Manganese Porphyrin Protects Neonatal Pig Islets against Oxidative Stress and Enhances their Function in Diabetic Mice

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ABSTRACT

Significant numbers of pancreatic islets are lost during isolation and early transplantation period due to oxidative stress. Neonatal pig islets are being considered to overcome the shortage of human donors. Manganese (III) tetrakis (N-ethylpyridium-2-yl) porphyrin (MnP) is a synthetic antioxidants that was shown to protect human and murine islets from oxidative stress.

We report that pre-treatment of neonatal pig islets with MnP resulted in up-regulation of gene expression of antioxidant enzymes such as superoxide dismutase and antiapoptotic molecules such as survivin. We found that MnP is not toxic to neonatal pig islets. Further, MnP provided protection to neonatal pig islets against H_2O_2 induced damage. In addition, NSG mice that received islets pre-treated with 34 μM MnP achieved normoglycemia earlier and had significantly lower average blood glucose level compared to other groups. We report that MnP could be beneficial to neonatal pig islets provided protection against oxidative stress and enhanced their function *in vivo*.

To my beloved parents,

Mohammed Reda and Khatoon;

My wonderful aunt Ameera and uncle Shaker;

My soul mate and only sister,

Reham;

My amazing brothers,

Feras, Salam, and A`alla.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 DIABETES MELLITUS

1.1.1   Definition and Classification of Diabetes Mellitus

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycemia with disturbance of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action or both [1]. It has been estimated that around 285 million individuals suffer from diabetes worldwide, and this number is expected to increase to 438 million by 2030. In Canada, more than 9 million people are living with diabetes mellitus or prediabetes [2]. Adult diabetic patients are two times more likely to die prematurely compared to non-diabetic adults. Diabetes mellitus contributes to the death of 41,500 Canadians per year. In addition, diabetic patients incur medical costs two to three times more than non-diabetic individuals. Treatment of diabetes mellitus in Canada can cost up to $15,000 per year. By the year of 2020 it is estimated that diabetes mellitus will cost Canadian healthcare system $16.9 billion per year. Besides the economic burden of diabetes mellitus, it is associated with long term complications which negatively affect the quality of life of patients with diabetes mellitus [2].

Most patients with diabetes mellitus fall into one of the two major categories: type 1 and type 2 diabetes mellitus. Type 1 diabetes mellitus, previously referred to as insulin

dependent diabetes or juvenile onset diabetes, characterized by deficiency in insulin

secretion due to autoimmune mediated destruction of beta cells of the pancreas. Type 2

diabetes mellitus, previously known as non-insulin dependent diabetes or adult onset

diabetes, is characterized by insulin resistance, usually combined with reduction in insulin

secretion [3, 4].Type 2 diabetes mellitus is more common and accounts for 90-95% of

diabetes cases. The risk of having this type of diabetes increases with age, obesity, physical

inactivity, family history of diabetes mellitus, and prior history of gestational diabetes

mellitus. In this form of diabetes mellitus, patients may remain undiagnosed for many

years, due to the gradual development of the disease process. Treatment modalities of type

2 diabetes mellitus include: diet, weight loss, oral hypoglycemic drugs, and insulin [3, 5].

Gestational diabetes mellitus is another type of diabetes which develops or diagnosed

for the first time during pregnancy. It affects approximately 2-5% of all pregnancies.

Treatment includes diet, exercise, and some patients may need insulin. Gestational diabetes

mellitus is associated with increased risks of pregnancy-induced hypertension, cesarean

delivery due to fetal macrosomia. Both mother and baby are at increased risk of developing

type 2 diabetes mellitus in the future [4, 5].

Other forms of diabetes include genetic defects of β-cell function such as maturity

onset diabetes of the young (MODY), genetic defects in insulin action such as Rabson-

Mendenhall syndrome, endocrinopathies for example acromegaly, disease of exocrine

pancreas like pancreatitis, cystic fibrosis, hemochromatosis, trauma, and tumors [1, 3, 4].

Some drugs and chemicals are implicated in the development of diabetes such as

pentamidine, glucocorticoids, thiazides, and diazoxide. Certain viruses have been identified

as triggering agents in β-cell destruction like congenital rubella and cytomegalovirus. Genetic syndromes associated with diabetes include Down syndrome, Turner syndrome, Klinefelter syndrome, Pradder-Willi syndrome, Huntington chorea, and Friedreich ataxia [1, 3, 4] .

1.1.2   Anatomy and Physiology of the Pancreas

The pancreas is lobulated structure covered by a thin fibrous capsule. It is divided into four main parts: head, neck, body and tail (Figure1.1). It is located in the retroperitoneal space except the tail. The head of the pancreas is located in the C-curve of the duodenum. The neck of the pancreas is located in front of the portal vein. The body of the pancreas crosses the midline anterior to the aorta, the splenic vein, the left adrenal gland, left renal blood vessels, left kidney, and the left crus of the diaphragm. The tail of the pancreas is located within the splenorenal ligament [6, 7]. The pancreas has a rich blood supply provided by the splenic artery, the pancreatic branches of the gastroduodenal and superior mesenteric arteries (Figure1.2). Venous blood of the pancreas drains into the portal vein. The splenic vein which runs posterior to the pancreas receives the inferior mesenteric vein, joins the superior mesenteric vein to form the portal vein. The pancreatic lymphatic drainage is carried from pancreaticosplenic, pancreaticoduodenal, subpyloric, and hepatic lymph nodes into the celiac lymph node. Then the lymph drains through the intestinal lymphatic trunk and thoracic duct into the junction of the left jugular and the left subclavian vein. The pancreas is innervated by the vagus nerve, the celiac and the superior mesenteric nerveplexuses [6-8].
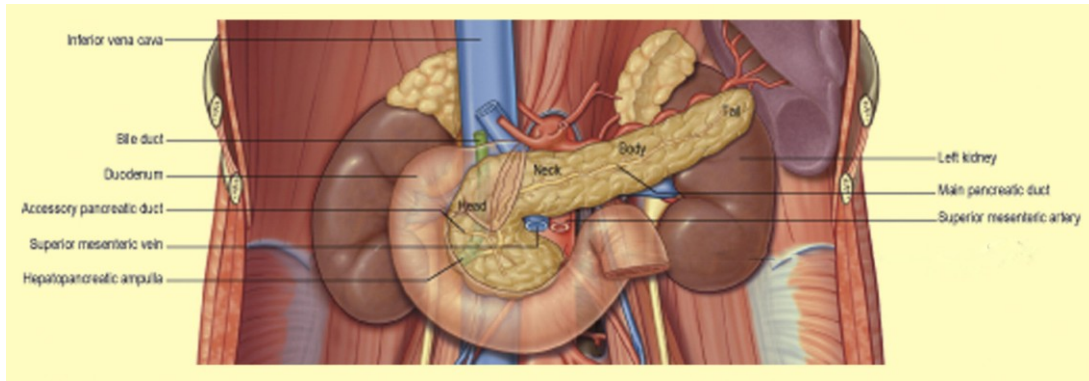
Figure 1.1: Anatomy of the pancreas. This demonstrates the parts of the pancreas and their relation to the nearby structures. This figure is adapted from Ellis, H., *Anatomy of the pancreas and the spleen. Surgery (Oxford)*, 2013 [6].
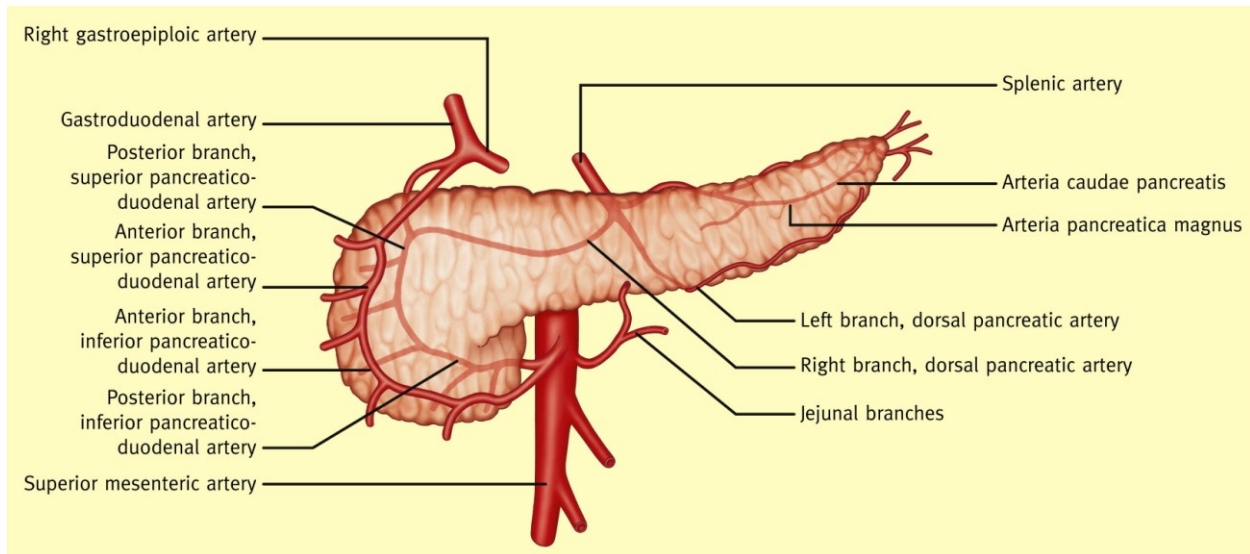
Figure 1.2: The pancreatic arterial blood supply. This figure is adapted from Ellis, H., *Anatomy of the pancreas and the spleen. Surgery (Oxford)*, 2013 [6].

The pancreas has mixed exocrine and endocrine functions. The endocrine part of the pancreas consists of islets of Langerhans which are scattered throughout the exocrine tissues (Figure1.3). Islets of Langerhans are well vascularized through glomerular-like network of capillaries, they receive 10-20% of the pancreatic blood flow, although they account for only 1-2% of the total pancreatic volume [8]. They consist primarily of four cell

types: alpha cells, beta cells, delta cells, and pancreatic polypeptide producing cells. Alpha cells produce glucagon, beta cells produce insulin, while delta cells produce somatostatin. The beta cells accounts for 60-70% of the cells within the islet of Langerhans , 5% are delta cells, and 15-20% either alpha cells or pancreatic polypeptide producing cells [8, 9]. Glucose homeostasis is maintained by insulin and glucagon which are released into the portal vein. Beta cells secrete insulin in response to high blood glucose level , while alpha cells secrete glucagon in response to low blood glucose level. The action of these hormones maintain the blood glucose level within a narrow range (5-8 mM) throughout the day [10]. Insulin reduces glucose production, protein degradation, and lipolysis, while glucagon increases the release of glucose by the degradation of glycogen [11]. The glucose-induced insulin secretion occur through regulated five steps: 1) entery of glucose into the beta cells through glucose transporter-2 (GLUT-2), 2) metabolism of glucose leading to adenosine triphosphate (ATP) production, 3) increment of the adenosine triphosphate (ATP) to adenosine diphosphate (ADP) ratio leading to closure of the ATP-sensitive potassium (K^+) cell-surface channels (KATP), resulting in depolarization of cell membrane, 4) entry of calcium (Ca^{+2}) into the beta cells, 5) increased intracellular calcium (Ca^{+2}) leading to release of insulin through exocytosis of insulin granules [10, 12, 13].
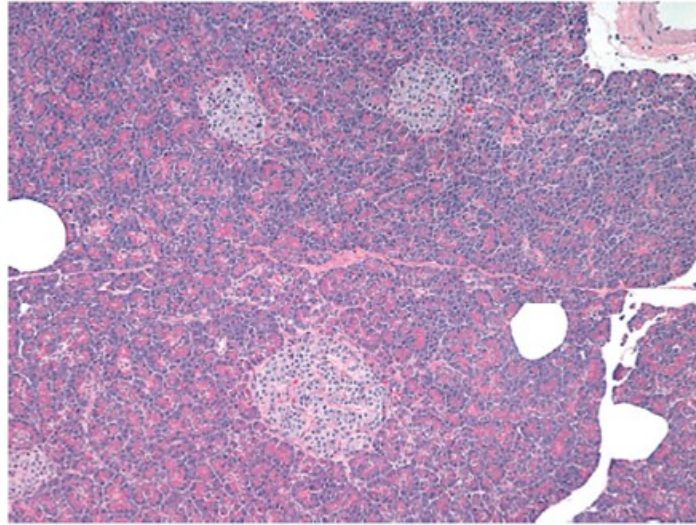
Figure1.3: A photomicrograph of a section of human pancreas. This image showing the islets of Langerhans surrounded by exocrine tissues. This figure is adapted from Bonner-Weir S, *Endocrine Pancreas*, in *Encyclopedia of Gastroenterology*, 2004 [8].

Insulin synthesis begins in the cytoplasm as the insulin mRNA translated into preproinsulin, which is composed of signal peptide, insulin A-chain, insulin B-chain, and C-peptide. Then preproinsulin crosses the endoplasmic reticulum membrane, where the signal peptide is cleaved by signal peptidase, producing proinsulin[14, 15]. Within the lumen of endoplasmic reticulum proinsulin folds forming disulfide bonds, then the properly folded proinsulin forms dimers and transported to Golgi complex. The proinsluin passes through Golgi complex into the immature secretory granules where the C-peptide is removed releasing the bioactive insulin that is stored in the mature secretary granules pending its release. It has been estimated that a single beta cell contains about 5,000-6,000 insulin secretary granules. Upon glucose stimulation some of these secretary granules undergoes exocytosis releasing the required insulin[14, 16]. Defects in insulin secretion or action result in development of diabetes mellitus[12, 17]. Type I diabetes mellitus is

characterized by the deficiency in insulin secretion due to autoimmune destruction of beta cells of the pancreas. While type 2 diabetes mellitus is characterized by insulin resistance with relative or complete insulin deficiency [17].

1.1.3 Clinical Features and Long Term Complications of Diabetes

The clinical presentation of hyperglycemia are polyuria, polydipsia, sometimes polyphagia, blurred vision, weight loss, and fatigue. Patients with poorly controlled diabetes mellitus are more susceptible to acute life-threatening conditions which are diabetic ketoacidosis (DKA) or hyperglycemic hyperosmolar state (HHS) [5]. Both conditions caused by high blood glucose levels, diabetic ketoacidosis is characterized by acidosis and ketonemia, while hyperglycemic hyperosmolar state is characterized by hyperosmolarity and dehydration. Diabetic ketoacidosis most commonly occur in type 1 diabetic patients who might have inadequate insulin treatment, or forget to take their insulin injection. In addition, patients with type 1 diabetes mellitus may develop diabetic ketoacidosis at times of stress such as myocardial infarction, pneumonia, urinary tract infection, or other medical or surgical conditions. However, hyperglycemic hyperosmolar state most commonly occur in patients with type 2 diabetes mellitus, who received inadequate treatment, or undergoing surgical procedures, or had underling illness like ischemia and infection[18]. Patients usually present with symptoms of hyperglycemia, nausea, and vomiting as shown in Table 1.1. Diabetic ketoacidosis and hyperglycemic hyperosmolar state are medical emergencies that require immediate intervention, admission, and close monitoring. Treatment of these conditions include replacement of the

fluid and electrolytes, insulin, and treatment of precipitating conditions such as infection [18].

Clinical features of DKA and HHS		
Clinical Presentation	DKA	HHS
Development of Symptoms	Hours to Days	Days to Weeks
Polydipsia/polyuria	+	+
Nausea/vomiting	+	+
Abdominal pain	+	−
Anorexia	+	+
Fatigue/malaise	+	+
Neurologic abnormalities	±	+ +
Hyperventilation	+	−
Dehydration	+	+ +

Table 1.1: Clinical presentation of diabetic patients with diabetic ketoacidosis (DKA), and hyperglycemic hyperosmolar state (HHS). This table is adapted from Maletkovic, J. and A. Drexler, *Endocrinol Metab Clin North Am,* 2013 [18].

Long term diabetes complications are divided into 2 main categories: microvascular and macrovascular. Microvascular complications are retinopathy, nephropathy, and neuropathy. Macrovascular complications include coronary heart disease, stroke, and peripheral vascular disease. The risk of developing such complication is related to the severity and duration of high glucose levels as well as the presence of other diseases like hypertension and hyperlipidemia [19].

Microvascular Complications

Diabetic retinopathy is the leading cause of blindness in individuals less than 65 years of age. It accounts for about 10,000 of newly diagnosed cases of blindness each year in the

United States. It is classified into background retinopathy with or without edema and proliferative retinopathy [19, 20]. Eighty percent of type 1 diabetic patients develop diabetic retinopathy after 10 years of diagnosis. Screening for diabetic retinopathy is recommended in type 2 diabetic patients at the time of diagnosis and in 3-5 years after diagnosis in patients with type 1 diabetes mellitus. Patients who develop macular edema or proliferative retinopathy need to be treated with laser photocoagulation to prevent loss of vision [20].

Diabetic nephropathy is the leading cause of renal failure. It has been estimated that 25-45% of type 1 diabetic patients may develop diabetic nephropathy. Patients usually develop microalbuminuria, which progress into proteinuria leading eventually to renal failure. It is recommended to screen for microalbuminuria which is usually asymptomatic at the time of diagnosis in type 2 diabetic patients and 5 years after diagnosis in type 1 diabetic patients. Reduction of disease progression can be achieved by better control of hyperglycemia and the administration of medication such as angiotensin-converting enzyme inhibitors or angiotensin receptor blockers [19, 20] .

Peripheral diabetic neuropathy may manifest as sensory, focal, multifocal, and autonomic neuropathies. Chronic sensorimotor distal symmetric polyneuropathy is the most common form of diabetic neuropathy. It is manifested as burning sensation, numbness, and electrical pain that get worse at night. Fifty to seventy percent of non-traumatic amputation is attributed to sensorimotor diabetic peripheral polyneuropathy. Other forms of neurological dysfunctions include gastroparesis, vomiting, diarrhea, bladder

dysfunction, erectile dysfunction, and orthostatic hypotension. Sensory examination of lower extremities should be conducted at least annually [19, 20] .

Macrovascular Complications

Macrovascular complications manifested as coronary heart disease, stroke, or peripheral vascular disease. Cardiovascular disease is the major cause of death in diabetic patients. Diabetic patients have atypical symptoms of coronary heart disease and they are at increased risk of developing heart failure and sudden death. Control of hyperglycemia, hypertension, and hyperlipidemia reduce the risk of having these complications. Diabetic patients are at greater risk of developing stroke and stroke related dementia. Incidence of ischemic stroke is 2.5 times higher in persons with diabetes mellitus. Patient with peripheral vascular disease experience intermittent claudication, rest pain, foot ulcers, and gangrene. Screening ankle to brachial index (ABI) should be performed to susceptible individuals [5, 19, 20] .

1.1.4   Type I Diabetes Mellitus

Type 1 diabetes mellitus accounts for 5-10% of all cases of diabetes. Worldwide, the highest incidence was reported in Finland (about 50 cases per 100, 000 per year), while China and Japan have the lowest incidence (in China around 0.57 cases per 100,000 per year) [21, 22]. In Canada, the highest incidence of type 1 diabetes mellitus was reported in Newfoundland and Labrador. Leigh *et al.* demonstrated increased incidence in the period from 2007 to 2010 up to 49.9 cases per 100,000 per year in children 0-14 years of age [23]. Type 1 diabetes mellitus is characterized by immune mediated destruction of β-cells of the pancreas. There are 2 forms of type 1 diabetes mellitus: type 1A which results from

autoimmune process damaging the β-cells, whereas type 1B which is known as idiopathic diabetes mellitus, is less common, and patients have varying degrees of insulin deficiency between ketoacidosis episodes. Type 1B is more common in individuals of African or Asian descent. This type of diabetes mellitus is characterized by the destruction of β-cells in the absence of autoimmunity [3, 24]. Patients with type 1B diabetes mellitus test negative for islet auto-antibodies at the time of diagnosis. Examination of sections of the pancreas under the microscope showed atrophic, disfigured islets, with no evidence of insulitis, necrosis, suppuration, hemorrhage, or cyst formation [24]. Characteristic features of this type of diabetes mellitus are rapid onset of diabetes, short duration of hyperglycemic symptoms, presence of acidosis at the time of diagnosis, elevation of serum pancreatic enzyme levels, and decreased C-peptide secretion [24, 25]. The mechanism of β- cell death is not known, and needs to be explored further. Viral infection might be implicated in the pathogenesis of type 1B diabetes mellitus given the abrupt onset of the disease and the presence of flu-like symptoms in some patients [24, 25].

Individuals at risk of developing type 1 diabetes mellitus have genetic susceptibility. It has been suggested that the combination of genetic susceptibility and the environmental triggers initiate an autoimmune reaction attacking specifically β-cells of the pancreas. The genetic determinant of type 1 diabetes mellitus is related to major histocompatibility (MHC) alleles, which has three subregions: human leukocyte antigen (HLA) class I, HLA class II, and HLA class III. Its main function is to present antigens to the immune system. The genetic predisposition to type 1 diabetes mellitus is related to HLA class II alleles on chromosome 6. Highest risk of having type 1 diabetes mellitus is associated with HLA class

II haplotype DR3/4-DQ8. On the other hand HLA class II haplotypes DR15-DQ6, DQB1 provide protection against type 1diabetes mellitus. Children with HLA haplotype DR3/4-DQ8 and have more than one type 1 diabetic family members are at 50% increased risk of developing type 1 diabetes mellitus. However, only 1 % of children with HLA haplotypes DR15-DQ6, DQB1 at risk of developing type 1 diabetes mellitus [26-28]. Environmental triggers that may play a role in initiating the autoimmune destruction of β-cells include viral infections (congenital rubella, coxsackie B , entroviruses), dietary factors (early exposure to cow's milk proteins, short duration of breastfeeding), deficiency of vitamin D, and toxins (nitrosamines) [22, 26, 29, 30].

Several inflammatory cells and molecules have been implicated in the development of type 1 diabetes mellitus. Antigen presenting cells such as macrophage, and dendritic cells, have 2 roles: to process and present the antigens to T-cells, and to release inflammatory mediators like cytokines. Macrophages activate T-cells via secretion of interleukin (IL)-12, which stimulate T-helper-1 CD4 T-cells. Activated CD4 T-cells produce IL-2 and interferon-gamma (IFN)-γ, which in turn assist in potentiating the activation of CD8 T-cells. The activated T-cells mediate destruction of β-cells of the pancreas via Fas/Fas ligand interactions [26, 27, 30, 31].

The role of B-cells is to act as antigen presenting cells and to produce auto-antibodies against β-cell antigens. The major auto-antibodies have been identified are islet cell antibody (ICA), glutamic acid decarboxylase (GAD) antibody, insulin antibody (IAA), protein tyrosine phosphatase insulinoma antigen 2 antibody (IA-2), and zinc transporter 8 antibody [31]. Animal study using non-obese diabetic (NOD) mice demonstrated the

development of diabetes mellitus in B-cell deficient recipients when they received T-cells from diabetic NOD mice. This indicates that B-cells are not essential for the autoimmune destruction of islets. It has been suggested that B-cells contribute to islet destruction via antigen presentation, rather than through the auto-antibodies produced by them [27]. These auto-antibodies may be present for years before the clinical onset of type 1 diabetes mellitus [22]. Auto-antibodies can be used as biomarkers for diagnosis and prediction of the disease [27]. Patients positive for 2 or more auto-antibodies are 50-100% at risk for having type 1 diabetes mellitus in 5 to 10 years period [30].

Type 1 diabetes mellitus is characterized by severe insulin deficiency, around 70-90% of β-cells are destroyed at the time of diagnosis. It is most common in childhood and adolescence, but can occur at any age. Patients present with history of polyuria, polydipsia, and weight loss. Other patients may initially present with diabetic ketoacidosis, and it has been estimated that 20-40% of patients less than 20 years of age present with diabetic ketoacidosis [5, 18]. Diabetic ketoacidosis is a medical emergency which results from severe insulin deficiency characterized by hyperglycemia associated with acidosis and ketonemia. Patients need to be hospitalized to receive urgent treatment and close monitoring. Treatment includes administration of fluids, insulin, and electrolytes [21, 25].

Treatment of type 1 diabetes mellitus consists mainly of daily insulin injections. Long term diabetes related complications such as retinopathy and neuropathy can be reduced by tight glycemic control. Diabetes control and complication trials (DCCT) confirmed the relation between the glycemic control and the development or progression of complication related to diabetes mellitus. Although intensive insulin therapy was shown to be effective

in reducing the long term complications, it was associated with greater risk of severe hypoglycemia [32]. Hypoglycemia is defined by low plasma glucose levels (less than 70 mg/dl or 3.88 mmol/L) that activates the autonomic nervous system, which in turn result in release of epinephrine and glucagon as well as the production of glucose in the liver to raise glucose level in the blood [33]. Patients present with symptoms like sweating, tremor, nausea, anxiety, and palpitation [20]. Recurrent episodes of hypoglycemia lead to unawareness of hypoglycemia, in such case patients may develop seizure or coma in the absence of usual warning symptoms. Unawareness of hypoglycemia results from repeated episodes of hypoglycemia which impair the body's ability to release stress hormones such as glucagon. Hypoglycemia is detrimental causing cardiac arrhythmias and reduced cognitive functions [19, 20, 33]. Cardiac arrhythmias may lead to sudden cardiac death [33]. It has been reported that up to 10% of death in type 1 diabetic patients are attributed to hypoglycemia [34]. A study investigating the association between brain lesions and hypoglycemia using magnetic resonance imaging (MRI) showed that patients with recurrent episodes of hypoglycemia had more brain lesions [35] .

Another approach in treating type 1 diabetic patients is the replacement of β-cells of pancreas via whole pancreas or islet transplantation providing more physiological way to supply insulin. However, the replacement of β-cells therapy is applied to patients with liable type 1 diabetes mellitus who suffer from severe hypoglycemic episodes [36]. At first the success of pancreas transplantation was low but increased over time as a result of the advances in both surgical techniques and immunosuppressive medications. In 1966, the first pancreas transplantation was performed by William and colleagues where a pancreas

was simultaneously transplanted with a kidney in patients with end-stage diabetic nephropathy. The recipient achieved insulin independence, however he died 2 months later due to sepsis. There are three categories of pancreas transplantation: 1) simultaneous pancreas and kidney transplantation in diabetic patients with uremia, 2) pancreas after kidney transplantation in patients that had renal failure corrected with kidney transplant, 3) pancreas transplantation alone in non-uremic diabetic patients [37, 38]. Pancreas transplant can reverse or slow the progression of diabetes related complications such as nephropathy and neuropathy. Other benefits include the avoidance of daily multiple insulin injections and frequent blood glucose monitoring to control the hyperglycemia. However, these advantages are accompanied with the risks related to surgery and immunosuppressive drugs. Complications related to surgery are vascular thrombosis, hemorrhage, anastomotic leak, pancreatitis, and infections [37-39]. On the other hand, islet transplantation, a less invasive procedure done via infusion of pancreatic islets into the portal vein, is a promising alternative to whole pancreas transplantation [36, 40]. It has been reported that morbidity of islet transplantation is 20 times lower than the morbidity related to the whole pancreas transplantation [41].

1.2 ISLET TRANSPLANTATION

1.2.1 *History of Islet Transplantation*

In 1889, Minkowsky and Von Mering discovered the association between the removal of pancreas and development of diabetes mellitus. They noticed that the dog become polyuric with evidence of glucose in the urine after removal of the pancreas. This lead to the trial of using pancreatic fragments to treat diabetes mellitus prior to the

discovery of insulin. In 1892, Minkowski transplanted a portion of the pancreas subcutaneously into the abdominal wall of the dog, which resulted in reversal of diabetes [42, 43]. In 1893, Watson Williams and his colleague Harsant treated a 15 year old diabetic boy suffering from diabetic ketoacidosis, by transplanting fragments of sheep's pancreas into subcutaneous tissue. There was temporary improvement in glycosuria, but the boy died 3 days later due to the rejection of the pancreas graft [44].

Advances in the field of islet isolation lead to the application of pancreatic islet transplantation in the treatment of type 1 diabetes mellitus. In 1964, Hellerstroem isolated intact islets from a rodent pancreas using free hand microdissection technique [45]. The use of collagenase combined with mechanical disruption to digest a chopped guinea pig pancreas to improve the yield of islets, was first described by Moskalewski in 1965 [46]. In 1967, Lacy and Kostianovsky, were able to isolate 300 intact islets from a single rat pancreas by injecting the pancreatic duct with Hank's solution prior to collagenase digestion of the pancreas revealing that sufficient amount of pancreatic islets could be isolated [47].

The first attempt of islet transplantation was performed by Younoszai and his colleagues in 1970. They transplanted islets isolated from rodent pancreas into diabetic rat, which resulted in temporary improvement of hyperglycemia [49]. Two years later, Ballinger and Lacy reported the first sustained reversal of hyperglycemia, by intraperitoneal or intramuscular transplantation of 400-600 isolated rat islets into streptozocin-induced diabetic rat. They showed that when the intramuscular islet transplant was removed, the rat become hyperglycemic again [50]. Reckard and Barker in

1973, transplanted larger number of islets 800-1,200 into the peritoneal cavity of diabetic rats, it was the first time that islet transplantation completely reversed chemically induced diabetes [51]. In the same year, Kemp *et al.* reported successful treatment of diabetes via intraportal injection of rat islets, normoglycemia was maintained 2 months later. They provided evidence that portal site for islet transplant was superior to subcutaneous (no change of glucose level) and intraperitoneal sites (amelioration of blood glucose) [52]. Different islet transplant sites were investigated in several experimental animal models (rat, mouse, dogs, and non-human primates) with variation in the degree of success [53]. These sites include subcutaneous tissue [52,54], intramuscular [50, 54], intraperitoneal [50, 52], omental pouch [55, 56], kidney capsule [57, 58], portal vein [52, 59], pancreas [60], spleen [61], testis [62], thymus [63], brain [64], bone marrow [65], anterior chamber of the eye [66], and gastric submucosa [67].

Earlier in 1973 Kemp *et al.* demonstrated that the use of the intraportal site for islet transplant, allowed the reversal of diabetes using less number of islets compared to subcutaneous and intraperitoneal sites [52].The International Islet Transplant Registry reported that clinical islet transplantations have been performed in majority of cases by infusion of pancreatic islets into the portal vein. The transplantation of islets into the portal vein is minimally invasive, providing islet's insulin secretion into the portal circulation, which is similar to the physiological state [53, 68]. Although the portal vein is the site of choice for islet transplantation, it is not the ideal site due to several reasons. The intraportal infusion of pancreatic islets associated with complications such as bleeding, portal vein thrombosis, portal hypertension, transient elevation of liver enzymes, hepatic

steatosis, gallbladder puncture, and arteriovenous fistulae [69-73]. Islets infused into the portal vein are exposed to recipient blood which triggers a detrimental reaction, known as instant blood mediated inflammatory reaction (IBMIR).This reaction results in activation of complement and coagulation cascade with eventual destruction of the transplanted islets [74]. In addition, islets are exposed chronically to toxic substance such as the diabetogenic immunosuppressive drugs and waste product from the gastrointestinal tract [75-77]. Lastly, the ability to monitor the islets by biopsies are limited due to technical difficulties [68].

The significant progress achieved in the field of islet isolation and the improved immunosuppressive medications led to the application of islet transplantation in human. The first clinical islet transplantation was performed by Najarian and colleagues in 1977. They transplanted pancreatic islets in diabetic patients with prior renal transplant immunosupressed with azathioprine and corticosteroids. None of the patients were off exogenous insulin, however insulin requirement decreased [78]. In 1978 in Switzerland, it has been reported that a patient with type 1 diabetes mellitus and renal failure was off exogenous insulin, maintaining normoglycemia for one year after successful simultaneous kidney and intrasplenic pancreatic islet allotransplants [79]. In 1989, Warnock and colleagues at the University of Alberta demonstrated the ability of islets isolated from cadaveric donor pancreata to secrete insulin by measuring the level of C-peptide. Two type 1 diabetic patients with end-stage nephropathy received islet and kidney allotransplant from the same donor. This study was unique in reporting for the first time sustained C-peptide secretion after simultaneous islet and kidney allotransplantation [80]. Long term

follow up which involved 2 more patients who received fresh and cryopreserved islets from multiple donors, revealed that the first two cases were insulin dependent with C-peptide secretion maintained for 8 months. One of the two patients who received fresh and cryopreserved islets, became insulin independent, had normal fasting glucose, and glycosylated hemoglobin at 1 year after discontinuation of exogenous insulin [81]. In addition, Lacy's group at the University of Washington, transplanted islets from pancreata of brain dead donors into the portal vein of type 1 diabetic patient, who previously had received renal transplant already on immunosuppressive medication, became insulin independent for 12 days after islet transplantation [82]. Also in 1989, a group of surgeons at the University Health Center in Pittsburgh, provided evidence on long term insulin independence after human islet allotransplantation into non-autoimmune diabetic patients. Nine patients with extensive abdominal tumors became diabetic after upper abdominal exenteration, received liver and islet transplants. Five out of nine patients became insulin independent and C-peptide was detectable in the plasma of all the patients [83]. The International Islet Transplant Registry reported that a total of 405 islet allotransplants have been performed worldwide between 1893 and 1998, with variable outcomes [84].

A renewed interest in islet transplantation research occurred in 2000, after the success reported by the Edmonton group at the University of Alberta [40]. Shapiro and colleagues performed intaportal islet allotransplantation in seven patients known to have type 1 diabetes mellitus with undetectable serum C-peptide levels (Figure 1.4). These patients had type 1 diabetes mellitus for more than five years and suffered from recurrent

severe hypoglycemia. All patients received islets from two or more donor pancreases, becoming insulin independent with no recurrence of hypoglycemic episodes. These patients had normal glycosylated hemoglobin and detectable serum C-peptide. The success was attributed to several factors, which include adequate number of islets were transplanted (a mean of 11,547± 1,604 islets per kilogram of the recipient's body weight), the use of glucocorticoid free immunosuppressive regimen (sirolimus, tacrolimus, and daclizumab), minimizing the duration of cold ischemia, and islet preparation using the human albumin instead of xenoprotien products [40]. A five year follow up study after islet transplantation revealed that islet graft survival was about 80%, while insulin independence rate was close to 10%. However, exogenous insulin doses decreased, hypoglycemia and glucose control were improved [85]. The success of the Edmonton group led to an international multicentre trial of islet transplantation using the Edmonton protocol. Nine international centers (three in Europe and six in North America) participated in the study [69]. Thirty six patients with type 1 diabetes mellitus who had severe glycemic liability or severe recurrent hypoglycemic episodes, underwent islet transplantation. Forty four percent were insulin independent one year after the last islet transplantation. Although insulin independence was not maintained, patients had improved glycosylated hemoglobin and were protected from severe hypoglycemic episodes. This study demonstrated the feasibility and reproducibility of islet transplantation using the Edmonton protocol and emphasized the importance of experience in islet transplantation [69].
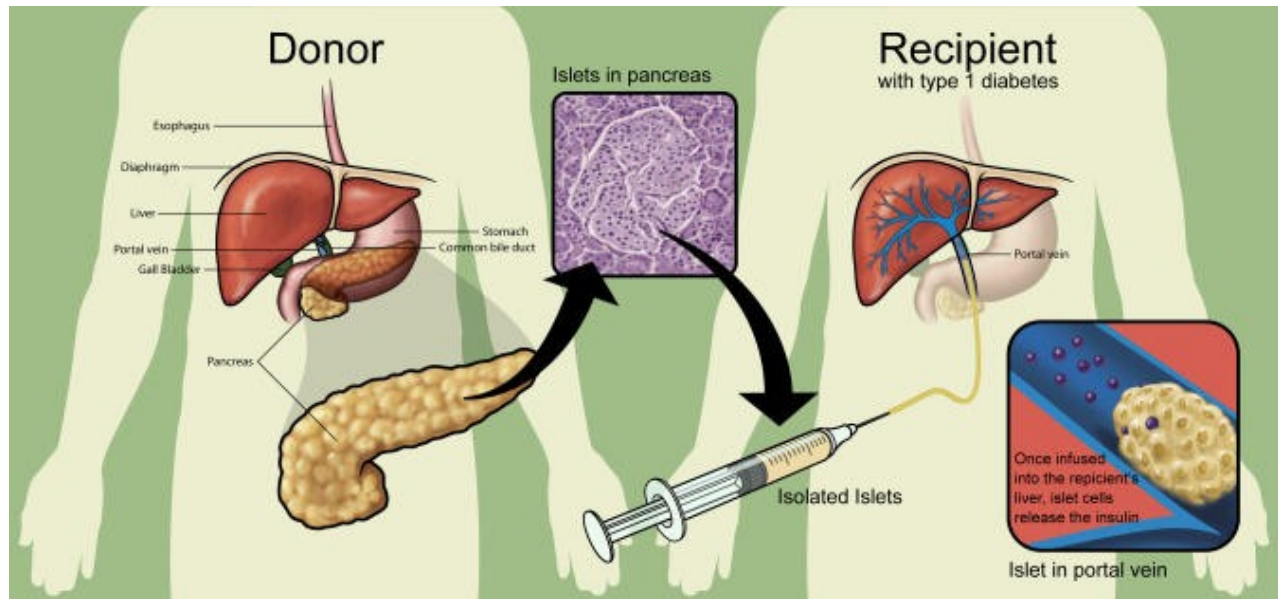
Figure 1.4: The process of clinical pancreatic islet transplantation. This figure is adapted from Naftanel, M.A. and D.M. Harlan, *PLoS Med*, 2004 [86].

1.2.2 Obstacles to Islet Transplantation

Currently islet transplantation treatment for type 1 diabetic patients have been limited to selected patients with severe hypoglycemic episodes. The major obstacles preventing the application of islet transplantation to many diabetic patients are the shortage of human pancreas donor, the need for immunosuppressive medications to prevent rejection, and the possible recurrence of autoimmunity.

1.2.2.1 Shortage of Human Pancreas Donor

Similar to organ transplantation, the demand far exceeds the available organs. This is aggravated in islet transplantation since a single patient may receive islets from several pancreas donors and some patients may require more than one transplant to achieve insulin independence. The use of insulin producing tissues from the animal source, which is

called xenotransplantation, could solve the critical shortage of human donors [40, 87, 88]. Xenotransplantation will be discussed later.

1.2.2.2 The Need of Immunosuppressive Medications

All organ transplant recipients are kept on long term immunosuppressive regimen to prevent graft rejection. Several medications have been used in islet transplantation including corticosteroids, sirolimus, calcineurin inhibitors (tacrolimus), anti-interleukin 2 receptor monoclonal antibody (daclizumab), mycophenolate mofetil, and azathioprine. The toxicity and side effects associated with these drugs have limited the use of islet transplantation as treatment for all type 1 diabetic patients. Many side effects have been reported such as mouth ulcers, gastrointestinal upset, acne, edema, ovarian cysts, weight loss, pneumonia, leukopenia, and impairment of renal function. Steroids are one of the diabetogenic drugs that can cause non-autoimmune diabetes. A clinical study reported the development of non-autoimmune diabetes in up to 20% in non-diabetic organ recipient as a result of calcineurin inhibitor / steroid therapy [40, 69, 73, 85].

1.2.2.3 The Possible Recurrence of Autoimmunity

Sutherland *et al.* demonstrated the recurrence of autoimmunity in type 1 diabetic patients who received pancreas from non-diabetic identical twins without immunosuppression. These patients had recurrence of hyperglycemia associated with isletitis and selective β-cell destruction was seen in the graft biopsies. These findings were not observed in patients who received pancreas transplant accompanied with adequate immunosuppressive regimen [89].

Tyden and colleagues reported two cases of pancreas graft failure due to autoimmunity despite immunosupression. The immunohistochemical examination of pancreatic grafts showed no signs of rejection, normal exocrine tissue and small number of cells staining for insulin. One patient had positive serum finding of islet cell auto-antibodies and glutamic acid decarboxylase (GAD) antibodies [90]. Another study showed correlation between the presence of the auto-antibodies and the graft survival. Patients with type 1 diabetes mellitus with positive auto-antibodies (islet cell auto-antibodies, GAD antibodies) had earlier impairment of islet graft function than those who were auto-antibodies negative despite the immunosuppression [91].

1.3 XENOTRANSPLANTATION OF PIG ISLETS

The history of clinical xenotransplantation begun in the sixteenth century, when a fifteen year old boy suffering from severe fever received blood from a sheep [92]. Interestingly the history of islet xenotransplantation preceded the discovery of insulin by 29 years. In 1893 Watson William and colleagues transplanted fragments of a sheep pancreas to treat a boy with diabetic ketoacidosis. The boy had transient improvement of glucose level in the urine, but he died few days later because of graft rejection [44].

Several animal sources of insulin producing tissues have been studied, such as fish Brockman bodies [93], cows [94], and pigs [95-97]. Pig is the preferred animal source due to several reasons including the similarity between the human and porcine insulin structure, which differ only by one amino acid. The porcine insulin had been used as treatment of diabetic patients for many years. Pigs are readily available, and relatively inexpensive [98]. The use of pigs in islet transplantation can provide unlimited source of tissues and avoid the detrimental effects of brain death on the organs used for

transplantation. In addition, they can be modified genetically to prevent rejection and enhance the graft survival [87].

1.3.1 Optimal Age of Pig Islet Donor

Pig is an attractive source for islet xenotransplantation, many researchers studied the use of porcine islets of different age groups to treat diabetes in experimental animal models. Despite the extensive research, the optimal age of porcine islet donor remains controversial [99]. There are three main age groups that have been investigated: fetal, neonatal, and adult. The advantages and disadvantages of each group will be discussed in the following sections [99].

1.3.1.1 Adult Pig Islets

In 1990, Ricordi *et al*. described a technique to isolate large numbers of intact adult porcine islets, using a modified automated method for isolation of human islets [97]. They were able to obtain 255,000 ± 32,407 islets per pancreas of an adult pig, with 80 - 95% purity. Six out of seven nude mice with diabetes were normoglycemic after pancreatic islets have been transplanted into renal subcapsular space [97].

Adult porcine islets are able to release insulin in response to glucose *in vitro* and *in vivo* within hours. A study done by Bottino *et al.* demonstrated the capability of adult porcine islet transplant to achieve normoglycemia in streptozotocin induced diabetic mice without delay [100]. Although adult porcine islets provide large amount of islets and respond to high glucose levels within hours, they have their disadvantages. They are fragile, difficult to isolate and maintain in culture, and they have poor growth potential [99, 101, 102].

Adult porcine islets are more immunogenic than neonatal porcine islets, which may increase the requirement of immunosuppressive medication [103]. In addition, a study performed by Emamaullee *et al.* showed that adult porcine islets are susceptible to hypoxia *in vitro* and *in vivo*. They were found to have high rates of apoptosis (50 ± 10.33 %), with majority of cells dying after 24 hours of hypoxia (94.66 ± 1.76 %) [104]. Further, housing the pigs for long periods until they reach the age for donation in pathogen free environment could be costly [99].

1.3.1.2 Neonatal Pig Islets

Neonatal pig islets are attractive source for islet xenotransplantation. They are less fragile, easy to isolate and maintain in culture compared to adult pig islets. In 1996, Korbutt *et al.* developed a method to isolate 50,000 viable islet cell aggregates per pancreas of 1-3 day old neonatal pig [96]. The cell aggregates are composed of 57% epithelial cells and 35% pancreatic endocrine cells [96]. Neonatal pig islets are able to proliferate and differentiate in the post-transplantation period [105]. In addition, they are capable of restoring normoglycemia in both small and large experimental animal models including non-human primates within 6-8 weeks post-transplantation [98, 106]. In comparison to adult porcine islets, neonatal pig islets are less immunogenic and more resistant to hypoxic injury [103, 104].

1.3.1.3 Fetal Pig Islets

Islet-like cell clusters (ICC) were isolated from the pancreas of a fetal pig, they are immature group of cells composed predominantly of epitheloid cells with only about 5% of β-cells. Groth *et al.* reported that an average of 10, 000 ICCs can be obtained per pancreas

of a pig fetus. As a result of this small number several fetuses will be needed for a single islet transplant recipient. Korsgren *et al.* demonstrated the delayed functionality of fetal porcine ICCs, when they transplanted fetal porcine islets beneath the kidney capsule of alloxan-induced diabetic mice, normoglycemia was restored 2 months later. In addition to their immaturity, delayed functionality, the fetal porcine ICCs have poor insulin response to glucose [95, 107].

However, fetal porcine ICCs have the potential to grow, mature and differentiate into insulin producing cells. A study performed by Foster and colleagues using ICCs isolated from fetal pigs at the gestational age of 83-92 days, demonstrated the ability of these cells to grow and differentiate in the post-transplantation period [108]. Chemically induced diabetic severe combined immunodeficient mice were transplanted with 5,000-38,000 encapsulated fetal porcine ICCs. These mice had normal blood glucose levels within 13-68 days following intraperitoneal transplantation of encapsulated fetal porcine ICCs. The percentage of insulin positive cells within an ICC before transplantation was around 7 ± 0.8%. However, the encapsulated fetal porcine ICCs which were removed at 25 weeks of transplantation had increased percentage of insulin positive cells reaching up to 70.7 ± 5.5%. The function of fetal porcine ICCs was assessed using 20 mM glucose as stimulus. Before transplantation 20 mM glucose stimulation did not significantly increase the insulin secretion. However, 6 months after transplantation insulin secretion in response to 20 mM glucose stimulation was increased about 96 fold [108].

1.3.2 Mechanism of Pig Islet Xenograft Rejection

Rapid destruction of porcine islets after xenotransplantation occur by activation of both innate and adaptive immune systems, which involve instant blood mediated

inflammatory reaction, humoral mediated immune response, and cell mediated immune response.

1.3.2.1 Instant Blood Mediated Inflammatory Reaction (IBMIR)

IBMIR is an innate immune response that occurs after the direct contact of donor islets with the recipient blood. It has been reported to be present in islet xenotransplantation as well as islet allotransplantation [109, 110]. IBMIR involves the activation of platelets, complement, and coagulation cascades. Coagulation cascade has intrinsic and extrinsic pathways, each can be stimulated by different factors. The intrinsic pathway is activated by the collagen residues on islet surface which is not normally in contact with blood. The extrinsic pathway is activated by tissue factor (TF) which is expressed in α -cells, β-cells, and the contaminating ductal structures. Due to the molecular incompatibilities between the donor and the recipient, the mechanisms which limit the effect of coagulation such as porcine TF pathway inhibitor do not occur. Platelets are also activated by interaction with collagen, von Willebrand factor or thrombin. Activation of coagulation cascade and platelets lead to clot formation, ischemia, and necrosis of islets. The mechanism by which the complement activation occurs is not clear, but possibly it involves the activation of alternative pathway. Within one hour of activation of these three pathways, islets will be infiltrated by neutrophils and macrophages. These infiltrating cells cause significant destruction of islets via several ways such as release of cytokines, phagocytosis, enzymatic digestion of islets, and antigen presentation to T-cells which is involved in cellular rejection of islets [110].

IBMIR causes significant loss of islets in the early transplant period which limits the success of islet transplantation. Rapid destruction of pancreatic islets after intraportal

transplantation was associated with release of insulin with peak of C-peptide, leading to the development of hypoglycemia in recipient animals [110, 111]. To overcome the damaging effect of IBMIR several pharmacological agents are being studied including the use of heparin (in islet preparation as in clinical islet allotransplantation), low molecular weight dextran sulphate, and compstatin [111-114].

The significant rapid loss of pancreatic islets after intraportal transplantation due to IBMIR led the researchers to explore alternative sites for islet transplantation such as pancreas, peritoneum, kidney, skeletal muscle, and gastric submucosa [53, 67, 75]. Although pancreas is the physiological site for islets, only few studies investigated the feasibility of pancreas as a site for islet transplantation. Pancreas is highly vascularized providing proper sensation of glucose level. Several factors limited the application of islet transplantation in clinical practice such as invasive surgical procedure, the leakage of digestive enzymes from the exocrine cells of the pancreas, and the risk of autoimmune recurrence [53, 115]. The peritoneum has the advantages of unrestricted transplant volume and it has been shown to accept encapsulated islets. The disadvantages for this site are the need of large number of islets to reverse hyperglycemia and the lack of parasympathetic re-innervation of islets transplanted into the peritoneum which is associated with abnormal glucose tolerance test [53, 115]. Kidney capsule site is the most commonly used in rodents. It has been reported that less number of islets were able to reverse hyperglycemia in chemically induced diabetic mice when transplanted under the kidney capsule, in contrast to intraportal site where larger number of islets are needed to restore normoglycemia. The success of islet transplantation under the kidney capsule could be limited in human due to poor blood supply, low oxygen content, invasive surgical

procedure, limited space, and the diseases affecting the kidney like diabetic nephropathy provide poor environment for islet engraftment [53, 115]. Intramuscular infusion of pancreatic islets has been investigated in human. Many reasons make this site attractive for islet transplantation like easy accessibility, good vascularization, ability to take biopsies, and to perform multiple infusions [115]. However, intramuscular implantation of pancreatic islets have been reported to be associated with fibrosis and necrosis, especially if they are transplanted as clusters. Disseminating islets longitudinally along the skeletal muscle may reduce the risk of fibrosis [115]. Gastric submucosa could be considered as an alternative site for islet transplantation, providing more physiological sensation of glucose level in the blood, high vascular supply, and accessibility to biopsy. In addition, islet transplantation into the gastric submucosa can be done using laparoscopy or endoscopy which are minimally invasive, however it needs to be explored further before application in human [67, 115].

1.3.2.2 Hyperacute Rejection

A major problem in pig to human xenotransplantation of vascularized solid organ is an antibody-complement mediated destruction of the transplanted organ, which is known as hyperacute rejection. Xenoreactive preformed natural antibodies present in the recipient blood induces hyperacute rejection by binding to antigens present on the surface of vascular endothelium of the xenograft, which in turn cause complement mediated destruction of the xenograft. A major target for the xenoreactive antibodies has been identified as the terminal carbohydrate galactose α 1, 3 (Gal) epitope. Most mammals have

the enzyme α 1, 3 galactosyltransferase, which transfer a terminal galactose residue to a subterminal galactose, forming Gal oligosaccharide epitope. Human, apes, and Old World monkeys lack this enzyme [98, 116-119].

Human xenoreactive antibodies develop as result to exposure to carbohydrate antigens on gastrointestinal flora [117, 120]. Oriol *et al.* reported the expression of α 1, 3 Gal epitope in different porcine tissues, including vascular endothelium, kidney, lung, skin, and pancreas (epithelial ducts) [121]. The expression of α 1, 3 Gal carbohydrate residue on the pancreatic endocrine cells is controversial, several studies have shown that α 1, 3 Gal is not expressed on porcine islets [121, 122]. However, Rayat and colleagues demonstrated that the expression of α 1, 3 Gal antigen on porcine islets is age dependent [123]. As the porcine islets mature, the expression of α 1, 3 Gal epitope decrease. It has been shown that 20% of neonatal porcine islets express α 1, 3 Gal on their surface, compared to 5% of adult porcine islets [123].

In vitro study demonstrated that both α 1, 3 Gal positive and α 1, 3 Gal negative porcine islets were susceptible to human antibody-complement mediated destruction [124]. In addition, McKenzie *et al.* showed that porcine islets were susceptible to anti-porcine antibodies other than α 1, 3 Gal antibodies. The porcine islet xenograft was destroyed over 48-72 hours post-transplantion in Gal deficient mice [106]. These studies suggest that α 1, 3 Gal epitope is not the only antigen inducing hyperacute rejection [106, 124].

1.3.2.3 Cell Mediated Rejection

T-cells play a major role in mediating graft rejection, their activation requires two signals. The first signal is antigen specific, where the T-cells interact with the foreign

antigen presented by antigen presenting cells (APCs). The second signal known as antigen non-specific co-stimulatory signal, which in the absence of such signals T-cells either undergo a programmed cell death (apoptosis) or become unresponsive [125]. There are 2 pathways of antigen presentation direct and indirect. In the direct pathway, recipient T-cells recognize an antigen on the surface of donor APC, where in the indirect pathway, recipient T-cells recognize donor antigen processed and presented by recipient's APC [125]. In the direct pathway (donor APC dependent) the antigen presented by the donor APC through major histocompatibility complex (MHC) class I and class II molecules interacts with CD8 T-cell and CD4 T-cell, respectively. While in the indirect pathway (host APC dependent) the antigen presented by host APC mainly through MHC class II molecules, leads to CD4 T-cell predominant response [119].

Islet allograft rejection is believed to be mediated by both pathways, although the direct pathway appears to be critical in initiating the rejection. However, islet xenograft rejection occurs mainly through the indirect pathway [98, 119, 126]. It was demonstrated in MHC class II deficient mice, which lack an indirect response, that islet allografts were acutely rejected while rat and porcine islet xenografts had prolonged survival. The xenograft rejection occurs predominately through an indirect pathway, which is believed to be due to the phylogenetic disparity between the donor and recipient [126].

CD4 T-cells have crucial role in both allograft and xenograft rejection [127]. Athymic mice, which are deficient in T and B-cells, were unable to reject porcine islet xenograft, demonstrating the need for T-cells to initiate rejection [128]. One study showed that rat islet xenografts were rejected in CD8 knockout mice, while CD4 knockout mice did not

reject the rat islets. This study illustrates that CD4 T-cells not CD8 T-cells are critical in mediating rejection of islet xenograft [127].

1.3.2.4 Potential Risk of Autoimmunity

Thomas *et al.* demonstrated that porcine islet xenografts are resistant to recurrence of autoimmunity in non-obese diabetic (NOD) mice [129]. Group 1 NOD mice received islets from C57BL/6 mice, whereas group 2 NOD mice received islets from adult pigs. Non-obese diabetic mice in both groups were given immunosuppressive medications for 14 days. The islet allografts were susceptible to severe damage with a mean survival of 20 days after discontinuation of immunosuppressive medications. However, islet xenografts had prolonged survival for more than 200 days after discontinuation of immunosuppressive medications [129].

1.3.3 Risk of Pathogen Transmission

Transmission of infection is a major concern when pigs are used as a source of islets for transplantation. The risk of infection can be decreased using specific pathogen-free animal colonies, however this is not applicable for porcine endogenous retrovirus (PERV) due to the presence of PERV in the germline of every pig [130].

The subtypes of porcine endogenous retrovirus are PERV-A, PERV-B, PERV-C, and PERV-A/C. PERV-A and PERV-B are integrated in the genome of every pig, while PERV-C is not present in all pigs. The retroviruses that have the ability to replicate in human cells *in vitro* include PERV-A, PERV-B and PERV-A/C, while PERV-C is restricted to pig cells. PERV-A/C has a high replication rate, which represent a potential risk in pig to human xenotransplantation. To reduce the risk of infection, pig donor needs to be screened for PERV expression [131].

Heneine *et al.* reported that there were no PERV infection detected in all the ten patients who received fetal porcine pancreatic islets [130]. Another study reported the absence of PERV transmission in all of the seven diabetic patients transplanted with neonatal porcine islets [132]. Furthermore, the risk of PERV infection could be prevented by knock down of PERV expression using small interfering RNA (siRNA) technology [133, 134].

1.4   CATALYTIC ANTIOXIDANT

1.4.1   *Non-Immune Mediated Destruction of Pancreatic Islets*

Unlike solid organ transplantation where the donor organ blood vessels are connected to the recipient vasculature resulting in immediate restoration of vascularization after transplantation, pancreatic islets are cellular transplant without direct linkage with the recipient blood vessels. Pancreatic islets have oxygen rich environment provided by an extensive network of capillaries that resembles the renal glomeruli [135]. Although the pancreatic islets constitute 1-2% of the pancreas, they receive around 10-20 % of the whole pancreas blood supply [8, 136]. The rich blood supply of the pancreatic islets allows appropriate sensation of glucose level, secretion of hormones, and dispersal of the secreted hormones to target organs [137]. The vascularization of pancreatic islets is interrupted during isolation and early post-transplantation period. As a result, pancreatic islets depend on the diffusion of oxygen and nutrients from the surrounding blood vessels until the formation of new blood vessels that may takes up to 2 weeks [136, 138, 139]. Thus, the interruption of vascularization exposes the pancreatic islets to hypoxia that has been reported to contribute to pancreatic islet death and dysfunction that occur during isolation and soon after transplantation [137]. Davalli *et al.* demonstrated in syngeneic

murine islet transplant the susceptibility of islets to damage during the immediate post-transplantation period [139]. In their study, the morphological analysis of the kidney bearing the islet graft that was harvested 1, 3, 7, and 14 days post-transplantation was performed using electron microscopy. On day 1 post-transplantation, necrotic pancreatic islets were detected, which was increased on day 3 post-transplantation. In addition, they noticed on day 3 post-transplantation infiltration of the islet graft with blood cells (mainly red blood cells). On day 7 post-transplantation, they observed blood vessels formed within the islet graft and on day 14 post-transplantation, they saw improvement of vascularization. The damage observed in islet graft was accompanied by reduction of insulin content on day 1 after transplantation, and further reduction detected on day 3 and 7 after transplantation [139].

It has been estimated that more than 50% of pancreatic islet graft are damaged during the early post-transplantation period. This substantial early cell death could be attributed to hypoxia, since this significant cell loss was found in both syngeneic and immunodeficient transplantation models, reflecting the possibility of non-immune mediated destruction [140, 141]. Previous studies on experimental pancreatic islet transplantation demonstrated that the blood supply of the transplanted pancreatic islets is different from the blood supply of the endogenous pancreatic islets. This was proven by the observation of reduced partial pressure of oxygen (Figure 1.5), vascular density (Figure 1.6), and blood flow in the transplanted pancreatic islets [136, 142]. As a result, pancreatic islets are exposed to hypoxia during isolation and during the process of revascularization.

Hypoxia/reoxygenation leads to the generation of reactive oxygen species (ROS) through activation of cascade of biochemical reactions [143]. Reactive oxygen species are

chemical compounds that contain atoms with one or more unpaired electron involving oxygen molecules. Reactive oxygen species includes superoxide anion (O_2^-), hydroxyl radical (OH^-), hydroperoxyl (HO_2^-), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and ozone (O_3) [144].
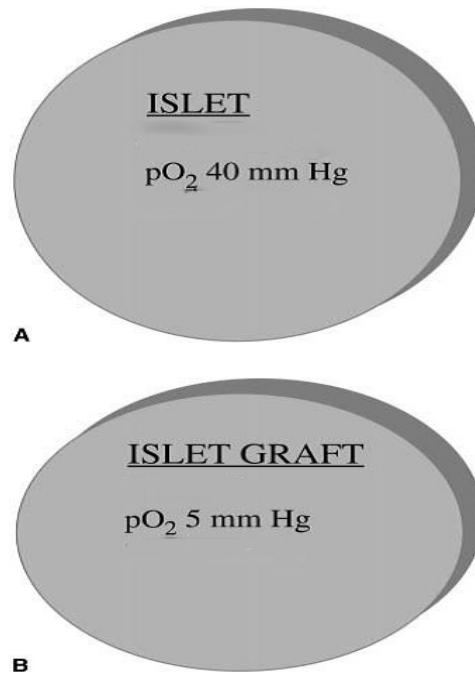
Figure 1.5: Schematic representation demonstrating the reduced partial pressure of oxygen (pO$_2$) in pancreatic islet graft. A: The partial pressure of oxygen (pO$_2$) in endogenous pancreatic islet. **B:** The partial pressure of oxygen (pO$_2$) in pancreatic islet graft. This figure was adapted from Jansson, L. and P.O. Carlsson, *Diabetologia,* 2002 [136].

I)
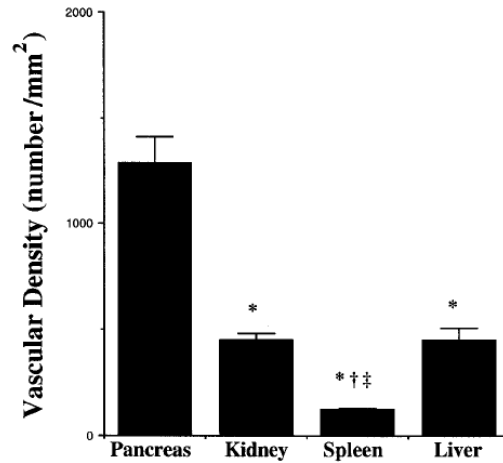
II)
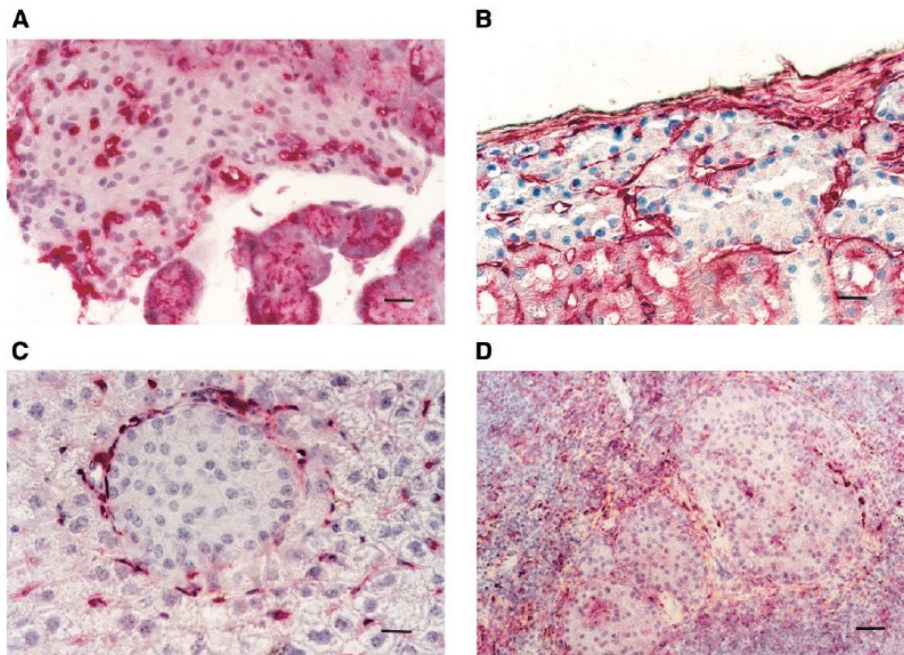
Figure 1.6: Reduced vascular density in pancreatic islet graft in comparison to endogenous pancreatic islets one month post-transplantation. I) Vascular density in endogenous pancreatic islets and in pancreatic islets transplanted into renal subcapsular space, liver, or spleen. Values are expressed as the mean ± SE of the number of the blood vessels per graft area (mm^2), *P <0.05. **II)** Photomicrographs showing the microvascular endothelium (stained red) within the endogenous pancreatic islets and pancreatic islet graft. **A:** Section of mouse endogenous pancreatic islets. **B:** Section of mouse pancreatic islets transplanted under the kidney capsule. **C:** Section of mouse pancreatic islets transplanted intraportally into the liver. **D:** Section of mouse pancreatic islets transplanted into the spleen. This figure was adapted from Mattsson, G., L. Jansson, and P.O. Carlsson, *Diabetes*, 2002 [142].

Oxidative stress occur when the reactive oxygen species produced exceeds the scavenging ability of antioxidant enzymes. Accumulation of excessive reactive oxygen species cause damage to cellular proteins, carbohydrates, lipids, and nucleic acids leading eventually to cell death [145]. The susceptibility of a tissue to oxidative stress mediated damage is related to the antioxidant defense of the tissue. Pancreatic islets have low level of antioxidant enzymes compared to other tissues, which make them more susceptible to oxidative stress induced damage (Table 1.2) [146]. The major antioxidant defense enzymes include superoxide dismutase (SOD), glutathione peroxide (GPx), and catalase (CAT). There are 2 isoforms of superoxide dismutase, copper-zinc- superoxide dismutase (Cu-Zn-SOD) and manganese-superoxide dismutase (Mn-SOD). Cu-Zn-SOD is located in the cytoplasm and the nucleus, while Mn-SOD is located in the mitochondria. Superoxide dismutase convert superoxide which is generated through electron transport chain into hydrogen peroxide. Glutathione peroxidase is found in the cytoplasm, the nucleus, and the mitochondria, while catalase is found in the peroxisome. Both glutathione peroxidase and catalase convert hydrogen peroxide into water and oxygen [147].

Tissues	CuZn-SOD (% Liver)	Mn-SOD (% Liver)	CAT (% Liver)	GPx (% Liver)
Liver	100 ± 7	100 ± 17	100 ± 10	100 ± 5
Kidney	99 ± 7	125 ± 19	78 ± 8	91 ± 9
Brain	77 ± 8	67 ± 16	36 ± 10	39 ± 8
Lung	80 ± 12	66 ± 17	50 ± 10	58 ± 9
SkeletalMuscle	59 ± 7	95 ± 14	41 ± 12	40 ± 10
Heart Muscle	70 ± 10	142 ± 9	72 ± 11	39 ± 7
Pituitary Gland	79 ± 19	47 ± 11	23 ± 2	66 ± 11
Adrenal Gland	175 ± 16	239 ± 25	45 ± 7	77 ± 12
Pancreatic Islets	38 ± 9	30 ± 5	Not detectable	15 ± 6

Table1.2: The gene expression profile of antioxidant enzymes in several tissues from albino mice. Values are represented as the mean ± SEM and expressed as a percentage of the liver gene expression. The table adapted from Lenzen *et al.*, *Free Radical Biology & Medicine*, 1996[146].

A marked improvement in the outcome of clinical islet transplantation was observed after the application of the Edmonton protocol. Achievement of insulin independence requires a large number of pancreatic islets (minimum of 9,000 IEQ / kg body weight) [40, 148]. The scarcity of available pancreatic islets strongly limits the number of type I diabetic patients who could be treated with islet transplantation. As a result, methods to reduce the number of pancreatic islets lost during isolation and post-transplantation are needed. Since pancreatic islets have inherently low level of antioxidant defense mechanism, one way that could improve the islet yield and quality is to boost their antioxidant defense enzymes [146]. Several studies investigated the effect of using antioxidants on pancreatic islets` viability and secretory capacity. Antioxidant treatment was used in different forms such as

exogenous administration, systemic delivery, or genetic manipulation. Various antioxidants were tested such as vitamins (e.g. ascorbic acid, tocopherols), trace elements (e.g. selenium, zinc), enzymes (e.g. SOD mimetic compounds), and others (e.g. herbs, folic acid) [149, 150]. Previous studies on overexpression of genes in charge of antioxidant enzymes of pancreatic islets demonstrated protection of islets against reactive oxygen species mediated damage. These studies provided evidence that strengthening of the pancreatic islets antioxidant defense enzymes could be a valuable approach to improve islet quality and quantity, thereby reducing the number of islets lost during isolation and transplantation procedures [150, 151]. Adenoviral-based overexpression of glutathione perioxidase in insulin secreting cells (INS-1, rat β-cell line) enhanced their resistance to cytotoxicity induced by both reactive oxygen and reactive nitrogen species [152]. Another study has shown that overexpression of manganese-superoxide dismutase in INS-1 cells provided protection against oxidative damage [153]. In addition, it has been reported that overexpression of catalase reduced the islet susceptibility to oxidative stress induced damage in human, pig, and rat pancreatic islets [154]. These studies demonstrated that enhancing the pancreatic islet antioxidant defense could block the oxidative stress resulting in reduction of islet death, increased islet survival, and could have a considerable impact on islet transplantation outcome.

1.4.2 *Manganese (III) Tetrakis N- Ethylpyridium-2-yl Porphyrin: Manganese Porphyrin (MnP)*

Enzymes that catalytically convert superoxide radical (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2) are known as superoxide dismutases (SODs). They are metalloproteins that are classified depending on the metal center they contain into four

groups: iron superoxide dismutase (Fe-SOD), manganese superoxide dismutase (Mn-SOD), copper-zinc superoxide dismutase (Cu-Zn-SOD), and nickel superoxide dismutase (Ni-SOD). Mammals have two classes of SODs, copper-zinc superoxide dismutase and manganese superoxide dismutase. The Cu-Zn-SOD is found in the cytoplasm, the nucleus, and the extracellular space, while Mn-SOD is found in the mitochondria. The mechanism of catalytic dismutation of superoxide radicals involves the sequential reduction and oxidation of the metal center, with concomitant oxidation and reduction of superoxide radicals [155-158]. The catalytic antioxidant we used in our study is manganese (III) tetrakis *N*- ethylpyridium-2-yl porphyrin (MnP), which is a synthetic antioxidant that mimics the action of superoxide dismutase.

SOD mimics were developed more than 2 decades ago, they have been subjected to many modifications to enhance their bioavailability and efficacy. It is not completely understood how SOD mimics function *in vivo,* since the superoxide dismutase activity observed *in vitro* is not always translated into function in *in vivo* studies, and vice versa. Studies have demonstrated the inability of manganese containing SOD mimics to functionally replace the superoxide dismutase activity in SOD-deficient strains of *Escheriachia coli*, while these complexes reported to have superoxide dismutase activity *in vitro*. Surprisingly, manganese (III)-tetra (4-pyridyl) porphyrin that was inactive *in vitro*, was found to be active *in vivo*. Thus, further studies are needed to explore the molecular mechanism by which SOD mimics might exert their action *in vivo* [158-160]. In our study we used manganese (III) tetrakis *N*- ethylpyridium-2-yl porphyrin which is a cationic manganese based porphyrin SOD mimic which has electron-deficient metal center that easily accepts and donates electrons. Thus, it binds with electron rich anionic radicals such

as superoxide anion and peroxynitrite (ONOO⁻). Manganese porphyrin have a broad

spectrum antioxidant specificity, scavenging superoxide anion, nitric oxide, peroxynitrite,

and lipid peroxyl radicals. Its chemical structure as shown in Figure 1.7 is characterized by

the positive metal center that allows it to accumulate within crucial cellular compartments

specifically the mitochondria, which has a negative membrane potential that could attract

such compound [161-164]. In addition, manganese porphyrin is stable, cell permeable

compound, with a history of reproducible outcomes, low toxicity and ease of application

[163, 165].
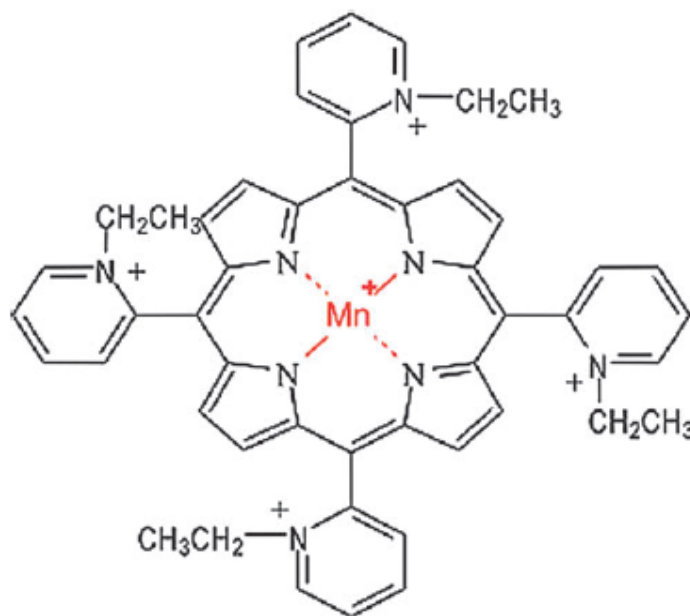
Figure 1.7: The chemical structure of manganese porphyrin (MnP). This figure was adapted from Delmastro Greenwood, *et al., Antioxidants & redox signaling,* 2013 [163].

Pancreatic islet viability is negatively affected by several factors before, during, and

after isolation, and transplantation procedures. These stressful factors include donor

characteristic, storage time, the isolation process itself, and the immunosuppressive

medications. The pancreatic islet isolation involves mechanical and enzymatic digestive

processes, which could negatively impact islet yield and functional capacity. It has been documented that pancreatic islets undergo structural changes during isolation procedure leading to islet death. In addition, it has been demonstrated that pancreatic islet mass decreased during culture [166-169]. Piganelli *et al.* reported the beneficial effect of manganese porphyrin in the field of pancreatic islet transplantation [165]. They demonstrated that addition of manganese porphyrin during the process of isolation of human pancreatic islets resulted in significantly higher (70% vs. 40%) islet cell mass than these obtained without the addition of manganese porphyrin. In addition, they showed that addition of manganese porphyrin during the culture of human pancreatic islets lead to reduction of islet loss, however, this protective effect was not observed in the first 24 hours of culture. Within 24 hours after isolation there was a similar reduction in islet mass in untreated and manganese porphyrin treated pancreatic islets. Further, they revealed that treatment of pancreatic islets with manganese porphyrin did not affect their insulin secretory capacity as MnP-treated islets were able to restore normoglycemia in immunodeficient diabetic mice [170].

Streptozotocin (STZ) causes pancreatic islets destruction through the formation of free radicals (superoxide and nitric oxide radicals)[171]. Previous studies demonstrated that pre-treatment with superoxide dismutase protected pancreatic islets against streptozotocin induced damage [172, 173]. Similar finding was reported by Piganelli *et al*. when they treated human pancreatic islets with 68 μM manganese porphyrin 20 minutes prior to exposure to streptozotocin lead to the protection against streptozotocin - induced cell death. In addition, they revealed that systemic delivery of manganese porphyrin inhibited streptozotocin induced diabetes in mice [165]. Treatment of pancreatic islets

with 68 μM manganese porphyrin for 24 hours prior to syngeneic (mouse model), allogeneic (mouse model), or xenogeneic (human pancreatic islets transplanted into diabetic mice) transplantation resulted in improved function in comparison with untreated controls. Diabetic mouse recipients of syngeneic islets pre-treated with manganese porphyrin had improved glycemic control at 30 and 60 minutes time points when subjected to an intraperitoneal glucose tolerance test on post-operative day 9 compared with recipients of untreated islets. Although diabetic mouse recipients of suboptimal syngeneic islets pre-treated with manganese porphyrin did not achieve normoglycemia, they had significantly lower blood glucose levels compared with recipients of untreated islets [163, 165]. Additionally, it has been shown that diabetic murine recipients of manganese porphyrin pre-treated allogeneic islets exhibited delayed allograft rejection compared to recipients of untreated islets. All allograft recipients restored normoglycemia, however, recipients of manganese porphyrin pre-treated islets retained normoglycemia longer (31.8 ± 7.1 days) than recipients of untreated islets (21.4 ± 1 days).On the other hand, they demonstrated that systemic treatment of diabetic mouse recipients with manganese porphyrin extended the delay in allograft rejection. Further, diabetic mouse recipients of human islets pre-treated with manganese porphyrin had significantly lower blood glucose levels compared to recipients of untreated human islets in the absence of immunosuppressive medications. These data indicates that manganese porphyrin could have immunomodulatory effects besides its antioxidant action, which might account for the delay in islet allograft rejection [163, 165]. Piganelli and colleagues demonstrated that metalloporphyrin SOD mimics have immunomodulatory effect, where systemic treatment with manganese porphyrin prevented the adoptive transfer of T-cell mediated diabetes in

young NOD. SCID recipients. Manganese porphyrin treated mice had an intact pancreatic architecture with absence of leukocytes infiltration, in contrast to control mice which developed pancreatitis with the loss of the normal pancreatic architecture [174]. They suggested that manganese porphyrin treatment resulted in the failure of antigen presenting cells to activate T-cells and in the inhibition of interferon-γ (IFN-γ) production by T-cells [174]. Thus, using manganese porphyrin would improve the islet survival, and function, possibly leading to reduction of the number of pancreatic islets needed for transplantation.

1.5 HYPOTHESIS, OBJECTIVES, AND OUTLINES OF THESIS PROJECT

Previous studies reported the beneficial effects of manganese porphyrin on human and murine pancreatic islets, however, no study examined the effect of manganese porphyrin on neonatal pig islets. In this study, we hypothesize that pre-treatment of neonatal pig islets with manganese porphyrin will protect them from oxidative stress, and improve their function in immunodeficient diabetic mice. Our objectives were to investigate the effect of manganese porphyrin on neonatal pig islets` morphology, and viability *in vitro* and to determine whether pre-treatment of neonatal pig islets with this antioxidant will improve islet function after transplantation into immunodeficient diabetic mice.

Pancreatic islets were isolated from 3-day-old pigs and they were cultured for 7 days. On day 7, neonatal pig islets were cultured overnight with 0, 34 or 68 μM manganese porphyrin at 37º C, 95% air, and 5% CO_2. They were then collected for qRT-PCR analysis to determine the gene expression of some antioxidant and antiapoptotic molecules such as hemeoxygenase-1 (HMOX-1), glutathione peroxidase-1 (GPx-1), superoxide dismutase-1

(SOD-1), B-cell lymphoma-2 (Bcl-2), and survivin. Islet viability was also determined using a two-color fluorescence assay. In addition, aliquots of islets were stained with CellRox Green Reagent after 2 hours incubation with hydrogen peroxide to examine the effect of oxidative stress on neonatal pig islets.

To examine whether pre-treatment of neonatal pig islets with manganese porphyrin will improve their function *in vivo*, streptozotocin induced diabetic (≥20 mmol/L) NSG (Non-Obese-Diabetes-severe combined immunodeficient –gamma, NOD.SCID γ) mice were transplanted with neonatal pig islets (2,000 IEQ) pre-treated with 0, 34 or 68 μM manganese porphyrin. Blood glucose levels of these mice were monitored once a week. When mice showed stable normal blood glucose levels, they were injected intraperitoneally with one bolus of glucose to determine how well the islet transplant can respond to glucose challenge. At the end of the study (>100 days post-transplantation), the kidney bearing the islet transplant was surgically removed to assure that the maintenance of normal blood glucose level relies on the presence of neonatal pig islet graft. Finally, sections of the kidney bearing the graft were stained with hematoxylin & eosin, in addition to immunohistochemistry staining for insulin and glucagon.

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CHAPTER 2

MANGANESE PORPHYRIN PROTECTS NEONATAL PIG ISLETS AGAINST OXIDATIVE STRESS AND ENHANCES THEIR FUNCTION IN DIABETIC MICE

2.1 INTRODUCTION

Type 1 diabetes mellitus is characterized by insulin deficiency due to immune mediated destruction of β-cells of the pancreas [1, 2]. It is a systemic disease affecting multiple organs, leading eventually to renal failure, heart disease, blindness, and amputation which reduce the quality of life of diabetic patients [1, 3]. The main treatment for type 1 diabetes mellitus is daily insulin injections [1, 2]. Although the discovery of insulin changed the fate of type 1 diabetes mellitus from a deadly disease into a chronic treatable disease, it has its disadvantages. Patients with type 1 diabetes mellitus need to frequently monitor their blood glucose levels to control the hyperglycemia [1, 3]. In order to prevent these complications diabetic patients should maintain tight glycemic control. Although intensive insulin therapy was shown to be effective in reducing the long term complication, it was associated with greater risk of severe hypoglycemia [4]. Hypoglycemia could cause cardiac arrhythmias and death [5, 6].

After the improved clinical outcome reported by the Edmonton group in 2000, pancreatic islet transplantation became an option to treat type 1 diabetic patients. Pancreatic islets are infused into the portal vein, which provide a more physiological way to supply insulin, thus, avoiding the risk of developing severe hypoglycemia [7]. In order to achieve insulin independence, a large number of pancreatic islets are needed. A single

patient may receive pancreatic islets from 2 or more donors, and may require multiple

infusion of pancreatic islets [7-9]. Pancreatic islet transplantation is similar to other organ

transplantation, where the demand for pancreas donors far exceeds the available donors.

This limits the application of pancreatic islet transplantation to selected patients. To

overcome the shortage of human donors, the use of animal tissues have been investigated

[10, 11]. Pig has been considered as the animal of choice providing unlimited source of

tissues. They are readily available, relatively inexpensive, and they can be genetically

modified [10, 12]. Neonatal pig islets in particular are easy to isolate and maintain in

culture. In addition, they have the ability to proliferate and differentiate, and they are

capable of reversing diabetes in both small and large experimental animal models [12-15].

However, it has been reported that significant number of islets are lost during

isolation and early post-transplantation period due to oxidative stress [16, 17]. Pancreatic

islets have low level of antioxidant enzymes making them more susceptible to oxidative

stress induced damage [18]. Previous studies demonstrated that the strengthening of

pancreatic islets` antioxidant defense enzyme protected them from oxidative stress

mediated destruction. Several methods were investigated to enhance pancreatic islet

antioxidant enzyme such as exogenous administration of antioxidant, systemic delivery,

and genetic overexpression of antioxidant enzymes [19, 20]. For instance, it has been

demonstrated that overexpression of glutathione peroxidase or manganese-superoxide

dismutase in insulin secreting cells (INS-1, rat β-cell line) provided protection against

oxidative damage [21, 22]. Further, it has been shown that the treatment of pancreatic

islets with antioxidant such as manganese porphyrin prior to syngeneic (murine model),

allogeneic (murine model), and xenogeneic (human pancreatic islets transplanted into diabetic mice) transplantation improved their function compared to untreated control [23]. Since the application of genetic modification in the clinical setting could be difficult, the use of manganese porphyrin antioxidant seems to be more practical [24]. Further, the effect of manganese porphyrin on neonatal pig islets was not reported previously. In our study we were interested in investigating the effect of manganese porphyrin on neonatal pig islets` viability, morphology, and function. In addition, we were interested to examine if pre-treatment of neonatal pig islets with manganese porphyrin would induce changes in the gene expression of selected antioxidant and antiapoptotic molecules.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Three day old Duroc male or female neonatal pigs (University of Alberta Farm, Edmomton, Canada) were used as a source of pancreatic islets for transplantation. Non-obese-diabetic-severe combined immunodeficient gamma (NSG) male mice (7 week old) were used as recipient of islet transplant (The Jackson Laboratory, Bar Harbor, ME, USA). Diabetes was induced in these mice chemically *via* intraperitoneal injection of 180 mg/kg body weight of streptozotocin (Sigma, St. Louis, MO, USA) 5-7 days prior to transplantation. Mice were considered diabetic when they have two blood glucose readings more than 20 mmol/L before islet transplantation. Blood glucose levels (BGL) were monitored once per week using a One Touch Ultra glucometer (Lifescan Inc., Milpitas, CA, USA). All mice received standard care and food according to the Animal Care Use Committee at University of Alberta and Canadian Council on Animal Care guidelines.

2.2.2 Neonatal Pig Islet Isolation and Culture

We isolated neonatal pig islets using a method developed by Korbutt *et al* [14]. Isoflurane was used to anesthetize neonatal pigs which underwent laparotomy. Blood was drained from the abdominal aorta using 16 gauge needle, then pancreas was harvested and placed in cold Hank`s Balanced Salt Solution (HBBS). After which, the pancreas was cut into small pieces using sterile scissors and digested using type XI collagenase at a concentration of 1 mg / ml (Sigma). Then the digested pancreatic tissue was filtered through 500 μm nylon mesh and washed three times with cold HBSS. Finally, the pancreatic tissue was cultured at $37\,^{o}$ C, 5 % CO_2, and 95% air for 7 days in Ham`s F10 media supplemented with 10 mmol/L D-glucose, 50 μmol/L isobutylmethylxanthine (ICN, Biomedicals, Montreal, QC, Canada), 0.5% bovine serum albumin (BSA), 2 mmol/L L-glutamine, 3 mmol/L $CaCl_2$, 10 mmol/L nicotinamide (BDH Biochemical, Poole, England). On days 1, 3, and 5 media was changed. On day 7 post-isolation neonatal pig islets were collected and 50 μl aliquots were taken to count the islets. After counting the islets in islet equivalent (IEQ), 1,000 IEQ were placed in 6 well plate in the presence of 0, 34, or 68 μM of manganese porphyrin and they were cultured overnight at $37\,^{o}$ C, 5 % CO_2, and 95% air. For transplantation into diabetic NSG mice approximately 4,000-6,000 IEQ were cultured overnight in Ham`s F10 in the presence or absence of 34 or 68 μM manganese porphyrin at ordinary culture conditions.

2.2.3 Determination of the Gene Expression Level of Selected Antioxidant and Antiapoptotic Molecules by Quantitative Real –Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (1 ml; Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from neonatal pig islets (1,000 IEQ) (Figure 2.1). After homogenization of the neonatal pig islets in TRIzol (1ml), chloroform (0.2 ml) was added and the tube was shaken vigorously by hand for 15 seconds. After incubation for 2 minutes at room temperature the tube containing the mixture was centrifuged at 12,000 x g for 15 minutes. The homogenate was separated into a clear upper layer (which contain the RNA) and a red lower layer (which contain the DNA and protein). The upper layer containing RNA was transferred into a new tube, where the RNA was precipitated by addition of 100% isopropanol (0.5 ml). Then, the sample was incubated at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes. The supernatant was removed and the RNA pellet was washed with 75% ethanol (1 ml), mixed by gently inverting the tube (3 times), and then centrifuged at 10,000 x g for 10 minutes. The supernatant was removed and the tube was centrifuged again at 10,000 x g for 5 minutes, to ensure the removal of almost all of the ethanol. The RNA pellet was dissolved in 100 μl of RNase-free water (diethylpyrocarbonate-DEPC-treated water). Following these steps, the RNA concentration was measured using NanoDrop device ranging from 38.60 to 429.5 ng/μl.

Total RNA (300 ng) was used to synthesize cDNA using qScript cDNA Supermix (Quanta, Biosciences, Gaithersburg, USA). Prior to the cDNA synthesis, genomic DNA in the RNA was removed by DNase digestion. One unit of DNase was added into the RNA (300 ng /10 μl) and incubated at 25° C for 30 minutes. Then EDTA (1 μL of 50 mM EDTA) was

added, and the tubes were incubated at 65°C for 10 minutes to inactivate the DNase. The

qScript cDNA SuperMix (10 µl) was added to each tube and then RNase-free water was

added to a final volume of 20 µl. The tubes were vortexed gently and centrifuged briefly.

After which, they were incubated at 25°C for 5 minutes, then at 42°C for 30 minutes,

followed by incubation at 85°C for 5 minutes.

PerfeCta qPCR FastMix (Quanta, Biosciences, Gaithersburg, USA) and MicroAmp Fast

Optical PCR Plates were used for setting up the real time PCR reaction. TaqMan probes

(Lifetechnologies, Bethesda, USA) were used to determine the level of expression of

selected genes including; hemeoxygenase-1 (HMOX-1), glutathione peroxidase-1 (GPx-1),

superoxide dismutase-1 (SOD-1), B-cell lymphoma-2 (Bcl-2), and survivin. The 18s rRNA

was used as a reference gene. A master reaction mix for each TaqMan probe was made

consisting of the following: PerfeCta qPCR FastMix (5 µl), nuclease-free water (0.5 µl), and

TaqMan probe (0.5 µl). Then the master reaction mix (6 µl) was mixed with the cDNA (4 µl)

in the PCR plate. The PCR plate was sealed using an optical adhesive cover, then

centrifuged briefly (1,000 rpm for 2 minutes). After which, the PCR plate was loaded into

the ABI 7900HT realtime PCR machine. The following thermal cycling condition was used:

initial denaturation at 95°C for 30 seconds, and then 45 cycles at 95°C for 2 seconds

followed by 60°C for 20 seconds.
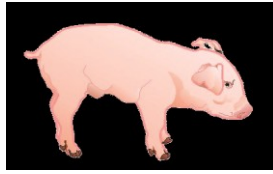
3 Day old neonatal pig
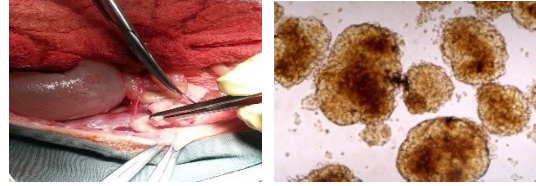
Isolate islets
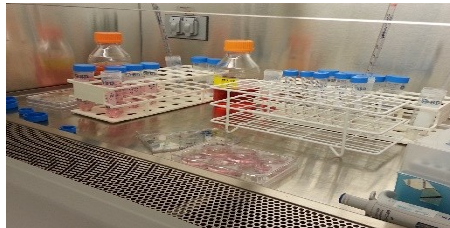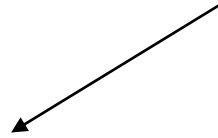
Place islets in culture ± MnP
(34 μM or 68 μM) overnight at 37°C, 5 % CO$_2$
and 95% air
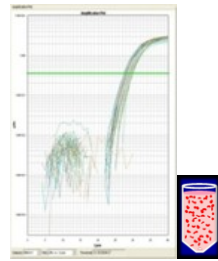
qRT-PCR analysis

Measure fold change in HMOX-1, SOD-1, & GPx-1 gene expression
Measure fold change in Bcl2 & Survivin gene expression

Figure 2.1: Experimental design of qRT-PCR analysis to determine the level of gene expression of selected antioxidant and antiapoptotic molecules.

2.2.4 Examination of Neonatal Pig Islet Morphology

Following 7 days of culture at 37° C, 5 % CO_2, and 95% air, neonatal pig islets were collected and centrifuged at 1,000 rpm for 1.5 minutes. Islets were then suspended in 50 ml of Ham`s F10 then aliquots of 50 μl were collected and islets in these aliquots were counted. One thousand IEQ were either treated with 34 μM or 68 μM manganese porphyrin or left untreated, then cultured overnight under ordinary culture conditions. Islets from these three experimental groups were visualized under the light microscope and pictures were taken.

2.2.5 Assessment of Neonatal Pig Islet Viability

The LIVE/DEAD Viability/ Cytotoxicity Kit (Molecular Probes, Invitrogen, Eugene, USA) was used to assess the neonatal pig islet viability. This kit includes 2 probes: calcein AM a cell permeable nonfluorescent dye which is converted by intracellular esterase into green fluorescent in live cells (green= live cells), and ethidium homodimer-1 which cannot enter the cells with intact plasma membrane but it enters the cells with damaged plasma membrane and binds to nucleic acid producing a red fluorescent in dead cells (red=dead). Working solution was prepared by adding 2 μl of calcein AM and ethidium homodimer-1 into 1ml of Hank's Balanced Salt Solution (HBSS). Aliquot of 1,000 IEQ of neonatal pig islets in 2 ml of HBSS was placed into micro-tube, centrifuged at 1,000 rpm for 1.5 minutes. Then, the media was aspirated, 200 μl of the fluorescent dye working solution was added, and incubated in dark at room temperature for 15 minutes. After the incubation, the tubes were centrifuged at 1,000 rpm for 1.5 minutes, the dye was aspirated, and the cells were washed 2 times with HBSS. Then, the cells were suspended in 50 μl of HBSS, fixed with 4%

glutaraldehyde (500 µl), and incubated in the dark at room temperature for 1 hour. Islets were then washed and suspended in HBSS (500µL). At the end of the experiment, a cell suspension was placed on a microscope slide, then islets were visualized using traditional fluorescence microscope (Figure 2.2),

2.2.6 Oxidative Stress Experiment

Oxidative stress was assessed using CellRox Oxidative Stress Green Reagent (Lifetechnology, Carlsbad, CA, USA). The CellRox Green Reagent is a cell permeable DNA dye, non-fluorescent and upon oxidation exhibit green fluorogenic signal. After 7 days of culture at ordinary culture conditions, neonatal pig islets were collected and counted. Aliquots of 500 IEQ were transferred into 6-well plate and were cultured overnight in the presence of 0, 34, or 68 µM of manganese porphyrin at ordinary culture conditions. Then, neonatal pig islets were incubated with 1 mM hydrogen peroxide (H_2O_2) for 2 hours. After which, neonatal pig islets were collected, centrifuged at 1,000 rpm for 1.5 minutes, and the media was aspirated. Then, the CellRox Green Reagent was added to the islets at a final concentration of 5 µM and the cells were incubated for 30 minutes in the dark at room temperature. Next, the cells were washed 3 times with PBS, were fixed by adding 4 % formaldehyde for 15 minutes, and then the cells were washed again with PBS. At the end of the experiment, the cells were placed on a microscope slide, visualized using traditional fluorescence microscope, and pictures were taken (Figure 2.2).
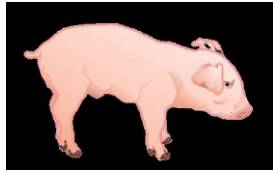
3 Day old neonatal pig   **Isolate islets**
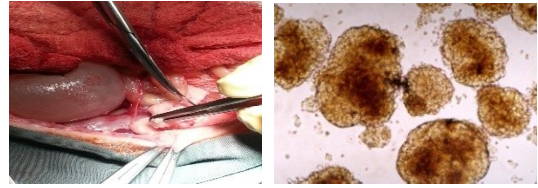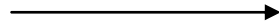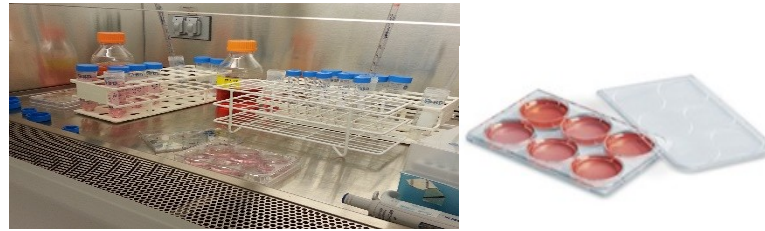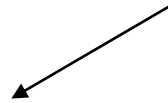
Place islets in culture ± MnP
(34 μM or 68 μM) overnight at $37^{o}C$, 5% CO_2
and 95% air

Fluorescence cell viability staining
(LIVE/DEAD Viability Kit)
CellRox Oxidative Stress Green Reagent Staining
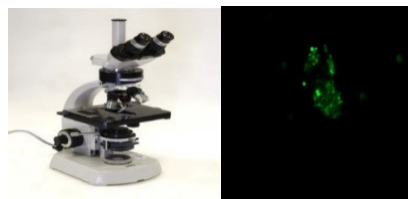
Visualize neonatal pig islets under the microscope
Take pictures of neonatal pig islets

Figure 2.2: Experimental design of neontal pig islet viability assessment and oxidative stress detection.

2.2.7 Transplantation of Neonatal Pig Islets into Diabetic Mice

After 7 days of culture, neonatal pig islets were either left untreated or treated with 34 or 68 μM manganese porphyrin overnight at 37° C, 5% CO_2, and 95% air. Then, islets were collected, counted, and aliquots of 2,000 IEQ were transplanted under the left kidney capsule of streptozotocin-induced diabetic NSG mice. The 2,000 IEQ of neonatal pig islets were transferred into polyethylene tube (PE-50), then centrifuged. Following centrifugation the tube was placed in the subcapsular space of the kidney, with the aid of a micromanipulator syringe islets were delivered into the subcapsular space, and cautery was applied at the puncture site.

The blood glucose levels of recipient mice were monitored once a week using a One Touch Ultra glucometer (Lifescan Inc., Milpitas, CA, USA) for more than 100 days after transplantation. Normoglycemia was defined as blood glucose level equal or less than 8.4 mmol/L. The kidney bearing the islet graft was removed (>100 days post-transplantation) to ascertain that the normal blood glucose level was achieved and maintained due to the presence of the islet graft. This was confirmed by reoccurrence of high blood glucose level (Figure 2.3).
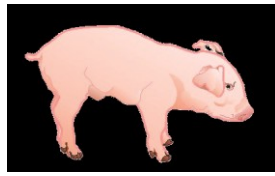
3 Day old neonatal pig
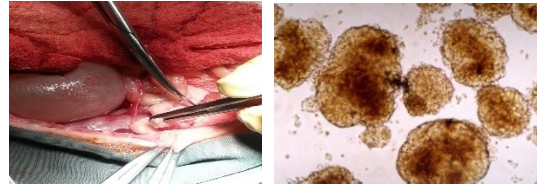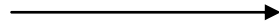
Isolate islets
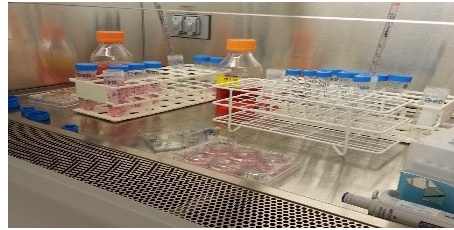
Place islets in culture ± MnP

(34 μM or 68 μM) overnight at 37°C, 5% CO_2 and 95% air

Transplant islets under the kidney capsule (2,000 IEQ) of STZ-induced diabetic NSG mice

Monitor blood glucose level for >100 days post-transplant

Perform Survival nephrectomy
Collect islet graft for immunohistochemistry staining

Figure 2.3: Experimental design of the assessment of neontal pig islet function in diabetic immunodeficient mice .

2.2.8 Intraperitoneal Glucose Tolerance Test (IPGTT)

After achievement of normal blood glucose level, an intraperitoneal glucose tolerance test was performed at more than 100 days (133 days) post-transplantation. Mouse recipients of neonatal pig islets were subjected to 2-hour fasting, after which 50% dextrose solution (3mg/g of body weight) was injected into the intraperitoneal space of the recipient mice. Blood glucose levels were determined at 0, 15, 30, 60, and 120 minutes *via* blood samples collected from the tail vein.

2.2.9 Immunohistological Staining of Graft Sections

Following survival nephrectomy (>150 days post-transplantation), the kidney bearing the islet graft was collected, fixed with 10% formalin, and embedded in paraffin. Five-micrometer thickness graft sections were stained for insulin and glucagon. The graft sections were quenched with 10% hydrogen peroxide in methanol, then 20% normal goat serum (Cedarlane, Burlington, ON, CA) was used for blocking the non-specific binding sites. For detection of β-cells (insulin secreting cells), the graft sections were incubated with guinea pig anti-insulin antibody (1:1,000 diluted in PBS, Dako Laboratories, Mississauga, ON, CA) for 30 minutes. Then, the graft sections were incubated with biotinylated goat anti-guinea pig secondary antibody (1:200 diluted in PBS, Vector Laboratories, Burlington, CA, USA) for 20 minutes. For detection of α-cells (glucagon secreting cells) the graft sections were incubated with guinea pig anti-glucagon antibody (1:500 diluted in PBS, Sigma-Aldrich, Missouri, USA) for 30 minutes. Then the graft sections were incubated with biotinylated goat anti-guinea pig secondary antibody (1:200 diluted in PBS, Cedarlane, Burlington, ON, CA) for 20 minutes. Following the incubation with the primary and

secondary antibodies, the graft sections were washed three times with PBS, then incubated

with avidin-biotin complex/horseradish peroxidase (ABC/ HP, Vector Laboratories) for 40

minutes. Thereafter, 3,3- diaminobenzidine chromogen solution (DAB, BioGenex, San

Ramon, CA, USA) was applied for 10 minutes. Finally, the graft sections were

counterstained with Harris` hematoxylin and eosin.

2.2.10 Statistical Analysis

STATA12 was used to perform statistical analysis and P-value < 0.05 was considered

statistically significant. Fold changes in gene expression and blood glucose levels (mmol/L)

were shown as mean ± SEM and compared using one-way ANOVA, among three

experimental groups (untreated control, 34 μM MnP, and 68 μM MnP) pairwise

comparisons were done using Bonferroni method (post hoc test).

2.3 RESULTS

2.3.1 Treatment of Neonatal Pig Islets with Manganese Porphyrin Resulted in Changes in the Level of Gene Expression of Antioxidant and Antiapoptotic Molecules

Analysis of the gene expression of antioxidant and antiapoptotic molecules were done

using qRT-PCR. The antioxidant molecules analyzed were hemeoxygenase-1 (HMOX-1),

glutathione peroxidase-1 (GPx-1), and superoxide dismutase-1 (SOD-1). The antiapoptotic

molecules were B-cell lymphoma-2 (Bcl-2) and survivin. RNA samples were obtained from

the neonatal pig islets following the treatment with 0, 34, or 68 μM manganese porphyrin,

and the total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). The

treatment of islets with manganese porphyrin increased the expression of SOD-1 (P= 0.04),

and survivn genes (P= 0.03) in a dose dependent manner compared to untreated islets

(Figure 2.4). In addition, treatment with manganese porphyrin increased the expression of

HMOX-1 gene by 1.5 fold in islets treated with 34 µM MnP and increased the expression of

HMOX-1 gene by 2 fold in islets treated with 68 µM MnP in comparison to untreated islets,

but these changes did not reach statistical significance (Figure 2.4). Further, the level of

GPx-1 gene expression was increased significantly (P= 0.006) in islets treated with 68 µM

MnP compared to untreated islets and islets treated with 34 µM MnP (Figure 2.4).

However, the up-regulation of Bcl-2 gene expression in islets treated with 68 µM MnP

compared to other experimental groups did not reach statistical significance (Figure 2.4).
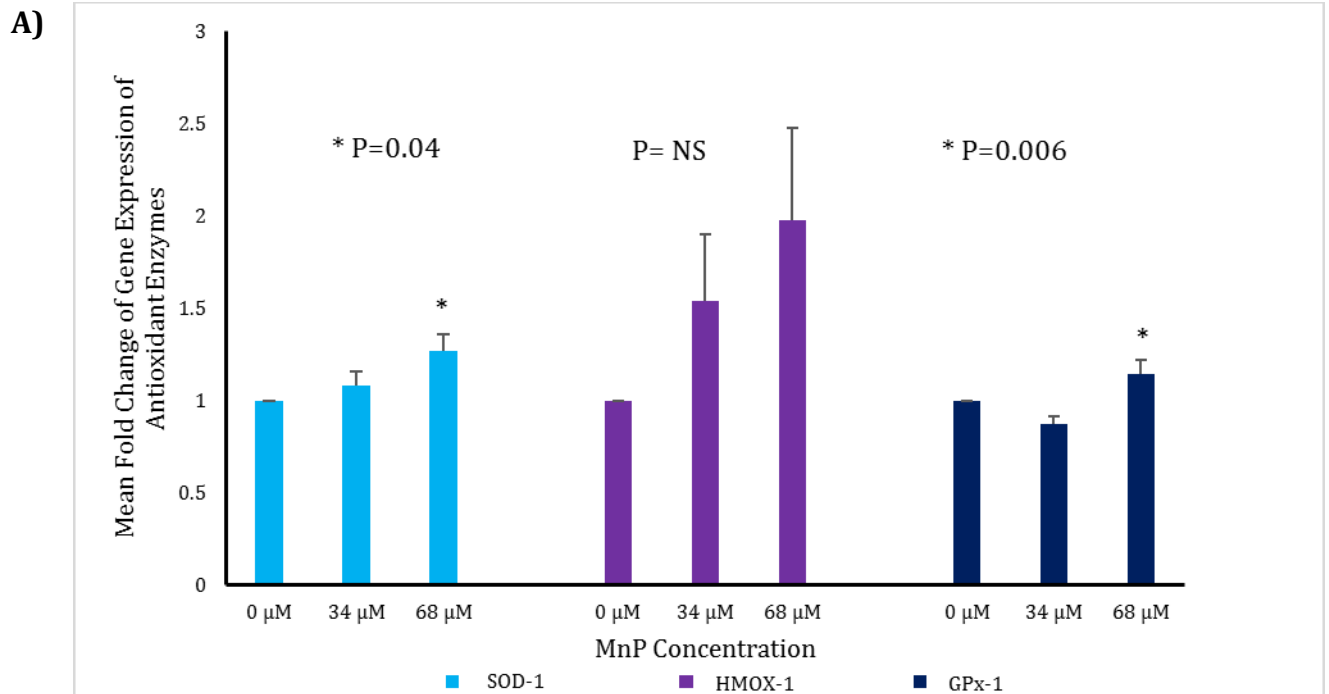
B)
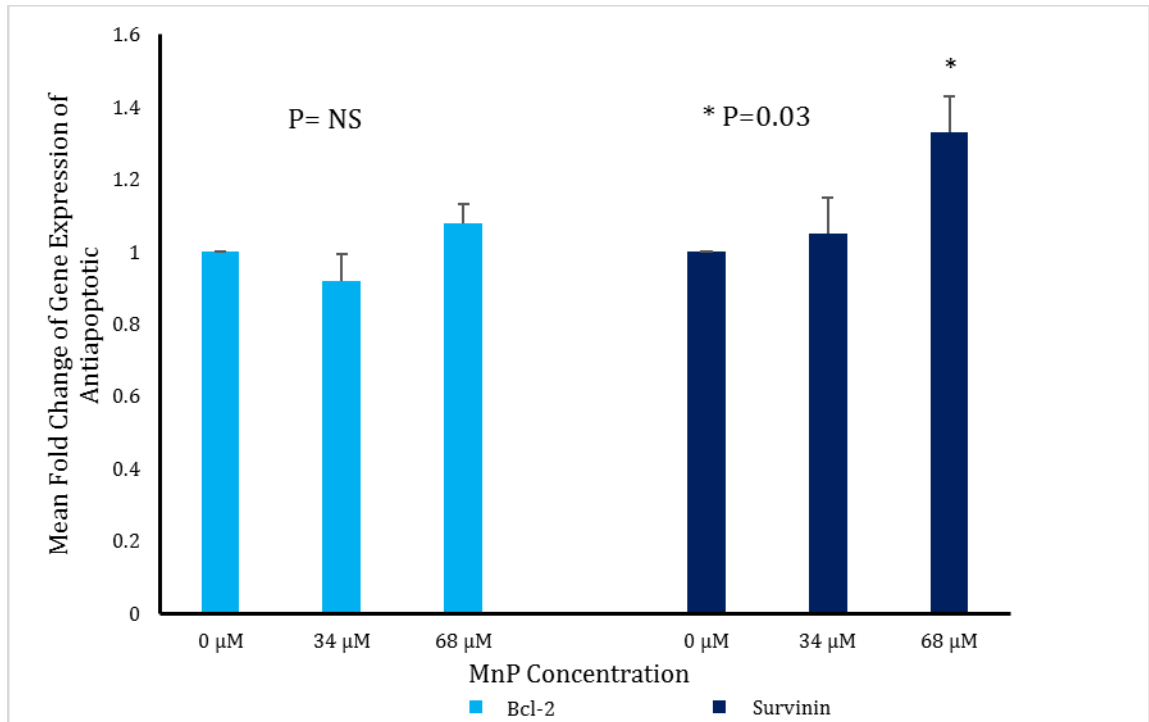
Figure2.4: Manganese porphyrin induced changes in the level of gene expression of selected antioxidant enzymes (A) and antiapoptotic proteins (B). Neonatal pig islets (A and B) were treated with 0, 34 or 68 µM MnP overnight. mRNA expression of the antioxidant enzymes (A) and antiapoptotic proteins (B) was assessed by qRT-PCR (n=7, duplicate) and18s rRNA was used as a reference gene.

2.3.2 No Morphological Changes were Observed between Untreated Neonatal Pig Islets and those Treated with Manganese Porphyrin

After 7 days of culture at 37° C, 5% CO_2, and 95% air, neonatal pig islets were collected and counted. One thousand neonatal pig IEQ were placed in 6 well-plate incubated overnight in the presence or absence of 34 µM or 68 µM MnP. No morphological changes were observed when islets from the three experimental groups were visualized using the light microscope (Figure 2.5).

Untreated islets **Islets treated with 34 μM MnP** **Islets treated with 68 μM MnP**
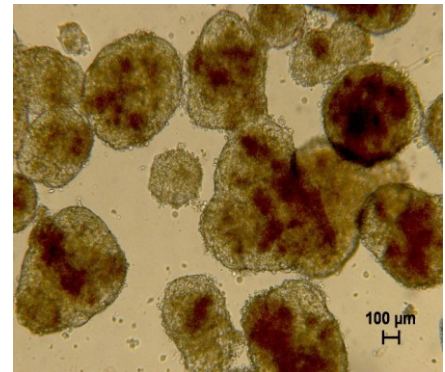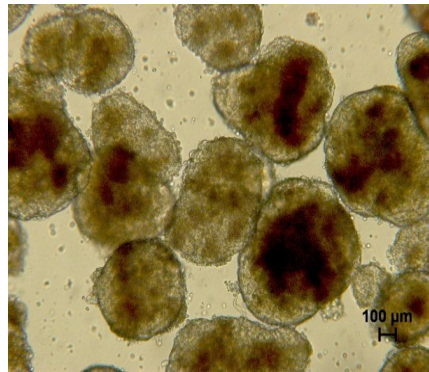
Figure 2.5: Light photomicrographs of neonatal pig islets incubated in the presence or absence of MnP. Images were taken following 8 days of culture in Ham`s F10 at ordinary culture conditions. Images showing no changes in islet morphology in all experimental groups.

2.3.3 Manganese Porphyrin is Not Toxic to Neonatal Pig Islets

A two color-fluorescent viability assay was used to assess islet viability. Simultaneous staining of live and dead cells was done using calcein AM which stains the live cells green, and ethidium homodimer-1 stains dead cells red. In the untreated and the MnP-treated islets, the majority (around 80%) of islets were alive (stained green) as shown in Figure 2.6. From the images taken there was no differences between the untreated neonatal pig islets and the MnP-treated islets.

Untreated islets   **Islets treated with 34 µM MnP**   **Islets treated with 68 µM MnP**
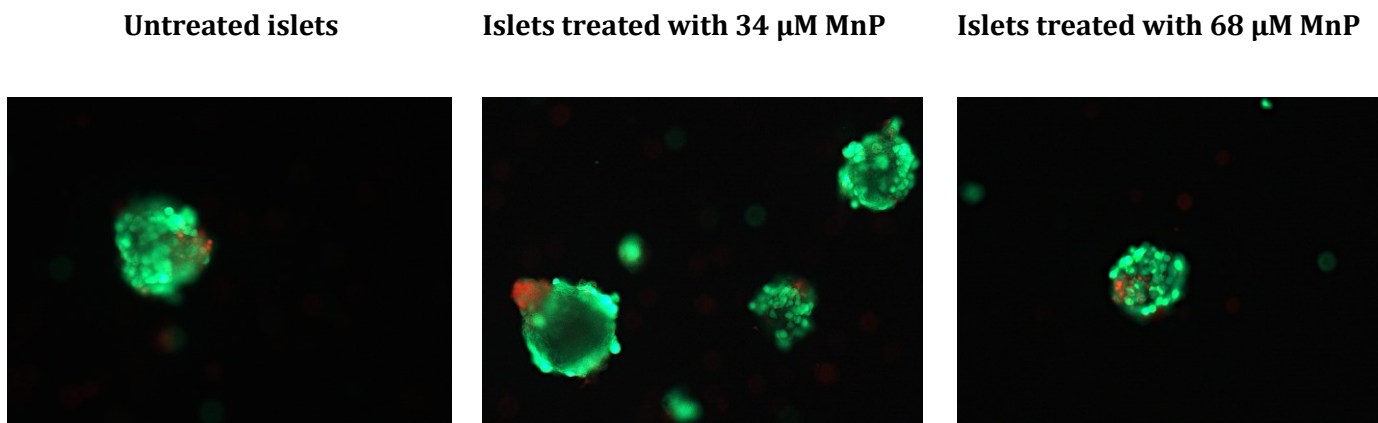
Figure 2.6: Photomicrographs of neonatal pig islets stained with green-fluorescent calcein-AM (green/live cells) and red-fluorescent ethidium homodimer-1 (red/dead cells). Neonatal pig islets were visualized under a fluorescence microscope at 10X magnification following the treatment with different concentrations of MnP.

2.3.4 Manganese Porphyrin Protects Neonatal Pig Islets against Hydrogen Peroxide (H$_2$O$_2$) Induced Oxidative Stress

Following 7 days of culture, aliquots of neonatal pig islets were treated with 0, 34 or 68 µM manganese porphyrin overnight at ordinary culture conditions. Neonatal pig islets then were incubated with 1mM hydrogen peroxide for 2 hours at 37° C, 5% CO$_2$ and 95 % air. Following the incubation with hydrogen peroxide neonatal pig islets were stained with CellRox Green Reagent, then visualized under the fluorescence microscope. CellRox Oxidative Stress Green Reagent (Lifetechnology, Carlsbad, CA, USA) is a cell permeable dye which upon oxidation by reactive oxygen species produces a green fluorogenic color. We found that the pre-treatment of neonatal pig islets with 34 µM or 68 µM manganese porphyrin protected islets from H$_2$O$_2$-induced injury. We observed that majority of untreated islets incubated with H$_2$O$_2$ were degraded and it was difficult to visualize intact islets (Figure 2.7). In contrast to islets pre-treated with manganese porphyrin and were

incubated with H_2O_2, many intact islets were observed (Figure 2.7). This observation indicates that pre-treatment of neonatal pig islets with manganese porphyrin protected them from H_2O_2-induced injury.

	Untreated islets	**Islets pre-treated with 34 μM MnP**	**Islets pre-treated with 68 μM MnP**
No H_2O_2 incubation			
After H_2O_2 incubation			
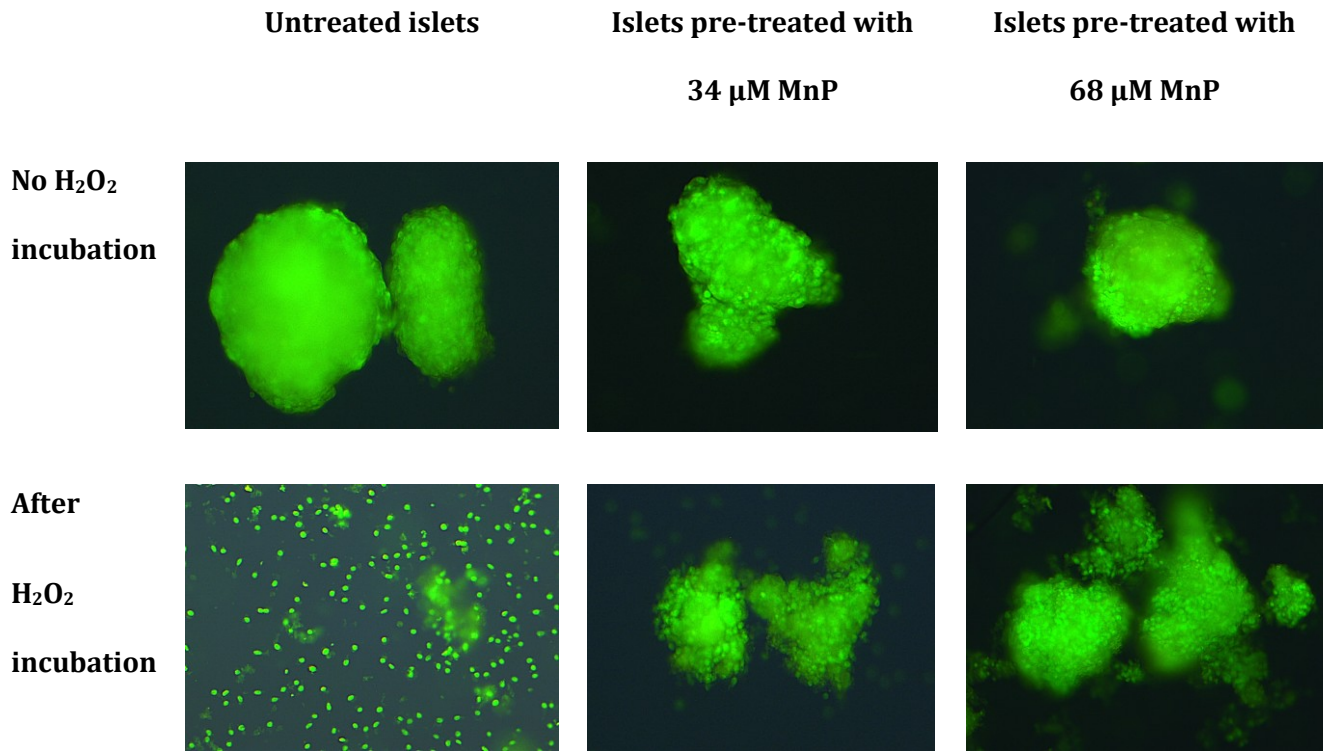
Figure 2.7: Photomicrographs of neonatal pig islets stained with CellRox Green Reagent after the incubation with hydrogen peroxide and visualized under a fluorescence microscope. Images were taken at 10X magnification. CellRox Green Reagent is a fluorogenic ROS sensor, it is nonfluorescent in its reduced state and upon oxidation by ROS, exhibits green fluorescence color.

2.3.5 Pre- treatment of Neonatal Pig Islets with 34 μM Manganese Porphyrin Improves their Function in Diabetic Immunodeficient Mice

Neonatal pig islets were incubated overnight in the presence or absence of manganese porphyrin (34 μM or 68 μM) at 37º C, 95% air and 5% CO_2. Two-thousand IEQ were transplanted under the left kidney capsule of STZ-induced diabetic NSG mice (n=15,

blood glucose level > 20 mmol/L) and the blood glucose levels were monitored once a

week. One animal from the 34 μM manganese porphyrin group was euthanized (black

arrow) before the completion of the experiment (37 days post-transplantation) due to

sickness (penile prolapse, hunched posture, and failure to gain weight). STZ-induced

diabetic NSG mouse recipients of neonatal pig islets pre-treated with 34 μM manganese

porphyrin (n=4) achieved normoglycemia earlier (53 ± 4 days post-transplantation, P=

0.0268) than NSG mouse recipients of untreated (n=5, 115 ± 19 days post-transplantation)

and 68 μM manganese porphyrin pre-treated neonatal pig islets (n=5, 110 ± 12 days post-

transplantation). The mean blood glucose level of NSG mouse recipients of neonatal pig

islets pre-treated with 34 μM manganese porphyrin was significantly lower (P=0.0248) in

comparison to NSG mouse recipients of neonatal pig islets untreated or pre-treated with 68

μM manganese porphyrin (Figure 2.8).
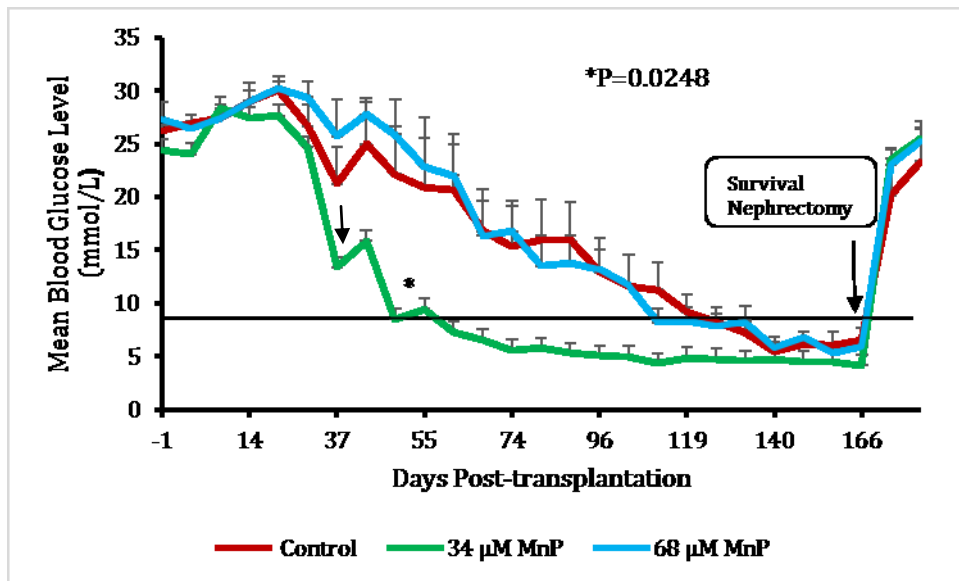
Figure 2.8: Mean blood glucose levels of NSG mice transplanted with neonatal pig islets. NSG mice were transplanted with 2,000 IEQ of neonatal pig islets pre-treated with 0, 34 or 68 μM MnP. Blood glucose levels were monitored one a week for more than 100 days post-transplantation.

At 133 days post-transplantation, an intra-peritoneal glucose tolerance test (IPGTT) was performed on the transplanted mice (n=12). Following 2 hours of fasting, dextrose (3mg/g of bodyweight) was injected into the intraperitoneal space. Then, the blood glucose level was monitored at different time points (0, 15, 30, 60, and 120 minutes). We observed an improved glycemic control at 15, 60, and 120 minutes in mouse recipients of islets pre-treated with 34 µM manganese porphyrin in comparison to other experimental group, but this did not reach statistical significance (Figure 2.9).
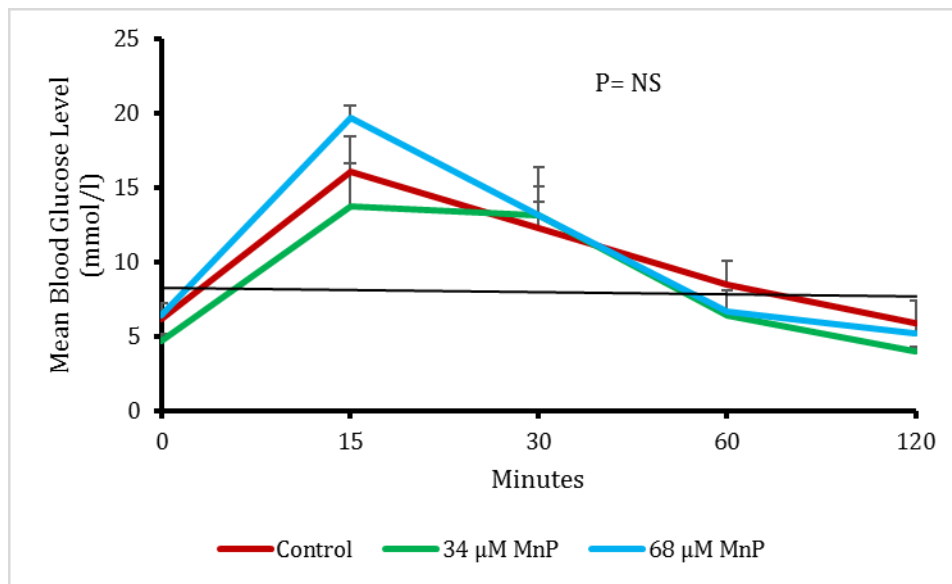
Figure 2.9: Mean blood glucose level of NSG mice during an intraperitoneal glucose tolerance test (IPGTT). When NSG mice showed stable normal blood glucose levels, they were injected intraperitoneally with one bolus of glucose and their blood glucose levels were recorded at different time points.

To confirm that the achievement of normoglycemia was due to the presence of neonatal pig islet graft, the kidney bearing the islet graft was removed at the end of the experiment. After removal of the islet graft, all of the mice reverted to hyperglycemia.

Mouse kidneys bearing the islet graft were fixed in 4% formaldehyde (z-fix, buffered zinc formalin fixatives) and embedded in paraffin. Sections of the islet grafts were stained for the presence of insulin- and glucagon-secreting cells. Examination of the islet grafts by immunohistochemistry under the light microscope revealed positive immunoreactivity to insulin as well as to glucagon (Figure 2.10).
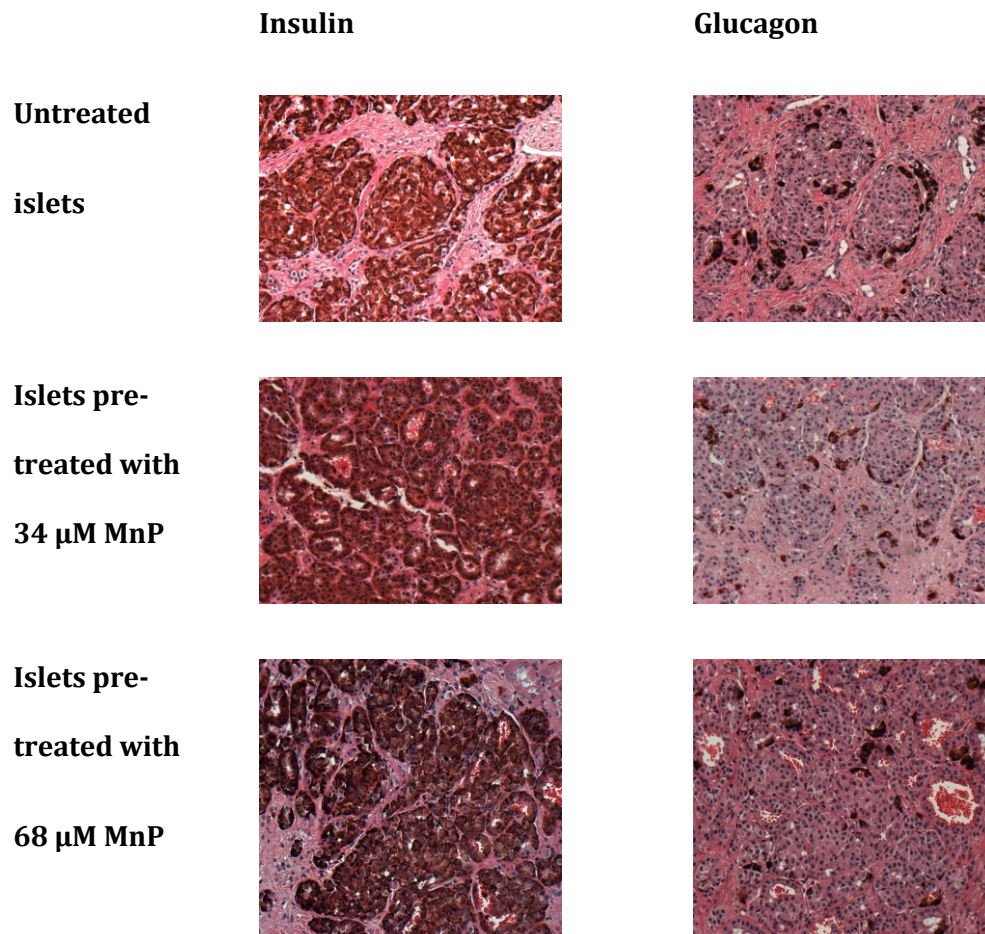
Figure 2.10: Light photomicrographs of islet grafts of mouse recipients of neonatal pig islets untreated or treated with 34 or 68 μM MnP. The graft sections were immunohistochemically stained for insulin and glucagon, then countered stained with Harris`s hemotoxylin and eosin. The brown color indicates the presence of insulin or glucagon positive cells. Images were captured at 10X magnification using a light microscope.

2.4 DISCUSSION

Islet transplantation became one of the treatment options for type I diabetic patients after the success achieved by the Edmonton group [7]. However, the widespread application of islet transplantation is still limited to selected patients due to the shortage of human donors. This is aggravated in the field of islet transplantation since most patients receive islets from more than one donor [7, 11]. In addition, significant number of islets are lost during the isolation procedure and early transplantation period. It has been suggested that hypoxia is the major cause of islet death [17, 25]. Unlike solid organ transplantation where the donor organ blood vessels connected to the recipient vasculature, islets are avascular cellular transplant, they receive nutrient through diffusion from the nearby blood vessels. They are exposed to hypoxia from the time of isolation until the formation of blood vessels which takes up to 2 weeks after transplantation [26-28]. Hypoxia initiates a series of biochemical reactions leading to the formation of reactive oxygen species and their accumulation causes cell death. Oxidative stress result from an imbalance between the level of reactive oxygen species and the level of antioxidants [29, 30]. Islets have low level of antioxidant enzymes which make them more susceptible to hypoxia induced damage [18]. In this study, we used neonatal pig islets to overcome the shortage of human donors, and to protect them from oxidative stress we investigated whether the pre-treatment of these islets with antioxidant would be beneficial.

It has been reported that significant number of islets are lost during the early post-transplantation period even in the absence of immune mediated destruction likely due to hypoxia induced stress since the transplanted islets are devoid of intrinsic vasculature and the revascularization process takes up to 2 weeks [17, 25-27]. An effort to reduce the

number of islets lost and to enhance their engraftment during the early transplantation period is crucial in the field of islet transplantation. Different methods were used to improve islet antioxidant defenses such as incubation of islets with antioxidant and overexpression of antioxidant genes [19, 20]. Since the application of genetic manipulation could be difficult in the clinical setting, in our study we thought to use exogenous administration of antioxidant [24]. We pre-treated neonatal pig islets with catalytic antioxidant prior to their transplantation into immunodeficient chemically induced diabetic mice. We used catalytic antioxidant that mimic the action of superoxide dismutase specifically manganese (III) tetrakis (N-ethylpyridium-2-yl) porphyrin (MnP) provided by Dr.Jon Piganelli from the University of Pittsburgh. It has been shown by Piganelli *et al.* that pre-treatment of human islets with manganese porphyrin during isolation preserved islet viability and insulin secretion capacity [23]. Furthermore, pre-treatment of human and murine islets with manganese porphyrin protected them from ischemia reperfusion injury. Improvement of the blood glucose level was observed in diabetic mice who received allogeneic islet transplant pre-treated with manganese porphyrin. Our choice to use 34 μM and 68 μM manganese porphyrin was based on Dr. Piganelli`s research paper which stated that 34 μM concentration of manganese porphyrin is required to have similar activity as the endogenous mitochondrial superoxide dismutase. They thought to use double the dose of 34 μM of manganese porphyrin and they reported that treating human and murine islets with 68 μM dose was not toxic [23]. In this study, we wanted to investigate if manganese porphyrin would be beneficial for neonatal pig islets.

Oxidative stress occurs when the reactive oxygen species produced exceeds the scavenging ability of antioxidant enzymes. Since islets have low antioxidant capacity, they

are susceptible to damage by oxidative stress. The major antioxidant enzymes of islets are superoxide dismutase, glutathione peroxidase, and catalase [18, 30, 31]. Isoforms of superoxide dismutase are present in different locations within the cell. Cu-Zn-SOD is located in the cytoplasm and the nucleus, while Mn-SOD is located in the mitochondria. Superoxide dismutase converts superoxide anion to hydrogen peroxide. Glutathione peroxidase is located in the cytoplasm, the mitochondria, and the nucleus. However, catalase is found in the peroxisomes. Both glutathione peroxidase and catalase convert hydrogen peroxide to water and oxygen [32]. Several studies reported that the overexpression of antioxidant is beneficial to islets. For instance, insulin producing INS-1 cells overexpressing glutathione peroxidase were resistant to reactive oxygen species cytotoxicity [21]. Overexpression of superoxide dismutase protected insulin producing INS-1 cell against oxidative damage [22]. In addition, it has been demonstrated that the induction of hemeoxygenase-1 protected mouse islets from apoptosis and enhanced their functional performance after transplantation. Hemeoxygenase-1 is a stress intracellular enzyme that is induced by oxidative stress and other stimuli [33]. We were interested to study whether the treatment with manganese porphyrin would induce changes in the levels of gene expression of superoxide dismutase, glutathione peroxidase, and hemeoxygenase-1 on neonatal pig islets. Additionally, we investigated whether the treatment with manganese porphyrin would induce changes in level of gene expression of antiapoptotic molecules namely Bcl-2 and survivin. B-cell lymphoma-2 (Bcl-2) is a member of a family of apoptosis regulatory molecules that includes death inhibitory and death inducing members [34]. Bcl-2 prevents cell death by inhibiting the mitochondrial release of cytochrome c, thereby preventing the effector caspases (e.g. caspase-3) activation. It has

been demonstrated that human β-cells overexpressing Bcl-2 were protected against cytokine-mediated destruction [35]. Survivin belongs to inhibitor-of-apoptosis proteins (IAPs) family which prevent apoptosis through the inhibition of effector caspases. Overexpression of survivin protected mouse islets against cell death caused by staurosporine [36, 37]. Our quantitative real time-polymerase chain reaction (qRT-PCR) analysis showed that the pre- treatment of neonatal pig islets with manganese porphyrin increased the expression of SOD-1 (P=0.04), and survivin genes (P=0.03) in a dose dependent manner compared to untreated islets. However, the level of GPx-1 (P=0.006) gene expression was increased only in neonatal pig islets pre-treated with 68 μM manganese porphyrin compared to untreated islets and islets treated with 34 μM manganese porphyrin. These findings could contribute to the beneficial effects of manganese porphyrin on neonatal pig islets, however, this needs to be confirmed by western blot or immunohistochemical staining.

Furthermore, we demonstrated that the pre-treatment of neonatal pig islets with either 34 μM or 68 μM manganese porphyrin did not affect the islet morphology nor its viability, indicating that manganese porphyrin is not toxic to neonatal pig islets and can be used safely. Untreated islets and islets pre-treated with 34 or 68 μM manganese porphyrin were stained with CellRox Green Reagent following 2 hours of incubation with 1 mM hydrogen peroxide. Zhang *et al.* showed that 1 mM hydrogen peroxide induced oxidative stress resulting in significant reduction of INS-1 cells (rat pancreatic β-cell line) viability [38]. We found that pre-treatment with either 34 μM or 68 μM manganese porphyrin overnight prior to incubation with 1 mM hydrogen peroxide (H_2O_2) protected neonatal pig islets from H_2O_2 – induced oxidative stress in contrast to untreated group where the

majority of islets were destroyed. These results show how islets are susceptible to oxidative stress due to their inherent lower level of antioxidant enzymes. When islets were treated with manganese porphyrin which up-regulated the gene expression of antioxidant enzymes such as superoxide dismutase-1, they were protected from H_2O_2 – induced oxidative stress. Similar protection of human islets pre-treated with 68 μM manganese porphyrin was reported when they were exposed to streptozotocin that mimic the ischemia/reperfusion injury [23]. Dohi *et al.* demonstrated that survivin transgenic mouse islets were protected from cell death induced by staurosporine, in contrast to non-transgenic islets. In addition, they showed that diabetic mouse recipients of suboptimal number of survivin transgenic islets reversed diabetes at 78 days post-transplantation in contrast to diabetic mouse recipients of non-transgenic islets which failed to correct the high blood glucose levels [37]. In our study we speculate that the protection against H_2O_2 – induced oxidative injury provided by manganese porphyrin could be due to the combination of the up-regulation of the gene expression of both the antioxidant enzymes such as SOD-1 and the antiapoptotic molecules such as survivin.

In this study, we also observed that pre-treatment of neonatal pig islets with 34 μM manganese porphyrin improved their function in immunodeficient STZ - induced diabetic NSG mice. Diabetic mouse recipients of neonatal pig islets pre-treated with 34 μM manganese porphyrin achieved normal blood glucose earlier (P= 0.0268) and had significantly lower average blood glucose levels (P= 0.0248) compared to recipients of untreated islets or islets pre-treated with 68 μM manganese porphyrin. In addition, recipient of islets pre-treated with 34 μM manganese porphyrin showed improved glycemic control when subjected to an intraperitoneal glucose tolerance test, however this

improvement did not reach statistical significance perhaps due to the small number of animals in each group. It has been reported by Emamaullee *et al.* that neonatal pig islets are more resistant to hypoxia-induced apoptosis in comparison to adult pig islets and the apoptosis observed in the neonatal pig islet grafts were limited to the cells that were strongly insulin positive [39]. We hypothesize that the earlier achievement of normoglycemia in mouse recipients of neonatal pig islets pre-treated with 34 μM manganese porphyrin could be attributed to the preservation of these few strongly insulin positive cells , however, this should be confirmed by immunohistochemical analysis of neonatal pig islet grafts at earlier time points such as 24 hours and 7 days post-transplantation. Although the pre-treatment of neonatal pig islets with 68 μM manganese porphyrin resulted in significant elevation of superoxide dismutase-1, glutathione peroxidase-1, and survivin gene expression as shown in our qRT-PCR analysis in comparison to other experimental groups, the enhancement of neonatal pig islet graft function *in vivo* using this concentration of manganese porphyrin was not observed. This indicates the importance of maintaining an accurate balance between the level of antioxidant induction and the level of reactive oxygen species production. It is possible that the increased antioxidant level could block the ROS signaling necessary for glucose stimulated insulin secretion [40]. At the end of the study (> 150 days post-transplantation) the kidney bearing the islet graft was removed to ascertain that normal blood glucose level obtained was due to the presence of the islet graft. All mice returned to the diabetic state after removal of the kidney bearing the islet graft and the immunohistochemical staining of the islet grafts showed similar immunoreactivity for insulin and glucagon in the untreated and manganese porphyrin pre-treated islets.

Previous studies have reported the beneficial effect of manganese porphyrin on human and murine islets. In our study, we demonstrated that manganese porphyrin had beneficial effect on neonatal pig islets as well. Pre-treatment of neonatal pig islets with either 34 μM or 68 μM manganese porphyrin protected them from hydrogen peroxide induced injury. However, our *in vivo* study showed that only diabetic mouse recipients of neonatal pig islets pre-treated with 34 μM manganese porphyrin achieved normoglycemia earlier and had significantly lower average blood glucose levels in comparison to diabetic mouse recipients of neonatal pig islets untreated or pre-treated with 68 μM manganese porphyrin. In addition, to the antioxidant action of manganese porphyrin it is possible that the beneficial effects we have observed could be due to the up-regulation of the gene expression of antioxidant enzymes and antiapoptotic proteins.

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CHAPTER 3

GENERAL DISCUSSION AND CONCLUSIONS

3.1 GENERAL DISCUSSION

Type 1 diabetes mellitus is a chronic progressive disease characterized by hyperglycemia which occur due to insulin deficiency [1, 2]. Poor glycemic control can lead to the development of long term complications such as heart disease, stroke, chronic kidney disease, and blindness, which reduce the quality of life of patients suffering from diabetes mellitus [3, 4]. In order to achieve and maintain glycemic control, diabetic patients need to frequently monitor their blood glucose levels and to receive multiple insulin injections. It was proven that tight glycemic control achieved through intensive insulin therapy reduces the long term complications related to diabetes, however, it was associated with increased risk of hypoglycemic episodes [5]. Frequent severe hypoglycemic episodes can lead to the development of unawareness of hypoglycemia where the diabetic patients may develop seizure or coma in the absence of the warning symptoms. In addition, hypoglycemia causes cardiac arrhythmia and reduced cognitive functions [3, 4, 6, 7]. Approximately 10 % of death in type 1 diabetic patients is attributed to hypoglycemia [8]. Type 1 diabetic patients who were not responding well to the conventional therapy, having severe hypoglycemic episodes interfering with their daily activities were improved after receiving pancreatic islet transplant [9, 10].

Pancreatic islet transplantation became an option to treat patients with type 1 diabetes mellitus following the success reported by the Edmonton group in 2000, where seven type 1 diabetic patients who suffered from severe hypoglycemic episodes received

intraportal infusion of pancreatic islets [11]. These patients became insulin independent

and were protected from the severe hypoglycemic episodes. One of the factors that lead to

this success is the transfusion of large numbers of pancreatic islets (a mean of 11,547 ±

1604 IEQ per kilogram of the recipient`s body weight). A single patient receives pancreatic

islets from more than one donor, in addition, patients may receive multiple infusions of

pancreatic islets [9, 11, 12]. Pancreatic islet transplantation is a less invasive procedure

compared to whole pancreas transplantation [13-15]. Pancreatic islet transplantation

provides remarkable glycemic control with correction of hemoglobin A_{1c} (HbA$_{1c}$), without

the risk of developing life threating hypoglycemia [9, 10]. In addition, it was reported that

pancreatic islet transplantation was superior to intensive medical therapy in slowing the

progression of diabetic nephropathy and diabetic retinopathy [16]. However, the broader

application of pancreatic islet transplantation is limited mainly due to the shortage of

human donors [10, 17]. Thus, an alternative source of insulin producing cells is needed to

be explored. Using animal tissues for transplantation which is known as

xenotransplantation could solve the shortage of human donors [18]. Several animal sources

of insulin producing cells were investigated, pigs seem to be the preferred animal source

due to several reasons [19, 20]. Pigs have large litter size, breed rapidly, have short

gestation period, and can be genetically modified. Pig insulin has been used as a treatment

for diabetic patients for many years [18, 21-23]. The controversy regarding the optimal age

of pig islet donor still exists, however, the use of neonatal pig islets seems promising [21,

24]. Neonatal pig islets are easy to isolate, maintain in culture, and are able to proliferate

and differentiate. Neonatal pig islets were able to reverse diabetes in non-human primates

within 6-8 weeks post-transplantation [21, 25, 26]. In addition, a clinical trial conducted in

New Zeeland where insulin-dependent diabetic patients received encapsulated neonatal pig islets have improved blood glucose levels. The preliminary results of 8 out of 14 patients have been reported, showing decreased exogenous insulin dose, improved blood glucose levels, reduced hemoglobin A_{1c} (HbA_{1c}), with reduced incidence of severe hypoglycemic episodes [23, 27, 28]. In addition, no PERV infection was detected in these patients [28].

Considerable number of pancreatic islets are lost during isolation and early post-transplantation period due to hypoxia [29-31]. Pancreatic islets are disconnected from their blood supply during the process of isolation and during the early transplantation period. They depend on oxygen and nutrient diffusion from the surrounding blood vessel until the formation of new blood vessels which takes up to 2 weeks [32-34]. This exposes pancreatic islets to oxidative stress where the accumulation of reactive oxygen species within the cell causes death. Pancreatic islets are more vulnerable to oxidative stress induced- damage because they have low antioxidant levels [35-37]. Previous studies investigated the use of antioxidant to improve islet viability and function [38-40]. Overexpression of antioxidant enzymes such as superoxide dismutase in INS-1 cells provided protection against oxidative damage [41]. Since the application of genetic modification in the clinics could be difficult, the exogenous administration of chemical agents would be more feasible [42]. Manganese porphyrin which is a synthetic antioxidant that mimics the action of superoxide dismutase, converts the superoxide radicals to hydrogen peroxide. It is a nontoxic, cell permeable compound with the ability to scavenge broad spectrum of reactive oxygen species [31, 43-45]. It has been demonstrated that

manganese porphyrin protected human islets viability during isolation and preserved their function following isolation [46]. Moreover, it has been reported that human pancreatic islets were protected from streptozotocin induced death when they were pre-treated with manganese porphyrin. In addition, the pre-treatment with manganese porphyrin resulted in improved function of pancreatic islets in syngeneic, allogeneic, and xenotransplantation (human pancreatic islets transplanted into diabetic mice) models [31].

In our study, we have demonstrated that manganese porphyrin is not toxic to neonatal pig islets, and it did not affect the neonatal pig islet morphology. In addition, neonatal pig islets pre-treated with manganese porphyrin were protected against hydrogen peroxide induced damage. We also found that the treatment of neonatal pig islets with manganese porphyrin resulted in changes in the levels of gene expression of antioxidant enzymes like superoxide dismutase and antiapoptotic molecules like survivin. Furthermore, diabetic immmunodeficient mouse recipient of neonatal pig islets pre-treated with 34 µM manganese porphyrin had superior function compared to recipients of untreated neonatal pig islets and neonatal pig islets pre-treated with 68 µM manganese porphyrin.

Based on our results, a number of questions still exists, such as: I) the up-regulation of the gene expression of the antioxidant enzymes such as superoxide dismutase and glutathione peroxidase, and antiapoptotic molecules such as survivin, need to be confirmed by western blot and/or immunohitochemical analysis, II) investigating whether the pre-treatment of neonatal pig islets with 34 µM manganese porphyrin enhanced the survival of the few insulin positive cells at earlier time points, through immunohistochemical analysis

of the islet grafts harvested 24 hours and 7 days post-transplantation, III) exploring if the beneficial effect found in our study could lead to reversal of diabetes using reduced number of neonatal pig islets in diabetic mice, IV) examining if the addition of manganese porphyrin during the isolation procedure would improve the islet yield, V) investigating whether the treatment of neonatal pig islets with manganese porphyrin would delay the rejection of islet xenograft in immunocompetent diabetic mice.

Finally, prior to clinical application several issues should be addressed such as the source of the donor pigs, the appropriate dose of neonatal pig islets needed, the risk of pathogen transmission, site of transplantation, patient selection criteria, and appropriate immunosuppressive medication regimen. In order to use neonatal pig islets in the clinics, donor animals should be tested routinely for specific infectious agents to establish a designated pathogen-free herds, and good manufacturing practices (GMP) should be established [22, 28].

3.2 CONCLUSION

In our study, we found that pre-treatment of neonatal pig islets with manganese porphyrin is not toxic and does not alter the pancreatic islet morphology. Manganese porphyrin provided protection to neonatal pig islets from hydrogen peroxide induced damage in comparison to untreated neonatal pig islets where the majority of pancreatic islets were destroyed. In addition, the pre-treatment of neonatal pig islets with 34 μM manganese porphyrin enhanced their function in diabetic immunodeficient mice. These mice achieved normoglycemia earlier, and had significantly lower average blood glucose levels in comparison to recipients of untreated neonatal pig islets and recipients of

neonatal pig islets pre-treated with 68 µM manganese porphyrin. The use of manganese

porphyrin prior to transplantation could be a feasible strategy to protect neonatal pig islets

from oxidative stress induced damage and improve their function after transplantation.

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