Investigating Ectopic Sites for Islet Transplantation

by

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ABSTRACT

Diabetes is a chronic metabolic condition characterized by hyperglycemia resulting from impaired glucose metabolism. Type 1 diabetes mellitus (T1DM) is a result of progressive autoimmune destruction of insulin-producing pancreatic beta (β)-cells. By far, parenteral insulin replacement is considered to be the most reliable replacement therapy to control hyperglycemia, and to delay the inevitable complications associated with diabetes. Nonetheless, insulin hardly imitates the physiological secretion pattern of endogenous insulin, and fails to control glycemic excursions in subset of diabetic patients suffering from what is known as "brittle diabetes"

Pancreatic islets transplantation represents a conceivable alternative to exogenous insulin administration. Virtually, it provides a real-time response to fluctuating blood glucose levels, replacing the lost pancreatic control on glucose metabolism. In 2000, seven patients attained long-term insulin independence after being transplanted with fresh islet allografts in the liver, using non-steroidal immunosuppressive therapy. Since then, this transplant protocol – known as the Edmonton Protocol – has been utilized by many clinical islet transplantation centers worldwide. Yet, many obstacles (such as the restricted availability of human cadaveric islets, and hostile transplantation microenvironment) have impeded its widespread clinical application.

Porcine islet xenografts offer a logical alternative tissue source for islet transplantation. Till recent time, many diabetic patients were relying on porcine insulin as a life-saving replacement therapy to control hyperglycemia. Neonatal porcine islets (NPI) possess numerous merits as an alternate clinical β-cell source, compared to their adult or

juvenile counterparts. They can be isolated and cultured easily in a large-scale setting, and they are capable of controlling hyperglycemia after proliferating within the recipient, despite being immature at the time of transplantation.

Finding a more suitable transplant site is as important as finding a virtually unlimited donor tissue source. The hepatic microenvironment contributes greatly to the acute post-transplantation graft loss. Instant blood-mediated inflammatory response leads to loss of more than half of the initial transplanted islet mass. Other sites have been investigated in order to prevent graft attrition and loss of insulin independence. An optimal ectopic site should be easily accessible with minor perioperative morbidity, can accommodate large volume of islets or engineered insulin secreting cells, and allows graft retrieval for cellular monitoring. It also should be well vascularized to enhance graft survival, glucose sensing, and insulin secretion. Most importantly, the microenvironment in that proposed site should enhance graft maturation and differentiation, with minimal host's reaction towards transplanted tissue.

This doctoral thesis provides the reader with results from multiple studies aimed to investigate two proposed ectopic transplant sites. Also, it underscores the impact of the recipient's sex on the behaviour of islet graft post-transplantation. **I hypothesize that augmented transplantation of islets subcutaneously or in the intra-abdominal fat folds can control hyperglycemia and reverse diabetes mellitus in diabetic animal models. Also, recipient's sex would have a profound influence on graft function due to difference in circulating gonadal hormones.**

This thesis is organized in a paper-based format, based on original experimental manuscripts published or under review. Initially, the reader is provided with a thorough literature review including historical background, clinical applications, current limitations, and recent attempts to circumvent those obstacles.

Next, we explore an attractive and clinically translatable extra-hepatic site for islet transplantation; we investigate the subcutaneous space and its potential to accommodate islet xenografts. Due to its poor vascularity, it is expected that islets will not survive without amending its local microenvironment. Hence, we used fibrin as a biocompatible scaffold for cell delivery and local vascular enrichment. We also demonstrated that intra-peritoneal fat folds (such as epididymal fat pads in mice) could support islet grafts. We used alginate (proven immune barrier) as another form of scaffold to deliver human islets in the fat pad. Shifting focus to the global recipient's influence on the graft, we demonstrated the effect of host's sex hormones on graft function and response to glucose-stimulated insulin secretion.

Taken together, this thesis offers clear results pertaining the capability of two different ectopic transplantation sites in supporting islet grafts, aiming to improve post-transplantation outcomes.

PREFACE

This thesis entitled **"Investigating Ectopic Sites for Islet Transplantation"** contains the original experimental work by Bassem Fayez Salama, and is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Surgery at the University of Alberta. This work aims to shed more light on the potential clinical application of two extra-hepatic sites for islet transplantation as a treatment for type 1 diabetes mellitus.

A concise version of **Chapter 1** was published as a literature review in ***Current Diabetes Reports*** (Salama BF, and Korbutt GS. ***Porcine Islet Xenografts: a Clinical Source of β-cell Grafts.*** Curr Diab Rep (2017) 17:14). This literature review provided a concise background about T1DM and conventional therapeutic trends, and focused mainly on islet xenotransplantation and exploring extra-hepatic transplantation sites. My role for this publication was manuscript designing, writing, designing and providing tables, and performing the bibliographic review. GSK performed final edits as the senior corresponding author.

Chapter 2 in this thesis is titled as "**Reversal of Hyperglycemia in Diabetic Mice after Subcutaneous Transplantation of Neonatal Porcine Islets Using Fibrin Scaffolds**". This chapter illustrates results obtained from original work of Bassem Salama, investigating the feasibility of the subcutaneous space as an alternative transplant site for islet xenotransplantation. It has been formatted as an article and being submitted to ***Transplantation*** journal (Salama BF, Seeberger KL, and Korbutt GS. ***Fibrin Supports Subcutaneous Islet Transplantation without the Need for Pre-Vascularization.*** June

2019, manuscript number: TPA-2019-0730). BFS performed NPI collection, designing transplantation method, animal transplantation, data collection and analysis, writing the manuscript, designing figures and tables, and performing the bibliographic review. KS analyzed plasma samples for insulin and/or C-peptide, and performed manuscript editing. GSK was involved with concept formation and experimental design, data analysis and interpretation, performing final edits for submission, and securing funding. He is the senior corresponding author.

Chapter 4 is titled as "**The Impact of Sex Difference on Neonatal Porcine Islet Xenografts Transplanted in Diabetic Mice**". This chapter demonstrates original experimental work of Bassem Salama underscoring the effect of recipient's sex on the behavior of porcine islets xenografts post-transplantation. It has been formatted as an article and being submitted to my Principle Investigator for final revisions, under this title: Salama BS, Polishevska K, Kelly S, Seeberger K, and Korbutt GS. ***The Impact of Sex Difference on Neonatal Porcine Islet Xenografts Transplanted in Diabetic Mice.*** BFS suggested the experimental rational, performed NPI collection and transplantation, data collection and analysis, writing the manuscript, designing figures and tables, and performing the bibliographic review. KP and SK performed glucose tolerance testing and plasma collection from naïve control mice, KS analyzed plasma samples for insulin and/or C-peptide. GSK was involved with concept formation and experimental design, data analysis and interpretation, and securing funding. He is performing final edits for submitting the article for peer-review, as the senior corresponding author.

*To my wonderful wife, **Monica**, who engulfs me with unconditional love, support, and motivation. May the Lord reward you for all your personal sacrifices and toils.*

This scientific work is dedicated to the everlasting memory of **Dr. Isaac Latif** – the father, the mentor, and the role model. Thank you for everything you taught me.

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I would like to take this opportunity to express my sincere gratitude to all individuals here at the **University of Alberta** who contributed to the success of this journey, through guidance, support, and mentorship.

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I am very obliged to the members of my supervisory committee, **Dr. Adetola Adesida** and **Dr. Peter Light**, for their support and encouragement. Throughout my graduate training, they have offered me honest advices, direction, and constructive criticism. I extend my thankfulness to **Dr. Cristina Nostro** for kindly serving as the External Examiner, and to **Dr. Jessica Yue** for being the Arm's Length Examiner, during my final dissertation. I am really honored to have you reviewing my thesis, and participating in the oral defense. I am in deep gratitude to **Dr. Tom Churchill**, former director of Graduate Education within the Department of Surgery, and to **Dr. Fred Berry**, who serves as the current director. Thank you for supporting all graduate students in the department. Thank you **Dr. Gina Rayat** for being the External Examiner for my Candidacy Exam. Your kindness and support are unforgettable.

I want to thank all my laboratory colleagues, both past and present, for their

assistance and support during my doctoral studies. I especially would like to thank **Dr. Purushothaman Kuppan** for his help and support. Puru, you are an amazing example for the true humble scientist. Huge genuine thanks **Ms. Karen Seeberger**, who has been always willing to listen, help, advise, and answer any question at any time. Karen, you are the secret ingredient for every successful experiment and project.

I would like to recognize the vast army who contributed to the success of this endeavor. I am thankful for the support and teaching efforts of the **Surgical Medical Research Institute** (SMRI). **Greg**, **Deb**, **Ryan**, and **Katie**: thanks for time and effort exerted in educating and assisting graduate and medical students. Your knowledge and technical standards are indispensable. Thanks to **Mr. James Lyon** for providing our laboratory with excellent research-grade human islets, graciously donated by individuals and families who believe in what we do on the bench. We would not be able to proceed to the bedside translation without their precious donations. Thanks to **Ms. Lynette Elder** for teaching me the foundations of tissue processing, and for providing that superior quality of work. Thanks to the **HSLAS** staff for their dedication and the utmost efforts exerted to look after the animals used throughout these studies. Thanks to the **Juvenile Diabetes Research Foundation**, for supporting me financially during my postgraduate studies, as well as the travel awards from the **Alberta Diabetes Institute** and **Department of Surgery** for providing me with the chance to showcase my research projects in national and international venues.

Again and always, I would like to express my gratitude and heartfelt thanks to parents, **Fayez** and **Nabila**, and my parents-in-law, **Farid** and **Salwa**: your prayers, support, and love are the motive behind this endeavor.

To **Monica**, the *"virtuous wife, whose worth far above rubies"*: I offer you the fruit of your patience and toils. I dedicate this to you and to our gorgeous sons, Demetrius and Maximus. I would not be able to reach that far without your kindness, patience, and encouragement.

Give thanks to the Lord, for He is good!

For His mercy endures forever.

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LIST OF ABBREVIATIONS

α-GAL	Galactose-α-1,3-galactose
β-cell	Pancreatic Beta-cell
AA	Amino acid
AAGP	Anti-aging glycopeptides
Ag	Antigen
APC	Antigen presenting cells
ArKO	Aromatase knockout
ATP	Adenosine tri-phosphate
AUC	Area under curve
BMI	Body mass index
cAMP	Cyclic adenosine mono-phosphate
DCD	Donor after circulatory arrest
DM	Diabetes mellitus
E2	17-β-estradiol
ECM	Extra-cellular matrix
EPF	Epididymal fat pad
ER	Estrogen receptor
ESC	Embryonic stem cells
GAD65	Glutamate decarboxylase 65
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GPCR	G protein coupled receptor
GSIS	Glucose-stimulated insulin secretion

GTKO	Alpha (α)-1,3-galactosyltransferase gene knockout
GWAS	Genome-wide Association Studies
HbA1c	Glycated hemoglobin
hCRPs	Human complement-regulatory proteins
hESC	Human-derived embryonic stem cells
HLA	Human leukocyte antigens
IA-2A	Insulinoma-associated protein 2
IBMIR	Instant blood-mediated inflammatory response
IEq	Islet equivalent
IF	Immune fluorescence
IHC	Immunohistochemistry
IL	Interleukin
IP	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
iPSC	Induced pluripotent stem/stromal cells
K_{ATP}	ATP-sensitive potassium channels
KC	Renal subcapsular space – Kidney capsule
kDa	Kilo Dalton
mAbs	Monoclonal antibodies
MODY	Maturity-onset diabetes of the young
MSB	Marius Scarlet Blue staining
MSCs	Mesenchymal stem cells
NDD	Neurological determination of death
NHP	Non-human primates
NPI	Neonatal porcine islets

OGTT	Oral glucose tolerance test
PAK	Pancreas after kidney transplantation
PEG	Polyethylene glycol
PERV	Porcine Endogenous Retrovirus
PTA	Pancreas transplantation alone
RGD	Arginine, glycine, and aspartic amino acids motif
SC	Subcutaneous space
SI	Stimulation index
SNPs	Single nucleotide polymorphisms
SPK	Simultaneous pancreas and kidney transplantation
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TF	Tissue factor
X-IAP	X-linked inhibitor of apoptosis
ZnT8A	Zinc transporter 8

CHAPTER 1.

GENERAL INTRODUCTION

CHAPTER 1 – ISLET TRANSPLANTATION: INVESTIGATING ALTERNATIVE DONOR(S) AND OPTIMAL IMPLANTATION SITES

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IMMUNOLOGY, TRANSPLANTATION, AND REGENERATIVE MEDICINE (L PIEMONTI AND V SORDI, SECTION EDITORS)

Porcine Islet Xenografts: a Clinical Source of ß-Cell Grafts

Bassem F. Salama[1,2] · **Gregory S. Korbutt**[1,2]

Abstract
Purpose of Review Diabetes is medical and social burden affecting millions around the world. Despite intensive therapy, insulin fails to maintain adequate glucose homeostasis and often results in episodes of hypoglycemic unawareness. Islet transplantation is a propitious replacement therapy, and incremental improvements in islet isolation and immunosuppressive drugs have made this procedure a feasible option. Shortage of donors, graft loss, and toxic immunosuppressive agents are few of many hurdles against making human allogenic islet transplantation a routine procedure.
Recent Findings Xenografts—especially pig islets—offer a logical alternative source for islets. Current preclinical studies have revealed problems such as optimal islet source, zoonosis, and immune rejection. These issues are slowing clinical application.
Summary Genetically modified pigs, encapsulation devices, and new immune-suppressive regimens can confer graft protection. In addition, extrahepatic transplant sites are showing promising results. Notwithstanding few approved clinical human trials, and available data from non-human primates, recent reports indicate that porcine islets are closer to be the promising solution to cure diabetes.

This article is part of the Topical Collection on *Immunology, Transplantation, and Regenerative Medicine*

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Introduction

Diabetes mellitus (DM) is "a group of metabolic disorders, characterized by hyperglycemia due to deficient insulin release, peripheral insulin resistance or both." The distinctive feature in type 1 diabetes (T1D) is the absolute lack of insulin secretion due to immune destruction of ß-cells. Hence, it is essentially accompanied with long-term complications, and the most prominently affected organs are the heart, kidneys, eyes, nerves, and blood vessels [1]. The International Diabetes Federation (IDF) estimated the number of adults suffering from DM in 2015 by 415 million: this number is expected to increase to 642 million patients in 2040 [2]. During 2015, it was estimated that one in 11 adults became diabetic, and half of these diabetic patients were undiagnosed and unaware of the complications related to chronic hyperglycemia. Moreover, the health expenditure on diabetes worldwide was estimated to be at least USD 673 billion and expected to spike towards USD 802 by 2040, showing that diabetes became a pandemic, and associated morbidities are not the only impacting factors; there are also socioeconomic influences that affect both patients and health care systems [3, 4]. Despite the recent advancements in exogenous insulin manufacturing and the wide array of glucose monitoring systems, unaware hypoglycemia, and glucose level excursions are still the most undesirable complications of diabetes that cannot be fully ameliorated, even with the tight insulin regimens and extensive monitoring.

A concise version of this section was published as a **Literature Review in *Current Diabetes Reports* as part of the Topical Collection on *Immunology, Transplantation, and Regenerative Medicine* 2017 Mar 1;17(3):14.** Additional topics have been included, and the expanded literature review is in this thesis reported as a general introduction to the experimental work.

ORIGINAL ARTICLE

Title: Porcine Islet Xenografts: A Clinical Source Of β-cell Grafts

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1.1 – ABSTRACT

Diabetes is a medical and social burden affecting millions globally. Type 1 diabetes mellitus (T1DM) results from autoimmune destruction of pancreatic beta (β)-cells, leading to diminished insulin secretion and sustained hyperglycemia. Despite intensive therapy, exogenous insulin administration fails to halt the progression of the neurovascular complications associated with hyperglycemia, and often results in episodes of hypoglycemic unawareness.

Islet transplantation is a propitious replacement therapy, and incremental improvements in islet isolation and immunosuppressive drugs have made this procedure a feasible option. Shortage of donors, suboptimal engraftment site, gradual graft loss, and toxic immunosuppressive agents are few of many hurdles against expanding human allogenic islet transplantation in the clinical setting. Xenografts – especially pig islets – offer a logical alternative source for islets. Genetically modified pigs, encapsulation devices, and new immune-suppressive regimens can confer graft protection against host's immune response. Yet, finding the ideal implantation site for any islet graft represents a major challenge in the clinical setting. Providing a near-native local environment at the transplantation site is essential, as it enhances the graft sustenance and permits frequent tissue monitoring.

With the available preclinical reports about xenografts or engineered insulin secreting cells and the progressing pursuit of alternative optimal implantation site, islet transplantation is closer to be well-established routine procedure to cure type 1 diabetes.

1.2 – INTRODUCTION

Diabetes mellitus (DM) is defined as a "*heterogeneous metabolic disorder characterized by the presence of hyperglycemia due to impairment of insulin secretion, defective insulin action or both".*[1] Autoimmune destruction of insulin secreting beta (β)-cells in the islets of Langerhans is a hallmark of type 1 diabetes mellitus (T1DM). In addition to loss of glycemic homeostasis, T1DM is often associated with numerous neurovascular complications related to the chronic nature of this metabolic disorder. The discovery and extraction of insulin by the young Canadian physician Frederick Banting has changed the quality of life for millions of patients around the globe.[2] Yet, insulin did not cure T1DM despite being capable of controlling hyperglycemia and slowing the progression of vascular complications. The pursuit of a definitive cure for T1DM that would restore the endogenous secretion of insulin started by the end of 18th century, even before the discovery of insulin.[3] However, contemporary methods of pancreatic islet isolation and portal vein infusion were developed based on Dr. Lacy's work starting from 1960s.[4] Since 1967, the islet transplantation field has been evolving experimentally and clinically to improve pancreatic isolation yield, pre-transplant islet survival, and post-implantation graft endurance. Unfortunately, the scarcity of donors, variability in allogeneic islet grafts function, and the progressive graft attrition that starts immediately after transplantation limits the expansion of islet transplant to be a standard clinical procedure for curing T1DM.

In this chapter, we provide a literature review about pancreas, diabetes, and available treatment options for T1DM. Next, we outline the challenges facing islet transplantation as an attractive surgical treatment for diabetic patients. Finally, we explore

the proposed strategies to enhance post-transplant graft survival and function, focusing on alternative implantation sites and xenografts as an alternative insulin-producing tissue source.

1.3 – THE PANCREAS

1.3.1 – Pancreas from historical perspective

Despite the comprehensive descriptions mentioned by Ancient Egyptians regarding sophisticated anatomical structures, they did not identify the pancreas and its crucial role in glucose homeostasis.[5] Herophilus (335 B.C. – 280 B.C.), the Chalcedonian "Father of Anatomy" was the first anatomist and surgeon to describe the pancreas as an abdominal structure, though he did not name it.[6] The name PANCREAS was not declared till the end of the first century A.D., when Rhuphos of Ephesus coined that term [Greek for *All Flesh*] in his famous work "*On the names of various parts of the human body*".[7] Due to few misbelieves, the physiological role of the pancreas was not investigated meticulously throughout more than four centuries. Herophilus, Hippocrates, Eudemous and Ruphous believed that the pancreas is a special type of a gland that might contribute to food digestion through specific type of glandular "juice" similar to saliva. Galen (Claudius Galenus, 129 – 216 A.D.) assumed that the *"pancreas might be a protective cushion for the stomach or most probably, for protecting the mesenteric vessels"*.[8] Unfortunately, that assumption was the most adopted undisputable notion about the pancreas' function till the 17th century due to the fame and prestigious position of Galen.

The German doctor Johann Georg Wirsüng (1589 – 1643) is credited for the detection of the Pancreatic Duct in 1642, during an autopsy performed for anatomical lecturing.[9] Despite not being able to identify its function, Wirsüng documented that discovery by engraving his findings on copper plates. This discovery has abolished the "protective cushion function" theory instituted by Galen, and highlighted the role of the pancreas in digestion.

1.3.2 – Anatomy and development of the pancreas

The pancreas is one of the gastrointestinal adnexa, with the liver, the gall bladder and the spleen. It is defined as a lobulated gland that rests retroperitonealy surrounded by fat in the upper portion of the abdomen (transpyloric plane).[10] The pancreas is 14-25 cm long; it is divided into head, body and tail, and weighs about 100 g on average.[10-12] The pancreas drains its exocrine secretions through the main pancreatic duct of Wirsüng into the major papilla (where it joins the common bile duct few millimetres before the ampulla of Vater), and the accessory pancreatic duct of Santorini that pierces the duodenum at the minor papilla. The arterial supply of the pancreas is derived from branches of the celiac trunk and the superior mesenteric artery, while its venous drainage is carried to the superior mesenteric vein, that later turn into the portal vein when it unites with the splenic vein.[13]

The embryological development of the pancreas starts at 4 – 6 weeks of gestation from an endodermal origin, as a large dorsal duodenal bud and a small ventral bud from the side of the gall bladder. At the 7th week of gestation, the ventral bud rotates posteriorly to fuse with the lower aspect of the dorsal bud, forming the final structure of the pancreas. The ducts of the two diverticula communicates together; the duct of the smaller bud becomes

the main (Wirsüng) duct of the pancreas, while the original duct of the larger portion turns to form the accessory (Santorini) duct.[10,14] The pancreas serves as a bi-functional gland; it secretes digestive exocrine juice into the duodenum, and endocrine hormones that maintain the glycemic homeostasis into bloodstream. For that purpose, the histological cytoarchitecture of the pancreas is composed of four functionally different components: exocrine acinar tissue, ducts, endocrine cells, and connective tissue.[13]

1.3.3 – Endocrine pancreas

The discovery of pancreatic islets is attributed to the German physician Paul Langerhans (1847 – 1888). He described the islets during microscopic examination of macerated pancreatic fragments as "*small irregular-polygonal structures with a cytoplasm perfectly shiny*".[15] Twenty-four years later, the French histologist Edouard Laguesse (1861 – 1927) confirmed that finding regarding the endocrine structures, naming them "Islets of Langerhans".[16]

Islets of Langerhans are well-vascularized endocrine cellular aggregates responsible mainly for upholding glucose homeostasis. An average human pancreas is estimated to have between 3 and 14 million islets dispersed throughout the acinar tissue, representing about 2% of its total cellular mass.[17] Microscopically, they appear as spherical structures with a thin fibrous capsule separating them from the exocrine parenchyma, and supplied by dense capillary network that provides the islets with about 15% of the total pancreatic blood supply.[18] The individual islet diameter varies significantly; it ranges between 50-400 μm, with an average of 150 μm in human islets.[19] In order to orchestrate a continuous glycemic homeostasis, islets comprise five different hormone-secreting cell types that

interact together through paracrine control, in addition to the external nutrient, neural, and hormonal stimuli. Those cells are known as α- (Glucagon-producing), β- (Insulin-producing), δ- (Somatostatin-producing), PP- (Pancreatic polypeptide-producing), and ε- (Gherlin-producing) cells.[20] This review will focus mainly on β-cells, and ailments associated with its dysfunction, i.e. Diabetes.

1.3.4 – β-cells

β-cells represent ~ 60% of the total cell count of a mature human islet.[18,21] Intra-islet percentage, distribution, and organization of β-cells vary widely between species. Rodents' islets are the most frequently studied specimen, and our common knowledge and understanding regarding the islet cytoarchitecture stems from that animal model. However, the increasing availability of human pancreatic tissue – graciously offered by human deceased donors – helped us to establish inter-species comparative studies that highlighted major differences between human and rodent islet cellular arrangements. Human islets contain less β-cell percentage (~ 70% in mice), and they do not follow the central arrangement as seen in their rodents' counterpart (a core of dense β-cell population, surrounded by a thin mantel of α-cells).[18,21-23] β-cell mass changes during development and aging in order to match the alterations in insulin requirements during physiological – such as pregnancy – or to a lesser extent the pathological metabolic changes.[24] Factors that influence the overall β-cell mass include cellular maturation, differentiation, individual size, and rate of apoptosis.[25] During pregnancy for example, the β-cell mass increases in an average of 50% of it size before pregnancy, showing both hypertrophy and hyperplasia.[26]

1.3.5 – Insulin

Insulin is a 51-Amino acid (AA) peptide with a strong hypoglycemic action, derived from biologically inactive precursor – proinsulin – that is secreted exclusively from β-cells. Proinsulin is composed 81 AA, in the form of three peptide chains (A; 21 AA, B; 30 AA, and a connecting C chain; 30 AA). Post-translational proteosomal cleavage of proinsulin in Golgi bodies results in mature insulin peptide (A and B chains), and C-peptide chain that is released with insulin in an equal molar ratio.[25] Together, insulin and C-peptide are packed in secretory granules that get translocated towards the inner surface of cell membrane. Insulin exists as a 6 KDa monomer in the peripheral circulation, and the two non-polar regions in its tertiary structure aggregates with other insulin monomers to form a hexamer crystal that binds with two zinc ions in the centre.[25]

Insulin secretion occurs mainly in response to glucose in a biphasic manner. Upon uptake by GLUT-2 transporter, β-cell responds via a rapid release of readily available insulin granules in the form of a sharp peak in circulating insulin's level known as first phase response that lasts for about five minutes. As this phase plummets, another slower (but longer) upsurge of insulin secretion follows and known as the second phase of insulin secretion.[27] GLUT-2 is a bi-directional transporter that allows uptake and metabolism of glucose in β-cell unrelated to intra/extracellular glucose concentration; this plays a key role in rapid glucose sensing and insulin release.[28] Intracellular ATP levels increase as β-cell metabolizes glucose, triggering the closure of ATP-sensitive potassium (K_{ATP}) channels. This leads to cellular depolarization, influx of calcium through voltage-gated calcium channels, ending by translocation and exocytosis of insulin granules.[29] Insulin exerts its hypoglycemic and anabolic effect through enhancing the peripheral uptake and storage of

glucose in three main stores: skeletal muscles, liver, and adipose tissue, in addition to suppressing glycogenolysis and gluconeogenesis.[30]

1.4 – DIABETES MELLITUS

1.4.1 – History of diabetes

Diabetes is one of the few observed ailments since antiquity. The earliest description of diabetes symptoms was recorded in an ancient Egyptian medical papyrus written around 1536 B.C. This papyrus is considered one of the oldest and most important medical literatures – among other rare and well-preserved scrolls – kept to our modern days. German Egyptologist George Ebers (1837 – 1898) purchased this scroll at Luxor, Egypt in 1873, and published an English/Latin translation for its introduction and vocabulary by 1875.[31,32] Five years later, Heinrich Joachim published a full German translation for the scroll in his book "*Papyrus Ebers: the oldest book on medicine*".[33] However, Paul Ghalioungui (1908 – 1987) argued that symptoms like polyuria and polydipsia stated in that document are not enough to suggest that the author was referring to diabetes.[34] Eastern physicians such as the Indian surgeon Sushruta also described the clinical picture of diabetes in his book "*Samhita*".[35] Sushruta reported symptoms and signs of the disease as "*large volume of sweet, sticky and honey-like urine (madhumeha) that attract flies and ants; persistent thirst; and the tendency for infections and boils formation*".[35] About seven centuries later, Aretaeus of Cappadocia (81 – 150 A.D.) gave a detailed description of diabetes and its associated morbidities in his famous work "*On the causes and symptoms of acute and chronic diseases*".[36] He also introduced the name DIABETES; a Greek term that translates "*to pass through a siphon*". English anatomist

Thomas Willis (1621 – 1675) referred to diabetes as *"pissing evil"*, and was credited for adding MELLITUS to diabetes – a Latin term that means "*sweet as honey*" – confirming Sushruta's observation regarding glycosuria.[37]

Since the 15th century B.C., numerous physicians and scientists described the symptoms, signs, and associated comorbidities (such as gangrene, poor eye sight, and impotence) of diabetes, but none of them established the correlation between that disorder and the pancreas. It was not exposed till the 19th century, when Oskar Minkowski (1858 – 1931) performed a total pancreatectomy on a dog to help Joseph von Mering (1849 – 1908) to investigate the importance of free fatty acids for lipid absorption.[38] At that time, von Mering drew an uncertain link between the pancreas and fat absorption but he lacked the evidence to prove his theory as he failed to ligate the pancreatic ducts successfully. When they met in 1889, Minkowski suggested that total pancreatectomy would offer an undisputable answer for his question, and he suggested performing that procedure himself if von Mering would provide him an experimental animal. After a successful surgery, Minkowski observed the signs of diabetes: polyuria, severe thirst, increased hunger, marked weight loss, and glycosuria. Minkowski also noticed that re-implantation of the excised pancreatic grafts subcutaneously improves glycosuria and the overall clinical picture of the diabetic animal, referring to a "*pancreatic product*" that aids in sugar metabolism.[38]

1.4.2 – Pathophysiology of Diabetes

Diabetes is a metabolic disorder manifested by glucose intolerance and hyperglycemia. Due to its heterogeneous etiology, diabetic patients can be classified based

on the causation of the disorder.[1] Type 1 diabetes mellitus (T1DM) refers to absolute deficiency in insulin secretion predominantly due to autoimmune β-cell destruction, while Type 2 diabetes mellitus (T2DM) describes a metabolic condition that results of a defect in insulin action on peripheral tissues. Gestational diabetes mellitus – the third major type of DM – signifies glucose intolerance that develops or manifests during pregnancy. In addition to the former major three categories, there are other less common etiologies for diabetes such as monogenic diabetes, and diabetes secondary to other medical conditions. Monogenic DM results from a single genetic mutation, causing improper glucose homeostasis. Maturity-onset diabetes of the young (MODY) is an example of monogenic mutation that leads to DM. Pancreatitis, pancreatic neoplasms, cystic fibrosis, various endocrineopathies, and drug- or chemically-induced diabetes are other conditions that result in DM as well.[39] According to the International Diabetes Federation, the number of patients diagnosed with diabetes in 2017 was estimated to be 425 million cases, about 1 in 11 adults. This number is predicted to jump to 629 million cases by 2045 – a 48% increase in 28 years.[40]

1.4.3 – T1DM

T1DM is a chronic metabolic condition manifested primarily by sustained hyperglycemia due to diminished insulin secretion from pancreatic β-cells. Mainly, it occurs as a result of immunological destruction of β-cells orchestrated predominantly by T lymphocytes. However, there is a small percentage of T1DM cases (<10%) which have no documented evidence of immunological causes, and known as idiopathic T1DM or type 1-b diabetes.[41] Globally, the number of T1DM cases are estimated to be about 10% of the total population of diabetic patient.[40] The process of physiological immune destruction of β-cell

is now more comprehensible based on the immunohistological analysis performed on samples obtained from human pancreases, in addition to experimental animal models. Exposure of naïve T lymphocytes to β-cell-derived self-antigens carried by antigen presenting cells (APC; mainly pancreatic dendritic cells) primes and activates T cells. The leukocytic invasion of pancreatic islets – known as insulitis – recruits other inflammatory and phagocytic cells to the islets, exacerbating the faulty immune response and causing more injury to the insulin producing cells. As β-cell death progresses (resulting from direct necrosis, and indirect T cell-induced apoptosis), the secreted insulin becomes more reduced and insufficient for proper glycemic control.

At the time of clinical manifestation, ~80% of the pancreatic β-cell mass is abolished already, and the immunological progress cannot be halted to prevent further attrition.[42,43] Despite the thorough mapping of cellular events that end by β-cell death, the triggering culprit that initiates insulitis and cause the hostile shift of T lymphocytes or other immune cells towards insulin-producing pancreatic β-cells is not fully elucidated yet.[43,44] The main two conceivable theories explaining T1DM etiology attribute genetic predisposition, and environmental exposures to the immunological hostile shift against self-antigens.

1.4.3.1 – Genetics

Recent advances in chromosomal mapping helped in linking genetic alteration and incidences of multiple diseases.[45,46] So far, Genome-wide Association Studies (GWAS) have identified numerous high-risk genes and/or loci associated with diabetes, and their colocalization is believed to increase the risk of T1DM occurrence, by affecting the proper immune response and/or function and integrity of β-cells.[47]

1.4.3.1.1 – Human Histocompatibility complex

Human leukocyte antigens (HLA) are cell-surface markers encoded by their genes – known as Human Histocompatibility complex – located on the short arm of chromosome 6 (6p). This complex is divided into three major areas: Class I, II, and III. Genes in this complex are responsible for antigen (Ag) presentation on professional APC, and regulating proper immune response. HLA Class I include A, B and C subtypes, while Class II includes DP, DQ, and DR sub types. Among those, eight regions/genes are highly polymorphic and believed to play a significant role in immune response that leads to T1DM. Those are HLA-A, HLA-B and HLA-C from class I loci, and HLA-DPA1, HLA-DPB1, DQA1, HLA-DQB1 and HLA-DRB1 on class II loci. Polymorphic class II genes represent the strongest genetic association to the pathology with an approximate 20 fold increased susceptibility for T1DM in individuals with the HLA-DR3-DQ2/DR4-DQ8 polymorphic genotype, compared to the general population.[48]

Polymorphism or variability in AA residues in those genes might cause structural and morphological changes in Ag-binding sites on APC, leading to improper/ inefficient Ag binding affinity, presentation, and activation of various subsets of T lymphocytes. Several islet antigens are believed to provoke the autoimmune response associated with T1DM, such as insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma-associated protein 2 (IA-2A), and zinc transporter 8 (ZnT8A). Those self-antigens stimulate the formation autoantibodies by B lymphocytes, which can be used as preclinical markers for the risk of developing T1DM, and testing for these markers at the time of diagnosis is now considered as a standard practice.[49,50]

1.4.3.1.2 – Polymorphic Non-HLA Loci

Numerous studies have proposed multiple non-HLA polymorphic loci that contribute to the genetic predisposition of T1DM. To date, there are more than 50 non-HLA candidates identified using GWAS genotyping performed of diabetic patients.[51-53] Although they have small contribution risk to the disease when they exist individually, those single nucleotide polymorphisms (SNPs) colocalizations increase the risk of T1DM development significantly. Despite the increasing list of non-HLA candidate genetic polymorphism that might contribute to T1DM pathogenesis, they represent marginal risk compared to HLA-DR/DQ genes.[53]

1.4.3.2 – Environment

There are strong evidence correlating environmental factors to the pathogenesis of T1DM; the rising incidence, and the seasonal pattern of the newly diagnosed cases of T1DM confirm – yet unexplain – that relationship.[42,48] It is challenging to identify or exclude key factors of the environmental hypothesis, probably due to the heterogeneous environmental components and exposures. Nonetheless, infections and dietary factors are the most prominent suspected culprits in the environmental theory. Infection seems to trigger islet autoimmunity via several mechanisms; since both islets and exocrine tissue drain via same lymph nodes, it is conceivable that any viral infection in exocrine tissue would trigger immune response against Islets.[54,55] Molecular mimicry is another hypothized mechanism relating infection to the development of T1DM; antigens of infectious (non-self) pathogen might share similar/near-identical epitopes with islets self-antigens, resulting in non-discriminating immune destruction for both.[56,57]

Infant dietary habits (such as the period of exclusive breastfeeding and/or early administration of certain food elements like cow's milk and/or gluten-containing food) is another focal point in T1DM research. Some studies claim that infants who received short period of breastfeeding (< 3 months) are more prone to develop T1DM later in their lives, compared to other infants who were breastfed exclusively for longer periods. It was also noted that early administration of cow's milk (before the age of 4 months) increases the incidence of developing T1DM.[58-60]

1.5 – TREATMENT OPTIONS OF T1DM

Re-establishing the diminished physiologic insulin profile is the paramount therapeutic goal of any successful diabetes cure. This restores the glycemic homeostasis as a short-term target, and prevents the deleterious long-term neurovascular complications resulting from sustained uncontrolled hyperglycaemia. Treatment options for T1DM can be broadly classified into parenteral hormonal administration, whole organ transplantation, or cellular replacement therapy.

1.5.1 – Parenteral insulin administration

The association between diabetes and pancreas established by von Merring and Minkowsky prompted scientists to pinpoint the process that controls glucose homeostasis. From feeding pancreas to diabetic patients[61,62] to injecting animals with aqueous pancreatic extracts,[63] investigators tried to decipher the crucial role of pancreas in carbohydrate metabolism. The ineffectiveness of those attempts was attributed to conceivable damage of the "glucose-lowering agent" by other proteolytic enzymes of the pancreas. In early 1920s,

Moses Barron (1884 – 1974) published a report demonstrating the possible relation between the non-acinar tissue of the pancreas (islets of Langerhans) and diabetes, as he found intact islets with normal glycaemic levels in a patient with atrophied pancreas due to pancreatic duct obstruction.[64] Building on former scientific observations, Frederick Banting and Charles Best were able to obtain a crude unpurified aqueous "internal extract" from a dog's pancreas after deliberate pancreatic duct ligation for ten weeks. Injecting completely diabetic dogs with this internal extract led to prolongation of the animals' lives, and improvement of symptoms and signs of diabetes. Utilizing the expertise of James Collip in purification of fetal calf pancreatic extracts, the Toronto investigators were able to treat the first human T1D patient in Toronto General Hospital using insulin, in January 1922.[65]

Parenteral insulin administration aims to replace both basal and stimulated endogenous insulin secretion. Till the 1980's, all the available commercial insulin injections were extracted and purified from bovine or porcine pancreases. Unfortunately, that was associated with numerous allergic and immunological disadvantages related to xeno-proteins, which affected the pharmacological efficacy of the hormone.[66] The advent of DNA sequencing and protein recombination techniques enabled scientist to synthesize human recombinant insulin, hence replacing the xenogeneic insulin source with human compatible protein. The modern commonly used insulin preparations are represented by three categories: unmodified recombinant human insulin, insulin analogues, and premixed preparations.[67] Each group is classified based on their action's onset, peak, and duration as shown in **Table 1.1**.[66] As pointed out earlier, insulin monomers tend to form hexamers and crystalline in the presence of zinc or other basic proteins. When injected subcutaneously, those hexamers cannot act on the insulin receptors immediately; instead, they require

longer time for breaking down back into monomers, which delays the absorption and extend their action. Exploiting this fact, physicians prescribe crystalline long-acting insulin as a replacement for basal endogenous insulin, while rapid- or short-acting preparations are given after meals to replace the stimulated insulin secretions.[66,67]

Exogenous insulin replacement occurs mainly through subcutaneous injections, and tight glycemic control requires multiple daily injections. Therefore, it is conceivable that patient non-compliance is the primary reason for ineffective control, due to fear of needles and/or pain. In order to avoid human errors in dose calculation, and multiple needles pricks, devices that provide continuous subcutaneous insulin infusion present an alternative hormone delivery modality. Insulin pumps deliver multiple and variable daily doses of rapid-acting insulin through a subcutaneous cannula attached to programmable pump and insulin reservoir. Patients can adjust the doses based on glycemia measurements and carbohydrate intake.[68,69] Multiple studies have displayed the effectiveness of insulin pumps in lowering HbA1c levels, and improving the quality of life for patients.[70] To ensure more accurate insulin replacement based on glycemic requirements, closed-loop insulin delivery systems (also known as "Artificial Pancreas") infuse insulin subcutaneously based on continuous glucose monitoring (CGM) obtained by the pump itself.[71] The artificial pancreas minimizes the human input to the minimum; it utilizes real-time glucose monitoring to provide exact insulin units required for maintaining euglycemia through intricate algorithms.[72] Nonetheless, parenteral insulin injections fail to replace the precise physiological role of β-cells, and hypoglycaemia is not uncommon complication associated with insulin therapy.

Type of insulin	Action (hours)		
	Onset	Peak	Duration
Rapid acting	0.2 - 0.5	0.5 - 2	3 - 4
Short acting	0.5 - 1	2 - 4	6 - 8
Intermediate acting	1 - 4	4 - 10	16 - 20
Long acting	1 - 3	No peak	24

Table 1.1 Common types of parenteral injectable insulin used for treatment of T1DM

1.5.2 – β-cell replacement therapy

A potentially effective alternative to daily insulin injections in T1DM is to transplant insulin-producing tissue to reestablish the natural physiological coordination for glucose homeostasis. Ideally, this will confer endogenous insulin production that resembles the pancreas's response to glucose excursions, eventually leading to long-term insulin independence; normalization of HbA1c levels; prevention of hypoglycemic episodes; and might even reverse the metabolic and neurovascular complications of diabetes.

1.5.2.1 – Pancreas Transplantation

Orloff carried out the earlier trials of whole or partial pancreas transplantation (often with simultaneous kidney transplants);[73] he also outlined at least two major conditions where T1DM patients might benefit from a pancreas transplant. The first group includes patients who need to receive a kidney transplant due to end-stage renal disease, and in this case, a pancreas transplant was considered to be a proposed method to prevent the

inevitable deterioration of the new kidney. Secondly – and far more common – are patients with long standing T1DM, and exhibiting chronic diabetic complications.[73] Pancreas is usually transplanted simultaneously with kidney (SPK), after previous kidney transplantation (PAK), and can be transplanted alone (PTA) in preuremic patients (patients who did not manifest renal failure due to diabetes-induced nephropathy).

In 1966, William Kelly and Richard Lillehei performed the first whole pancreas transplant simultaneously with kidney transplant at the University of Minnesota. The recipient remained insulin-independent for six days, after that the patient had to receive exogenous insulin due to the massive dose of steroids prescribed to prevent graft rejection. Later, that patient developed more postoperative complications that led to removal of both grafts, and eventually died from pulmonary embolism.[74] By the late 1970s, surgeons started to examine the feasibility of segmental pancreas transplantation to mitigate the immune reaction towards the graft. The longest duration where the recipient remained insulin-independent was 18 years.[75] Advantages of whole organ transplantation are numerous: rapid vascular reperfusion, preserving the organ native microenvironment, and using a single donor tissue per recipient. Nonetheless, the massive perioperative burden – such as sepsis, thrombosis, and anastomosis leakage – in addition to complications associated with pancreatic ductal drainage[76-78] render whole organ transplantation a least favourable replacement therapy.

1.5.2.2 – Transplantation of purified pancreatic islets

In contrast to whole pancreas replacement, islet transplantation is a minimally invasive procedure that ameliorates the disease similarly (by eliminating the need for exogenous daily insulin injections) without the risk of major surgical procedures.

Minimizing the host's inflammatory response towards the graft (through removal of non-endocrine tissue components) is an additional privilege of purified islet transplantation. Practical islet isolation witnessed drastic improvements from the 1960s till the beginning of 2000s. In 1967, Paul Lacy established the practice of intraductal injection of collagenase prior to pancreas procurement and digestion;[4] that improved the yield of rodents' islets compared to digestion-after-chopping method used earlier.[79] Later, further enhancement of islet isolation occurred by developing the concept of density gradient purification using Ficoll[80,81] to separate islets from exocrine tissue fraction. The isolation and purification techniques kept improving to serve animal studies, till 1989 when Lacy and his team performed the first islet transplantation trial in a diabetic patient, where they transplanted human islets into the liver via the portal vein.[82] Despite the short-lived insulin independence period, the success of that trial and other trials followed[83] provided the proof of concept to pursuit islet transplantation as a conceivable therapeutic option for T1DM.

From 1987 to 1998, 300 patients with T1DM received human islet transplants.[84] The International Islet Transplant Registry reported that 40% of the islet grafts lost their function within weeks of the transplant, and only 8% of patients remained off insulin for 1 year.[84] Yet, reports from some centers, including Edmonton, demonstrated a long-term insulin-independence by a small percentage of islet recipients.[85-87] As reported in 2000, seven islet transplant recipients in Edmonton attained insulin independence by receiving freshly isolated islets from multiple donors, and steroid-free anti-rejection therapy - a procedure known as the Edmonton Protocol.[88-90] This protocol set the standard worldwide, and now many other groups have attained similar success.[91-93] Current data are showing an increase in median insulin independence periods, associated with massive reduction of

hypoglycemic episodes,[94,95] and recent reports show that five-years insulin independence rates achieved by islet cell infusions are comparable to rates obtained by solid organ transplantation.[76] Despite the incremental improvements in human islet isolation and transplantation, the widespread clinical application as a standard long-term cure for T1DM is still hindered. Chronic need for immune suppression, donor shortage, post-transplant graft attrition, and associated procedural risks are still unresolved obstacles that need to be addressed in order to improve the graft survival and extend the insulin independence period.

1.6 – CHALLENGES AGAINST ISLET TRANSPLANTATION

Islet transplantation represents an exciting treatment option for T1DM, especially for a subset of patients where hyperglycemia cannot be controlled by conventional strict insulin therapy, or preuremic patients with rapidly progressing complications. As concluded from current reports of clinical allotransplantation, the key for any successful transplant can be concluded in three main goals:

1. Finding an unrestricted donor tissue source, with similar physiological characteristics
2. Achieving better immune protection and/or tolerance for donor tissue
3. Finding the optimal islet graft implantation site

In current transplant studies, researchers are trying to fulfill this triad to overcome the multiple challenges against expansion of this therapeutic option, in order to be a routine

procedure utilized for more patients. In the coming section we will discuss the common challenges, and proposed strategies to overcome them.

1.6.1 – Donor scarcity and clinical application of porcine islet xenografts

Islet mass required to reverse diabetes in a patient is calculated according to his/her body weight. On average, a recipient will require pooled islets from at least two donors, and it is not uncommon that a single recipient might demand more than one islet infusion procedure. Since islet transplantation relies on obtaining the tissue from deceased donors, finding a consistent, virtually unlimited, and reliable source for tissue requires different donor rather than human cadavers. The World Health Organization (WHO) defines xenotransplantation as "*any procedure that involves transplantation, implantation or infusion into a human recipient of (i) live cells, tissue or whole organs from non-human source, or (ii) human body fluids, cells, tissues or organs that had ex vivo contact with live non-human animal cells, tissues or organs*".[96] Xenotransplantation has been practiced since the 17th century, and Cooper[97] referred in his report to clinical attempts where animal cells/tissues were used in treatment of human patients. While some demonstrated unexpected success, most of those trials had failed. For more than 90 years, porcine insulin has been used as a routine replacement therapy for patients with diabetes; with only one different amino acid residue from the human counterpart, porcine insulin was considered the optimal exogenous replacement therapy for diabetes. Novo Nordisk introduced the unpurified porcine insulin in 1920s, and was able to produce it glucagon-free by 1950s. At the late 1970s, the "very pure insulin" was introduced to clinical practice, diminishing the problems of lipoatrophy/hypertrophy.[98] In addition to insulin; porcine biological heart valves have been used for heart valve replacements.[99]

There is a strong rationale to pursue the use of porcine donors for clinical islet xenotransplantation, including: 1) unlimited availability of porcine islets, which increases the access to islet transplants and eliminating waiting time, 2) the reproducibility and quality of preparing porcine islets, predictably high and not compromised by comorbidity, brain death and ischemia related to human islets, 3) porcine insulin has been used to treat human diabetes for more than 60 years, 4) porcine islets respond to glucose in the same physiological range as do human islets, 5) new techniques allow genetic manipulation and cloning of pigs, if it proves necessary or advantageous to do so, and 6) porcine islets are a potential therapy for highly allosensitized patients.[100] Thus, the risk-benefit ratio of porcine islet grafts make them a major therapeutic option to the currently used human islet grafts. Reproducible isolation of large numbers of islets from adult pigs has been challenging, since adult porcine islets are fragile and difficult to maintain in culture. In recent years however, there have been some improvements in the methodology and reagents used to isolate adult pig islets.[101-106] Yet, the potential disadvantages of adult porcine islets such as inefficiencies and variability of the isolation process, and the practical considerations of maintaining large adult herds make them less desirable transplant donors. Because of those difficulties, many researchers have focused on developing a translational strategy to use neonatal porcine islets (NPI) instead, to treat patients with T1DM.

In 1996, Korbutt and his group reported a simple, inexpensive and reproducible method to isolate large numbers of neonatal porcine islets.[107] These islets are comprised of differentiated endocrine and endocrine precursor cells that both *in vitro* and *in vivo* have the potential for proliferation and differentiation and have been shown to reverse hyperglycemia in immune deficient mice,[107] allogeneic outbred pigs,[108] and in non-human

primates (NHP).[109,110] Furthermore, NPI possess numerous advantages over their adult counterparts, as they exhibit resistance to hypoxia,[111] human pro-inflammatory cytokines,[112] hyperglycemia,[113] and islet amyloid deposition,[114] as well as their inherent ability to differentiate and proliferate[107] and achieve transplant tolerance induction in diabetic mice.[115] Taken together, these observations clearly indicate that neonatal porcine islets are a promising tissue source for clinical islet xenotransplantation.

In 1995, a Swedish group led by Groth and colleagues transplanted ten patients with T1D with fetal porcine islets.[116,117] Although no patient became insulin independent, serum porcine C-peptide was detected[116] as well as surviving islet cells within graft biopsies.[117] Moreover, subsequent follow-up of these patients demonstrated no infection of porcine viruses as a test of the safety of this procedure.[118,119] Living Cell Technologies initiated limited clinical trials conducted by Elliot and colleagues for the transplantation of NPI in patients with T1DM (http://www.lctglobalcom). To date, this group has transplanted 14 non-immunosuppressed T1DM patients with microencapsulated neonatal porcine islets and was able to alleviate hypoglycemic unawareness in these patients.[120,121] In addition to this metabolic improvement, none of the recipients exhibited any evidence of infection with porcine viruses, thereby further demonstrating the safety of this procedure.

Taken together, these studies provide evidence for the clinical feasibility of neonatal porcine islet transplantation. Despite the strong rational to pursuit porcine islets as an alternative clinical candidate for islet transplantation, some concerns (such as donors' age, host reaction towards xeno-proteins, and possible zoonosis) have to be extensively studied before standardizing the clinical use of porcine islets.

1.6.1.1 – Choosing appropriate porcine donor age

The age of the donor pig is one of the debatable factors when it comes to choose what islets should be used in xenotransplantation. As denoted previously, many research groups favor adult pigs as they can yield high number of adult porcine islets (up to 800,000 islet equivalents per isolation from a single pig's pancreas).[122] These islets are mature, and expected to correct diabetes immediately, or within few days, in the recipient.[123] Nonetheless, difficulties in isolation and fragility of the islets during culture make them challenging to use. On the other hand, neonatal porcine islets are resistant to hypoxia, hyperglycemia and pro-inflammatory cytokines, and with a reproducible and simple protocol for isolation, relatively low cost of herd housing, and feasibility of raising in a designated pathogen-free facility, neonatal porcine islets have many benefits over adult porcine islets.[107] When implanted in mice, it is noted that neonatal porcine islets require at least 6-8 weeks to correct diabetes,[107] however when implanted in allogeneic pigs[108] or NHP[109] neonatal porcine islets can correct diabetes with 2 to 3 weeks. This difference in the time to correct diabetes is likely related to the poor efficacy of porcine insulin in mice.[124]

Some studies suggest the benefits of fetal porcine islets, such as the incomplete formation of contaminating exocrine tissue, and resistance to hypoxic/ischemic injury makes their isolation easy.[125] However, they pose the same shortcoming of delayed function due to their immaturity. Also, their expression of Galactose-α-1,3-Galactose (α-Gal) is extremely high, which makes them a target for imminent rejection. Finally, recovery of fetal porcine islets is very low relative to neonatal or adult pancreas, a single transplantation will require more than one fetus, and the compulsory sacrifice of the sow makes this approach more expensive.[125] Another potential islet source are juvenile pigs (8-

10 weeks old); their housing logistics are easier than adult pigs, and they are capable of reversing diabetes in preclinical animal models.[126] However, *in vitro* secretory stimulation assays revealed that there are no added significant benefits when compared to the adult porcine islets.[127] **Table 1.2** demonstrates briefly some of the pig-to-primate preclinical experiments relevant to adult vs. neonatal porcine islets.

1.6.1.2 – Galactose-α-1,3-Galactose (α-Gal) and the role of genetic modification

α-Gal is a carbohydrate present on the cell membrane of most of living cells, with the exception of primates, including humans. Exposure to α-Gal typically occurs at an early stage of life, via gut flora, resulting in formation of humoral immunity and reactive xenoantibodies.

	Year – Group	Transplant site	Immune protection	Graft survival (Days)
Adult:				
	1996 – Sun (128)	Intraperitoneal	Microencapsulation	~ 800
	2006 – Hering (104)	Intraportal	Pharmacological	~ 180
	2006 – Dufrane (129)	Subcapsular space	Microencapsulation	~ 180
	2007 – Cardona (130)	Intraportal	Pharmacological	< 345
	2009 – van der Windt (131)	Intraportal	Pharmacological + GTKO	< 400
	2010 – Dufrane (132)	Subcutaneous	Macroencapsulating device	~ 180
	2014 – Veriter (133)	Subcutaneous	Coencapsulation with MSC	~ 210
	2014 – Bottino (134)	Intraportal	Pharmacological + GTKO	Up to 365
	2015 – Shin (106)	Intraportal	Pharmacological	150 – 600
Neonatal:				
	2005 – Elliot (135)	Intraperitoneal	Microencapsulation	~ 250
	2006 – Cardona (109)	Intraportal	Pharmacological	140 – 260
	2011 – Thompson (110)	Intraportal	Pharmacological	90 – 340
	2011 – Thompson (136)	Intraportal	Pharmacological + GTKO	~ 250
	2012 – Thompson (137)	Intraportal	Pharmacological	< 100
	2014 – Hawthorne (138)	Intraportal	Pharmacological	~ 200

(MSC): Mesenchymal Stem cells, (GTKO): α-galactosyltransferase knockout, (~): An average

Table 1.2. Relevant Pig-to-Primate preclinical experiments using porcine islets obtained from adult and/or neonatal pigs

α-Gal is highly expressed on porcine endothelial cells, and the occurrence of hyperacute or acute immune rejection is the eventual outcome in pig-to-primate whole organ xenotransplants. It has been demonstrated that α-Gal expression is mostly present in immature fetal and neonatal porcine islets, and its expression is lower in more mature and adult porcine islets; moreover its expression is not restricted to non-endocrine cells and can thereby be present on islet endocrine cells.[139-141] A strategy to overcome this impediment is the generation of α-1,3-galactosyltransferase gene knockout (GTKO) pigs produced by targeted gene modification technology[142] that has been applied to neonatal porcine islets implanted in non-human primates.[136] This allows the production of α-Gal-free donor pigs, preventing this antigen from being targeted by the host's immune system. Although decreasing the load of xenoantigens by using islets obtained from GTKO pigs has shown better graft survival than wild type islets, it failed to provide long-term protection against host response.[138] Additional gene manipulation and targeting using GTKO background pigs can be adventitious, if combined with expression of human complement-regulatory proteins (hCRPs). Transgenic pigs have been generated to produce islets that express hCD46 a complement-regulatory protein and have demonstrated function when implanted into non-human primates.[131]

1.6.1.3 – Zoonosis and cross-species contagion

A considerable limitation to bring porcine xenografts to the clinic is cross-species contamination and introduction to additional morbidities to the recipients. WHO has published a consultation report, listing the possible pathogens that might pose risks to human recipients.[143] Porcine Endogenous Retrovirus (PERV) is by far the most concerning pathogen in xenotransplantation, as it is present in all porcine cells. PERV is an

endogenous viral element that reversely transcripts its RNA into DNA sequence and embeds it into the host genome. There are three classes of PERV: A, B and C. Both classes A and B are polytropic – they can infect and replicate in non-porcine cells – while class C possesses an ecotropic characters with narrow infectious spectrum.[144] Although multiple studies have been published regarding the capability and positivity of *in vitro* infection of human cell lines by PERV,[145,146] evidence and conclusions drawn from the few clinical trials and preclinical studies contradict these *in vitro* findings.[116,147-149] Cheng[150] discussed the effect of Canadian and Australian public's opinions regarding their socio-ethical point of view towards xenotransplantation, and expanding the clinical use of animal cells and organs. Unfortunately, the outcome resulted in a ban in Australia on clinical xenotransplantation.

Nonetheless, development of designated-pathogen free facilities for raising herds is feasible, and can control the introduction of unwanted pathogens to the "sterile" animals. In addition, the widespread use of Good Manufacturing Practices techniques, facilities, and protocols guarantee the uniform and reproducible quality control required for production of cell products for human use. WHO recommended as well some guidelines for donor/recipient screening, and stressed on the benefits of the communication between centers to exchange information as good tools to examine cross-species contamination. Finally, follow-up for patients and preclinical experimental subjects is a paramount, and all together, are encouraging steps towards more clinical trials of xenotransplantation.[151]

1.6.2 – Protection against host's reaction

Even with the significant improvement of procurement techniques, long-term

survival of islet graft inside the host is greatly unpredictable. Cellular stress occurring during digestion, purification, and culture are few of many factors that affect sustenance of live tissue in vivo that cannot be simulated on the bench. Collectively, those factors can lead to acute loss of major portion of transplanted islet mass, or even primary non-function of the graft. Extensive efforts are made to minimize the cellular stress during isolation and transplantation, such as preconditioning of the islets, or adding agents that prevent stress induced apoptosis. Moreover, the inflammatory/immune response of the host remains a major hurdle against graft sustenance over time, even with the state of art immunosuppressive agents. Below we will demonstrate few of the issues that impede the long-term graft survival and function in the host.

1.6.2.1 – Instant blood-mediated inflammatory response

Instant blood-mediated inflammatory response (IBMIR) describes the nonspecific and nonimmune-mediated inflammatory response that results in islet destruction when transplanted directly in the blood stream. It is a significant obstacle in human islet allotransplants, as the islets are infused intravascular to settle into the hepatic portal tree through the portal vein.[152] Tissue factor (TF) expressed by isolated islets is believed to be the main trigger for IBMIR, as it stimulates clotting cascades, platelet aggregation, and complement activation. A recent report proposes that xenogeneic-induced IBMIR is platelet-independent, and involves multiple simultaneous mechanisms and activation pathways, leading to eventual leukocyte/macrophage infiltration, and the ominous fate of inevitable graft loss.[153,154] Despite the possibility of experimental control of complement activation via Cobra venom factor, or attenuating the platelet aggregation and coagulation by anti-platelet agents and low molecular weight heparins respectively, these *in vitro*

strategies are not proven safe to be used clinically, and other methods should be investigated to make sure that it is clinically applicable.[155,156]

1.6.2.2 – Pharmacological immunosuppression and the benefits of modern biological agents

Earlier, corticosteroids were considered the backbone of any chemical immunosuppressive regimen due to their superior capabilities to inhibit the immune system. However, that came on the cost of other deleterious global side effects, especially when used chronically in multi-morbidity patients. Newer agents and regimens used in immunosuppression exclude corticosteroids, enabling more survival of islet allografts in recipients. Nonetheless, they are still not patient-friendly agents that pose numerous undesired adverse side effects. Ironically, an effective new agent prescribed routinely (the calcineurin inhibitor tacrolimus) is known to be nephrotoxic, diabetogenic, and may adversely affect islet vascularization post-transplantation.[157]

The concept of adding protective agents in tissue culture medium and the preconditioning of the islets prior to transplantation is suggested to protect the islets from stress-induced apoptosis and tacrolimus-related toxicity. A study published recently by Gala-Lopez reports that using anti-freeze proteins analogs, also known as Anti-Aging Glycopeptides (AAGP) enhances the survival of engrafted islets. AAGP reduced oxidative stress and Interleukin (IL) -1β and -6 expressions, lowered apoptosis, and enhanced insulin secretion in both human and murine islets.[158] using unique and specific monoclonal antibodies (mAbs) is an alternative method of selective immune suppression; it employs the concept of “selective targeting” of receptors and/or ligands involved in the process of graft immune rejection. Examples of this method were reported in 2006: in two separate studies,

Cardona,[109] and Hering[94] used specific CD154/CD40L mAbs, to suppress activated T cells in NHP transplanted with islet xenografts, derived from adult and neonatal pigs respectively. Although its efficacy, there are reports about the thrombotic effects of CD154 mAb, that precludes its usage in the clinic. Adopting this concept, more selective agents that target specific pathways with more safety outcomes are being explored.

1.6.2.3 – Immune isolation and physical graft protection

Immune isolation or containment of individual/few islets by microencapsulation, or numerous islets by macroencapsulation in a polymer or a chamber is considered as a valuable method of immune isolation. An ideal isolation chamber would allow exchange of nutrients and oxygen between graft tissue and surrounding interstitial space, and simultaneously protects against direct exposure to immune/inflammatory cells of the host. Early studies of islet macroencapsulation where islets were placed in hollow synthetic capillaries were reported in mid 1970s. These capillaries were later connected to vascular system of diabetic rats, and blood flowed inside the capillaries allowing oxygen, nutrients, wastes, and cell products to be exchanged across the capillary walls. However, over time the capillaries became occluded due to thrombosis, and the islet grafts subsequently failed.[159] Over the past 20 years, extensive research has been done to develop better macroencapsulation devices without having adverse side effects on the islet grafts, and many prototypes have been investigated such as vascularized bioartificial pancreas devices or diffusion chambers. TheraCyte™ is an example of a diffusion chamber that possesses the advantage of having a double layer of polyester-Biopore membrane that allows it to be implanted subcutaneously thereby permitting diffusion from the neovascularization occurring at the surface. TheraCyte™ devices have been shown to reverse diabetes in

rodents and prolong protect islet allograft rejection.[160,161]

The concept of islet microencapsulation started in 1964 by Thomas Chang[162] when he proposed the theory of an "artificial cell"; he hypothesized that microencapsulation would not only protect cells against immune rejection, but also increase the exchange surface area between the encapsulated cells and surrounding environment, thus enhancing the exchange process.[162] The usage of alginate as a common biopolymer in islet microencapsulation was reported in 1980,[163] where encapsulated islet xenografts were capable of survival and controlling elevated blood glucose in diabetic rats. Since then, multiple reports regarding usage of alginate-encapsulated islet xenografts have been published,[164-168] demonstrating the proof of concept. Purity of alginate, cross-linking molecules, surface coatings, and transplantation site are few of many factors that are involved in success and survival of microencapsulated islets. Although the concept of microencapsulation relies on physical separation between donor tissue and host immune system, it does not provide a complete protection against immune-mediated graft loss. Cellular breakdown inside the containment chamber or microcapsule promotes homing of host's inflammatory cells to aggregate at transplant site, releasing more cytokines and propagating more inflammatory response towards the graft. Fibrous overgrowth refers to the accumulation of inflammatory/immune cells on the surface of capsule/device; it is known to abolish adequate diffusion of oxygen/nutrient between graft tissue and surrounding environment.[169-172] Recently, a group in Massachusetts, USA, published a report about size and shape of microcapsules influence the foreign body immune response in rodents and NHP.[173] Against what was expected, larger diameter islet-containing alginate capsules showed significantly lesser fibrous overgrowth and foreign body immune reaction,

compared to their smaller counterparts. This enforces the need to perform similar trials using human patients to investigate this paradox, and to see whether the capsules will show the same results or not.

Conformal coating is another technique intended for individual immune isolation of islets or other cellular aggregates; it relies on the concept of interfacial or step-growth polymerization, versus droplet capsule formation used in alginate microencapsulation. Layers of biopolymer are deposited over the surface of islets through crosslinking, providing a uniform coating layer. This will minimize the polymer thickness allowing less tissue volume during transplantation, and less incidence of graft core hypoxia.[174] Early studies[175,176] used PEG as a biopolymer for conformal coating, and the first pig-to-rodent xenotransplantation demonstrated successful graft survival in immune competent host.[177] Continuous incremental improvements are sought to find more biocompatible coating hydrogel to enhance the immune protection, and minimize the host's inflammatory response.[178,179]

1.6.3 – Islet engraftment site and effect of local microenvironment on graft survival

Success of any cellular/tissue replacement therapy depends heavily of the transplantation site and its local microenvironment. Within the pancreatic parenchyma, islets of Langerhans are nurtured via dense network of capillaries that supply them with roughly 15% of total blood supply delivered to the pancreas. This ensures that enough oxygen and nutrients are supplied to islets so they can perform their crucial metabolic role. The process of procurement and isolation pancreatic islets severs vascularization and damages the native stromal architecture of the donor tissue. Loss of cell-to-cell contact or

attachments to basement membrane promotes dedifferentiation, apoptosis due to cellular stress signals, and progressive graft attrition. Moreover, islets depend mainly on oxygen diffusion from the surrounding implantation site during the early post-transplantation period; depending on the location, new microcirculation starts to develop within the first few days, and might require a longer period in order to get well established.[180] Strategies to confer a near native transplant site include finding an alternative optimal implantation location, enhancing the local microenvironment to be more suitable for donor tissue, or using biological/biocompatible scaffolds for cell delivery.

1.6.3.1 – Portal vein and other transplant sites

Portal vein is the standard engraftment site in clinical islet transplantation. Under radiological guidance, portal vein is accessed percutaneously and islets suspension is infused using gravity flow to embolize into the terminal sinusoids of the hepatic portal venous tree. Despite being a minimally invasive procedure, intrahepatic islet infusion is often associated with life-threatening intraperitoneal bleeding,[181] portal vein thrombosis, and focal hepatic steatosis.[182,183] Moreover, the hypoxic environment inside the portal vein does not fulfill the high oxygen demand of islets for survival and proper endocrine functions.[184] Additionally, IBMIR, peri-islet blood clot formation, and inflammation are inevitable, since islets are infused intravascular. Hence, portal venous infusion of islets is not the optimal engraftment site, and pursuit for an alternative, safer site for islet transplantation is therefore desirable and an important issue to address.[185] Ideally, the alternative transplant site should be easily accessible through a minimally invasive procedure; this will reduce the mean operative time and subsequently the perioperative morbidities and/or mortalities, and allows easy access for biopsy and retrieval. In addition,

it should be spacious enough to accommodate large tissue volumes and/or scaffolds, and does not elicit excessive inflammatory response towards the graft. Above all, it must be well vascularized to promote graft angiogenesis, allow real time glucose sensing, and has a physiological drainage for rapid response and control of glycemic fluctuation.

Kidney capsule – or subcapsular space of the kidney – is the standard transplant site in animal model,[186,187] and has been used as well in human trials during simultaneous islet and kidney transplantation.[116] Nonetheless, it is considered to be a limited space for the large volumes of islet transplants, and graft implantation or routine biopsy might lead to kidney injury and nephropathy. Striated muscles are another proposed site; skeletal muscles are well vascularized, highly oxygenated,[189] and easily accessible percutaneously, which allows easy implantation and retrieval for the graft. This site has been employed for clinical autotransplantation, and showed long-term survival of islet graft, associated with normal response to glucose challenge test.[189] Yet, results obtained from animal studies displayed extensive fibrosis with loss of graft at transplant site.[188] Intra-abdominal fat folds are promising sites for islet transplantation; the thin and highly vascular peritoneal reflections have large surface areas to accommodate grafts, and can be accessed using laparoscopic techniques. Animal[190] and human[191] trials are showing promising results regarding islet survival and function.

1.6.3.2 – Subcutaneous space as a promising implantation site

The subcutaneous space is an attractive ectopic transplant site with numerous merits: it can accommodate large-sized grafts, less invasive surgical access, feasibility of frequent sampling for graft monitoring and/or whole graft removal. This site is proven successful in the clinical setting, where salvaged parathyroid glands are autotransplanted

following thyroidectomy. Grafts have shown remarkable recovery of function, with normal peripheral blood parathyroid hormone levels in 96% of patients within 3 months after surgery.[192] Nevertheless, it is poorly vascularized, which is often associated with modest islet tissue survival.[193] It is clear that the subcutaneous site requires optimization to enhance the process of neovascularization, thereby minimizing cell loss in the early post-transplant period. Multiple approaches and strategies are investigated to render the subcutaneous space a more nurturing environment for insulin secreting graft tissue. Recently, the Shapiro group suggested a 'device-less' approach to transform the inhospitable subcutaneous tissue into a viable and vascularized engraftment location, through the temporary implantation of an angiocatheter[194] that stimulates foreign body reaction and neovascularization around the catheter. After few weeks, simultaneous catheter withdrawal and graft transplantation extinguishes this reaction, yet retaining a vascularized bed for the newly transplanted graft. The Shapiro group has demonstrated that this site is efficacious in reversing diabetes post-transplant of both human and rodent islets, even in models of aggressive foreign body reaction and alloimmunity.[194,195]

Fibrin is another useful tool in amending the host's microenvironment. Additional to its crucial hemostatic role, fibrin promotes cell maturation through the interaction between cell surface integrins and RGD AA motif present in fibrin and other ECM components.[196,197] The feasibility of in vitro crosslinking of fibrin allows the seeding of islets during scaffold construction, and facilitates its handling and implantation. The contact between islets and the surrounding fibrin matrix leads to upregulation of cell surface integrins, followed by strong activation of downstream intracellular kinases that collectively increase cell survival, increase insulin production/secretion, and reduce cell

death. Cotransplantation of other cell lines or tissues with islet graft is an alternative way to improve the conditions at transplant site, to promote angiogenesis, enhance graft maturation, or confer immune protection. Stem cells are excellent candidates for this purpose; they are progenitor cells that can proliferate *in vitro* or *in vivo* and give rise to various differentiated cell types. Mesenchymal stem cells (MSCs) play a crucial role in tissue repair through differentiation, or by releasing soluble trophic factors to affect surrounding cellular environment.[198] These properties enhance the survival of transplanted islets through direct or indirect actions of MSCs on graft re-vascularization[199,200] or by downregulating immune/inflammatory responses.[201,202]

1.6.3.3 – Using scaffolds for graft delivery

The use of porcine islets or β-cell grafts derived from human embryonic or pluripotent stem cells would require close monitoring and frequent biopsies to check for any undesirable cellular transformations. This prerequisite severely limits the liver's capacity to house future xenogeneic β-cells and engineered insulin-producing cells safely. Therefore, the concept of developing retrievable scaffolds and devices is a key component for β-cell replacement therapy for T1DM. This model involves "seeding" individual islets onto three-dimensional (3-D) scaffolds that are often made of biopolymer fibers that provide a 3-D support structure for the islets that is deprived during the isolation procedure, and thereby mimics the natural pancreatic microenvironment. It is conceivable that islets will engraft more effectively in a 3-D than 2-D environment; scaffolds are expected to improve graft viability by promoting cell adherence and nutrient diffusion, thereby increasing islet survival immediately after transplantation. In addition, a polymer scaffold will prevent direct exposure to blood in the first few weeks after transplantation thereby

attenuating IBMIR. Three-dimensional synthetic scaffolds have been reported to provide a protective environment during *in vitro* culture, by preventing islet aggregation and thereby enhancing viability and function.[203,204] Similar beneficial effects were observed when human islets were cultured on decellularized lung-derived micro-scaffolds.[205] In 2005, Dufour demonstrated that syngeneic mouse islets seeded onto scaffolds composed of poly-(glycolide-lactide) co-polymer fibers successfully corrected diabetes.[206] Ellis has also developed a highly vascularized matrix for the ectopic transplantation of neonatal porcine islets into the subcutaneous space of mice.[207,208] Recent advances of polymeric 3-D scaffold fabrication facilitated the addition of proteins and other beneficial biological molecules. Those hybrid scaffolds loaded with various biomimics promotes islet maturation, graft vascularization, and minimize the foreign body reaction at the transplant site.[209]

1.6.3.4 – Recipient's sex and its influence on graft function

There is a growing interest in investigating the potential influence of host's sex hormones on the transplanted graft. Previously published reports point out the difference of tissue sensitivity to insulin between sexes,[210] or the effect of the recipient's sex on maturation of transplanted grafts.[211] Clinical studies carried on healthy individuals from both sexes revealed that women exhibit increased insulin/C-peptide plasma levels postprandially compared to men.[212] That observation applied as well during analyzing the residual β-cell function at the time of T1DM diagnosis.[213] The sex effect is not fully elucidated yet; while some studies offer a conceivable explanation for that effect,[214] the majority of other studies cannot pinpoint the exact pathway or key players. During pregnancy, interactions between female- and pregnancy-specific sex hormones and β-cell mass have been thoroughly investigated. Prolactin, serotonin, progesterone, and placental

lactogen enhance cellular hyperplasia and expansion of the β-cell mass to assist the mother to adapt for the perinatal metabolic needs. [215,216] Nonetheless, it is extremely challenging to orchestrate that hormonal interaction outside the host and attempt to mimic it in vitro. Additionally, applying the notion obtained from the animal models on human islets is unrealistic, due to difference in cellar-hormonal interactions among species.[217]

1.7 – CONCLUSION

Pancreas is one of the most studied glands in the human body; its crucial role in glucose homeostasis is undisputable. Diabetes represents a massive socio-economic burden, and the chronic nature of this ailment prompts investigators and clinicians to explore more therapeutic choices to halt the progression of its neurovascular complications. By far, exogenous insulin administration represents the widely used conventional treatment to control glycemic excursions. Nonetheless, it fails to precisely adjust the glucose levels similar to the pancreas. Moreover, serious adverse effects such as hypoglycemia are not uncommon. Pancreas transplantation is successful alternative to insulin injections; yet, the massive perioperative risks, the chronic administration of immune suppressive agents, and the shortage of donors limit this therapeutic option to a certain subset of T1DM patients. Intrahepatic islet infusion is another surgical option for T1DM treatment. Although less invasive compared to whole organ transplantation, it is still associated with procedural side effects, requires multiple donors for a single recipient, and suboptimal local environment leads to serious graft attrition.

Porcine islets represents a promising substitute donor tissue; the close homology of porcine insulin, and advances in animal housing, genetic modifications, and islet

procurement make it conceivable to use this xenografts in the clinical setting sooner than expected. Engraftment site plays a key role in determining the fate of the transplanted graft. Ideally, an optimal engraftment site will encompass procedural feasibility; minimal invasiveness; easy access; promote rapid neovascularization; and requires a marginal tissue mass to achieve euglycemia. Subcutaneous space is a promising candidate site; yet, hypoxia is the main deterring factor in clinical application. Numerous strategies and approaches are suggested to enhance the local microenvironment in that site, and the current data suggest more positive outcomes regarding graft sustenance and function. With the observed progress rate in investigating alternative donors and sites, islet transplantation is anticipated to be a routine clinical procedure for T1DM patients in the near future.

1.8 – OBJECTIVES AND GENERAL THESIS OUTLINE

The primary objective of this thesis is to explore ectopic extra-hepatic sites for islet transplantation. Although much work has been done in this field, we try to investigate alternative sites using materials that can be easily applied in clinical islet transplantation. Neonatal porcine islets represent a logical alternative to cadaveric human pancreatic islets. Based on many published reports, they are the most attractive candidates for clinical islet xenotransplantation. While other researchers attempt to investigate their potential in vitro, we are more interested in exploring their capability in correcting diabetes through in vivo xenotransplantation. By doing this, we try to optimize the graft and the transplant site in order to step towards more clinical translatable application. We hope that NPI xenotransplantation would move forward from bench side towards bedside in the near future.

In **Chapter 1**, we offered to the reader a concise – yet updated – literature review demonstrating historical and contemporary overview of pancreas and diabetes. We illustrated the conventional methods to treat hyperglycemia, and clinical/experimental surgical alternatives to exogenous insulin administration. Then we underscored the potential applications of porcine islet xenografts in preclinical animal models and clinical trials. Finally, we displayed the existing hurdles that impede the immediate widespread use in clinical setting.

Chapter 2 begins this thesis' experimental work by exploring the subcutaneous space as an alternative extra-hepatic transplant site. We demonstrate that subcutaneous space can accommodate islet grafts, and it is easily accessible through minor procedure with minimal perioperative burden. We highlight that porcine, human, or murine islet grafts cannot survive without amending that ectopic site. We explore the benefits of fibrin as a biocompatible scaffolding material that have an impressive impact on NPI graft survival, through amending the local microenvironment of subcutaneous space. Through post-transplantation metabolic follow-up and glucose tolerance testing, we proved the capability of this site to support islet graft survival, and its potential to be an alternative site to the liver in clinical islet transplantation.

Chapter 3 studies another extra-hepatic site for islet transplantation: intra-abdominal peritoneal folds or fat pads in experimental animal models. Omentum offers an excellent transplant site as it can accommodate large grafts, and offer more physiological portal venous drainage for the transplanted islets. In our experimental mouse model, we used epididymal fat pads instead of omentum due to the limitation of surface area of the later. When unfolded gently, fat pads can offer a transplantable site that can support islet

grafts. We used human islet grafts encapsulated in alginate as a model of islet xenograft. Our lab has published many reports showing that alginate microencapsulation can provide protection against cell-mediated graft destruction. In this experiment, we show a novel way of using alginate for constructing a scaffold that can support islets through micro- and/or macroencapsulation. After transplantation, animals displayed rapid and sustained control over hyperglycemia that lasted for 20 weeks post-transplantation. The site was able to support the graft survival and function as well, through the control of diabetes, and response to glucose challenge test. Our data prove the feasibility of this site as a clinically applicable ectopic transplant location, capable of supporting large encapsulated islet grafts.

In **Chapter 4** we underscore the undoubted impact of recipient's sex on islet graft function. Data from literature have shown the noticeable differences between males and females regarding lipid and carbohydrate metabolism. Studies on basal and postprandial glucose levels, or rate of glucose clearance after glucose tolerance test have revealed gender-related variances between sexes. We explore the possible sex influence on our porcine islet xenografts by transplanting islets to male and female diabetic mice. Through metabolic follow-up, glucose tolerance testing and graft characterization, we clearly display the sex-related differences of graft maturation, glucose clearance, and cellular insulin content in either sex diabetic animal model. This data elicit more questions regarding islet transplantation in the clinical setting, such as the importance of donor/recipient sex, and other criteria to be taken in consideration in choosing candidates for islet transplantation.

Taken together, this thesis offers a cohesive approach towards finding a clinically applicable alternative transplant site, such as subcutaneous space or intra-abdominal peritoneal folds. It also highlights the importance of amending those ectopic sites to make

them more suitable for graft survival and function, through using biocompatible scaffolds and materials. Nonetheless, there are more factors yet to be elucidated – such as the role of recipient's sex – that contributes to the survival and function of allo- and/or xenografts, and how those factors can be employed in clinical setting. We hope that this work contributes towards rendering islet transplantation to be an established and available clinical therapeutic option for more diabetic patients in the near future.

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CHAPTER 2.

REVERSAL OF HYPERGLYCEMIA IN DIABETIC MICE AFTER SUBCUTANEOUS TRANSPLANTATION OF NEONATAL PORCINE ISLETS USING FIBRIN SCAFFOLDS

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ORIGINAL ARTICLE

Title: Fibrin Supports Subcutaneous Islet Transplantation without the Need for Pre-Vascularization

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2.1 – ABSTRACT

The subcutaneous space is a potential extra-hepatic location for clinical islet transplantation. In this study, we explore the benefits of fibrin scaffolds in enhancing the viability and function of islet grafts in this ectopic site.

Male, inbred B6.129S7-Rag1^{tm1Mom}/J mice were rendered diabetic by administering streptozotocin. Mice transplanted with islets subcutaneous (SC) and under the kidney capsule (KC) were compared to mice that received islets imbedded in fibrin then transplanted subcutaneous (SC+F). Animals were monitored for reversal of hyperglycemia, and grafts were assessed for cellular viability, hormonal activity, and evidence of neovascularization.

Mice with streptozotocin induced diabetes in both the KC and SC+F experimental groups achieved normoglycemia between 5 and 22 weeks post-transplantation, and displayed normal physiological response towards glucose challenge test. Immunohistochemistry (IHC) staining of grafts displayed robust insulin staining in both groups, and newly formed capillaries surrounding and penetrating mature NPI in subcutaneous subjects.

Fibrin-embedded islet grafts remained viable, and were capable of reversing diabetes and maintaining euglycemia in diabetic mice, up to 22 weeks post-transplantation in subcutaneous space. Observed results were comparable to outcomes obtained from standard kidney capsule transplantation, without the morbidities associated with invasive intra-abdominal procedures.

2.2 – INTRODUCTION

Islet replacement therapy is a conceivable cure for type-1 diabetes (T1DM), as it offers a physiological real-time control of glycemic fluctuations.[1,2] The incremental improvements in allogeneic islet isolation techniques, and usage of modern non-steroidal immunosuppressive agents have shown startling outcomes.[3,4] Recently, post-transplant insulin independence rates have become comparable to whole pancreas transplantation – especially in preuremic patients – without the perioperative burden associated with major surgical procedures in subjects with multiple comorbidities.[5-7] Nonetheless, widespread clinical application is impeded by the progressive attrition of graft mass and viability over time, accompanied by eventual loss of insulin independence.[8,9]

Many research groups reported the efficacy of xenogeneic islets and insulin-producing cells induced from human embryonic[10] (ESC) or pluripotent[11] (iPSC) stem cells, as a potential alternative for allogeneic grafts obtained from cadaveric donors. Neonatal porcine islets (NPI) possess numerous advantages as a promising clinical source for islet replacement;[12,13] they are resistant to hypoxia[14] and pro-inflammatory cytokines,[15] in addition to their inherent capability of maturation after engraftment in ectopic transplant sites despite of sustained hyperglycemia.[16,17] Our lab[18] and other research groups reported the conspicuous success in normalization of hyperglycemia in diabetic animal models.[19-21] Additionally, limited preclinical pig-to-human islet xenotransplantation trials has already started, and results show sustained graft survival associated with better control in blood glycemia.[22-24]

Transplant site represents another challenge for successful long-term post-transplant insulin independence. Islet embolization in the hepatic portal venous tree is the standard – yet the least optimal – clinical transplant procedure.[25] Life-threatening intraperitoneal (IP) bleeding,[26] portal vein thrombosis,[27] sub-optimal oxygen levels,[9] non-specific blood-mediated inflammatory response,[21,28] peri-islet hepatic steatosis,[29] and chronic immunosuppressant-related side effects[30,31] are few of numerous limitations related to the hepatic site. Furthermore, undifferentiated and/or engineered tissue grafts require frequent post-transplantation cytological monitoring to avoid any undesirable cellular transformation, which is almost impossible in this particular setting. Hence, developing an ectopic site where grafts can be retrieved is a key concept, as important as finding alternative tissue source.

Amending local sub-optimal environment in any proposed ectopic transplant site is an effective strategy to circumvent hypoxia and other injurious factors that lead to progressive graft attrition;[32] increasing local blood supply via stimulation of new blood vessels formation would consequently improve oxygen diffusion and cell nutrition/metabolism.[33] Additionally, establishing a near-native extracellular matrix (ECM) is crucial for graft survival in any ectopic site, as contact between cell surface receptors and ECM components contributes significantly to islet sustenance. The 3-D arrangement of matrix fibers augments cellular adhesion, enhances angiogenesis, prevents islet cells dedifferention, and offers protection against inflammatory-mediated damage resulting from direct blood contact. This can be achieved by using of biocompatible scaffolds as a method of cellular delivery to the recipient.[34-36]

Fibrin embodies an optimal material for scaffold fabrication; it is biocompatible, biodegradable, can cross-link in vitro allowing cell seeding, and facilitates graft delivery. In this study, we hypothesize that using fibrin scaffolds for NPI transplantation in subcutaneous space would enhance the sustenance and function of islet grafts, through stimulation of angiogenesis, and offering a near-native ECM support for islets in the investigated ectopic site.

2.3 – MATERIALS AND METHODS

2.3.1 – Islet preparation

All procedures involving animals were carried out according to the guidelines established by Canadian Council on Animal Care. Donor pancreases were surgically removed from either sex neonatal piglets (Swine Research and Technology Center, University of Alberta, Edmonton AB CAN). Neonatal porcine islets were isolated and kept in Ham's-F10 tissue culture medium (Sigma-Aldrich, St. Louis MO USA), as previously described.[18] At the third day of culture, the incubation medium was switched to DMEM/F12 (Thermofisher, Burlington ON CAN) supplemented with 1% (vol/vol) pig serum (heat inactivated), 0.2 mM Insulin, 7 μM Transferrin, 4 μM Sodium Selenite (ITS; Thermofisher), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Cedarlane, Burlington ON CAN), 10 mM nicotinamide (Sigma-Aldrich), and 10 nM GLP-1 analogue (Exendin-4; Sigma-Aldrich). A total of four independent NPI isolations were used for the transplant experiments reported in this study. Mouse islets were collected from male inbred BALB/c mice (The Jackson Laboratory, Bar Harbor Main USA), and procured as previously described.[17] We also used human islets obtained from Alberta Diabetes Institute IsletCore

(University of Alberta) isolated from a brain-dead heart-beating donor, according to their established protocol.[37] Ethics approval for work with human tissue was obtained from the Health Research Ethics Board-Biomedical Panel, University of Alberta.

2.3.2 – Scaffold preparation

Fibrin scaffolds were constructed using a commercially available fibrin sealant kit (Evicel®; Johnson & Johnson Medical Companies Inc., Markham ON CAN). Equal volumes (150 µL) of fibrinogen and thrombin were combined in a single well of a 24-well tissue culture plate (Fisher Scientific, Burlington ON CAN) and allowed to cross-link for 15 minutes in a 37 C incubator. Islet containing fibrin scaffolds were constructed, with either 5000 NPIs, 5000 human islets or 500 syngeneic BALB/C mouse islets. To a single well of a 24-well tissue culture plate, islets were added to 150 µl each of fibrinogen and thrombin, and cross-linked for 15 minutes in a 37 C incubator. To verify β-cell integrity and incorporation into the scaffold, porcine islet fibrin discs not intended for transplantation were stained with dithizone reagent.[18] Briefly, 100 µL of dithizone was placed onto the islet-fibrin disc for 2-5 minutes then imaged.

2.3.3 – Transplantation and metabolic follow-up

In a preliminary set of experiments, we first assessed the *in situ* effects of fibrin scaffolds alone in the subcutaneous space of naïve BALB/c mice. A subcutaneous space was surgically created in the dorsal aspect and the scaffolds were gently placed under the skin in 6-8 week old BALB/c mice (n=4). Scaffolds were subsequently collected for morphological and histological assessment at 1, 2 and 4 weeks post-implant. Animals were

maintained under virus-antibody-free conditions in climatized rooms with free access to sterile tap water and pelleted food.

Male, inbred, B6.129S7-Rag1^{tm1Mom}/J mice (Jackson Laboratories) were used as recipients of either NPIs or human islets whereas BALB/c mice were recipients of syngeneic mouse islets. Mice were rendered diabetic by intraperitoneal injection of 185 mg/kg streptozotocin (freshly dissolved in acetate buffer; Sigma-Aldrich) 2-4 days before transplantation. Blood samples were obtained from the tail vein for glucose measurement (OneTouch UltraMini glucose meter, LifeScan Europe, Zug Switzerland). Mice were considered diabetic when the blood glucose level was greater than 20 mM. Porcine islets were transplanted into diabetic mice, either in, the subcutaneous space with fibrin scaffolds (SC+F; n=17) or without fibrin scaffolds (SC; n=7), and without fibrin under the kidney capsule (KC; n=12). Human islet fibrin scaffolds (SC+F) were transplanted in the subcutaneous space of diabetic mice (n=4) and islets without fibrin were transplanted under the kidney capsule (KC; n=5). Mouse islet fibrin scaffolds (SC+F) were transplanted into the subcutaneous space of diabetic BALB/c mice (n=4) and mouse islets without fibrin were transplanted under the KC (n=3). Naïve (non-diabetic non-transplanted) male, inbred, B6.129S7-Rag1^{tm1Mom}/J mice (n=5) were used as a control.

At the time of transplantation, a subcutaneous space was surgically created in the dorsal aspect of diabetic animals then the islet+fibrin scaffolds were gently placed under the skin. In the cohort that received islets alone (SC), islets were collected in 1 mL syringe, and injected subcutaneously using 23G needle. In the KC group, islets were aspirated into polyethylene tubing (PE-90; Becton Dickinson, Sparks MD USA) and pelleted by

centrifugation then gently placed under the kidney capsule with the aid of a micromanipulator syringe.[18]

All mice were monitored for non-fasting blood glucose levels within the first week post-transplant, and once a week thereafter. When the blood glucose level was ≤ 11.1 mM for two consecutive weeks, mice were deemed normalized, and an intra-peritoneal glucose tolerance test (IPGTT) was performed. After 12 hours fasting, D-glucose (3 g/Kg) was administered as a 50% solution IP into non-anesthetized mice, and blood glucose was measured at 0, 15, 30, 60, 90 and 120 minutes. Glucose clearance was compared to naïve mice, plotted and expressed as area under the curve (AUC). To determine the insulin release in response to elevated blood glucose, insulin (pg/mL) in blood samples collected at 60 minutes post glucose administration was compared to insulin levels in blood samples collected prior to fasting. Stimulated increase (SI) in insulin was calculated by dividing plasma insulin (pg/mL) at 60 minutes by plasma insulin (pg/mL) prior to fasting. Plasma insulin was measured using the Ultrasensitive Insulin ELISA (ALPCO, Salem NH USA) that does not detect mouse insulin. Therefore, negative controls for this assay included blood samples collected from naïve mice that also underwent an IPGTT.

To confirm the KC grafts had corrected diabetes, at weeks 22 post-transplant mice from the KC group underwent a survival nephrectomy of the graft-bearing kidney. These nephrectomized animals were monitored to confirm the recurrence of hyperglycemia within 48 hours. Some of the islet graft bearing kidneys were also assessed for total cellular insulin content.[18] To confirm the SC+F implants had corrected diabetes, 22 weeks post-transplant the implant and the pancreas were removed from this cohort. Since removal of the SC+F grafts often results in morbidities or mortalities, a survival surgery was not

possible. Therefore, total pancreatic cellular insulin content of these mice was determined to confirm no potential β-cell regeneration following STZ administration.[18] Pancreatic insulin content was then compared to the pancreatic insulin content of naïve mice (n=8). Total cellular insulin of graft bearing kidneys, total SC+F grafts, and total pancreatic cellular insulin content was measured according to the previously published protocol.[18] Briefly, tissue homogenates were sonicated in 10 ml of 2 mmol/liter acetic acid (0.25% BSA), incubated for 2 hours at 4 C, centrifuged (10,000 RPM, 25 min), then supernatants were collected and the pellets further extracted by sonication in an additional 5 ml of acetic acid. The second supernatant was collected after centrifugation, combined with the first supernatant and samples were assayed for insulin by metabolic assay (MesoScale Discovery, Gaithersburg, MD USA).[18]

2.3.4 – Characterization of harvested grafts

After morphological assessment the fibrin alone implants were fixed in 4% w/v paraformaldehyde (Thermofisher), embedded in paraffin blocks and subsequently stained using Martius Scarlet Blue (MSB) polychrome staining method.[38] Tissue sections (5 μm) were de-paraffinized, rehydrated, immersed in Celestin Blue solution (5 min), Mayer's Hematoxylin solution (5 min),1% Lithium Carbonate solution (30 sec) then placed in 95% Ethanol. Subsequently, slides were immersed in Martius Yellow (2 min), crystal scarlet (10 min.), phosphotungstic acid (5 min.), methyl blue (5 min.), and finally rinsed in 1% acetic acid. Slides were dehydrated and cover-slipped using clear mounting solution.[39] The MSB allows for the visualization of newly formed fibrin and erythrocytes, more mature fibrin and muscles (pink/red), old fibrin, collagen, elastic fibers and basement membranes (blue) and nuclei (blue/black).[38] Sequential tissue slides of islet containing grafts were processed

for histological assessment through insulin, glucagon, and trichrome staining. Primary antibodies for immunostaining included; 1/1000 anti-insulin (DAKO Mississauga ON CAN), 1/5000 anti-glucagon (Sigma-Aldrich). All appropriate species-specific biotinylated secondary antibodies were diluted 1/200 in 5% normal goat serum. Detection of labeled insulin and glucagon was done using the avidin-biotin complex method (Vectastain® ABC kit; Vector laboratories, CA USA) with peroxidase and diaminobenzidine (DAB; BioLegend, MA USA) as the chromagen.[18]

2.3.5 – Statistical analysis

Data is expressed as mean ± SEM of n independent observations. Statistical significance of differences was calculated with one-way ANOVA, Log-Rank (Mantel-Cox) sum test, or Student's t-test. A p-value of < 0.05 was required to deem results significant.

2.4 – RESULTS

2.4.1 – *In situ* effects of subcutaneous fibrin implants

Macroscopic observation of subcutaneous tissue revealed an increase in minute blood vessels converging towards the implantation sites of islet-free fibrin discs **(Fig. 2.1A-C)**. Micrographs of skin tissue stained using MSB polychrome dye showed that the scaffold maintained its architecture without breaking or fragmenting with no obvious inflammatory response towards fibrin, indicated by lack of nuclear staining or cellular infiltration within the disc. As time progresses, fibrin fibrils mature and become more organized displaying blue color when stained **(Fig. 2.1D-F)**. At 28 days, the presence of numerous small-sized

vessels around fibrin scaffold can be observed represented by the yellow colored erythrocytes surrounded by a capillary wall **(insert in Fig. 2.1F)**.

2.4.2 – NPI Transplantation and Metabolic Follow-up

Metabolic follow up of transplanted animals has shown a steady decrease in blood glucose over time **(Fig. 2.2A)**, and reversal of diabetes in all subjects in KC and SC+F experimental groups between 5 and 22 weeks post-transplantation **(Fig. 2.2B)**, with no significant statistical difference between those groups (p = 0.1). In contrast, none of the animals that received islets alone subcutaneously (SC) attained normoglycemia, and four animals had to be euthanized between 3 and 7 weeks post transplantation due to diabetic-related morbidities. Due to the continuous decline of SC cohort number as a result of morbidities and euthanasia, it was difficult to statistically compare between that cohort and other experimental groups after 5th week post-transplantation. Animals that received human or mouse islets subcutaneously loaded in fibrin scaffolds did not show any decline in blood glucose levels **(Fig. 2.2C)** whereas their KC respective counterparts have shown rapid improvement on blood glucose and overall health within the first week after transplantation, and continued till the experiment was terminated.

2.4.3 – Glucose tolerance testing

IPGTT was performed once the transplanted animals exhibited sustained normoglycemia (KC; SC+F groups). Results from an independent IPGTT executed on naïve Rag-1 mice (n=5) were included as a control **(Fig. 2.3A)**. KC and SC+F groups displayed rapid clearance of injected glucose within the first 60 minutes of the test, compared to naïve mice.

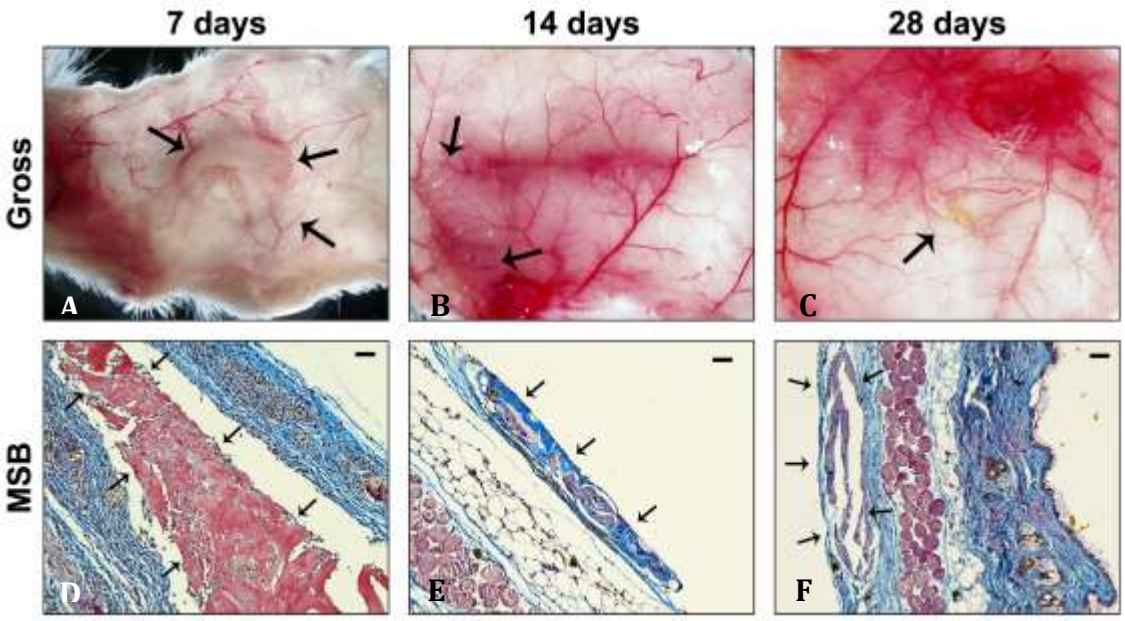

Figure 2.1. Skin obtained from naïve BALB/C mice transplanted with islet-free fibrin scaffold, 7, 14 and 28 days post-implantation.

(A – C) Macroscopic appearance of the undersurface of the skin, immediately after removal from animals. Overtime, more neovascularization can be visually observed around the site of the disc.

(D – F) Micrographs of tissue sections obtained from the corresponding skin samples (A – C) and stained using Martius-Scarlet-Blue (MSB) histochemical staining method (Asterisks represent fibrin discs). MSB technique stains fresh fibrin in yellow color, mature fibrin in pink/red color, and old fibrin in blue color. At 7 days, fibrin disc (pink/red) shows no fragmentation, with no apparent nuclear staining or cellular infiltration within the scaffold. In later time points, fibrin becomes more organized and reacts towards the dye in a bluish color.

Gross images were captured using a surgical dissecting microscope at 10x (A) and 20x (B,C) magnification. Scale bars are 100 μm and arrows denote the fibrin graft.

After 60 minutes, the blood glucose levels of naïve mice remained significantly higher than KC and SC+F groups ($p < 0.01$).

Computed area under curve (AUC) from IPGTT **(Fig. 2.3B)** revealed no significant difference between KC and SC+F transplant groups (1517.1 ± 168.1 and 1514.8.8 ± 70.6, mM.min respectively, $p = 0.99$). However, comparing either of those groups to Naïve groups revealed a statistically significant difference in computed AUC ($p < 0.001$; **Fig. 2.3B**). Analysis of plasma samples obtained during IPGTT has shown that post-injection circulating porcine insulin levels detected in SC+F and KC transplanted mice were higher than their basal levels (**Tab. 2.1**). The stimulation index (SI) was comparable between those two groups, with no significant difference ($p > 0.05$).

At experimental endpoint, nephrectomized KC subjects returned hyperglycemic within 48 hours after removing graft-bearing kidneys. Total insulin content in removed pancreases from SC+F group was calculated (1.0 ± 0.2, n=16) and compared to values obtained from pancreases of naïve mice (34.8 ± 2.0 µg, n=8). Analyzing the total cellular insulin content of the extracted grafts revealed no significant difference between KC and SC+F experimental groups (46.5 ± 5.8 and 38.9 ± 5.1 respectively; $p > 0.1$; **Fig. 2.3C**)

2.4.4 – Histological assessment

The external fabrication of NPI-fibrin scaffolds using multi-well tissue culture plates **(Fig. 2.4A)** preserved islet mass during fabrication, and facilitated the graft delivery to subcutaneous pockets. During graft extraction at the experimental endpoint, confluent newly formed blood vessels were seen converging towards transplantation site in the subcutaneous space in SC+F experimental group **(Fig. 2.4B)**.

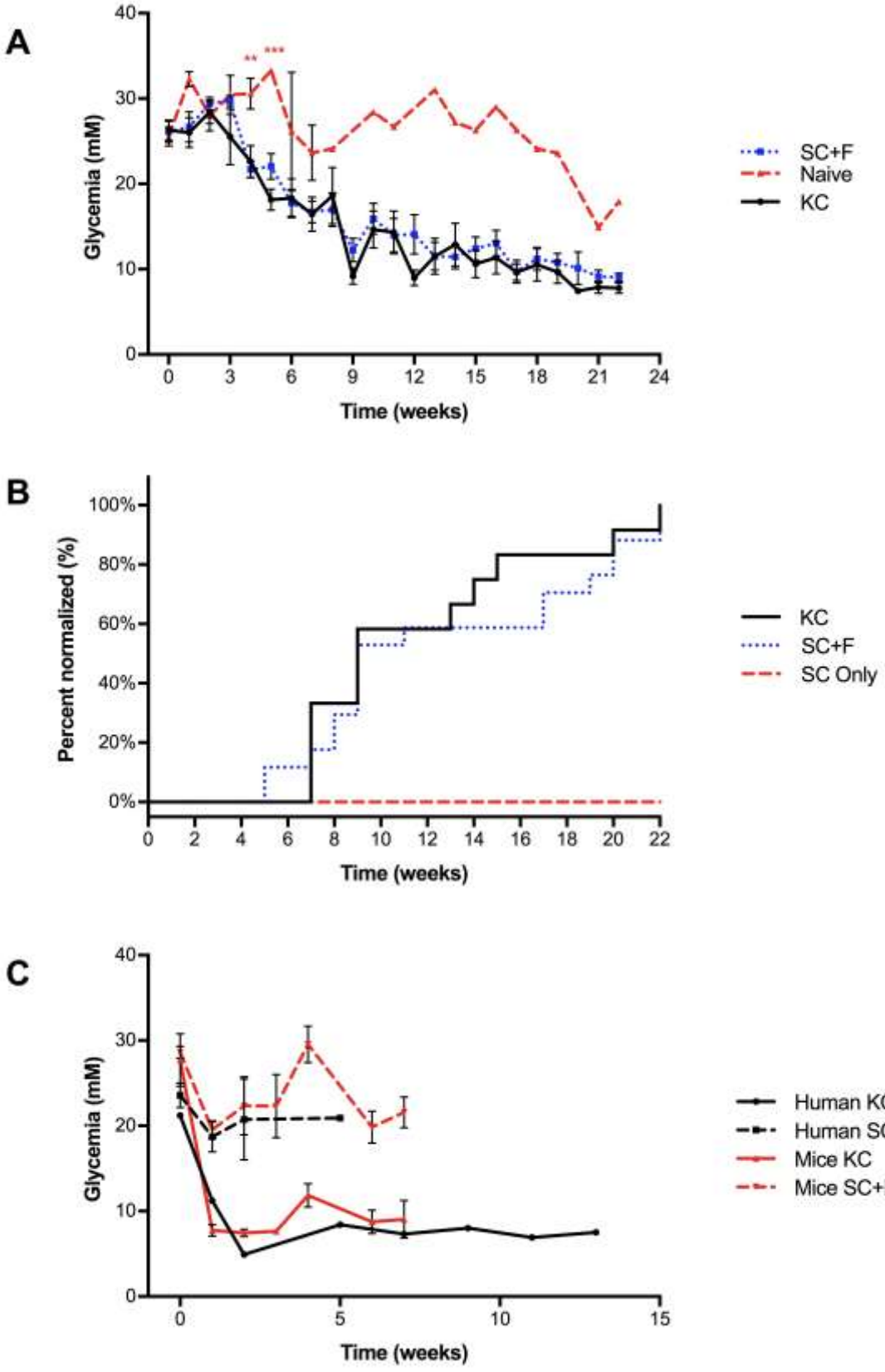
A
Glycemia (mM)
Time (weeks)
SC+F
Naive
KC
B
Percent normalized (%)
Time (weeks)
KC
SC+F
SC Only
C
Glycemia (mM)
Time (weeks)
Human KC
Human SC+F
Mice KC
Mice SC+F

Figure 2.2. Post-transplantation weekly follow-up of diabetic mice.

Diabetic B6/Rag$^{-/-}$ mice were transplanted with 5000 NPIs either in the renal sub-capsular space (KC; n=12; solid black line), subcutaneously embedded in fibrin (SC+F; n=17; dotted blue line), or subcutaneously without fibrin (SC; n=7; dashed red line).

(A) Non-fasting blood glucose levels assessed weekly from 0 to 22 weeks post-transplant using a hand-held glucometer.

(B) Percentage (%) of mice achieving normoglycemia (glycemia < 11.1 mM for 2 consecutive weeks. Percentage of mice reverted to normal blood glucose levels over time course of experiment.

(C) Weekly blood glucose levels of diabetic B6/Rag$^{-/-}$ mice transplanted with 5,000 human islets (KC, n=5 solid black line, SC+F n=4 dashed black line), or 500 syngeneic Balb/c mouse islets (KC n=3 solid red line, SC+F n=4 dashed Red line).

Values are expressed as mean ± SEM analysed using One-Way ANOVA with Bonferroni's post hoc correction, Log-rank (Mantel-Cox) test, or Student's *t* test with for multiple comparisons where appropriate. **P* < 0.05, ***P* < 0.01, ****P* < 0.005, *****P* < 0.0001.

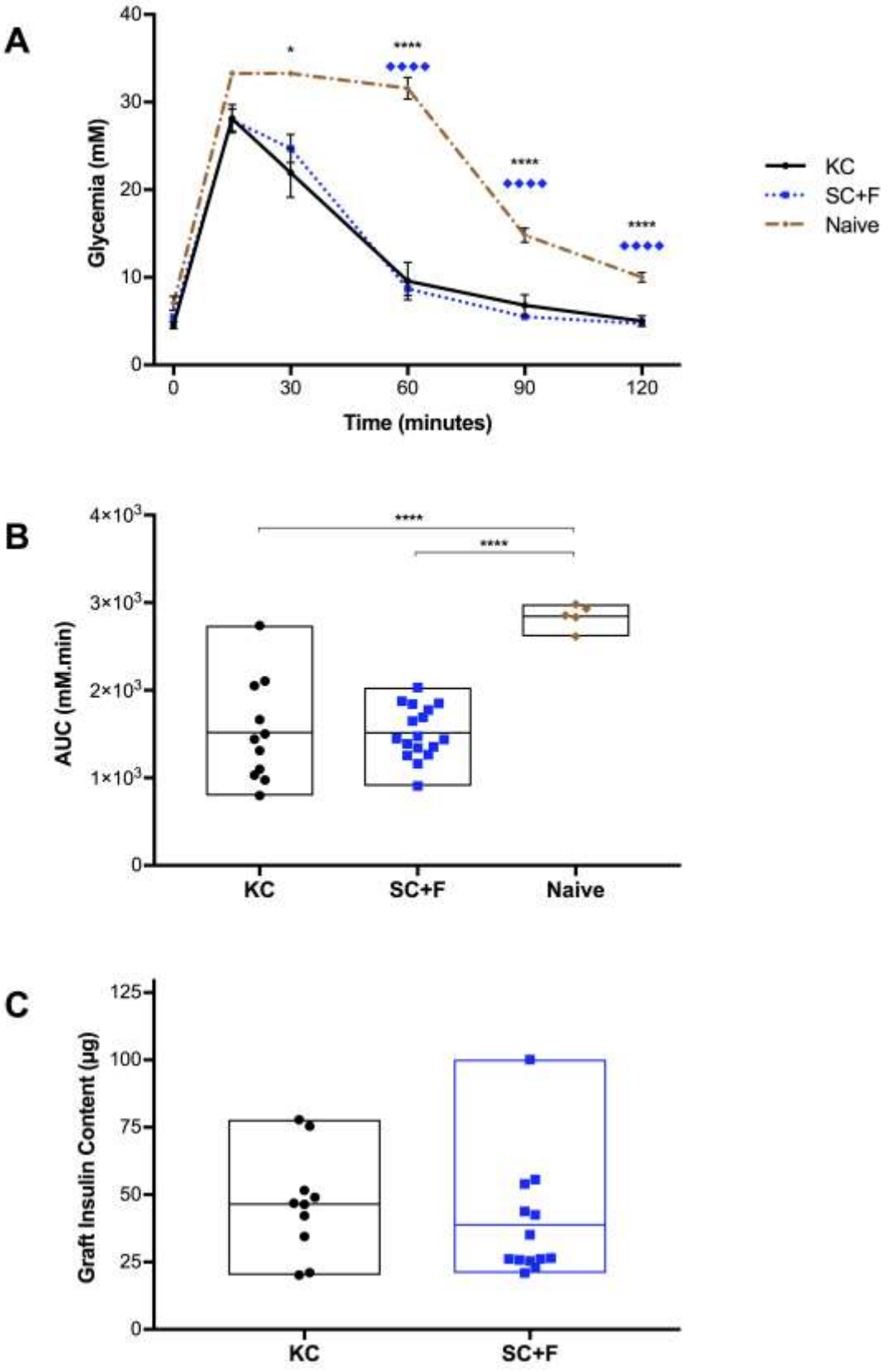
A
Glycemia (mM)
Time (minutes)
KC
SC+F
Naive
B
AUC (mM.min)
KC
SC+F
Naive
C
Graft Insulin Content (µg)
KC
SC+F

Figure 2.3. Glucose tolerance testing and total cellular insulin content

Post-normalization glucose challenge test on diabetic transplanted, and non-diabetic naïve mice

(A) Blood glucose values during an IPGTT (n=11 KC, n=17 SC+F, and n=5 naïve control mice).

(B) Computed area under the curve for respective IPGTT.

(C) Analyzed insulin content in grafts obtained from both experimental groups (KC n=10 black line, SC+F n=13 blue line) at the experimental endpoint.

Values are expressed as mean ± SEM analysed using One-Way ANOVA with Bonferroni's post hoc correction, or Student's *t* test with for multiple comparisons where appropriate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****/◆◆◆◆$P < 0.0001$.

	Insulin (pg/ml)		SI
	0 min	60 min	
KC (n=7)	86.6 ± 15.4	151.9 ± 16.5*	2.0 ± 0.2
SC+F (n=16)	102.3 ± 19.9	200.3 ± 26.9*	2.3 ± 0.3
Naïve (n=8)	N.D.	N.D.	N.D.

Table 2.1. Porcine insulin calculated in plasma samples collected during IPGTT done post-transplantation, or from naïve mice

Values are expressed as mean ± SEM of four independent experiments. No significant differences were found in the fasting (0 minutes) or stimulated (60 minutes) porcine insulin between both experimental groups. Yet, there is a significant increase in the stimulated porcine insulin, compared to basal insulin within the same cohort (*$p < 0.05$, using paired Student's t-test). Basal and stimulated blood samples were taken from naïve (non-diabetic, non-transplanted) mice as well to confirm that the used assay does not cross-react with murine insulin.

KC; Subcapsular site, SC; Subcutaneous site using fibrin, SI; Stimulation index (stimulated insulin ÷ basal insulin), N.D.; Not detected.

IHC staining for insulin-positive cells displayed weak staining at 3 weeks **(Fig. 2.5A)** and no insulin at 7 weeks **(Fig. 2.5B)** post-transplant, in grafts obtained from SC cohort. A similar outcome was observed in the grafts obtained from animas that were transplanted with human/mice islet-loaded fibrin scaffolds subcutaneously **(Fig. 2.5C,D)**. On the contrary, histological staining revealed abundant cells with robust insulin and glucagon staining in both experimental groups (KC: **Fig. 2.6A-D**, SC+F: **Fig. 2.6G-J**), in addition to the pronounced inter- and intra-islet neovascularization that is clearly seen using trichrome staining (KC: **Fig. 2.6E,F**, SC+F: **Fig. 2.6K,L**).

2.5 – DISCUSSION

Islet cell transplantation possesses numerous advantages as an emerging replacement therapy for T1DM. Despite the life-saving role of exogenous insulin injections, it is usually accompanied with numerous side effects or serious life-threatening risks. Islet transplantation on the other hand offers more physiological control of hyperglycemia, thus minimizing the long-term neuro-vascular complications in diabetic patients. Unlike whole pancreas transplantation, portal vein islet infusion is a minimally invasive procedure associated with marginal perioperative morbidities. However, this standard engrafting site remains least favourable, particularly with the increasing interest in non-allogenic insulin-secreting primary and/or engineered cells. Since physical graft assessment is indispensable, it is conceivable that a safer alternative transplant site must be established to allow serial sampling of the graft for cellular visualization and mapping of abnormal phenotypic transformations.

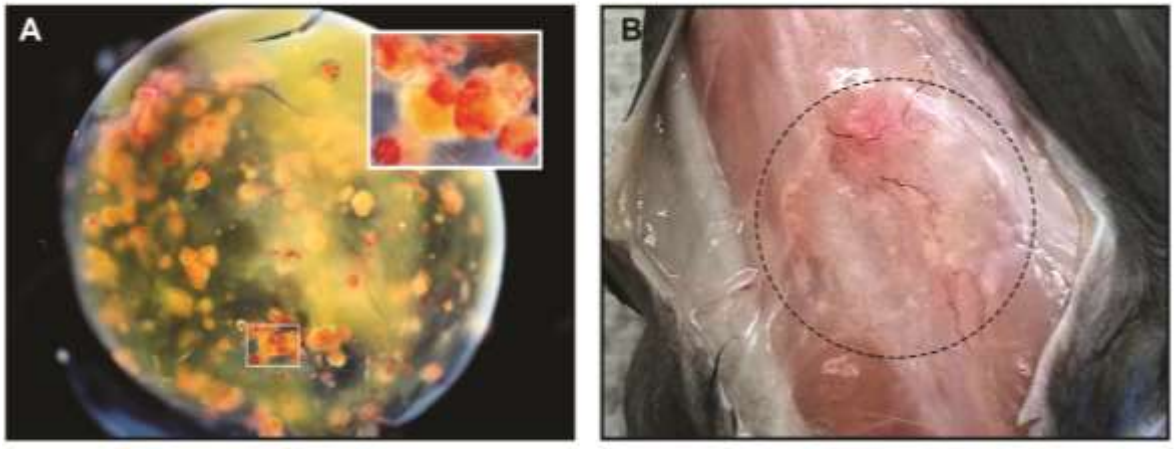

Figure 2.4. Pre-transplant appearance of NPI-loaded fibrin disc, and transplantation site at the experimental endpoint.

(A) Dithizone staining of a NPI graft embedded in fibrin prior to implantation subcutaneously Images were captured using surgical dissecting microscope, with 2x (6.3x for insert) power objective lens magnification.

(B) Macroscopic appearance of an NPI+Fibrin graft in the subcutaneous site 21 weeks post-transplant. Subcutaneous transplantation site (skin removed) showing circular shadow with newly-formed blood vessels on top (inside dashed circle), marking the site of degraded, NPI-loaded fibrin disc, 21-weeks post-transplantation. Images were captured using digital still camera, with 2X magnification.

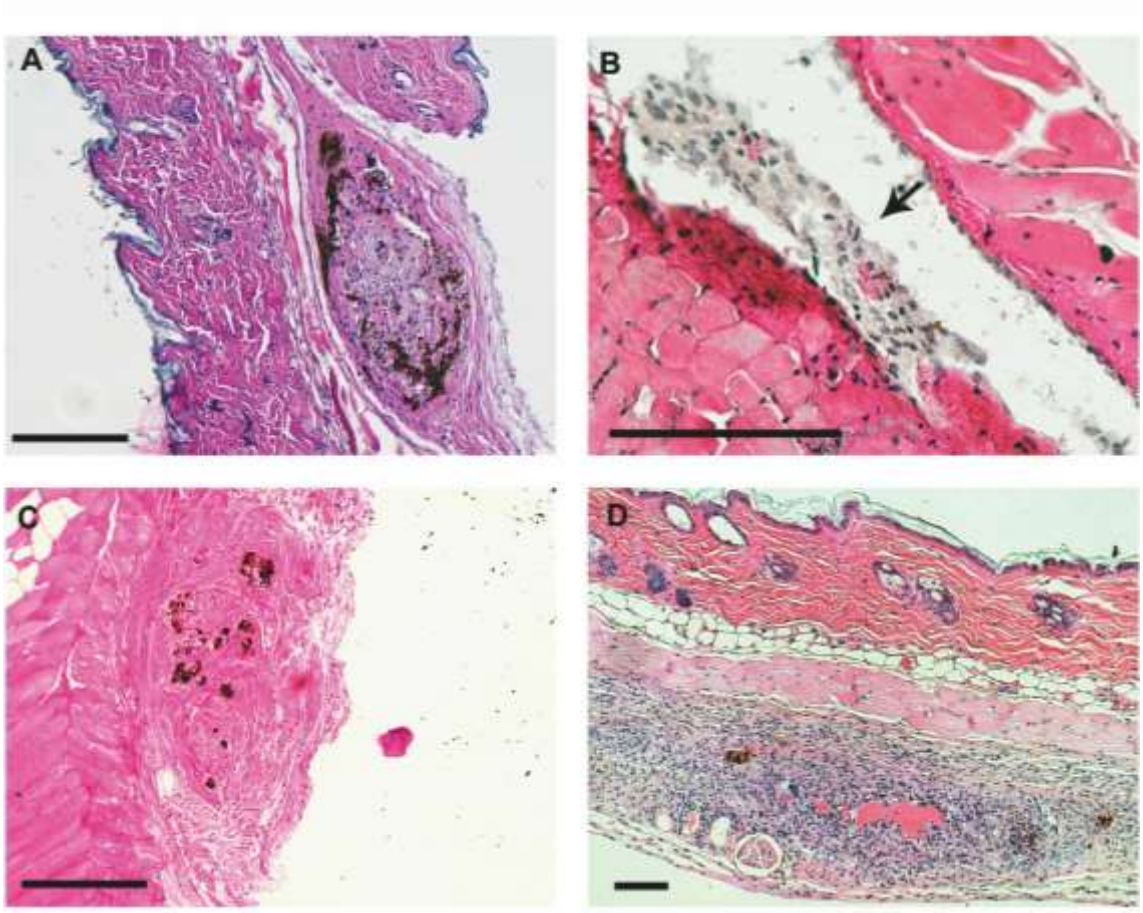

Figure 2.5. Histological assessment of explanted islet grafts at the experimental endpoint.

Representative immune staining of grafts for insulin obtained from islets transplanted under the kidney capsule or subcutaneously

(A – D) Grafts collected from B6/Rag$^{-/-}$ mice transplanted subcutaneously with NPI without fibrin were also immune-stained following 3 (A) and 7 (B) weeks post-transplantation.

Grafts were also stained for insulin from mice transplanted subcutaneously with fibrin with either human (C) or syngeneic mouse islets (D). Scale bars =100 μm, arrow denotes graft location.

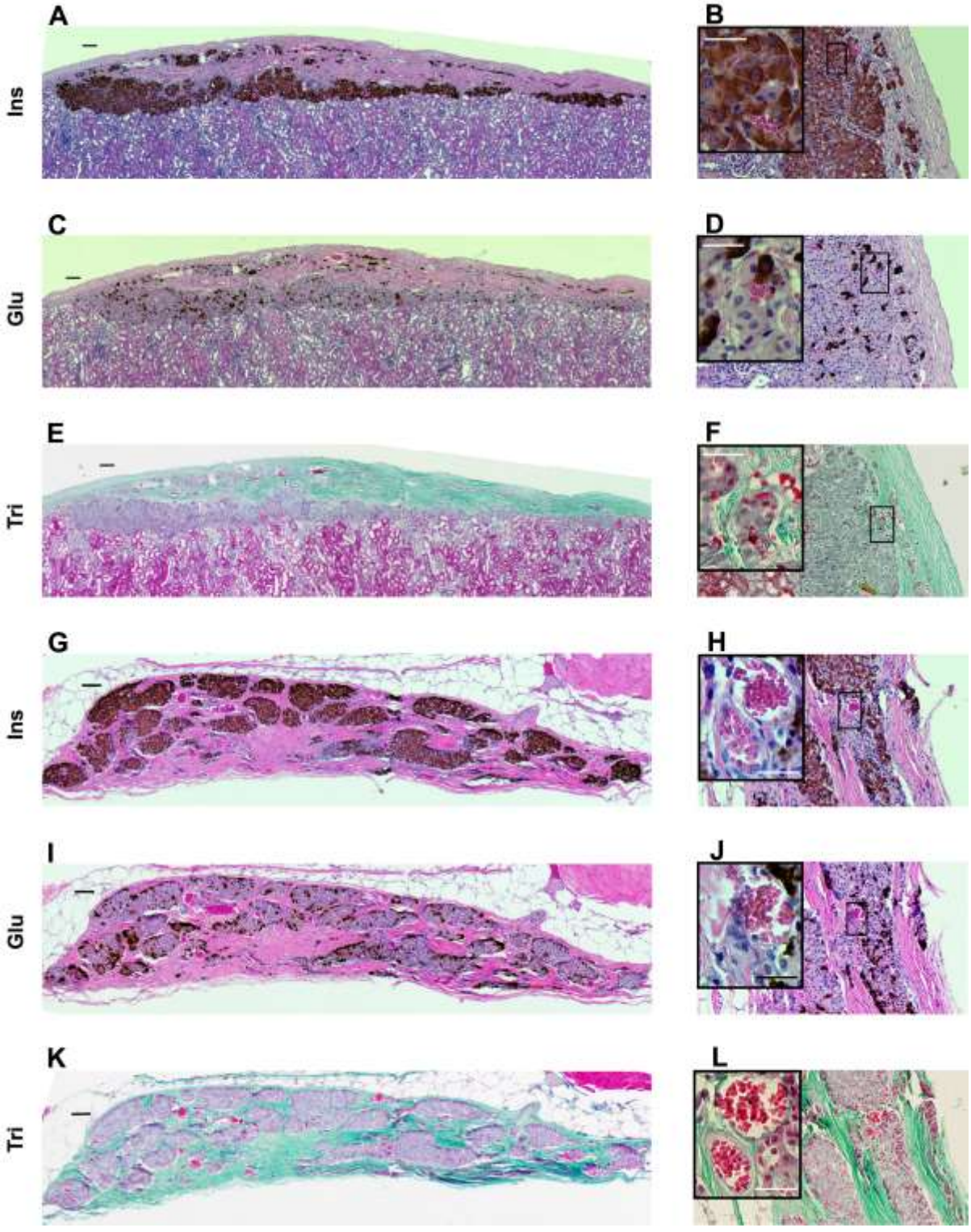
A
B
Ins
C
D
Glu
E
F
Tri
G
H
Ins
I
J
Glu
K
L
Tri

Figure 2.6. Histological assessment of explanted NPI grafts at the experimental endpoint.

Representative micrographs of grafts obtained from NPI transplanted under the kidney capsule (A – D), or subcutaneously using fibrin (E – L) from B6/Rag$^{-/-}$ mice at 21 weeks post-transplant. Tissue is stained for insulin (Ins), glucagon (Glu), or Mason's trichrome (Tri).

Images were captured at Images were captured at 4x (A, C, E, G, I, K), 10x (B, D, F, H, J, L), and 40x (inserts) power objective lens magnification. Scale bars = 100 μm.

In this report, we investigate the subcutaneous space as an ectopic site for islet transplantation, and the advantages of fibrin in enhancing islet graft viability and function, combined with the potential benefits of NPI as an alternative clinical source for β-cell replacement therapy. This is observed through reversal of hyperglycemia in STZ-induced diabetic mice transplanted with NPI embedded in fibrin scaffolds into subcutaneous space. Using an immune deficient animal model such as B6/Rag$^{-/-}$ eliminates the possibility of immune-mediated graft rejection, allowing us to study the role of the extrahepatic site in graft survival. Also, we induced diabetes chemically in the recipients, so graft function (manifested by achieving normoglycemia) can be observed clearly.

Results obtained from the two transplant groups (KC and SC+F) were comparable pertaining the steady decline of hyperglycemia, percentage of subjects normalized, and post-transplant time till normalization. The delay between transplantation and lowering of

blood glucose is attributed to the use of immature neonatal porcine islets; mice transplanted with mature human or murine islets under the kidney capsule showed a rapid correction of hyperglycemia, started from the next day after transplantation. Nevertheless, prolonged hyperglycemia did not affect the capability of NPI to mature or differentiate within the recipient.[17]

Both groups have shown similar physiological response towards IP glucose bolus within 60 minutes after injection, and returned to normal glucose level at 90 minutes. Statistical analysis of AUC from IPGTT curve has shown no difference between the two experimental groups (KC and SC+F); yet, when comparing either group to naïve mice, the later group showed a significant increase in AUC, which would be translated as a delayed glucose clearance. This difference in glucose clearance rate – in addition to euglycemia set point – was observed as well in other xenotransplants,[41] where glucose homeostasis is determined by the islets from donor species. Sixty minutes after glucose injection, levels of circulating insulin in both KC and SC+F mice groups were higher than what was detected before administration of glucose. This observation is an indicator of survival and responsiveness of islet grafts transplanted in either ectopic site. As mentioned earlier, all subjects attained normoglycemia between 5 and 22 weeks after transplantation, and except of two mortalities, remaining mice have reached to the experimental endpoint. The two mortalities reported were not related to diabetes or graft transplantation, and both mice have normalized and responded physiologically to IPGTT before being terminated. One mouse was euthanized at 19 weeks post-transplant – based on advice of veterinary care team – due to non-healing ulcerative bacterial dermatitis, and the other mouse was found cannibalized by cage mates at 20 weeks post transplant. When graft-bearing kidneys were removed from

KC group, all recipients returned diabetic again within 48 hours of graft removal; an undoubted sign of graft function. Survival graft removal from SC+F recipients would cause unnecessary morbidities and mortalities to mice. Instead, we collected their pancreases and compared their total cellular insulin content to pancreases from naïve mice.[41]

We attempted to observe similar outcome in human and murine islets, yet, when we transplanted fibrin scaffolds loaded with mature human or murine islets in the subcutaneous site, none of the animals achieved euglycemia. A conceivable explanation for that might be attributed to the high demand for oxygen by the metabolically active mature islets and the low pO_2 level at the transplant site, which might explain why those mature islets only survived under the kidney capsule. Unfortunately, subcutaneous graft loss occurs prior to neovascularization that is induced by fibrin. Metabolic follow-up and histological assessment of animals transplanted with NPI alone subcutaneously conforms to the previous results reported by other groups,[42,43] and supports our hypothesis that embedding NPI in a pro-angiogenic biocompatible scaffold would improve the outcome of subcutaneous islet transplantation drastically.

The preliminary testing of NPI-free fibrin scaffolds in naïve mice revealed macroscopic and microscopic increase of newly-formed minute capillaries in response to fibrin scaffolds, compared to skin examined at untreated area from same mice (not shown). Macroscopic observation of subcutaneous grafting site in SC+F group exhibited plenty of capillaries and small blood vessels developing at the remaining disc-shaped shadow of fibrin scaffold; this associates with the previous observations noted during preliminary fibrin testing. Finally, findings from IHC staining correlates with the observations during metabolic follow up.

The standard experimental islet transplant site in diabetic animal model is the renal subcapsular space,[44,45] and previous experiments have confirmed the capability of this ectopic site to support islet viability, and provide sufficient oxygenation comparable to the pancreas.[9] Nonetheless, procedural invasiveness, risks of organ injury or nephropathy, and difficulty of histological graft monitoring remain considerable obstacles. Subcutaneous space is an attractive ectopic transplant site with numerous merits; it can accommodate large-sized grafts, less invasive surgical access, feasibility of frequent sampling for graft monitoring and/or whole graft removal. In the clinical setting, salvaged parathyroid glands following thyroidectomy are autotransplanted in subcutaneous space, and have shown remarkable recovery of graft function, with normal peripheral blood parathyroid hormone levels in 96% of patients within 3 months after surgery.[46] Many research groups displayed myriad benefits of culturing or transplanting cells in three-dimensional (3-D) conditions.[47,48] The 3-D arrangements within these natural or bioengineered scaffolds mimic the native ECM, and interactions between cell adhesion molecules or membrane receptors and surrounding environment enhance cell maturation and function, and prevent phenotypic deterioration and dedifferentiation. Our group has demonstrated the advantages of islet-loaded biocompatible scaffolds in correcting hyperglycemia in diabetic mice, and creating a successful ectopic transplantation site using highly vascularized collagen matrix.[49-51]

Fibrin is a natural circulating glycoprotein that plays a pivotal role in hemostasis, and wound healing.[52] Additionally, it promotes cell maturation and maintains function through the interaction between cell surface integrins and RGD amino-acid motif present in fibrin and other ECM components.[53-56] Riopel et al.[56] investigated the cellular-level events

involved in the beneficial effects of fibrin as a carrier scaffold. The contact between islets and surrounding fibrin matrix led to upregulation of cell surface integrins that was associated with strong activation of downstream intracellular kinases. This interaction also increased the expression levels of some important transcription factors such as Pdx-1, in addition to the decreased levels of cleaved Caspase-3. Collectively, these events were believed to increase cell survival, increase insulin production/secretion, and reduce cell death.[54,56] Moreover, fibrin(ogen) is known to be a pro-angiogenic plasma transglutaminase[57] with a positive proliferative effect on fibroblasts.[58]

The clinical grade fibrin glue utilized in this experiment (Evicel®) is already approved by Health Canada to be used for human surgical applications as a local hemostatic agent and sealant for cutaneous incisions. We did not attempt to prepare fibrin scaffolds from raw components – as reported by other groups[42,59] – to eliminate any further approvals required for islet transplantation trials in diabetic patients. This also guarantees consistent quality control, availability, sterility and cross-linking properties between production lots. Ex-vivo crosslinking of fibrin scaffolds in a multi-well plate facilitates easier scaffold construction and handling, even distribution of islets, and more uniform thickness, with expected equal oxygen diffusion throughout the disk.

To our best knowledge, this is the first study to report the success of transplanted NPI using fibrin scaffolds to reverse hyperglycemia in diabetic mice. Moreover, we displayed the responsiveness of NPI grafts during glucose challenge test, which was not shown in the previously cited studies.[42,59] Other groups reported transplanting islets intramuscularly[60,61] or in abdominal fat folds.[62-63] Although deemed successful, it is still considered invasive when transplanted intra-abdominally, or may induce fibrosis

intramuscularly. Besides, post-transplantation physical graft monitoring would be challenging or even risky in those ectopic sites. In their recent article published in Diabetes,[41] Mesmaeker et al. reported similar findings when they transplanted encapsulated perinatal porcine islets subcutaneously in non-diabetic mice. Our results conform to their published work regarding the delayed reversal of hyperglycemia, lower than regular fasting blood glucose in transplanted mice (we highlighted the difference by including naïve mice during IPGTT), the observed expansion in β cell mass in the recipients, and the loss of mature human/porcine islets transplanted subcutaneously.

This study highlights the potential benefits of subcutaneous space as an ectopic transplant site. It also exhibits the advantageous role of fibrin as a biocompatible 3-D scaffolding material that offers a near-native extracellular microenvironment for NPI, in addition to its known role in angiogenesis.

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CHAPTER 3.

SURVIVAL AND FUNCTION OF HUMAN ISLETS TRANSPLANTED IN THE EPIDIDYMAL FAT PAD USING ALGINATE SCAFFOLDS.

A Proof-of-Concept for the Ectopic Site and Scaffolding Material

3.1 – INTRODUCTION

Diabetes mellitus defines the chronic impairment of glucose tolerance due to regression in insulin secretion, resistance to insulin's anabolic actions by peripheral tissues, or both. Type 1 diabetes mellitus (T1DM) results from autoimmune destruction of insulin-secreting pancreatic β-cells. Clinical islet transplantation is widely accepted as an efficient cure for T1DM; continuous incremental improvements in islet isolation and purification are contributing to the increasing insulin independence rates worldwide.[1] Nonetheless, transplantation site – one of the major causes for post-transplantation graft attrition – has not been perfected yet. Trans-cutaneous intra-hepatic infusion of allogeneic islets via the portal vein is the standard site in the clinical transplantation setting. Despite being minimally invasive, the procedure itself is associated with considerable perioperative risks, such as intraperitoneal haemorrhage, portal vein thrombosis, and peritonitis.[2] Moreover, intravascular islet embolization into the hepatic portal tree is the least optimal microenvironment for the survival and metabolic function of islet grafts.[3] The instant blood-mediated inflammatory response is the main culprit involved in acute graft attrition, and post-transplant loss of insulin independence.[4]

Other locations have been proposed,[5,6] or already tested[7] as alternative extra-hepatic sites for islet transplantation; yet, they either exhibited poor graft survival, or the procedure was technically challenging, and associated with considerable risks.[5] Intra-abdominal peritoneal reflections (such as the greater omentum) are plausible proposed experimental sites for islet transplantation; they are highly vascularized; their venous circulation drains into portal vein; and can be moved or folded freely to accommodate large grafts.[8-12] In rodents animal model (especially mice) the omentum is limited in its size, and does not

accommodate large cellular grafts or scaffolds used for islet transplantation.[13] Hence, we attempt to investigate the epididymal fat pad (EFP) of male mice as an ectopic transplant site, and compare it with the subcapsular location, which is the most extensively utilized site for experimental optimization of islet transplantation.[14-15]

Our group has been always interested in porcine islet xenotransplantation, as a logical alternative for the scarce cadaveric human islets.[16-19] In order to reduce the likelihood of immune-mediated graft rejection, physical immune barriers and/or immunosuppressive agents have to be employed with xenotransplantation. Microencapsulation or physical containment of individual or few cells in a porous microsphere is a widely accepted tool for isolating islet grafts from host's immune response.[20-24] The porous capsule will permit oxygen and nutrition exchange between the islets and surrounding microenvironment; yet, it prevents donor/recipient cell-to-cell interaction.[25,26]

In this chapter, we examine the feasibility of transplanting human islets into the EFP of male diabetic Rag-1 mice, using biocompatible scaffolds fabricated from alginate. This will provide us with a proof-of-concept that can be utilized for further investigations of intra-abdominal peritoneal folds.

3.2 – MATERIALS AND METHODS

3.2.1 – Animals

All procedures involving animals were carried out according to the guidelines established by Canadian Council on Animal Care. Male inbred B6.129S7-Rag1^{tm1Mom}/J

mice (The Jackson Laboratory, Bar Harbor Main USA) were used as recipients of human islets. Mice were rendered diabetic by intraperitoneal injection of 185 mg/kg streptozotocin (freshly dissolved in acetate buffer; Sigma-Aldrich, St. Louis MO USA) 2-4 days before transplantation. Blood samples were obtained from the tail vein for glucose measurement (OneTouch UltraMini glucose meter, LifeScan Europe, Zug Switzerland). Mice were considered diabetic when the blood glucose level was greater than 20 mM.

3.2.2 – Preparation and microencapsulation of islets

Human islets obtained from Alberta Diabetes Institute (ADI; University of Alberta Edmonton AB CA) IsletCore, from deceased donors after obtaining necessary next-of-kin consents. Ethics approval for work with human tissue was obtained from the Health Research Ethics Board-Biomedical Panel, University of Alberta. Pancreases were removed after circulatory arrest (DCD), or after Neurological Determination of Death (NDD), and islets were isolated and purified according to IsletCore established protocol.[27] A total of six independent human islet preparations were used in this experiment, and donor/isolation information are shown in **Tab. 3.1**. Upon receiving the human islet preparation, islets were incubated at least for 24 hours in Ham's-F10 tissue culture medium (Sigma-Aldrich) supplemented with 14.3 mM Sodium Bicarbonate (Fisher Scientific, Burlington ON CAN), 10 mM D-Glucose (Sigma-Aldrich), 2 mM L-Glutamine (Sigma-Aldrich), 0.5% (wt/vol) BSA (Sigma-Aldrich), 50 µM IsoButylMethylXanthine (IBMX; Sigma-Aldrich), 10 mM Nicotinamide (Sigma-Aldrich), 1.6 mM Calcium Chloride Dihydrate (Sigma-Aldrich), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Cedarlane, Burlington ON CAN), 5 µg/ml Caspase Inhibitor (R&D Systems MN USA), and 0.66 µl/ml of Protease Inhibitor Cocktail (Sigma-Aldrich), and were kept at 37°C ad 5% CO_2.

Table 3.1. Donor biometrics and isolation information of human islet preparations

Isolation	Donor Type	Age (Yrs)	Sex	BMI (kg/m^2)	HbA1c	Organ Weight (g)	Cold Ischemia (Hrs)	Total IEq	Ins/IEq (ng/IEq)	Purity (%)
R139	NDD	50	Male	24.8	5.9	16.5	16.5	270,267	2.1	90
R140	NDD	49	Female	21.9	5.6	21.5	21.5	389,566	19.3	95
R227	DCD	32	Male	19.9	4.9	18	18	278,573	23.1	95
R253	NDD	57	Male	25.5	5.0	14.3	14.3	693,295	9.9	90
R282	NDD	57	Male	26.4	6.0	9.5	9.5	456,631	15.6	90
R288	NDD	69	Female	27.6	5.7	22.3	22.3	279,900	13.8	95

NDD: donor after neurological determination of death; **DCD:** donor after circulatory arrest; **BMI:** body mass index; **IEq:** islet equivalent; **Ins:** insulin

Information provided by ADI IsletCore (www.epicore.ualberta.ca/IsletCore).

3.2.3 – Preparation of human islets discs

Diabetic Animals received human islets either microencapsulated then embedded in an alginate disc (*Encap*), or embedded directly in an alginate disc without microencapsulation (*Non-encap*). Aliquots of 5,000 human islet equivalents (IEq) were washed three times with Ca^{++} and Mg^{++} free HBSS (Fisher Scientific, Burlington ON CAN), and islets were left to sediment at the bottom of the tube in between washes by the effect of gravity. After that, islet aliquots were re-suspended in 0.44 ml of Ca^{++}/Mg^{++} free HBSS and mixed 0.55 ml of 1.5% (wt/vol) ultrapure, medium viscosity (> 200 mPas), high guluronic content (≥ 60% guluronic monomer) sodium alginate (PRONOVA™ UP MVG Alginate; NovaMatrix Norway) dissolved in Ca^{++}/Mg^{++} free HBSS. Alginate microcapsules were formed by electrostatic stripping, using an electrostatic droplet generator (designed by Metabolex Inc.) assisted by hydraulic pump. Capsules drop in 120 mM calcium chloride solution to crosslink for 5 minutes, then washed three times in HBSS using gentle agitation and gravity sedimentation. Individual aliquots of capsules were then cultured in previously described Ham's-F10 tissue culture medium for 24 hours before embedding in alginate discs and transplantation. On the transplantation day, individual aliquots of capsules were collected, washed with Ca^{++} and Mg^{++} free HBSS as described formerly, and mixed with 0.1 ml of alginate in a 48-well tissue culture plate. Cross-linking solution (120 mM $CaCl_2$) was added gently on the sides of the well to prevent disc deformation, and plates were shaken gently using an orbital plate shaker for 5 minutes. Afterwards, discs were collected and rinsed gently with HBSS, and taken for transplantation into diabetic animals. To fabricate *Non-encap* alginate discs, individual aliquots of non-encapsulated islets were

collected on the transplantation day, washed and embedded right away into alginate discs as described above.[23]

3.2.4 – Transplantation and metabolic follow-up

Islets were transplanted into diabetic mice, either intraperitoneal in the epididymal fat pad after being encapsulated then embedded into an alginate disc (*Encap*; n=10) or embedded directly in the alginate disc without prior microencapsulation (*Non-encap*; n=18), and under the kidney capsule (KC; n=14). At the time of transplantation, a midline laparotomy incision was made then the fat pad was spread gently on wet gauze. Discs were placed on the fat pad **(Fig. 3.1A)**, which was folded over afterwards and edges were sealed using 7.0 non-absorbable polypropylene sutures (Ethicon USA; **Fig. 3.1B**). In the KC group, grafts were aspirated into polyethylene tubing (PE-90; Becton Dickinson, Sparks MD USA) and pelleted by centrifugation then gently placed under the kidney capsule with the aid of a micromanipulator syringe.[16] All mice were monitored for non-fasting blood glucose levels within the first week post-transplant, and once a week thereafter.

3.2.5 – Glucose tolerance testing

When the blood glucose level was ≤ 11.1 mM for two consecutive weeks, mice were deemed normalized, and an intra-peritoneal glucose tolerance test (IPGTT) was performed. After 12 hours fasting, D-glucose (3 g/Kg) was administered as a 50% solution IP into non-anesthetized mice, and blood glucose was measured at 0, 15, 30, 60, 90 and 120 minutes.[16] Glucose clearance was compared to naïve mice, plotted and expressed as area under the curve (AUC). To determine the insulin release in response to elevated blood glucose, insulin (pg/mL) in blood samples collected at 60 minutes post glucose

administration was compared to insulin levels in blood samples collected prior to fasting. Stimulated increase (SI) in insulin was calculated by dividing plasma insulin (pg/mL) at 60 minutes by plasma insulin (pg/mL) prior to fasting. Plasma insulin was measured using the human specific insulin assay (MesoScale® human insulin assay; MesoScale Discovery, Gaithersburg, MD USA).

At week 12 post-transplant, mice from KC group underwent survival nephrectomy of the graft-bearing kidney, and nephrectomized animals were monitored to confirm the recurrence of hyperglycemia within 48 hours. Some of the islet graft bearing kidneys were also assessed for total cellular insulin content. Since removal of the grafts transplanted into the fat pad often results in morbidities or mortality, therefore, total pancreatic cellular insulin content of these mice was determined to confirm no potential β-cell regeneration following STZ administration.[16] Total cellular insulin of graft bearing kidneys, total fat pad grafts, and total pancreatic cellular insulin content was measured according to the previously published protocol.[16] Briefly, tissue homogenates were sonicated in 10 ml of 2 mmol/liter acetic acid (0.25% BSA), incubated for 2 hours at 4 C, centrifuged (10,000 RPM, 25 min), then supernatants were collected and the pellets further extracted by sonication in an additional 5 ml of acetic acid. The second supernatant was collected after centrifugation, combined with the first supernatant and samples were assayed for insulin by metabolic assay (MesoScale® Mouse/Rat Insulin Kit; MesoScale Discovery).[16]

3.2.6 – Characterization of harvested grafts

After macroscopic morphological assessment, implants were fixed in 4% w/v paraformaldehyde (Thermofisher), embedded in paraffin blocks and 5 μm tissue sections were prepared. Paraffin sections were prepared for immunohistochemistry detection of

insulin using the avidin-biotin complex (ABC) method with peroxidase and diaminobenzidine (DAB) as the chromogen, as described before.[16] Primary antibodies for immunostaining included; 1/1000 anti-insulin (DAKO Mississauga ON CAN), 1/5000 anti-glucagon (Sigma-Aldrich). All appropriate species-specific biotinylated secondary antibodies were diluted 1/200 in 5% normal goat serum. Detection of labeled insulin and glucagon was done using the avidin-biotin complex method (Vectastain® ABC kit; Vector laboratories, CA USA) with peroxidase and diaminobenzidine (DAB; BioLegend, MA USA) as the chromagen.[16]

3.2.7 – Statistical analysis

Data is expressed as mean ± SEM of n independent observations. Statistical significance of differences was calculated with one-way ANOVA, Log-Rank (Mantel-Cox) sum test, or Student's *t*-test. A *p*-value of < 0.05 was required to deem results significant.

3.3 – RESULTS

Metabolic follow up of transplanted animals has shown a steady decrease in blood glucose over time **(Fig. 3.2A)**, and reversal of diabetes in most of the subjects in three experimental groups the first 4 weeks post-transplantation (KC: 11/13, *Encp*: 10/10, *Non-encap*: 17/18, **Fig. 3.2B**). During the first week post transplantation, both animal cohorts that received islets in the fat pad displayed lower blood glucose than KC animal cohort (*Non-encap*: $p \leq 0.05$, *Encap*: $p \leq 0.005$, **Fig. 3.2A**). However, at the 7th week post transplantation, *Non-encap* group showed a statistically significant elevation in glycemic levels, relative to KC and *Encap* groups ($p < 0.05$).

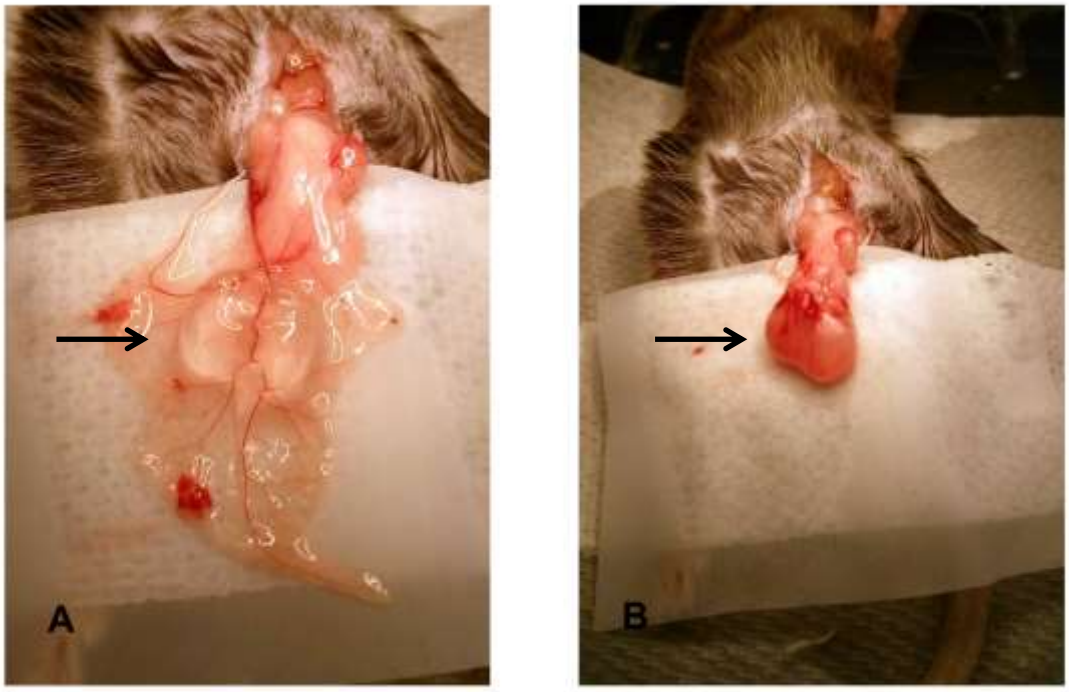

Figure 3.1. Transplantation of islet-loaded alginate disks to the fat pad of diabetic mice.

After spreading the fat pad gently on wet gauze (A), alginate disc was placed and the pad (arrow) edges were folded over and sealed using non-absorbable suturing material (B)

IPGTT was performed at 11th week post-transplant, after the majority of transplanted animals exhibited sustained normoglycemia. Mice from *Encap* cohort showed statistically significant elevated fasting blood glucose levels compared to KC and *Non-encap* counterparts ($p \leq 0.001$ and < 0.01 respectively, **Fig. 3.3A**). However, *Non-encap* group recorded higher glucose levels compared to KC group during 30 and 60 minutes time points ($p < 0.0001$, and < 0.05 respectively, **Fig. 3.3A**). Area under curve (AUC) computed

from IPGTT curve revealed no statistically significant difference when comparing *Encap* to KC or *Non-encap* groups. However, comparing KC to *Non-encap* displayed a significant difference in glucose clearance time ($p < 0.01$, **Fig. 3.3B**).

Analysis of plasma samples obtained from some animals during IPGTT (**Tab. 3.2**) has shown that fasting levels of circulating human insulin detected in *Non-encap* mice cohorts were higher than what was detected in other experimental groups ($p < 0.01$). Also, there is a statistically significant increase in stimulated insulin detected at 60 min in both KC and *Non-encap* groups ($p < 0.01$ and < 0.001, respectively) compared to their basal levels; however, the stimulation index is comparable with no significant statistical difference ($p > 0.05$, **Tab. 3.2**).

	Insulin (µg/L)		**SI**
	0 min	**60 min**	
KC (n=9)	24.9 ± 4.7	43.5 ± 6.4††	2.0 ± 0.3
Encap (n=3)	9.0 ± 1.6	33.8 ± 10.4	3.4 ± 0.7
Non-encap (n=5)	9.2 ± 1.3$^{\wedge\wedge\wedge}$	27.9 ± 4.1***	3.3 ± 0.4

Table 3.2. Calculated human insulin in plasma samples collected during IPGTT

Data is expressed in mean ± SEM. $^{\wedge\wedge\wedge}$ $p < 0.005$, *Non-encap* 0 min vs. other groups.

†† $p < 0.01$, *** $p < 0.001$ simulated vs. basal insulin.

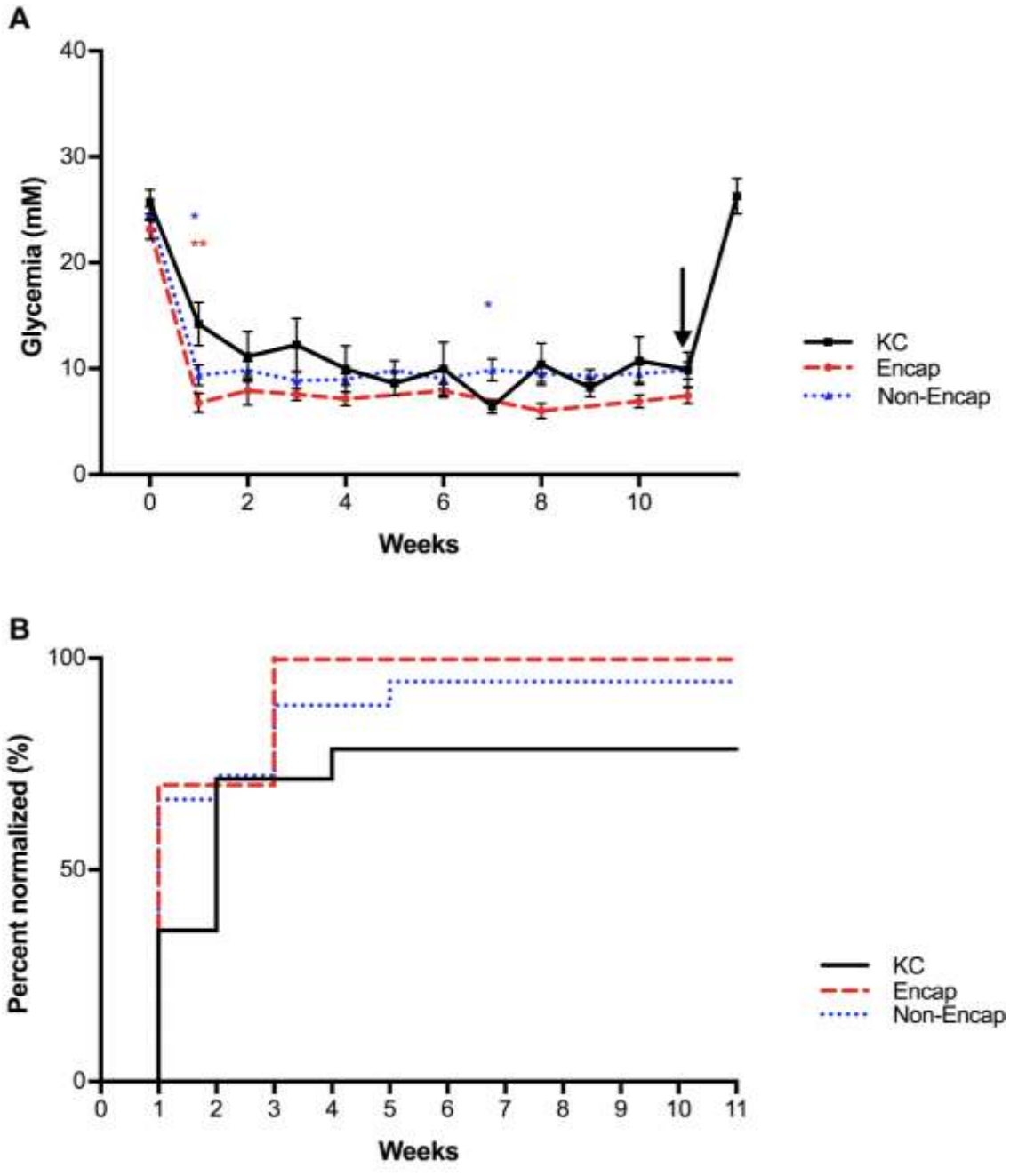
A
Glycemia (mM)
40
30
20
10
0
0
2
4
6
8
10
Weeks
KC
Encap
Non-Encap
B
Percent normalized (%)
100
50
0
0
1
2
3
4
5
6
7
8
9
10
11
Weeks
KC
Encap
Non-Encap

Figure 3.2. Metabolic follow up of diabetic mice transplanted with human islets

Mice were transplanted with ~5000 Human pancreatic islet equivalents (IEq), either in the renal sub-capsular space (KC; n=14; solid Black line) or in the intra-abdominal fat pads embedded in disc-shaped alginate scaffolds. The scaffold recipients were split into two experimental groups: in one group, islets were microencapsulated using alginate before constructing the disc (*Encap*; n=10; dashed Red line), while in the other group islets were seeded into the scaffold without initial microencapsulation (*Non-encap*; n=18; dotted blue line)

(A) Non-fasting blood glucose levels assessed weekly from 0 to 11 weeks post-transplant using a hand-held glucometer. Arrow represents reversal of diabetes after survival removal of the graft-bearing kidney in KC cohort at the 11^{th} week.

(B) Percentage (%) of mice reverted to normal blood glucose level (glycemia < 11.1 mM for 2 consecutive weeks.

Values are expressed as mean ± SEM analysed using One-Way ANOVA with Bonferroni's post hoc correction, and Log-rank (Mantel-Cox) test, where appropriate. $*p < 0.05$, $**p < 0.01$

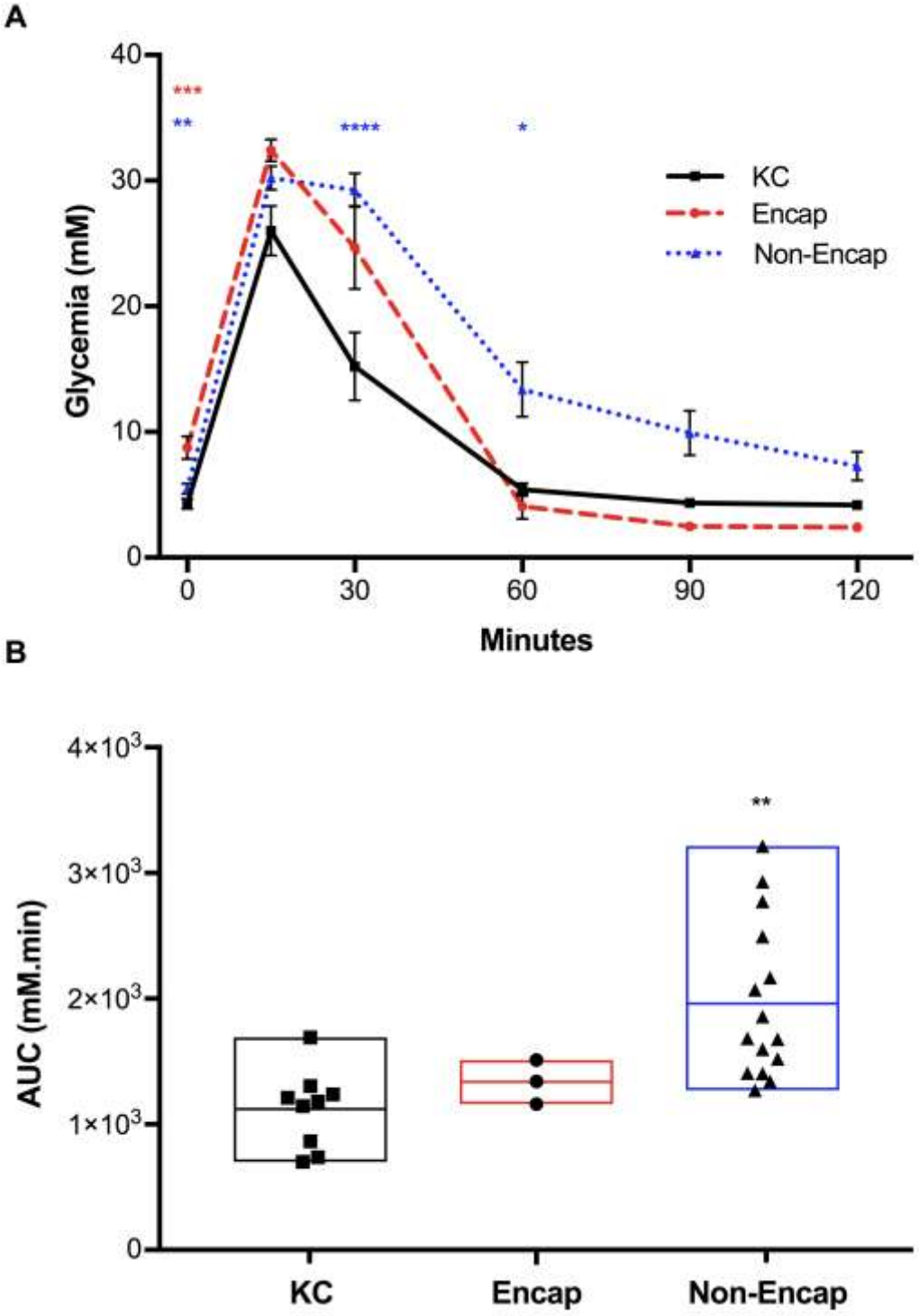
A
40
30
20
10
0
Glycemia (mM)
***
**
****
*
KC
Encap
Non-Encap
0
30
60
90
120
Minutes
B
4×10³
3×10³
2×10³
1×10³
0
AUC (mM.min)
**
KC
Encap
Non-Encap

Figure 3.3. Glucose tolerance testing

Post-normalization glucose challenge test performed on diabetic transplanted with human islets

(A) Blood glucose values during an IPGTT (n=9 KC, n=3 *Encap*, and n=15 *Non-encap*).

(B) Computed area under the curve for respective IPGTT.

Values are expressed as mean ± SEM analysed using One-Way ANOVA with Bonferroni's post hoc correction where appropriate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.0001$.

At experimental endpoint, nephrectomized KC subjects returned hyperglycemic within 48 hours after removing graft-bearing kidneys **(arrow, Fig. 3.2A)**. At the time of graft recovery, alginate discs remained secured in the fat pads **(Fig. 3.4A)**, and the whole pad had to be excised to harvest the graft **(Fig. 3.4B)**. Total insulin content in removed pancreases from *Encap* and *Non-Encap* groups was calculated (1.3 ± 0.2, n=9; 2.1 ± 0.4, n=14 respectively) and compared to values obtained from pancreases of naïve mice (34.8 ± 2.0 µg, n=8). Analyzing the total cellular insulin content of the recovered grafts from the three experimental groups revealed a statistically significant difference only between the KC and *Non-encap* experimental groups ($p < 0.05$). Insulin content from harvested grafts was as following: KC: 17.0 ± 3.0 (n=8), *Encap*: 15.5 ± 4.8 (n=6), *Non-encap*: 6.8 ± 1.5 (n=14). IHC staining for insulin-positive cells displayed abundant cells with robust insulin

staining in all experimental groups (KC; *Encap*; *Non-encap*; Fig. **3.5A-C**), confirming graft viability and function.

3.4 – DISCUSSION

Intra-hepatic islet embolization following portal vein infusion is the standard implantation site in experimental and clinical transplantation, despite all drawbacks mentioned before. Renal subcapsular space is the most extensively used site for islet transplantation in smaller experimental animal models; however, practical application of that ectopic site is less likely to happen in the clinical setting. Subcapsular space in human kidney is not as prominent as in rodents; the capsule is thinner, weaker, and tethered to the parenchyma of the kidney. Even with meticulous dissection, the space has limited capacity that cannot accommodate large islet mass. Gorth et al.[28] reported transplantation of a kidney, with fetal porcine islet aggregates placed under its capsule. That procedure resulted in short-term detection of porcine C-peptide in the patient's circulation, without achieving insulin independence.

In this chapter, we reported the survival and metabolic function of islet xenografts transplanted in fat pads of male diabetic mice, using alginate as a biocompatible material for scaffold fabrication. Shortly after transplantation, mice exhibited sharp decline in hyperglycemia, associated with overall health improvement manifested by animal activity, and shiny fur. Generally, an efficient ectopic site should be accessed easily with minimally invasive, less time consuming, and repeatable procedure; capable of accommodating large cellular volumes and/or scaffolds; highly vascularized to support graft viability and enhance its metabolic function; mimic as possible the native microenvironment of donor

tissue; and protect the graft from recipient's hostile immune/inflammatory response. Among many proposed sites, intra-abdominal peritoneal reflections stand out as a highly conceivable candidate. Our lab and others displayed successful outcomes when transplanted syngeneic or xenogeneic islets, using various techniques.[8-12] The Diabetes research Institution (DRI) and the Ricordi group have obtained the FDA approval for their BioHub Strategy, in which they transplant allogeneic islet imbedded into biocompatible/biodegradable scaffold into the greater omentum, using laparoscopic surgery.[29]

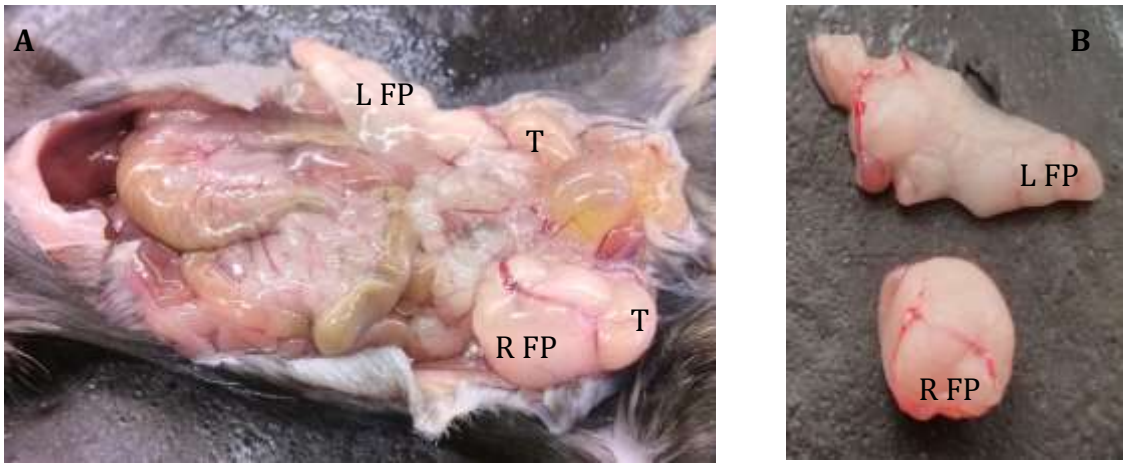

Figure 3.4. Macroscopic assessment of fat pad grafts at the time of recovery

A) At the time of graft recover, alginate discs were found secured in their transplant location

B) Excision of both fat pads for comparison. Images captured using digital still camera.

R FP; right fat pad, **L FP**; left fat pad, **T**; testicle

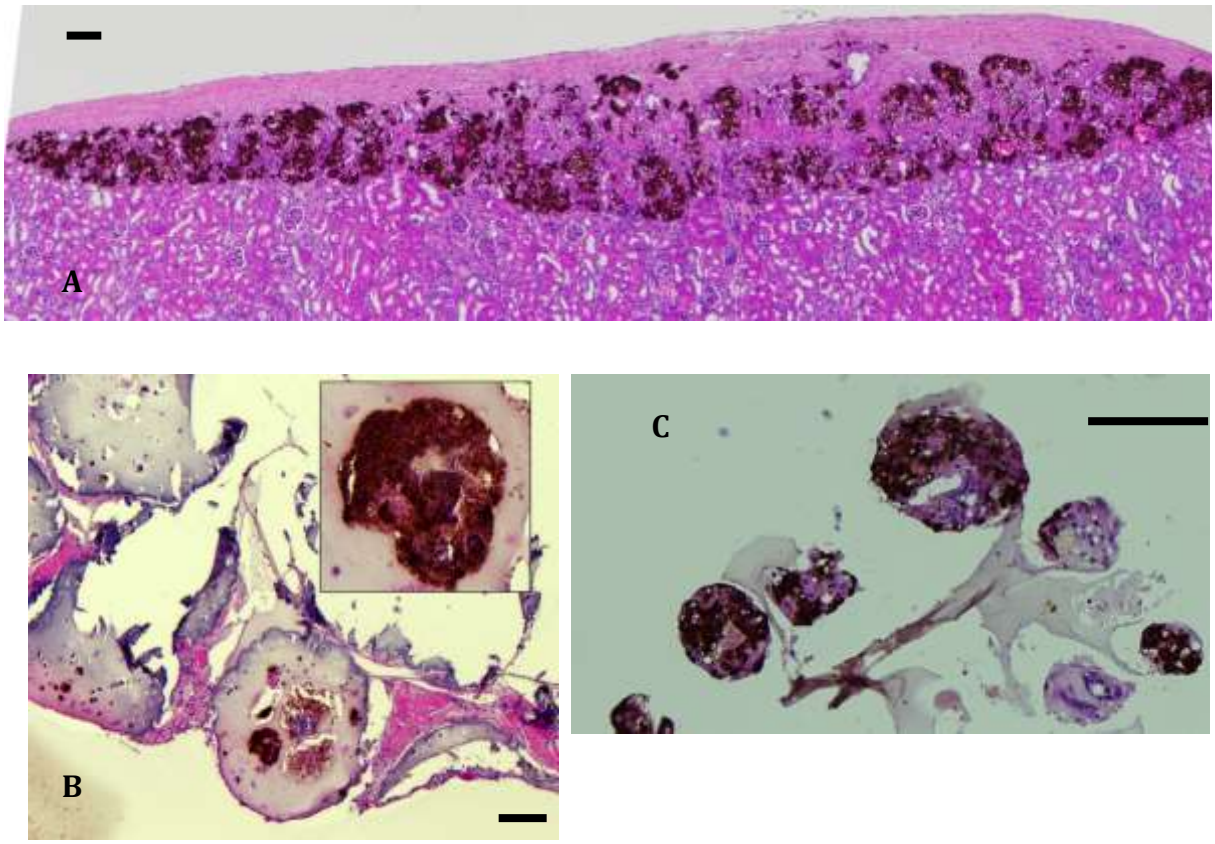

Figure 3.5. Immunohistochemistry staining of recovered grafts

Immune staining for insulin-positive cells (brown) in grafts recovered from KC (a), *Encap* (b), and *Non-encap* (c), 12 weeks post-transplantation. Scale bars = 200 µm

In this experiment we transplanted human islets to diabetic and immune deficient animal model. Post-transplantation metabolic function of human islets is variable, and depends on donor's age, sex, life style, cause of death, cold ischemia time…etc.[30-32] We exploited this fact as a simple and quick preliminary indicator for engraftment success; rapid and sustained control of hyperglycemia means that the ectopic site is capable of supporting fragile human islets when transplanted in alginate scaffolds. Over the course of 12 weeks, human islets were able to remain viable and to control glycemic excursions in diabetic mice. During glucose tolerance testing, the three transplant conditions were able to clear the elevated glucose; another indicator for proper metabolic function. However, the significant increase in AUC in *Non-encap* group, in addition to the low total cellular insulin content, suggests loss of some of the transplanted islet mass. A conceivable explanation would be that non-encapsulated islets are over-crowded with no proper oxygen diffusion, compared to encapsulated islets that have sufficient surface area, granted by the vicinity of the capsule.

Alginate is a linear polymer derived from brown seaweeds. It is based on two monomeric units: β-D-mannuronate (M unit) and α-L-guluronate (G unit). It can form chains of pure M units (M blocks), pure G units (G blocks) or a mixture of both (MG blocks); blocks are capable of forming 3-D structures by bonding together.[33] Alginate co-polymer gels instantly when it comes in contact with divalent cations such as calcium, magnesium, or barium. Alginate gels with higher content of G blocks yields stronger, more stable, and more porous capsules.[34] There are many factors that control the success of production of alginate beads; the purity of alginate, its dilution, the size and shape of the bead, and number of islets per bead. In this experiment, we used alginate in a novel way to

circumvent graft clumping and hypoxia pointed out in **Appendix A**. we fabricated a scaffold for graft delivery by embedding human islets (either encapsulated or non-encapsulated), using a 48-well plate as a template for disc forming. The scaffold was solid at the time of transplantation and recovery, with no breaking or fragmentation. Wang et al. published an online video protocol for syngeneic islet transplantation in the fat pad, using decellularized scaffold. Our experiment reports same technique and outcome, yet we demonstrated efficacy of alginate as an easy, simple, and affordable biocompatible material for scaffold fabrication.

One of the anticipated limitations of this study is using the fat pad not the omentum. Fat pads are intra-abdominal fat depot in rodents; continuous local insulin secretion might contribute to localized fat accumulation at the transplantation site. By time, the increased localized fat buildup will minimize the oxygen diffusion from the nearby capillaries, and eventually leads to graft hypoxia. This could be avoided by using an angiogenic scaffold material that would stimulate and enhance angiogenesis in the transplantation site (as shown in **Appendix B**). Another expected drawback for using the fat pad was the systemic venous drainage, compared to the portal drainage of omentum. Nonetheless, IPGTT results displayed rapid graft response and efficient glucose clearance, which implies real-time glucose sensing and insulin secretion.

In this report, we demonstrated the proof-of-concept of using intra-abdominal peritoneal folds and fat pads as ectopic sites for islet transplantation. Also, we demonstrated a novel way for employing alginate co-polymer in cell delivery and islet transplantation.

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CHAPTER 4.

IMPACT OF SEX DIFFERENCE ON NEONATAL PORCINE ISLET XENOGRAFTS TRANSPLANTED IN DIABETIC MICE

4.1 – ABSTRACT

Beta (β)-cell replacement therapy is a conceivable alternative for daily exogenous insulin injections in treating type 1 diabetes mellitus (T1DM). In 2000, seven patients achieved long-term insulin independence after receiving fresh islets isolated from multiple cadaveric donors. Patients were maintained on steroid-free immunosuppressive regimen, without any major or life-threatening perioperative complications. The Edmonton Protocol has been employed since then as a standard clinical procedure for intrahepatic islet transplantation in many centers worldwide. Global reports are showing more improvements in islet procurement and infusion, with longer periods of insulin independence postoperatively.

Usually, a single recipient would receive islets from more than one donor, and it is not uncommon to require more than one islet infusion in order to attain glycemic control. This is one of the hurdles that impede the clinical expansion of β-cell replacement therapy. There are multiple strict criteria for selecting recipients in order to decrease donors-per-recipient ratio. Nonetheless, recent reports underscore the possible influence of sex difference on glucose metabolism. Females tend to display better glucose tolerance and increased peripheral insulin sensitivity, compared to males. This might suggest that females might be better candidate for islet transplantation, or they might achieve normoglycemia using lower β-cell mass.

In this report, we explore the possible impact of recipient's sex on islet graft behaviour. We transplanted male and female diabetic mice with neonatal porcine islet (NPI) xenografts in the subcapsular space. Gender impact would be observed through time

taken to achieve normoglycemia, graft response during glucose tolerance testing, and graft characterization at the experimental endpoint. We hope that the outcome would shed more light on the impact of recipient's sex the outcome of islet transplantation.

4.2 – INTRODUCTION

Diabetes is a chronic metabolic condition characterised by sustained hyperglycemia, due to absolute deficiency of insulin secretion, resistance to insulin's action on peripheral tissues, or both.[1] Type 1 diabetes (T1DM) is an autoimmune disorder that incites progressive destruction of pancreatic β-cells, leading to insulin deficiency and loss of glucose homeostasis.[2] Pancreatic islet transplantation is an attractive alternative to parenteral insulin replacement, particularly in a subset of patients where strict insulin regimens fail to control glycemic excursions.[3]

In 2000, a group of seven patients achieved prolonged insulin independence after receiving freshly isolated islets from multiple donors, and using a non-steroidal immune suppression regimen.[4] Since then, The Edmonton Protocol (developed by the Islet Transplant Group in Edmonton) has been widely applied by multiple centres around the world, and post-transplant insulin independence rates are now comparable to results acquired by whole pancreas transplantation.[5,6] Unfortunately, limited cadaveric islet supply and the acute postoperative graft loss are few of many obstacles preventing the widespread application of this cellular replacement therapy.[7,8] Immediately after intrahepatic implantation, islets trigger non-immune-mediated coagulative inflammatory response that eventually leads to acute loss of more than 50% of the initial graft mass.[9,10] Hence, finding

a virtually unlimited donor tissue source is necessary for expanding islet transplantation, and making β-cell replacement therapy more accessible for more diabetic patients.

Porcine islets represent a promising tissue source for clinical islet xenotransplantation, and our group developed a simple and reproducible protocol for mass isolation[11,12] and differentiation[13] of neonatal porcine islets (NPI). These immature endocrine aggregates differentiate and become glucose responsive *in vitro* and *in vivo*, and capable of reversing diabetes in multiple animal models.[11,14-16] Moreover, NPI exhibit resistance to hypoxia-induced apoptosis[17] and hyperglycemia,[18,19] which make them a potential alternative for cadaveric human islets in experimental and clinical β-cell transplantation.

The impact of host's sex on disease pathophysiology [20-23] and/or treatments [24-27] is gaining more interest recently. Therefor, exploring the recipient's sex and its influence on islet survival is another strategy to address post-transplant graft attrition and subsequent loss of insulin independence. Pregnancy is one of the distinctive female-related periods where the whole body undergoes tremendous changes to accommodate the increased metabolic and circulatory workloads caused by the growing foetus.[28] Pregnancy-associated hormones promote a temporary increase in the pancreatic β-cells mass to maintain the glycemic homeostasis.[29-32] Even in non-gestational periods, females display a different hormone-related metabolic pattern compared to males.[33-35]

In a recently published article,[36] Saber et al. investigated the possible impact of pregnancy on human stage 4 pancreatic progenitors driven from induced embryonic stem cells (S4; hESC-derived pancreatic progenitors). They also looked at the probable effect of

the recipient's sex on the maturation of both S4 and S7 hESC-derived pancreatic progenitors. According to their reported results, both human S4 and S7 grafts have responded to glucose stimulation in female recipients prior than their male counterparts.[36] In this article, we transplanted NPI to male and female diabetic immune deficient mice to investigate the possible influence of recipients' sex on islet xenografts. This is another step taken in order to investigate the potential of porcine islets as possible alternative source for clinical islet transplantation, and to explore the possible effect of host's sex hormones on the outcome of pancreatic islet transplantation.

4.3 – MATERIALS AND METHODS

4.3.1 – Islet preparation

All procedures involving animals were carried out according to the guidelines established by Canadian Council on Animal Care. Donor pancreases were surgically removed from either sex neonatal piglets (Swine Research and Technology Center, University of Alberta, Edmonton AB CAN). Neonatal porcine islets were isolated and kept in Ham's-F10 tissue culture medium (Sigma-Aldrich, St. Louis MO USA), as previously described.[11] At the third day of culture, the incubation medium was switched to DMEM/F12 (Thermofisher, Burlington ON CAN) supplemented with 1% (vol/vol) pig serum (heat inactivated), 0.2 mM Insulin, 7 μM Transferrin, 4 μM Sodium Selenite (ITS; Thermofisher), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Cedarlane, Burlington ON CAN), 10 mM nicotinamide (Sigma-Aldrich), and 10 nM GLP-1 analogue (Exendin-4; Sigma-Aldrich). A total of four independent NPI isolations were used for the transplant experiments reported in this study.

4.3.2 – Transplantation and metabolic follow-up

Male and female, inbred, B6.129S7-Rag1^{tm1Mom}/J mice (The Jackson Laboratory, Bar Harbor Main USA) were used as recipients of neonatal porcine islets. Animals were maintained under virus-antibody-free conditions in climatized rooms with free access to sterile tap water and pelleted food. Mice were rendered diabetic by a single intraperitoneal injection of streptozotocin (STZ; 185 mg/kg for males and 200 mg/kg for females, freshly dissolved in acetate buffer; Sigma-Aldrich) 2-4 days before transplantation. Blood samples were obtained from the tail vein for glucose measurement (OneTouch UltraMini glucose meter, LifeScan Europe, Zug Switzerland). Mice were considered diabetic when the blood glucose level was greater than 20 mM.

Islets were transplanted into male (n=11) and female (n=11) diabetic mice under the kidney capsule (KC). After lateral laparotomy, grafts were aspirated into polyethylene tubing (PE-90; Becton Dickinson, Sparks MD USA) and pelleted by centrifugation then gently placed under the capsule of the left kidney with the aid of a micromanipulator syringe.[11] All mice were monitored for non-fasting blood glucose levels within the first week post-transplant, and once a week thereafter.

4.3.3 – Glucose tolerance testing

When the blood glucose level was ≤ 11.1 mM for two consecutive weeks, mice were deemed normalized, and an intra-peritoneal glucose tolerance test (IPGTT) was performed. After 12 hours fasting, D-glucose (3 g/Kg) was administered as a 50% solution IP into non-anesthetized mice, and blood glucose was measured at 0, 15, 30, 60, 90 and 120 minutes. Glucose clearance was compared to naïve mice, plotted and expressed as area

under the curve (AUC). To determine the insulin release in response to elevated blood glucose, insulin (pg/mL) in blood samples collected at 60 minutes post glucose administration was compared to insulin levels in blood samples collected prior to fasting. Stimulated increase (SI) in insulin was calculated by dividing plasma insulin (pg/mL) at 60 minutes by plasma insulin (pg/mL) prior to fasting. Plasma insulin was measured using the Ultrasensitive Insulin ELISA (ALPCO, Salem NH USA) that does not detect mouse insulin. An independent IPGTT was performed on non-diabetic, non-transplanted male and female naïve mice (n=9 each), and murine plasma C-peptide secreted at time 0 and 60 min was measured as well using a specific ultrasensitive murine insulin assay (ALPCO). [36]

4.3.4 – Termination and characterization of harvested grafts

At week 20 post-transplant, mice from both sexes underwent survival nephrectomy of the graft-bearing kidney, and nephrectomized animals were monitored to confirm the recurrence of hyperglycemia within 48 hours. Afterwards, animals were euthanized. Few randomly selected islet-containing grafts were processed for histological assessment/insulin staining. Explanted grafts were trimmed and fixed in 4% (wt/vol) paraformaldehyde (Thermofisher), embedded in paraffin blocks and subsequently stained for insulin using horseradish peroxidase immune-histo-chemistry technique.[11] Primary antibodies for immunostaining included; 1/1000 anti-insulin (DAKO Mississauga ON CAN), 1/5000 anti-glucagon (Sigma-Aldrich). All appropriate species-specific biotinylated secondary antibodies were diluted 1/200 in 5% normal goat serum. Detection of labeled insulin and glucagon was done using the avidin-biotin complex method (Vectastain® ABC kit; Vector laboratories, CA USA) with peroxidase and diaminobenzidine (DAB; BioLegend, MA USA) as the chromagen.[11]

To assess the effects of sex difference on graft vascularization, tissue sections from multiple grafts (3 individual sections per graft, 200 μm apart) from both groups were double immune-stained for insulin- and CD31-positive cells. Primary antibodies included 1/1000 anti-insulin (DAKO), and 1/50 anti-CD31 (AbCAM Cambridge MA USA). All appropriate species-specific secondary antibodies were AlexaFluor 488 or 594 conjugates (Thermofisher) and diluted 1/200 in 5% normal goat serum. To assess the graft vascularization difference between male and female transplanted mice, the CD31-positive areas and the DAPI-positive cell number were quantified using ImageJ Software (http://rsb.info.nih.gov/ij/download.html). The ratio of CD31+ vasculature was then calculated as described previously.[37] Negative controls included sections of the same tissues incubated only with the secondary antibodies, whereas positive controls included sections of neonatal porcine pancreas stained for both insulin and CD31.

The remaining grafts were assessed for total cellular insulin content according to the previously published protocols.[11] Briefly, tissue homogenates were sonicated in 10 ml of 2 mmol/liter acetic acid (0.25% BSA), incubated for 2 hours at 4 C, centrifuged (10,000 RPM, 25 min), then supernatants were collected and the pellets further extracted by sonication in an additional 5 ml of acetic acid. The second supernatant was collected after centrifugation, combined with the first supernatant and samples were assayed for insulin by metabolic assay (MesoScale Discovery, Gaithersburg, MD USA).

4.3.5 – Statistical analysis

Data is expressed as mean ± SEM of n independent observations. Statistical significance of differences was calculated with Log-Rank (Mantel-Cox) sum test, or

Student's *t*-test when appropriate. A *p*-value of <0.05 was required to deem results significant.

4.4 – RESULTS

4.4.1 – Male mice have significantly higher body weight than female mice

Weights of transplanted mice were recorded before chemical induction of diabetes (Pre) and once more before glucose tolerance test (GTT) after normalization. Also, weights of naïve mice that participated in the independent IPGTT were recorded as well (Naïve) for comparison **(Tab 4.1)**. Generally, weights of male mice (including naïve controls) were significantly higher compared to female mice of the same age ($p < 0.0001$; male vs. female, **Tab. 4.1**). Subsequently, female mice received more islets/g body weight compared to male counterparts, as we transplanted ~5,000 NPI graft per animal regardless their body weights. On average, male mice received ~ 172.8 ± 4.2 NPI/g, compared to ~ 219.5 ± 3.2 NPI/g for female transplants ($p < 0.0001$). Nonetheless, weights of male and female transplanted mice after achieving normoglycemia post-transplantation were comparable to their weights before induction of diabetes ($p > 0.05$, GTT vs. Pre; **Tab. 4.1**).

4.4.2 – Metabolic follow-up

Non-fasting metabolic follow up of transplanted animals has shown steady decrease in blood glucose over time for all subjects from both sexes **(Fig. 4.1A)**, with significantly lower blood glucose levels detected in female diabetic mice during most of the follow-up time points ($p \leq 0.05$). All female subjects have attained normoglycemia between 5 and 11 weeks post-transplantation **(Fig. 4.1B)**, while only 10 out of 11 male subjects have shown

reversal of hyperglycemia between 7 and 20 weeks post-transplantation ($p \leq 0.001$, **Fig. 4.1B**).

4.4.3 – Graft response to glucose challenge

IPGTT was performed once the transplanted animals exhibited sustained normoglycemia (two consecutive non-fasting blood glucose readings ≤ 11.1 mmol/L), between 14 and 20 weeks post-transplantation. Both sex groups displayed rapid, near-identical pattern of glucose clearance within the first 60 minutes of the test with no statistically significant differences between them, except in the fasting blood glucose reading (0 min) recorded before injection of glucose (lower fasting glucose levels in males vs. females, $p \leq 0.05$; **Fig. 4.2A**)

	Transplanted		Naïve
	Pre	GTT	
Male	29.1 ± 0.7 [a]	27.7 ± 0.5 [b]	26.8 ± 0.5 [c]
Female	22.8 ± 0.3	22.2 ± 0.2	20.9 ± 0.3

Table 4.1. Body weights of male and female transplanted and naïve animals

Body weights (in grams) of male and female transplanted and naïve mice recorded before induction of diabetes (Pre) or during glucose tolerance testing (GTT and Naïve). Data expressed in mean ± SEM, [a, b, c] $p < 0.0001$ (male vs. female during same weighing occasion), using non-paired and paired Student's *t* test when appropriate.

Computed area under curve (AUC) from IPGTT **(Fig. 4.2B)** revealed no significant difference between male and female transplants groups ($p > 0.05$; **Tab. 4.2**). Analysis of plasma samples obtained during IPGTT has shown that post-injection circulating porcine insulin levels detected in male and female transplanted mice were significantly higher than their basal levels ($p \leq 0.05$, **Fig. 4.2C**). Moreover, the stimulated porcine insulin levels detected in female transplanted subjects were significantly lower than their male counterparts ($p \leq 0.05$, **Fig. 4.2C**). The stimulation index (SI; computed by dividing stimulated insulin/basal insulin) was comparable between those two groups, with no statistically significant difference ($p > 0.2$, **Tab. 4.2**).

4.4.4 – Naïve mice response to glucose testing

When we compared results obtained from transplanted mice to the outcome of an independent IPGTT on male and female naïve mice, we found a close similarity pertaining the graft secretory response in females compared to males. However, naïve animals showed a prolonged glucose clearance time compared to transplanted animals **(Fig. 4.2D)**; that significant delayed clearance was underscored when we compared AUC **(Fig 4.2B,E)** between naïve and transplanted animals ($p \leq 0.0001$, **Tab. 4.2**). Analysis of circulating murine C-peptide in plasma samples obtained from naïve mice has revealed similar outcome to what was detected in transplanted animals **(Fig. 4.2F)**. Both sexes has shown a significant post-injection increase in the circulating murine C-peptide compared to basal levels, yet, stimulated C-peptide levels detected in male naïve mice were comparable to their female counterparts ($p > 0.05$, **Tab. 4.2**). We also compared basal and glucose-stimulated insulin/C-peptide between both sexes transplanted and naïve animals. Fasting insulin/C-peptide levels were comparable in either sex naïve/transplanted animals **(Tab.**

4.2). However, male and female transplanted mice have shown significantly less stimulated insulin/C-peptide compared to naïve animals ($p \leq 0.005$, **Tab. 4.2**). Lastly, the SI of female transplanted animals was significantly lower than female naïve counterparts ($p \leq 0.01$, **Tab. 4.2**)

At experimental endpoint, nephrectomized KC subjects returned hyperglycemic within 48 hours after removing graft-bearing kidneys **(Arrow, Fig. 4.1A)**. Total insulin content in removed grafts from male transplant group was significantly higher than what was calculated from female counterparts (48.7 ± 5.3 vs. 23.7 ± 2.8 μg respectively, $p \leq 0.01$)

4.4.5 – Histological assessment

IHC staining for insulin-positive cells revealed abundant cells with robust insulin staining in both experimental groups (**Fig. 4.3A, B**). We quantified the CD31-positive areas in relation to the number of DAPI-positive particles [37] as an indicator for the extent of graft vascularization, and to see if the host's sex would affect the degree of neovascularization or not. The quantification has revealed no significant difference regarding degree of vascularization between the two experimental groups (417.1 ± 37.6 vs. 364.6 ± 30.3 area/cell male vs. female, $p > 0.2$)

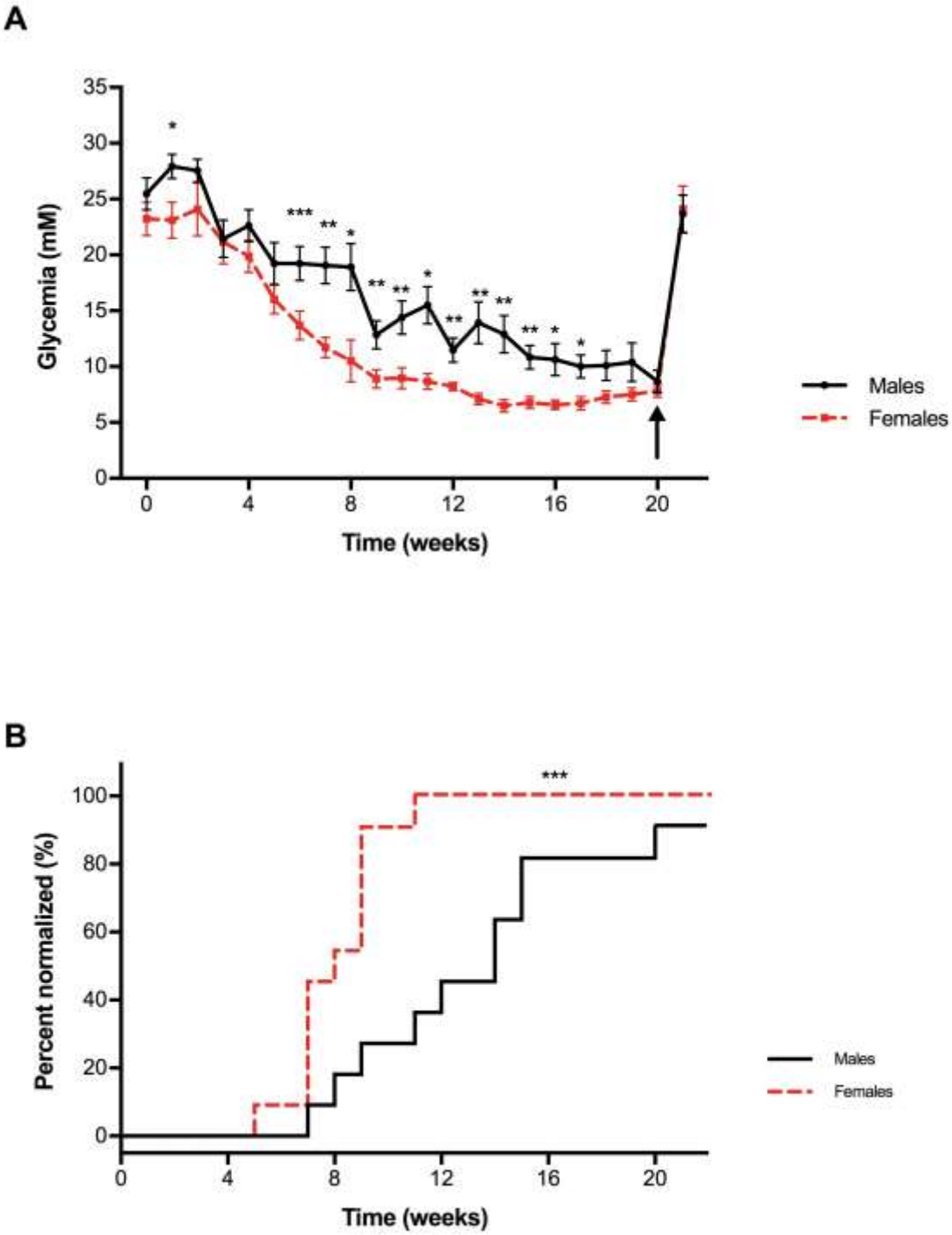
A
Glycemia (mM)
35
30
25
20
15
10
5
0
0
4
8
12
16
20
Time (weeks)
Males
Females
B
Percent normalized (%)
100
80
60
40
20
0
***
Time (weeks)
Males
Females

Figure 4.1. Post-transplantation metabolic follow-up of male and female diabetic transplanted mice

Weekly follow-up of male (n=11, Black line) and female (n=11, Red line) diabetic B6/Rag$^{-/-}$ mice transplanted with 5000 neonatal porcine islets under the kidney capsule. **A:** Blood glucose levels assessed weekly from 0 to 20 weeks post-transplant. **B:** Percentage (%) of mice reverted to normal blood glucose levels (glycemia < 11.1 mM for 2 consecutive weeks) over time course of experiment. At the endpoint of the experiment, the graft-bearing kidneys were removed via a survival nephrectomy procedure (arrow), and animals were monitored for return of diabetes. Data expressed in mean ± SEM, * $p<0.05$, ** $p<0.001$, *** $p<0.001$ using non-paired Student's *t* test, or Log-rank (Mantel-Cox) test for statistical analysis when appropriate.

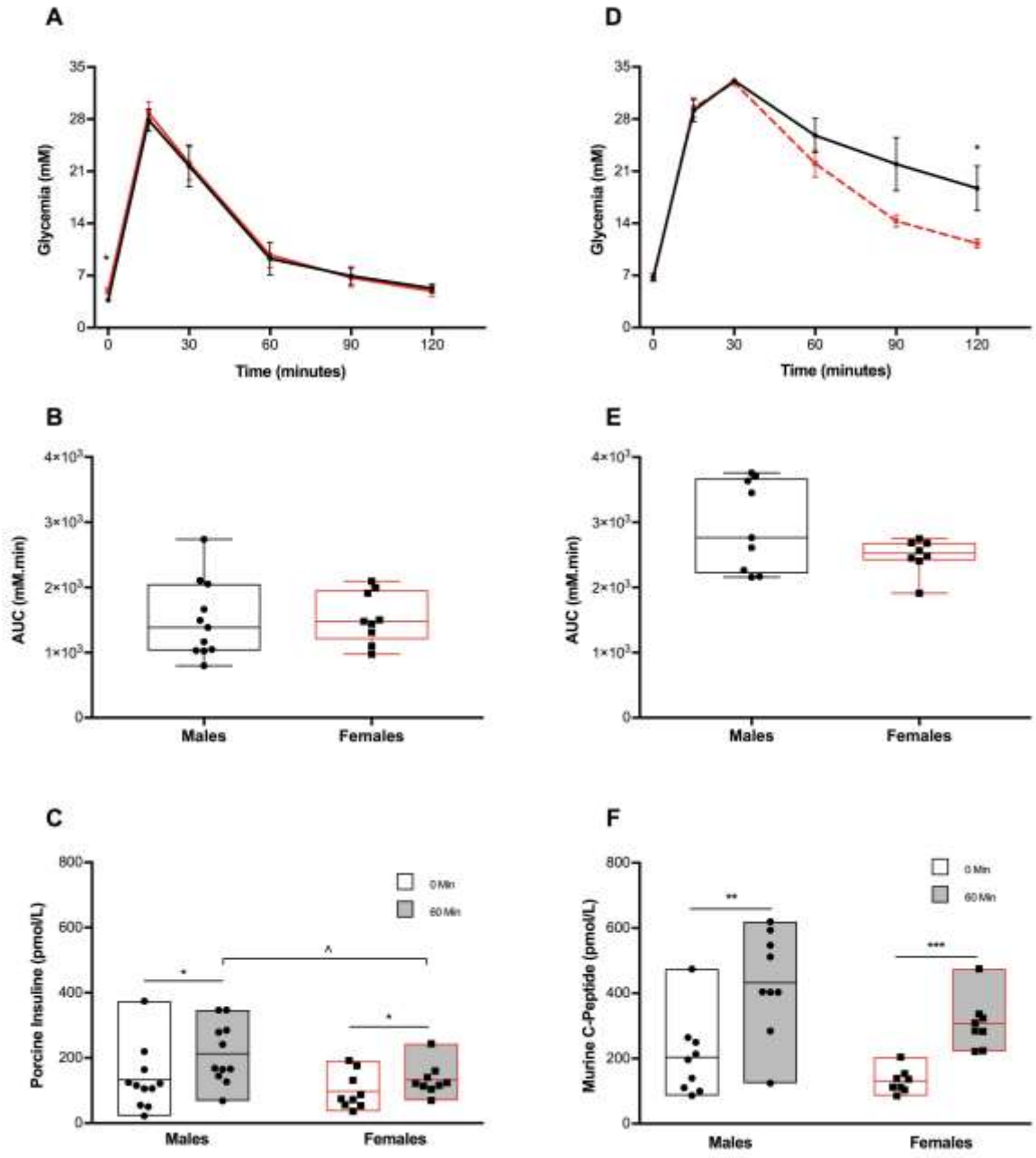
A
Glycemia (mM)
Time (minutes)
D
Glycemia (mM)
Time (minutes)
B
AUC (mM.min)
Males
Females
E
AUC (mM.min)
Males
Females
C
Porcine Insuline (pmol/L)
0 Min
60 Min
Males
Females
F
Murine C-Peptide (pmol/L)
0 Min
60 Min
Males
Females

Figure 4.2. Glucose tolerance testing in transplanted and controlled mice

Results from glucose tolerance test performed after mice have attained normoglycemia, between 14 and 20 weeks post-transplantation (A-C), or naïve control mice (D-E).

(**A)** Blood glucose values during an IPGTT (n=11 males, Black line; n=9 females, Red line).

(**B)** Computed area under the curve for respective IPGTT (A).

(C) Porcine insulin calculated in plasma samples collected from male and female diabetic transplanted mice at 0 and 60 minutes during respective IPGTT.

Similar IPGTT was performed on naïve male and female control mice (n=9 each), displaying blood glucose values **(D)**, computed AUC **(E)**, and calculated murine C-peptide in plasma samples obtained at 0 and 60 minutes **(F)**.

Data expressed in mean ± SEM, * $p<0.05$, ** $p<0.001$, *** $p<0.001$ using non-paired and paired Student's *t* test when appropriate

	Males		Females	
	Tx	**Naïve**	**Tx**	**Naïve**
AUC	1500.7 ± 169.9 [a]	2946.9 ± 216.9	1534.3 ± 123.6 [b]	2491.7 ± 87.4
T0	132.7 ± 27.9	203.8 ± 37.9	97.8 ± 17.6	130.9 ± 12.1
T60	212.7 ± 26.7 [c]	432.4 ± 49.6	132.7 ± 15.2 [d]	307.0 ± 26.5
SI	2.1 ± 0.3	2.6 ± 0.4	1.6 ± 0.2 [e]	2.4 ± 0.2

AUC; Area under curve, **Tx**; Transplanted animals, **Naïve**; Control animals, **T0**; Basal, **T60**; Stimulated, **SI;** Stimulation index

Table 4.2. Sex-related observed differences pertaining glucose clearance rate, basal and stimulate insulin/C-peptide, and stimulation index, during glucose tolerance testing

Glucose clearance rate (AUC; mmol.min) calculated during IPGTT. Basal or Stimulated porcine insulin (Tx mice) or murine C-peptide (naïve control mice) detected in plasma samples collected during glucose tolerance testing. SI calculated by dividing T60 over T0.

Data expressed in mean ± SEM, [a, b] $p < 0.0001$, [c] $p < 0.005$, [d] $p < 0.001$, [e] $p < 0.01$ (transplanted vs. control mice, within same sex), using non-paired and paired Student's *t* test when appropriate.

4.5 – DISCUSSION

Glucose homeostasis is crucial process achieved through precise coordination between various organs and tissues such as the pancreas, liver, muscles, brain, and adipose tissue. Nonetheless, multiple factors that affect euglycemia – either positively or negatively – are not elucidated yet. In recently published [36] report; Saber et al. has illustrated the effect of host's sex on the time taken by induced hESC to respond to glucose stimulation. In this study, we investigated the possible influence of recipients' sex on porcine islet xenografts behaviour. We were able to observe the undoubted host's effect on NPI grafts through the time taken to achieve normoglycemia, the response towards the standardized glucose challenge test, and post-mortem cellular graft characterization. NPI grafts show a delayed control over hyperglycemia [11,13] due to their immaturity at the time of transplantation,[11] and diabetic animals experience noticeable weight loss during that period. We showed in previous report [37] that mice regain the wasted body weight by the time they attain normoglycemia. In this report, we recorded animals' body weights before induction of diabetes, and once more after normalization of blood glucose levels to highlight the difference in their naïve weights, and to see if animal's sex would impact weight recovering. Males reported heavier weights in both occasions (naïve and post normalization). Nonetheless, female transplanted mice regained an average of 99% of their naïve pre-transplant weight, compared to 92% in their male counterparts. This highlights the anabolic efficacy of porcine insulin, and the great potential of NPI as an efficient alternative tissue source for clinical islet transplantation. On average, female diabetic mice achieved normoglycemia after 56 days post-transplantation versus 84 days in their male diabetic counterparts.

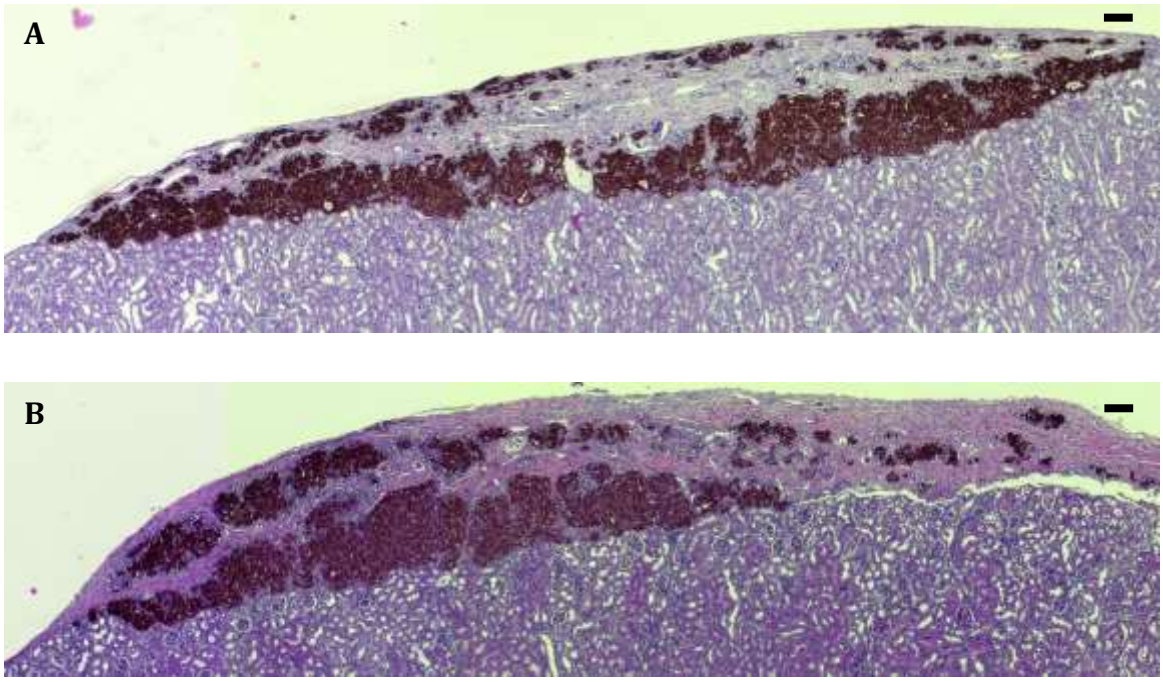

Figure 4.3. Histological assessment of NPI grafts

IHC staining of graft-bearing kidney slices obtained from male (A), and female (B) mice, at the experimental end point. Tissue was stained for Insulin (brown), then counterstained using haematoxylin and eosin histological staining. Image captured using 4x magnification objective lens. Scale bar = 100 μm

During the experimental period, female mice exhibited a steady decline of elevated blood glucose levels compared to the male cohort. The transplanted NPI grafts were able to reverse hyperglycemia – and remain euglycemic – in all animals participated in this study, except one subject in the male transplant group. That male subject displayed noticeable

decline of hyperglycemia (lowest recorded was 13.6 mmol/L), yet, did not show a full control on elevated blood glucose level. At the end of the experiment, all mice underwent survival nephrectomy of the graft-bearing kidney and subsequently returned diabetic, including the male mouse mentioned formerly. This confirms the sustained viability and in vivo maturation of neonatal porcine xenografts, and its capability of controlling hyperglycemia in diabetic animals. The faster normalization in females was thought to be a result of the significant discrepancy in transplanted β-cell mass between male and female mice. Since we transplanted fixed islet mass disregard to the body weight of the recipient, then it was expected that female mice received more islet mass per gram body weight. However, analyzed total cellular content of the explanted graft at the experimental endpoint revealed the opposite; grafts obtained from female mice contained on average 50% less insulin than grafts harvested from male mice (48.7 ± 5.3 and 23.7 ± 2.8 μg, male vs. female, $p \leq 0.01$). This observation suggests that earlier control of elevated glucose levels in female participants is not related primarily to the initial graft mass or body weight.

Euglycemia relies on a fine tuned balance between islet responsiveness –manifested by rapid insulin secretion, and peripheral tissue sensitivity demonstrated by swift glucose uptake. To investigate the NPI graft responsiveness, we performed glucose tolerance test on transplanted animals after attaining normoglycemia. Both male and female transplanted mice showed a rapid, near-identical response, and were able to clear the spiked glucose level within the first 60 minutes. When we analyzed the basal and stimulated circulating porcine insulin obtained during the IPGTT, we found that the graft secretory response (represented by the amount of stimulated porcine insulin) in male mice was almost double of what was detected in their female counterparts. This finding led us to run an independent

IPGTT on male and female naïve mice to see if the former finding was specific to porcine islet xenografts or not.

When we compared the AUC computed from IPGTT, naïve animals from both sexes took longer time to clear the glucose load compared to transplanted animals. That was expected as we demonstrated in a previous report (submitted for publication at the time of writing this article) that NPI-transplanted diabetic mice would show lower euglycemic level and faster glucose clearance rate, compared to naïve mice.[39] Nevertheless, female naïve mice displayed a significantly lower postprandial glucose levels than their naïve male counterparts, despite being almost identical during the first hour of the test. More importantly, when we analyzed the graft secretory response in naïve animals, we found it similar to what was observed from porcine xenografts in transplanted cohorts; lower stimulated murine C-peptide detected in female mice plasma compared to males.

The most conceivable explanation for this finding can be attributed to the effect of estrogen hormone in female mice. More research evidence highlights the role of estrogen on insulin biosynthesis, protection of β-cell against oxidative stress, enhancing peripheral insulin sensitivity, and improving glucose uptake and storage by skeletal muscles and adipose tissue.[39,40] Estrogen (17β-estradiol; E2) is a steroid sex hormones synthesized mainly by the ovary in females through the activity of Aromatase enzyme on gonadal/adrenal androgen. In males, E2 is synthesized as well in tissues that express aromatase enzyme, yet in lesser biological concentrations.[41] Similar to any steroid hormone, E2 diffuses easily through the cell membrane to bind with its intracellular receptors (ER) where it exerts its action. ER are distributed widely throughout multiple target tissues, including pancreatic islets.[42] In pancreatic β-cells, E2 enhances the

biosynthesis of insulin through its action on ERα mainly, modulating insulin gene expression (via ERK1/2 pathway) and increasing the cellular insulin content.[43] This "inverted U-shaped dose response" was abolished in islets obtained from ERα-knockout mice, as they failed to respond to the upregulating effect of E2 in vitro.[43] E2 is involved as well in glucose-stimulated insulin secretion (GSIS) via the action trans-membrane G protein-coupled receptor (GPCR) GPR30.[44-48] Both E2 and GPR30 agonist were able to enhance the GSIS in a dose-dependant manner from male and female mice islets, via increasing intracellular cAMP concentration.[49,50] Moreover, E2 exerts a cytoprotective effect on pancreatic β cells by reducing endoplasmic reticulum-induced apoptosis. It reduces the reticular oxidative stress by attenuating the expression of pro-apoptotic stress markers, increasing survival protective proteins, and enhancement of misfolded protein degradation.[50-52]

The effect of E2 extends to the peripheral insulin sensitivity and glucose uptake by storage tissues. Numerous studies highlighted the sex-related difference in glucose clearance, and have demonstrated the increased insulin sensitivity among females compared to males.[53-56] Skeletal muscle is the largest target for peripheral glucose storage; it is responsible for more than 70% of glucose clearance following intravenous glucose administration,[53,57,58] and it has been shown that E2 enhances insulin-mediated glucose uptake in skeletal muscle, mainly through ERα.[59,60] Aromatase deficiency in humans [61] or in animal models (such as aromatase knockout mice; ArKO) is associated with decreased insulin sensitivity in skeletal muscle, leading to glucose intolerance, insulin resistance, obesity, and hyperinsulinemia.[62,63]

The evidence-based effect of estrogen on β-cells and peripheral glucose uptake can explain our experimental findings. Neonatal porcine islets are immature – yet glucose responsive – at the time of transplantation, and they are capable of maturing within the recipient –despite the sustained hyperglycemic microenvironment.[18] It is conceivable to assume that the amount of insulin produced by NPI increases as the graft matures and differentiate within the host. Since female mice exhibit a natural sensitivity to circulating insulin (due to physiological concentrations of E2), then it is expected that they would show faster glycemic control as soon as the graft secretes enough insulin, sufficient to enhance glucose disposal in storage tissues. In male mice, sustained hyperglycemia (due to decreased insulin sensitivity in comparison to female counterparts) leads to more insulin synthesis and secretion to compensate for peripheral resistance. This explains the slightly elevated basal porcine insulin, the vigorous glucose-stimulated insulin secretion (detected after 60 minutes of glucose injection), and the total cellular insulin content detected in the grafts extracted from male transplanted animals.

To our best knowledge, this is the first report to underscore the sex difference effect on islet xenografts transplanted to diabetic animals. Saber et al. reported similar findings, and demonstrated that hESC-derived pancreatic progenitors mature faster in female versus male recipients.[36] hESC-derived pancreatic progenitors at both maturation stages were able to acquire glucose-stimulated C-peptide secretion in females earlier than males. Also, the less mature (S4) pancreatic progenitors lowered fasting blood glucose in females sooner than male animals, while the more mature (S7) showed similar response regardless the host's sex. Our report shows that even in diabetic microenvironment, the more mature and endocrine-dedicated NPI (compared to the hESC-derived pancreatic progenitors) were

influenced by the host sex hormones, and were able to control non-fasting hyperglycemia and reverse diabetes sooner in female transplanted mice. Moreover, we displayed clearly the similar glucose disposal response during IPGTT, highlighting the graft secretory difference between both sexes.

This study elicits more questions regarding sex difference in the clinical islet transplantation setting. Would grafts obtained from pre-menopausal donors show better survival and function than ones obtained from post-menopausal women? Do we need less β-cell mass per body weight in female recipients? Do we need to match the donor-recipient sex for optimal transplantation, and decreasing the donors-per-recipient ratio? Would pre-menopausal recipients show better glycemic control compared to post-menopausal females and/or males? Only ongoing research and evidence-based results might be able to answer these questions.

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CHAPTER 5.

GENERAL DISCUSSION AND CONCLUSIONS

5.1 – GENERAL DISCUSSION

The irreversible autoimmune damage that occurs in type 1 diabetes mellitus (T1DM) leads to enduring impairment in glucose tolerance. Longstanding hyperglycemia eventually progresses to inevitable macro- and micro-vascular complications that contribute to chronic morbidities associated with this ailment.[1] T1DM accounts for 10% of all diabetes cases, and the International Diabetes Federation estimated the number of T1DM patients globally to be around 43 millions in 2017; this number is expected to reach ~ 63 million cases by 2045.[2] The anticipated 48% hike is associated with huge socio-economic burden represented by increased load on the healthcare systems, and decreased workforce due to lingering morbidities.[2]

By far, the discovery of insulin remains the greatest event in the ominous history of diabetes. Work by Banting, Best, Collip, and other investigators led to insulin isolation and purification, that eventually saved – and still saving – millions of lives afflicted with that scourge.[3] To a far extent, exogenous insulin replacement is able to restore the impaired glycemic control, allowing tissues to uptake and utilize glucose as a source of energy needed for proper cellular functions. Since 1920s, continuous efforts have been invested to improve parenteral insulin in order to increase its efficacy, and to minimize its adverse effects.[4] From its original animal origins (such as porcine or bovine) to the current recombinant human form, insulin remains the most reliable treatment option for T1DM; nonetheless, exogenous insulin replacement is frequently accompanied by numerous – and occasionally life-threatening – drawbacks.

The concept of organ replacement for treating T1DM has been investigated since late 1800s. Surgical trials included feeding pancreas to patients, injecting animals with pancreatic extracts, surgical implantation of pancreas or pancreatic tissue.[5] Pancreas transplantation has been always associated with high perioperative morbidity risks; therefore, this therapeutic option was usually reserved for patients receiving kidney replacement, or patients with uncontrolled glycemic excursions despite strict insulin regimens. Nonetheless, anastomosis leakage, fistulas, and peritoneal infections are not uncommon complications associated with this procedure.[6] Moreover; whole organ transplantation requires intensive, steroid-based immunosuppressant agents to prevent rejection, which added a diabetogenic burden on the recipient.

After the renowned success of the Edmonton Protocol in 2000,[7] more centers worldwide adopted the approach of intrahepatic fresh islet transplantation that is followed by steroid-free immunosuppressive regimen. Exclusion of exocrine tissue from transplanted islets minimizes the immune response within the recipient, and reduces the need for stronger, steroid-based immunomodulation. However, despite all the ongoing attempts to optimize islet isolation and peri-transplantation conditions, immune suppression remains inevitable in any allotransplantation; this fact limits the broader application of this therapeutic choice for more T1DM patients. Chronic side effects of immunosuppressant agents, and the attenuation of healthy surveillance of the immune system render islet transplantation the least favourable option –especially for juvenile and young patients.[8] Therefore, additional attempts are needed to protect allografts against immune destruction, and to discard or minimize the use of harmful immunousppressants.

Furthermore, clinical islet transplantation relies on high quality cadaveric donor tissue that is usually obtained from brain-dead donors. Also, every recipient has to meet stringent transplantation eligibility criteria, requires pooled islets obtained from more than one donor, and often needs another islet infusion session(s) to attain insulin independence.[9] The former facts are few of many obstacles that impede the widespread application of clinical islet transplantation. Islet xenotransplantation is a conceivable alternative to cadaveric islets.

Tissues or organ components obtained from other species have been – and still – used in medicine and surgery; suture materials, insulin, and heart valves are few of many xenoproducts used by healthcare professionals for treating humans.[10-15] Porcine islets are virtually unlimited source for clinical-grade donor tissue. Pig herds can be raised in designated pathogen-free facilities to minimize cross-species contagion.[16] Auckland Island pigs represent a prime example for specific pathogen free organisms that carries a great potential for clinical use. In the late 1990s, wild type pig herd was relocated from Auckland Island to a designated pathogen-free facility in New Zealand. Testing those pigs revealed that they were free of pathogens that are commonly found in other domestic pigs.[17] Yet, rigorous monitoring for potential transmittable pathogens remains essential, before any clearance for large scale clinical application.[18,19] Using neonatal porcine islets (NPI) possesses numerous additional merits to the adult or juvenile counterparts. The logistical considerations of maintaining large adult herds make adult pigs less favourable compared to neonatal piglets; large herds of the later can be raised in a designated-pathogen free environment, with less economical impact.[16] Additionally, adult porcine islets are fragile

and difficult to maintain in culture, together with the variability of their isolation process, render them less desirable in clinical xenotransplantation.

Our group developed easy, simple, and reproducible procedure for large-scale procurement and isolation of NPI,[20] in addition to a practical *in vitro* multi-step differentiation protocol that aims to enhance the functional maturation of the isolated porcine islets.[21] One of the most striking qualities of NPI is their resistance to hypoxia-induced apoptosis, compared to adult porcine islets.[22] Emamaullee et al. demonstrated the survival and metabolic function of NPI after exposure to hypoxia and hypoxia/reperfusion *in vitro,* with limited apoptosis detected after 24 hours post-transplantation.[22] This natural capacity to tolerate hypoxia is attributed majorly to the high expression of a potent endogenous anti-apoptotic gene known as X-linked inhibitor of apoptosis (XIAP). High level of XIAP expression is observed in neonatal pig islets, and is diminished in adult islets.[22] Overexpression of XIAP in murine[23] and human[24] β-cells reduces the hypoxia/reperfusion cell injury, apoptosis, and subsequent graft loss.

Despite the convincing experimental data, two major issues need to be resolved before the employment of porcine islet xenografts in the clinical: zoonosis and xenogenicity. Porcine endogenous retroviruses (PERV) are DNA sequences driven from retroviruses, and embedded in the pig genome. Due to its insertion into germline cells, the viral sequence becomes endogenous and passes to the offspring.[25] There are three classes of PERV: A, B and C. Both classes A and B are polytropic – they can infect and replicate in non-porcine cells – while class C possesses an ecotropic characters with narrow infectious spectrum.[26] Nonetheless, the modern techniques for genetic manipulation enabled us to eliminate some of the naturally occurring xenoinfections such as PERV. Clustered

Regularly Interspaced Short palindromic Repeats/CRISPR-associated protein 9 (CRISPRCas9) endonucleases represent a valuable technique in genetic modification toolbox that enables researchers to target, insert, modify, or eliminate a gene (or set of genes) in xeno-cells.[27,28] In their recent work, a renowned genetics group in Harvard University was able to inactivate PERV in an immortalized pig cell line, using CRISPR-Cas9 technique.[29,30] Another challenge facing the widespread use of xenografts is the ubiquitous expression of xeno-antigens on cell surface, such as α-Gal antigens. α-Gal is not expressed in humans, or old world non-human primates; therefore, hyperacute rejection is anticipated after pig-to-human xenotransplantation due to the preformed antibodies during early years of life. Generation of α-1,3-galactosyltransferase gene knockout (GTKO)[31] pigs has been proven to be a conceivable method to mitigate the rejection of xeno-islets,[32] but failed to provide long-term protection against host response.[33] Additional gene manipulation and targeting using GTKO background pigs can be adventitious, if combined with expression of some immune modulatory molecules on porcine cells surface.[34]

5.2 – CONCLUSIONS AND FUTURE DIRECTIONS

In this thesis, I focused on investigating translatable extrahepatic sites for islet transplantation. I used NPI mainly as a donor tissue, as our laboratory is interested in optimizing porcine islets for clinical islet xenotransplantation. In order to minimize the factors that lead to primary graft failure or post-transplantation loss of function, I used an immune deficient mouse model. Rag-1 knockout mouse is an excellent, low maintenance, and widely-used animal model for testing xenografts survival and function, as it lacks functioning adaptive immune response due to absence of mature B and T lymphocytes.[35]

Rag-1 mouse is not a "leaky" model (compared to the SCID mouse), hence, immune-mediated graft loss is not expected during xenotransplantation. In my experiments, I transplanted islet grafts to diabetic mice (chemically-induced by Streptozotocin; STZ) to test the graft function; reverting to normoglycemia is a positive sure sign for transplantation efficacy. STZ is an antimicrobial/chemotherapeutic agent employed in inducing diabetes in animal models,[36] through DNA alkylation and subsequent death of β-cells.[37] We injected a single, high dose of STZ intraperitoneally, as described by Like et al.[38] Since, STZ is a biological agent synthesized by *Streptomycetes achromogenes*, we have to test every batch to determine the appropriate dose in mg per Kg animal weight to ensure that a single dose will achieve complete deficiency in insulin secretion, without acute toxicity that might lead to animal morbidities and/or mortalities. Kolb pointed out to the inconsistent outcome following diabetes induction in female mice following STZ administration.[39] This resistance is attributed to the effect of female sex hormone on β-cells; this resistance can be circumvented by increasing the dose of STZ per Kg animal weight. In our studies, we found that the appropriate dose for male and female mice is 185 and 200 mg/Kg, respectively.

The standard control site for my transplants was the subcapsular space;[40,41] the two investigated sites in the aforementioned experiments were compared to a control cohort that received equal islet mass under the kidney capsule. The survival of the graft was judged by its metabolic function, manifested by controlling hyperglycemia in diabetic recipients, and exhibiting a physiological response towards the glucose challenge test. I chose to perform intraperitoneal glucose tolerance test (IPGTT) rather than administering glucose via oral route (OGTT). Administration of glucose orally stimulates endogenous release of GLP-1,

which will enhance insulin secretion from the graft.[42] Our group is interested in the translational application of NPI in clinical islet xenotransplantation; hence, I wanted underscore the efficacy of the NPI graft in responding to rapid glycemic fluctuation, without the effect of an endogenous secretagogue such as GLP-1.[42] I included naïve mice some investigations (such as quantifying circulating insulin, and total pancreas insulin content). Naïve mice were non-diabetic, non-transplanted healthy animals. Our main anticipated/observed result is the reversal of hyperglycemia, which occurs in a delayed fashion when using NPI. Therefore, performing sham surgical procedure on animals would not highlight any possible difference on the long term. Also, using untreated diabetic animals as negative controls would result in numerous morbidities and mortalities, and affect the general animal welfare.

5.2.1 – Subcutaneous space for islet transplantation

By far, islet embolization in the hepatic portal tree following portal vein infusion is the standard successful location used in clinical islet transplantation. Yet, on the cellular level, intravascular transplantation predisposes to immediate loss for great portion of transplanted β-cell mass, due to instant blood-mediated inflammatory response (IBMIR) triggered by tissue factor (TF) expressed by islets.[43] The subcutaneous space is a virtually promising alternative transplantation site; it is easily accessible for graft implantation and retrieval using minor surgical procedure, can accommodate large volumes of transplanted tissue, and clinically tested for hosting endocrine[44] tissue or hormone-releasing devices.[45] Nevertheless, the limited subcutaneous vascularity does not seem appropriate for hosting metabolically active grafts such as pancreatic islets. In their native microenvironment, islets of Langerhans represents about 2% of the whole pancreatic tissue, yet they are nourished

with ~ 15% of total pancreatic blood supply through a dense, glomerular-like capillary network.[46] During procurement and islet isolation, this rich blood supply is severed, and restoration of sufficient blood supply in the implantation site starts after few days post-transplantation; yet, hypoxia-induced apoptosis seems to occur faster than the neovascularization. Therefore, amending the local microenvironment in the subcutaneous space is crucial for post transplantation islet viability.

5.2.2 – Enhancing local vascularization in the subcutaneous space

Augmenting the local vascular density of the subcutaneous site is one of the widely accepted approaches in order to maintain islet viability post-transplantation. Increasing the vascularity should improve oxygen and nutrients delivery to islets, and eventually would enhance cellular metabolism, glucose sensing, and insulin secretion. Pepper et al.[47] established an effective experimental technique for enhancing the subcutaneous vascularity, by exploiting the natural host's foreign body response towards an angiocatheter inserted subcutaneously. After removing the foreign body, a void is created capable of accommodating transplanted islets.

In **Chapter 2**, I investigated the subcutaneous space as an ectopic site for islet transplantation; I used an angiogenic biocompatible scaffold for islet delivery and stimulation of neovascularization, rather than stimulating the host's immune system towards a foreign body. Fibrinogen (also known as Factor I) is a glycoprotein produced by liver and circulates in the bloodstream, and serves primarily in hemostasis by contributing to the clot formation. Upon vascular injury and endothelial retraction, a series of events (know as coagulation cascade) start by the interaction of factor VII with sub-endothelial TF

and end by enzymatic cleavage of fibrinogen into fibrin. Thrombin activates soluble fibrinogen to become a non-soluble scleroprotein that polymerizes with other fibrin proteins forming a complex mesh network. The scaffold arrangement of fibrin fibrils enhances the precipitation of platelets and fibroblasts to the wound site, promoting wound healing.[48] In addition to its role in hemostasis, fibrin can enhance cellular proliferation and differentiation through acting as a 3-D scaffold that imitate extracellular matrix that enhances cell adhesion, migration, and vascular proliferation.[49] Moreover, fibrin contains Arg-Gly-Asp (RGD; arginine, glycine, and aspartic) amino acid sequence in its structure. It is widely known that RGD serves as an important ligand for many integrin receptors responsible for cell viability and proliferation. The interaction between fibrin and integrin receptors (mainly αvβ3) has a well-established influence on cell proliferation, protein synthesis, and stimulation of angiogenesis.

Exploiting those facts, we embedded NPI into fibrin scaffolds and placed them subcutaneously in diabetic mice. As demonstrated by us and other groups, islets failed to survive when they were transplanted alone (without amending the vascular conditions) subcutaneously.[40,51] On the contrary, when we transplanted them embedded into fibrin discs, they remained viable, and reversed hyperglycemia in diabetic animals. Nonetheless, attempting to use mature human or murine islets to obtain similar outcome has failed. As we pointed out earlier, NPIs are naturally resistant to hypoxia associated with the early postoperative phase. This inherent resistance is attributed to two main factors: being immature at the time of transplantation, and higher levels of XIAP expression. As immature cellular aggregates, metabolic activity of NPI is lower than their mature counterparts, hence requiring less oxygen tension and nutrients. More importantly, NPIs expresses high levels

of XIAP that shifts the BCL protein family towards promotion of cell survival instead of inducing apoptosis.[22] This underscores the vast potential of NPIs as an alternative source for β-cells in clinical transplantation setting.

To follow-up on the findings of this experiment, it is essential to investigate the fibrin's trophic effect on NPI more deeply. Riopel et al. provided an in depth investigation on the mechanism of action of fibrin, on immortalized β-cells line. Nonetheless, it would be more beneficial if we shed more light on the intracellular events that happens in porcine β-cells, when they are embedded in fibrin. *In vitro* studies would provide us with essential information about such as up- or down-regulation of receptor expression, DNA quantification, gene expression, nuclear proliferation, and survival pathways.

Additionally, I am interested in finding a technique to prevent graft loss, when using adult human or murine islets. On the top of the proposed experiments: subcutaneous prevascularization using thicker fibrin discs, followed by transcutaneous injection of mature islets after for weeks of initiating prevascularization. Also, it is possible to mix fibrin scaffolds with endothelial cells (e.g. porcine aortic endothelial cells) to stimulate more angiogenesis, and facilitate the establishment of the new capillary network for the transplanted graft.

5.2.3 – Intra-abdominal peritoneal folds and the use of scaffolds for islet transplantation

Promising outcome from the subcutaneous space motivated us to pursuit another extra-hepatic site for islet transplantation. Among many investigated sites, intra-abdominal peritoneal reflections are widely accepted as logical candidate. Our lab has previously

investigated omentum for the feasibility of islet transplantation, with or without scaffolds.[52,53] In **Chapter 3**, I used a different type of intra-abdominal peritoneal reflection due to the limitation of mouse model. Epididymal fat pad (EFP) is another experimental transplant site that upholds a lot of clinical potential, similar to the omentum. In this experiment, I used an unusual method to fabricate biocompatible porous scaffold for islet transplantation. I used alginate for forming a disc that will have encapsulated and non-encapsulated islets seeded into it. This scaffold was rigid enough to permit its manipulation during transplantation, yet remained porous allowing oxygen and nutrition exchange. The concept was developed to circumvent the outcome of previous experiments that displayed capsule over-crowding and clumping when transplanted subcutaneously. Moreover, attempting to place hundreds of slippery microspheres on a glistening surface such as the fat pad would end up by graft dispersion in the peritoneal cavity. Hence, fabrication of a porous rigid scaffold was the most logical thinking to overcome that technical obstacle.

Despite the successful proof-of-concept, EFP remains a surrogate for human omentum; its fat content, and its vascular drainage are different than human omentum. Nonetheless, the outcome of experiments in **Chapter 3** revealed that even with systemic vascularization, human islets were able to reverse diabetes and control the rapid fluctuation of blood glucose in diabetic animals. Yet, on the experimental level, the transplant procedure requires advanced surgical skills to gently manipulate the EFP without damaging it, and more importantly, the limited capacity of the EFP requires smaller grafts, and compact scaffolds. The next step pertaining EPF is to test the benefits of fibrin in the fat pad. We briefly investigated this approach, however we focused more on alginate scaffolds to generate more diverse studies.

After investigating two extra-hepatic sites for future islet xenotransplantation, a clearer comparison can be made to highlight the pros and cons of each location. Subcutaneous space offers an easy accessible transplantation site, through a minimally invasive procedure that can be done using local anaesthesia. It also permits the feasibility of obtaining biopsies for graft monitoring. Additionally, the proposed place to implant the graft can be hidden under clothes, to avoid any social anxiety. However, the major concern might be related to continuous localized insulin secretion, and the accompanied probability of lipodystrophy occurrence. On the other hand, intra-abdominal fat folds represent more spacious and near physiological location for islet transplantation. The omental venous drainage joins the portal circulation, which will resemble the pancreatic drainage. Yet, the procedure requires advanced surgical skills, and the need for general anaesthesia.

5.2.4 – The influence of sex hormones on islet grafts

During our experimental work, we noticed that islets transplanted in female recipients normalized glycemia faster than those transplanted in male counterparts. Reviewing previously published literature[54,55] and some of the recent reports,[56] we were able to develop a hypothesis suggesting that the impact of host's sex can extend to affect xenogeneic grafts as well. Testing this hypothesis has revealed a plausible sex influence on the graft control on hyperglycemia, glucose sensitivity, and insulin secretion. Again, a panel of in vitro and in vivo experiments are needed to gain a clearer insight about the actual mechanism of the effect of sex difference on NPI xenografts. Examples of in vitro investigations include immunohistochemical staining for ERα, in addition to quantification of its gene expression in grafts obtained from both sexes. Also, supplementing the tissue culture medium with physiological levels of E2 might mimic the host's hormonal

microenvironment; that would be followed again by assessing a panel of gene expression related to cellular proliferation and insulin synthesis.

Transplanting islets to female mice at various ages is another important arm in this hypothesis investigation. It is conceivable that the observed positive effect of female sexual hormones on islet grafts can diminish as the recipient animal progresses towards senescence. Some animal models such as non-human primates can provide us with a close simulation to the process of reproductive senescence occurring in humans. Yet, this preclinical model is less likely to be used routinely, due to complex social and ethical hurdles, financial obstacles, and to the long lifespan of the animal model.[57] Another way to compare between pre- and post-menopausal female recipients without waiting for natural senescence to occur is to perform surgical ovariectomy in young female mice. Removing the ovaries will result in cessation of estrogen action in the recipient.[58] However, acute deficiency of estrogen post-ovariectomy does not simulate the chronic, gradual hormonal regression that accompanies the natural reproductive aging.[59] In order not to undermine the role of the donor, I'm really interested in investigating the outcome of matching donors' and recipients' sex during transplantation. It is possible that prolonged exposure of donor islets to physiological levels and cyclic secretion patterns of E2, would have a great influence on the graft behaviour after transplantation.

This thesis contains original experimental work carried out by Bassem Salama, under the supervision of Dr. Gregory Korbutt. We ensured all scientific honesty and integrity during planning, execution, analysis, and presentation of this original experimental work. We also adhered to all ethical and moral requirements for scientific investigation,

that conform to policies of University of Alberta, and Canadian ethics. I hope that the work presented here would contribute to the improvement of experimental and/or translational islet transplantation, so more patients can enjoy their lives, as healthy as possible.

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APPENDIX A

SUBCUTANEOUS TRANSPLANTATION OF ENCAPSULATED NEONATAL PORCINE ISLETS FAILED TO REVERSE HYPERGLYCEMIA IN DIABETIC MICE

A.1 – OBJECTIVE

To determine whether or not microencapsulated neonatal porcine islets (NPI) have the ability to survive and reverse hyperglycaemia when transplanted subcutaneously in diabetic mice.

A.2 – RATIONALE

The aim of this thesis is to investigate an extra-hepatic site for islet transplantation. As pointed out previously, we used NPIs as a potential donor tissue to replace cadaveric human tissue in clinical islet transplantation. Subcutaneous site was the widely accepted proposed ectopic site for transplantation, mainly due to its ease of access, and its capability to house large volumes of β-cell mass. This experiment provided us with the necessary skills and knowledge to perform later experiments mentioned in **Chapter 2**.

Since we are proposing to use xenografts for clinical islet replacement, then it was conceivable to use an immune barrier in an attempt to minimize the likelihood of immune-

mediated graft loss. Therefor, we used microencapsulation technique as a physical immune barrier, and to test the viability and function of encapsulated NPI subcutaneously. Again, this experiments aided us to gain the knowledge and skills required for islet microencapsulation, which was translated and improved in experiments performed in **Chapter 3**.

A.3 – EXPERIMENTAL PROCEDURE

Neonatal porcine islets were isolated and cultured according to the previously published technique. Aliquots of 5,000 NPI were microencapsulated using 1.5% (w/v) PRONOVA UP-MVG sodium alginate, following the same protocol described in **Chapter 3**.

On the day of transplantation, individual encapsulated NPI aliquots were collected in 5 ml tubes and were left to sediment by the effect of gravity. Using 14G vascular cannula attached 3 ml syringe, encapsulated islets were aspirated from tubes, and then injected percutaneously in the subcutaneous space of twelve STZ-induced diabetic Rag-1 immune deficient mice. Injection site was sealed using a stainless steel surgical skin clip, and all mice were monitored for non-fasting blood glucose levels within the first week post-transplant, and once a week thereafter.

At the experimental endpoint (12 weeks post-transplantation), animals were euthanized and grafts were collected for histological characterization and immune staining for insulin.

A.4 – RESULTS

After transplanting microencapsulated NPI subcutaneously, none of the transplanted animals attained normal glucose level **(Fig. A1)**. Follow-up of mice weights revealed significant loss of body weight over the experimental period (4.6 ± 0.5 grams weight loss, $p < 0.00001$, **Fig. A1**). Upon graft recovery and macroscopic assessment, we found that the capsules were clumped together and surrounded by thin connective tissue capsule, with prominent vasculature around the clumped capsules **(Fig. A2.a,b)**. Immunohistochemistry staining for insulin-positive cells performed on paraffin sections displayed weak staining, with lots of capsules devoid of insulin-positive cells (**Fig. A3)**.

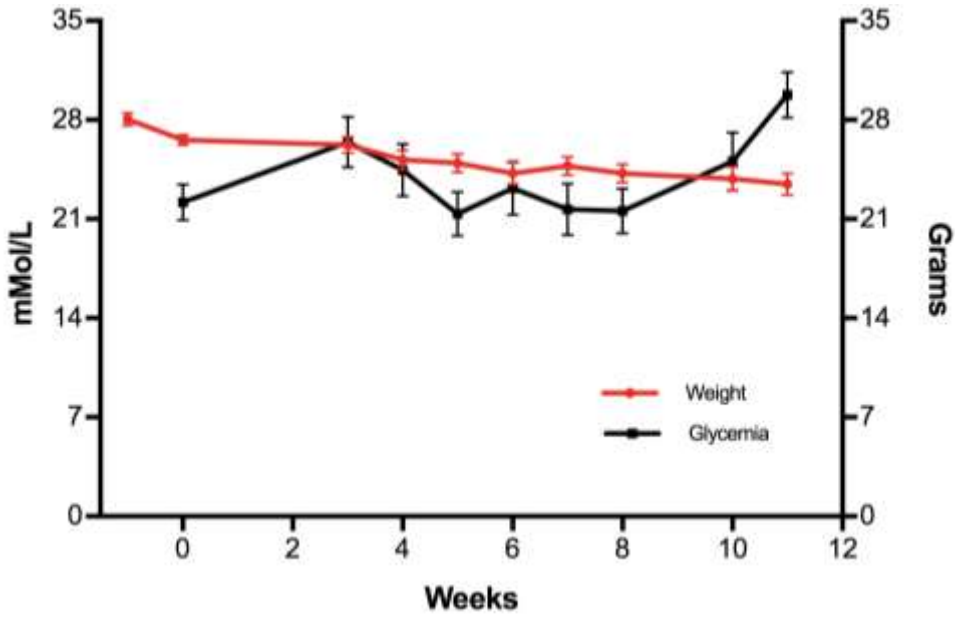

Figure A.1 – Metabolic follow-up of transplanted animals

Weekly non-fasting glucose (black line) and animals' body weights of STZ-induced diabetic animals transplanted with aliquots of 5,000 microencapsulated NPI subcutaneously. Data expressed in mean ± SEM on n=12 animals.

a

b

Figure A.2. Macroscopic appearance of encapsulated NPI grafts

(a) Encapsulated NPI were clumped together at the transplant site, surrounded by thin capsule of connective tissue. Image captured using digital still camera; 2x zoom.

(b) Examination of the undersurface of the skin with the clumped tethered capsules under a surgical dissection microscope revealed numerous minute capillaries surrounding the implantation site (1x magnification)

A.5 – CONCLUSION

In this experiment, microencapsulated NPI failed to correct hyperglycemia when transplanted in the subcutaneous space of diabetic Rag-1 mice. We have demonstrated in previous experiments that NPI exhibit delayed control on hyperglycemia due to their immaturity at the transplantation time; nonetheless, they still secrete enough insulin sufficient to prevent severe weight loss. In this experiment, there was significant loss of

body weight in diabetic animals (16.4 ± 2.0 % of pre-STZ weight, over 11 weeks). This implies loss of insulin secretion due to progressive graft attrition. The macroscopic clumped appearance of the graft can be attributed to the healing process of created subcutaneous pocket during transplantation. As the dissected subcutaneous space starts to heal from the periphery, capsules were forced to cram and clump, depriving the core of the graft from oxygen diffusion and nutrients. That was apparent during histological characterization of the graft. Immunohistochemistry staining of the tissue sections hardly displayed any insulin-positive cells inside the microcapsules. This strongly suggests that NPI were not able to survive; despite their inherent resistance to hypoxia. Fibrous overgrowth on the capsule surface can be excluded due to absence of cellular deposits on alginate capsules.

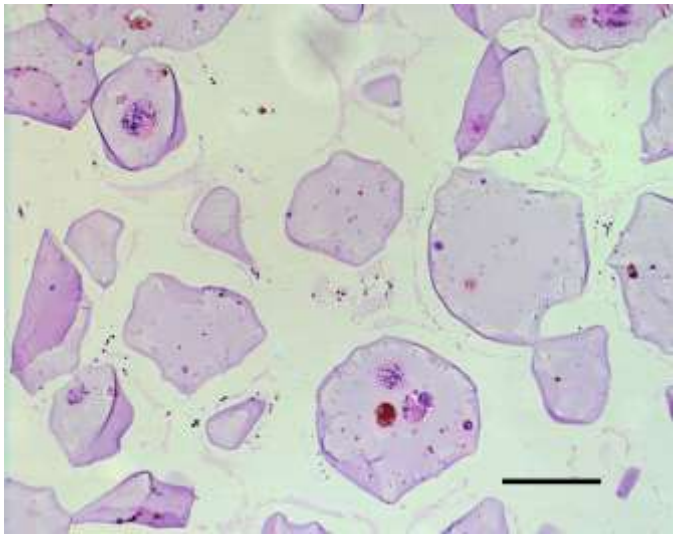

Figure A3 – Immunohistochemistry staining for insulin in grafts recovered after 11 weeks. Images captured using 10x objective lens magnification; scale bar = 200 μm

The discouraging outcome suggests that in order to maintain oxygen diffusion and graft viability, additional approaches have to be taken to prevent graft deformity and clumping. That was applied in experiments carried out in **Chapter 3**, where capsules were embedded in alginate disc to avoid clumping and central necrosis.

APPENDIX B

FIBRIN IMPROVED METABOLIC FUNCTIONS OF ENCAPSULATED NEONATAL PORCINE ISLETS TRANSPLANTED SUBCUTANEOUSLY IN DIABETIC MICE

B.1 – OBJECTIVE

To examine the effect of fibrin on the viability and metabolic function of microencapsulated neonatal porcine islets (NPI) transplanted subcutaneously in diabetic mice.

B.2 – RATIONALE

In **Appendix A**, we attempted to transplant encapsulated NPI subcutaneously in diabetic mice. After 12 weeks, none of the transplanted mice achieved normoglycemia, and the animals displayed significant weight loss (> 15% of initial body weight). During graft recovery, we noticed that capsules were clumped into a thin connective tissue sac. Histological observations revealed almost no insulin-positive cells in the core, and very weak staining in the peripheral capsules.

In **Chapter 2,** we demonstrated the exceedingly beneficial effect of fibrin on NPI survival and metabolic function in the subcutaneous space. Fibrin enhanced cellular proliferation and protein synthesis, by augmenting the poor subcutaneous circulation. **In Chapter 3**, we displayed a novel way to prevent over-crowding of encapsulated islets in the fat pad. We fabricated a scaffold using alginate in which human islets were seeded, either previously encapsulated, or directly without micro encapsulation. In this experiment we demonstrate the proof-of-concept of using fibrin discs (similar to what was mentioned in **Chapter 2**) to transplant encapsulated NPI subcutaneously.

B.3 – EXPERIMENTAL PROCEDURE

Neonatal porcine islets were isolated and cultured according to the previously published technique. Aliquots of 5,000 NPI were microencapsulated using 1.5% (w/v) PRONOVA UP-MVG sodium alginate, following the same protocol described in **Chapter 3**. On the day of transplantation, individual encapsulated NPI aliquots were collected and mixed with 0.1 ml of fibrinogen in 24-well tissue culture plate. Additional 0.1 ml of thrombin was added to each well and contents were stirred gently using sterile pipette tip. Scaffolds were left to crosslink for 15 min at 37 C in 5% CO_2 incubator. Five male diabetic Rag-1 animals received fibrin scaffolds subcutaneously (SC) as described in **Chapter 2**. Two control mice received non-encapsulated equal β-cell mass under the kidney capsule (KC) without fibrin.

Mice were monitored for fasting and non-fasting blood glucose levels within the first week post-transplant, and once a week thereafter. At the experimental endpoint (15

weeks post-transplantation), animals were euthanized and grafts were collected for histological characterization and immune staining for insulin.

B.4 – RESULTS

Starting from the 4th week post-transplantation, SC mice displayed lower fasting and non-fasting glucose, compared to KC group. Also, fasting SC glycemic values were significantly lower than non-fasting SC ($p < 0.05$) starting from the 4th week post-transplantation **(Fig. B1)**.

At the time of graft recovery, macroscopic assessment revealed that the capsules were still distributed uniformly over the back muscles, with no signs of clumping or calcifications. **(Fig. B2.a)**. Immunohistochemistry staining displayed intact islets with insulin-positive cells inside capsules **(Fig. B2.b)**.

B.5 – CONCLUSION

In this experiment, we transplanted encapsulated NPI embedded into fibrin scaffolds subcutaneously, in diabetic mice. There were two reasons for using fibrin as a scaffolding material:

1. To enhance neovascularization in the subcutaneous space, based on what we observed in **Chapter 2**;
2. To keep capsules spread under the skin, and prevent clumping and subsequent central necrosis.

Indeed fibrin had a positive effect when it was integrated with the encapsulated islets subcutaneously, displayed in the lowered fasting glycemic values, compared to non-fasting values. The experimental outcome emphasizes on the importance of amending any proposed ectopic site sought for islet transplantation, and the potential applications of fibrin in stimulating neovascularization in the subcutaneous space, so it could support the islet graft viability and improve its metabolic function.

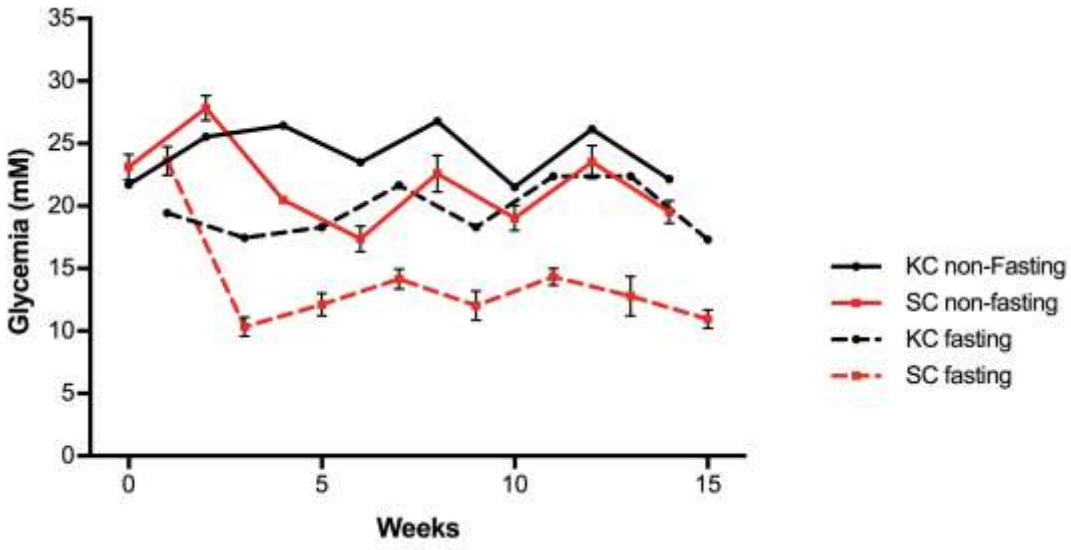

Figure B.1 – Metabolic follow-up of transplanted animals

Weekly glucose monitoring of STZ-induced diabetic animals transplanted that received aliquots of 5,000 microencapsulated NPI, embedded in fibrin discs subcutaneously (SC; n=5), or non-encapsulated islets under the kidney capsule (KC; n=2).

a **b**

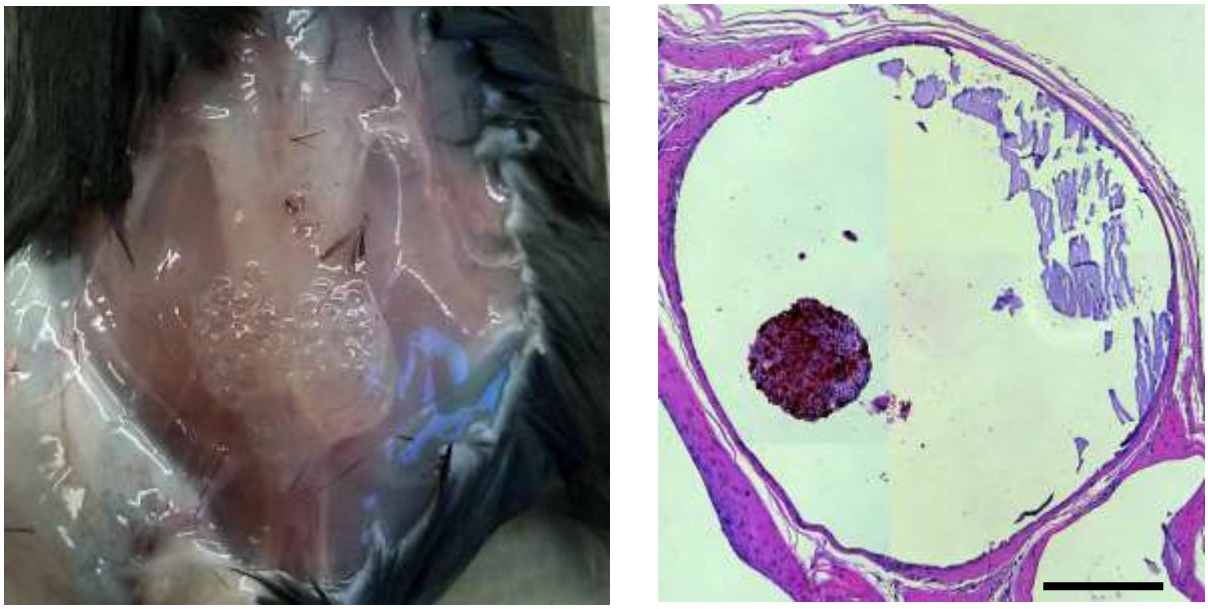

Figure B.2 – Characterization of encapsulated NPI grafts

a) Macroscopic appearance of encapsulated NPI transplanted subcutaneously with fibrin.

b) Immunohistochemistry staining for insulin in grafts recovered after 15 weeks. Images captured using 10x objective lens magnification; scale bar = 200 µm