islets was assessed via their response to an *in vitro* glucose challenge by measuring their insulin secretion following a 2-hour incubation in either 2.8 or 20 mM glucose. Increasing the length of culture time had a positive effect on the glucose responsiveness of islets as shown in Table 2.2. In control experiments, the stimulation index (ratio of insulin secreted at 20 mM glucose to that secretd at 2.8 mM glucose) increased with more days in culture suggesting islet maturation *in vitro*. Caspase and protease inhibition lead to an increase in stimulation index as well. However, interestingly, caspase and protease inhibition led to an over-stimulation of the islets where they showed a hyper-secretion of insulin to both 2.8 mM and 20 mM glucose challenge conditions. As a result, although there were considerable differences in islet activity in control and treated groups, the stimulation indices observed were not significantly different since stimulation index is ratio of the two values.

Table 2.2. The effect of longer culture duration on insulin secretion activity of neonatal porcine islets in control vs. experimental groups.

Treatment	Time in days	Insulin secretion (% release)		Stimulation Index
		2.8 mM glucose	20 mM glucose	High:low
Control				
	1	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.0
	3	1.3 ± 0.1	2.5 ± 0.2	1.9 ± 0.1
	7	0.9 ± 0.2	1.8 ± 0.4	1.9 ± 0.2
Caspase & Protease Inhibitor treated				
	1	1.7 ± 0.3	1.7 ± 0.3	1.1 ± 0.1
	3	2.4 ± 0.3	3.4 ± 0.8	1.3 ± 0.1
	7	1.5 ± 0.3	2.6 ± 0.4	1.9 ± 0.3

Results are expressed as mean ± S.E.M., n=6. Neonatal porcine islets were cultured in four plates per pancreas for 7 days under control conditions in one set of experiments. The second set of experiments included a caspase and protease inhibitor treatment followed by culturing in one plate per pancreas. Stimulation index of each was determined by dividing the amount of insulin released at 20 mM glucose to that released at 2.8 mM glucose.

2.3.3. Effect of culture duration of 1 day, 3 days and 7 days on insulin per β-cell in control NPI cultures and caspase and protease inhibitor treated NPI cultures.

As shown in Table 2.3, insulin per β cell levels decline during the culturing period, especially after 3 days of culture. For control experiments, from day 1 to day 3, an 18.6% decline in insulin per β cell levels occurred. Most notably, islets show decreasing insulin per β-cell levels following day 3 when insulin per β cell levels fall by 39.14% from 3 days to 7 days of culture.

However, the treatment group did not show this steep decline in insulin per β-cell levels. From day 1 to 3 days of culture, there was only a decline of 1.8 % in insulin per β-cell and from 3 days to 7 days of culture duration, the loss in insulin per β cell was 49.29 %.

Thus, interestingly, treatment of NPIs with caspase and protease inhibitors results in significantly (Table 2.5) higher insulin levels per β-cell compared to controls suggesting a decrease in β cell loss in particular or possibly a higher insulin content per β cell in general (Table 2.3). For caspase and protease inhibitor treated NPI cultures, the insulin per β-cell levels are 57.7% higher than controls for day 1, 47.8% higher for day 3 and 42.6% higher for day 7.

Table 2.3. The insulin/ β-cell levels for control as well as caspase and protease inhibitor treated NPI cultures decline over culture time.

Ins (ρg)/ β-cell		
Treatment	**Time in days**	**Mean ± SEM**
Control		
	1	5.7 ± 1.0∗
	3	4.6 ± 1.1∗
	7	2.8 ± 0.4
Caspase and Protease Inhibitor treated		
	1	9.8 ± 1.7∗
	3	9.6 ± 1.4∗
	7	4.9 ± 1.1

The insulin content per β-cell is expressed as mean ± SEM for 1 day, 3 days and 7 days of culture for two independent experiments, n=6. NPI cultures treated with caspase and protease inhibitor show an increase in the insulin content per β-cell compared to untreated NPI cultures. Statistical significance of difference between the two groups was calculated by *unpaired t-test* (two-tailed); ∗ denotes $p < 0.05$ vs. control.

Thus, interestingly, treatment of NPIs with caspase and protease inhibitors results in significantly (Table 2.5) higher insulin levels per β-cell compared to controls suggesting a decrease in β cell loss in particular or possibly a higher insulin content per β cell in general (Table 2.3). For caspase and protease inhibitor treated NPI cultures, the insulin per β-cell levels are 57.7% higher than controls for day 1, 47.8% higher for day 3 and 42.6% higher for day 7.

2.3.4. Effect of culture duration of 1 day, 3 days and 7 days on cell composition in case of NPIs from control experiments and caspase inhibitor and protease inhibitor treated experiments.

The assessment of cell composition of NPI cultures by immunohistochemistry showed that while the percentage of endocrine cells (β-cells an α-cells) increases during the duration of culture, the ductal cell population decreases in both control as well as caspase and protease inhibitor treated cultures (Table 2.4.). This can be determined from the increase in cells staining positive for insulin and glucagon and the drop in cells staining positive for CK-7. It should be noted that results from caspase and protease inhibitor treated NPIs cultured in one plate per pancreas indicate that this treatment leads to a decrease in the population of all cell types. It seems that these results reflect the decrease noticed in the obtained insulin and DNA values in view of a paired comparison. All in all, the p-values obtained from an unpaired t-test comparison between different variables from day 1, 3 days, and 7 days of cultures are listed in Table 2.5.

Table 2.4. The cell compositions per pancreas of the control and caspase and protease inhibitor treated NPI cultures.

	Control			Caspase & protease inhibitor treat.		
	Time in Days			Time in Days		
	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
% of positive cells						
Insulin	6.3 ± 1.2	10.4 ± 1.2	14.6 ± 1.0	5.4 ± 1.0	4.6 ± 0.3	10.7 ± 1.1
Glucagon	6.0 ± 0.9	10.0 ± 1.3	17.5 ± 1.0	4.1 ± 0.4	4.2 ± 0.6	12.2 ± 1.0
CK-7	22.5 ± 1.4	19.9 ± 1.7	12.5 ± 1.3	21.7 ± 1.0	19.1 ± 0.3	14.6 ± 0.9
Number of cells (10^6) per pancreas						
β-cells	13.6 ± 3.0	5.1 ± 0.8	3.6 ± 0.7	5.2 ± 1.6	1.4 ± 0.2	1.6 ± 0.2
α-cells	12.7 ± 2.0	4.9 ± 0.8	4.2 ± 0.8	3.7 ± 0.7	1.3 ± 0.2	1.9 ± 0.3
Ductal cells	47.9 ± 4.4	9.3 ± 0.8	3.2 ± 0.8	20.3 ± 4.2	6.0 ± 0.8	2.5 ± 0.5
Total cells	212.1 ± 12.0	47.9 ± 4.7	23.9 ± 3.6	90.5 ± 14.9	31.5 ± 4.2	16.3 ± 2.9

The mean ± SEM values of percentage of cells positive for insulin (β-cells), glucagon (α-cells), and CK-7 (ductal cells) are shown followed by total number of each cell type (n= 6).

Table 2.5. Statistical significance of differences between control and treated groups calculated by two-tailed, unpaired *t*-tests.

Comparison between control vs. experimental group	p-value
Insulin content change from day1 to day3	0.0258
% change in insulin content from day1 to day3	0.5999
Insulin content change from day1 to day7	0.0045
% change in insulin content from day1 to day7	0.2306
DNA content change from day1 to day3	0.0001
% change in DNA content from day1 to day3	0.0297
% change in DNA content from day1 to day7	0.1244
Insulin/DNA change from day1 to day3	0.3634
% Change in Insulin/DNA from day1 to day3	0.3048
Insulin/DNA change from day1 to day7	0.7323
% Change in Insulin/DNA from day1 to day7	0.9064
Insulin/β-cell for day 1	0.0502
Insulin/β-cell for day 3	0.0192
Insulin/β-cell for day 7	0.1103

2.4. Discussion

The great potential of clinical islet transplantation to treat T1DM (Shapiro AM et al., 2000) is hindered by the limited availability of human pancreatic donor tissue (Sheehy E, et al., 2003). The research shows that approximately 50,000 neonatal islet equivalents per kg are required for transplantation to successfully correct diabetes in non-human primates (Cardona K, Korbutt GS, Milas Z et al., 2006). Frequently, more than 60% of transplanted islet mass is lost due to increased apoptosis and necrosis (Biarnes M, Montolio M, Nacher V et al., 2002). Early islet cell death can contribute to increase in the beta cell mass required to achieve normoglycemia in diabetic recipients and can have negative effects on the long-term evolution of the graft after transplantation. Thus it can be expected that a reduction in initial beta cell death would improve the graft outcome. This can be accomplished by various methods such as blocking apoptotic pathways via pre-treatment of islets with synthetic caspase inhibitors, using protease inhibitors.

Culturing islet before transplantation has many advantages over immediate transplantation. Short-term culture is associated with loss of exocrine tissue and increase in purity of islet preparation with increase in metabolic efficiency and also reduces immunogenicity of islets (Stein E et al., 1994). Culturing can also help in removal of substantial percentage of apoptotic and necrotic islets, which are present post-isolation. Hypoxia experienced during isolation procedure and collagenase digestion has a profound effect upon the survival of islets and has powerful inhibitory effect upon insulin secretion. The islet cultures as a result are subject to necrotic and apoptotic cell death from hypoxia and stress experienced

during islet isolation (Brandhorst D et al., 1997; Giuliani M, Moritz W, Bodmer E, et al., 2005; Ko SH, Ryu GR, Kim S, 2008). The powerful stimulus of apoptosis by hypoxia is a caspase-mediated pathway. Also, caspases signal not only apoptosis but also antigen-induced activation of immune system cells. Since caspases are primary executioners of apoptotic cell death, the use of caspase inhibitors seems to be a plausible method to inhibit apoptosis.

Islet culture can also be affected by presence of residual proteases from digestion phase of islet isolation (Brandhorst H et al., 2003). These enzymes lead to destruction of islet's collagen matrix causing fragmentation and death. The addition of protease inhibitors to culture media may help counter these detrimental effects. This study was carried out in order to determine the appropriate culture length pre-transplantation and assess if caspase and protease inhibition has a positive influence on islet yield and function. Clearly, optimizing the ideal conditions/time for transplantation could potentially lead to a reduction in the number of islet equivalents required to achieve euglycemia, less chance of tissue contamination if not culturing for longer periods of time, consequently, also requiring fewer media changes, reducing overall costs. The results obtained from this study suggest that culturing neonatal porcine islets for a period of about three days offers the best advantages. The insulin and DNA content, islet responsiveness to glucose challenge decreases further by day seven of culture.

Many studies have reported decreased islet cell death with caspase inhibition leading to better graft outcomes in the recipients (Brandhorst H, Brandhorst D, Kumarasamy V, et al., 2003; Emamaullee JA. et al., 2005;

Emamaullee JA et al., 2008; Emamaullee JA. et al., 2007., Montolio M et al., 2005). Results from this study indicate that the exposure of islets to z-VAD-fmk, a pan caspase inhibitor leads to a significantly ($p<0.05$) enhanced cell survival as assessed by decreased DNA loss in neonatal porcine islet cell cultures within the treated group. However, this independent experiment had lower initial DNA and insulin content and reduced number of overall cell composition of each cell type compared to independent control study. Also, the islet function seems to be affected in the treated group where islets had an increased sensitivity in low (2.8 mM) glucose and an increased sensitivity to high (20.0 mM) glucose, however it was not a statistically significant finding. Interestingly, caspase inhibitor and protease inhibitor treated islets had higher insulin content per beta cell suggesting that this combination therapy was somehow selectively more protective towards beta cells in culture.

Overall, caspase inhibitor was unable to prevent all of the post-isolation decrease in islet DNA content and β-cell populations, indicating that extensive cell death following isolation may proceed through a caspase-independent mechanism as well. Alternatively, the moderate concentration of caspase inhibitor used in these experiments may have been insufficient to completely prevent apoptosis. In addition, it is not clear how the combination therapy of protease inhibition after caspase exposure affects overall results.

The results obtained from this study seem consistent with some of the observed phenomenon involved in induction of cell death pathways under cell stress. It should be noted that these pathways many times serve to achieve a state

of homeostasis resulting in positive rather than negative effects. For example, one of the positive effects of hypoxia-induced apoptosis is activation of Akt, which plays a central role in hypoxia-induced hypoxia-inducible factor-1 (HIF) - dependent vascular endothelial growth factor (VEGF) expression (Kunz M and Ibrahim SM., 2003). VEGF is a potent angiogenic factor causally linked to neovascularization (Aiello et al., 1995).

Now, there are three major cell death pathways, namely apoptotic, necrotic, and autophagic cell death. Of these cell death types, caspases have been primarily linked with apoptotic cell death. On the other hand, death domain-containing serine-threonine kinase RIP1 is important for necrotic cell death. Many processes such as injury caused by hypoxia, reactive oxygen species (ROS) are accompanied by poly (ADP-ribose) polymerase-1 (PARP-1)- mediated caspase-independent cell death (Kalai M et al., 2002). The most important fact to be noted is that caspase inhibitor zVAD-fmk modulates the three major types of cell death in different ways.

Addition of zVAD-fmk blocks apoptotic cell death while sensitizes cells to necrotic cell death. Moreover, autophagic cell death may also be triggered by zVAD-fmk. The mechanism of action of caspase inhibitor zVAD-fmk is also affected by concentration used. Temkin et al. (2006) study shows that zVAD-fmk at a concentration of 20 μM targets a cysteinyl residue in Adenosine Nucleotide Translocator (ANT) preventing the interaction with cyclophilin D, which is required for proper functioning of ANT. The direct consequence of improper functioning of ANT is enhanced ROS production and diminished ATP

production. A decreased concentration of ATP in itself has been reported to lead to induction of necrotic cell death (Nicotera et al., 1999). Caspase inhibition has also been directly linked to induction of catalase degradation and mitochondrial ROS accumulation suggesting a protective role of caspases, in particular caspase-8 against oxidative stress in mitochondria (Yu L et al., 2006). All in all, the eventual effect of zVAD-fmk administration appears to be an enhanced ROS production, leading to either necrosis or autophagic cell death. Thus, a better understanding of the interlinking molecular mechanisms of different cell death pathways is needed to devise the best method/ agent to combat cell death during the process of islet isolation and transplantation.

As determined from the declining insulin and DNA content values during culture duration, three days (and possibly four or five days) seem to be the optimum culturing time of neonatal porcine islets. The findings of this study indicate that the pretreatment of neonatal porcine islets with combination treatment of zVAD-fmk, a pan caspase inhibitor post-isolation and culturing in presence of protease inhibitor leads to a significantly better cell survival as determined by decreased DNA loss (Figure 2.2), and therefore this treatment can potentially reduce the islet mass necessary to restore normoglycemia in diabetic animals.

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CHAPTER THREE

General Discussion and Conclusions

3.1. Discussion and Conclusions

Diabetes is a disease that results either because of complete loss of the insulin production due to selective immune system destruction of β-cells in the islets of Langerhans of the pancreas (T1DM) or a combination of loss of insulin production and/or body's ineffective use of insulin (T2DM) (Devendra D, Liu E and Eisenbarth GS, 2004). There is an emerging global epidemic of diabetes due to increases in obesity and physical inactivity. According to World Health Organization, more than 180 million people worldwide have diabetes and total deaths by diabetes are projected to rise by more than 50% in the next ten years. There are many micro and macro vascular complications associated with diabetes leading to enormous economic burdens of treating the disease along with increased mortality.

Although lifelong insulin administration has been the main therapeutic option for T1DM, it still results in decreased life expectancy due to acute complications such as hypoglycemia episodes, and chronic secondary conditions such as cardiovascular disease or renal failure. Although intensive blood glucose regulation improves glycemic homeostasis, it results in increased risk of hypoglycemic episodes, which can lead to coma and death. As a result, the transplantation of insulin-producing tissue continues to be considered the ideal treatment for T1DM.

The advent of the Edmonton Protocol in 2000, which used steroid free immunosuppression, revived the field of islet transplantation owing to its high success rate where all of the seven patients achieved insulin independence for one year (Shapiro AM et al., 2000). Despite its early success, many obstacles hinder its widespread application. These include a shortage of donor tissue, loss in islet graft function, complications and side effects from aggressive chronic immunosuppressive regimen needed to prevent immune rejection of the graft in place (Ryan EA et al., 2005).

The large number of islets, often requiring two to three donor tissues to establish normoglycemia in diabetic patients compounds the problem of insufficient human organs for transplantation. This has lead to a search for alternative sources of insulin-producing tissue for transplantation. Some of these potential sources of insulin-producing cells include genetically engineered cells (Efrat S, 1999; Cheung AT et al., 2000), differentiation of embryonic and adult stem cells, expansion of pre-existing β-cell population, and xenogeneic sources of islet tissue. The use of genetically engineered cells is limited by their inability to respond to normal physiological changes in blood glucose. On the other hand, it is difficult to maintain long-term β-cell function. In regards to the application of stem cells, much work remains to be done to understand the mechanisms involved in the differentiation of stem cells into insulin-producing cells and their functional yield remains low. In addition, ethical controversy associated with used of stem cells, especially embryonic stem cells hinder progress in this area.

Perhaps the most attractive alternative to cadaver islet tissue is the use of xenogeneic islets. While many animal sources have been considered (Marchetti P et al., 1995; Korbutt GS et al., 1996), in particular neonatal porcine islets seem to be most promising alternative source of insulin producing tissue. Neonatal porcine islets have been shown to effectively correct hyperglycemia in mice (Korbutt GS et al., 1996), non-human primates (Cardona K et al., 2006) and humans (Valdes-Gonzalez RA et al., 2005). In spite of its effectiveness, pig islet xenotransplantation must still overcome many hurdles before becoming clinically applicable. These include the potential for cross-species disease transmission (PERV), controversy involved with public acceptance of xenogeneic tissue for transplantation into humans, risk of hyperacute immune rejection in response to porcine cell surface Gal-α-Gal antigen.

Another major cause of concern in the field of clinical islet transplantation is diminishing graft function over time. Recent follow up studies with the Edmonton Protocol have shown that after three years, only 50% of the recipients were insulin independent (Ryan EA et al., 2001) and by five years post-transplant, this number falls to approximately 10% (Ryan EA et al., 2005). A multitude of factors including subclinical graft rejection, chronic islet toxicity from the immunosuppressive drugs, and recurrent autoimmune attack of transplanted islets in T1DM patient likely account for this degeneration in islet function. Many studies have shown that during islet transplantation, up to 70% of the transplanted islet mass never becomes functional (Davalli AM et al., 1995; Biarnes AM et al., 2002; Ryan EA et al., 2002). The loss in graft function is mostly attributed to

many physiological stresses such as hypoxia and reperfusion injury, tissue factor release, instant blood-mediated inflammatory reaction during revascularization process especially during the immediate post-transplant period (Dionne KE et al., 1993; Giuliani M et al., 2005).

Thus, a substantial amount of transplanted tissue becomes nonfunctional very soon after transplantation. Apoptosis is the leading cause of hypoxic cell death and up to 60% of β-cell mass is lost due to apoptosis prior to revascularization (Davalli AM et al., 1996; Paraskevas S et al., 2000; Moritz W et al., 2002). Also, the presence of large amounts of apoptotic tissue can lead to increased immune system response in the recipient leading to elevated demand for pharmacological immunosuppression. It is clear that extensive refinement of the clinical protocols is needed to improve graft outcomes. One of the promising interventional strategies seem to be the use of anti-apoptotic agents that will help prevent cell death during transplantation, thereby improving graft function.

Considering this fact, the aim of this study as described in chapter two was to investigate the potential of pan-caspase inhibitor, z-VAD-FMK and general protease inhibitor to prevent cell death in neonatal porcine islet cell cultures. Chapter two also sought to determine the ideal length of culturing time under these conditions. Secondly in order to reduce the overall time and cost associated with the procedure, one plate per pancreas as opposed to four plates per pancreas was used in combination with treatment with caspase and protease inhibitors. This study demonstrated that the neonatal porcine islets cultured in one plate per pancreas and exposed to caspase inhibitor followed by culturing in media

supplemented with protease inhibitor resulted in a significantly lower loss in cellular DNA content during culture time compared to control group. There was not a significant difference in the insulin content between control and experimental groups. Key to the further success of this therapy is to carefully assess the ratio/concentration of caspase and protease inhibitor used. Additionally, the declining insulin content and cell function after day 3 indicates that 3 to 5 days is the ideal culturing time of neonatal porcine islets pre-transplantation.

Overall, these findings indicate that caspase and protease inhibitor therapy provides with a more efficient method of use of tissue available for islet transplantation. The better cell survival due to this treatment could have positive implications in reducing the amount of islet tissue required and length of time needed for the achievement of glucose homeostasis in diabetic recipients. Moreover, the decrease in apoptotic cell mass could relieve some of the dependence on immunosuppressive drugs, resulting in better graft outcome long-term.

These results need to be tested *in vivo* and other avenues for use of caspase and protease inhibitor need to be explored i.e. alternative methods to inhibit pro-apoptotic caspases, which might prove more useful. Also, in order to address the controversy associated with the use of caspase inhibitors that it leads to elevated levels of necrosis needs to be addressed. Consequently, measurement of ROS, mitochondrial membrane potential, tunnel assay, propidium iodide (PI) flow cytometric assay can aid in differentiating between apoptosis and necrosis. This will help elucidate whether caspase treatment leads to increased necrotic cell

death and also help quantify these results in general. In addition, morphological procedures can help in identification and discrimination of apoptotic cells (surface blebbing) from necrotic cells (lesions). Application of these methods in the future can also help shed light on causes and sequences of cell death over culturing time. Also, using methods aimed at assessing the individual beta cell populations for apoptotic/necrotic cell death via double staining will clarify whether this treatment is more protective towards beta cells in general or whether this effect is seen due to already expected higher levels of β-cells with culture duration due to exocrine cell death.

Altogether, this work adds to the continuing refinement of protocols for islet cell transplantation and reinforces the reliability of neonatal porcine islets as a highly attractive alternative source of insulin-producing tissue for islet transplantation.

3.2. Future Directions

The development of the Edmonton Protocol revolutionized the field of islet transplantation in 2000, with most recipients being able to become insulin-independent immediately after transplantation. β-cell replacement through islet transplantation presents the best method to treat T1DM that also inhibits associated long-term complications. However, the widespread application of this therapy is hindered by the limited cadaveric donor pool. Consequently, neonatal porcine islets have emerged as a highly attractive alternative source of insulin producing tissue. Recent studies have greatly advanced the field of

xenotransplantation by showing that neonatal and adult porcine islet transplantation leads to glucose homeostasis in diabetic non-human primates (Cardona K et al., 2006; Hering BJ et al., 2006).

However, according to current isolation procedure, a single neonatal porcine pancreas yields an average of approximately 40,000-50,000 islet equivalents (IEQ) (Korbutt GS et al., 1996). Clinical transplantation studies show that an average of 12,000 IEQ/kg are required to achieve insulin independence in diabetic patients (Shapiro AM et al., 2000). Therefore, it is estimated that for xenotransplantation, an average weighing patient of 70 kg will need tissue from approximately seventeen pig pancreases. Obtaining this enormous amount of tissue for each patient is a very costly and labor-intensive task. The use of one plate per pancreas as opposed to four plate per pancreas for culturing addresses some of these concerns by lowering costs and time involved during this procedure.

Increasing the β-cell mass *in vitro* is necessary if larger numbers of diabetic recipients are to be transplanted. Therefore, determination of the optimum culturing time is necessary. In addition, methods for efficient use of available tissue and research into decreasing the marginal islet cell mass required to achieve normoglycemia can go long ways in making xenotransplantation a feasibly clinical therapy for diabetes in future. Use of pan-caspase caspase inhibitor and protease inhibitors leads to a better cell survival *in vitro*. The next step should include testing this phenomenon *in vivo*. Also, alternative methods to

inhibit caspases, which might prove more useful, need to be explored and a reliable method to use this technique has to be established.

Overall, as yet many hurdles need to be overcome before xenogeneic tissue can move to the clinical exploration stage. Two of the main obstacles remain to be firstly, the diminishing graft function over time and secondly, the toxic side effects of immunosuppressive therapies used. Methods to increase and maintain the mass and functional capacity of transplanted islets as well as modification of immunosuppressive regimens used by implementing less β cell toxic drugs will likely result in a highly enhanced clinical outcome.

Strategies targeting better cell survival during the engraftment period, for example, the use of apoptotic inhibitors appear to be a novel method of enhancing long-term graft function and reduction of islet tissue mass necessary to reverse diabetes with additional benefit of possibly reducing immunosuppression. This method serves as a further step towards widespread application of islet transplantation.

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