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IMPROVING THE PRESERVATION OF THE NON-HEART-BEATING-DONOR PANCREAS

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To my family;

this means as much to them as it is to me.

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Abstract

Successful pancreatic islet transplantation depends on the number of islets transplanted, islet viability and extent of early islet loss due to ischaemia reperfusion injury. Novel pancreas preservation techniques which can improve these variables can increase the utilisation of non-heart-beating-donor pancreases for islet transplantation.

A non-heart-beating-donor rat pancreas preservation model was developed. Pancreases preserved by either static cold storage, hypothermic machine perfusion or portal venous oxygen persufflation underwent islet isolation and purification. The yield, viability and *in vitro* function of isolated islets were compared. Portal venous oxygen persufflation improved the islet yield, viability and morphology as compared to static cold storage. The percentage of pancreases with functional islets (stimulation index greater than 1.0) was also higher after oxygen persufflation as compared to static cold storage.

Severity of reperfusion injury in pancreases preserved by static cold storage or portal venous oxygen persufflation was compared after *in vitro* warm reperfusion. Amylase, lipase and glycerol levels in the portal effluent were measured. Lipid peroxidation and apoptosis in reperfused pancreas were measured using thio-barbituric acid reactive substances assay and caspase 3 assay respectively. Expression of genes relevant to ischaemia reperfusion was compared using RNA microarrays.

Severity of ischaemia reperfusion injury was similar in both groups. Microarray analysis revealed increased expression of genes related to apoptosis in the portal venous oxygen persufflation group. This group also showed up-regulation of pro-survival

cellular pathways and over-expression of genes related to cellular repair as compared to static cold storage.

This project has for the first time evaluated oxygen persufflation as a method of pancreas preservation and investigated changes in global gene expression after this form of preservation. Overall, the project suggests that portal venous oxygen persufflation improves the recovery of non-heart-beating-donor rat pancreas. Further investigation to examine its role in the preservation of large animal pancreases is needed.

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Abbreviations

ADP	Adenosine diphosphate
AKT/PKB	Protein kinase B
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease activating factor 1
ATP	Adenosine triphosphate
Bad	Bcl2-associated agonist of cell death
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma-2
Bcl2A1	Bcl-2 related protein A1
Bcl2L1	B-cell lymphoma-2 like 1
Bcl2L11	Bcl-2 like protein 11
BID	BH3 interacting domain death agonist
Birc2	Baculoviral IAP repeat-containing 2
Birc3	Baculoviral IAP repeat-containing 3
CBD	Common bile duct
Cflar	CASP8 and FADD-like apoptosis regulator
DAG	Diacyl glycerol
DCD	Donation after Cardiac Death
DMEM	Dulbecco Minimal Essential Medium
ERK	Extracellular signal-regulated kinase
Fadd	Fas-associated death domain
GO	Gene Ontology
GRB2	Growth factor receptor-bound protein 2
GSIS	Glucose stimulated insulin secretion
HBSS	Hanks' buffered salt solution
HIF	Hypoxia inducible factor
IEQ	Islet equivalent
IL1B	Interleukin 1
IQR	Inter-quartile range

IVC	Inferior vena cava
IκK	Inhibitor of Kappa Kinase
Iκβ	Inhibitor of Kappa Beta
JNK	cJun n-terminal kinase
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
NFκB	Nuclear factor Kappa B
NHBD	Non-Heart-Beating-Donor
NRF2	Nuclear factor 2
p90RSK	p90 ribosomal s6 kinase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
Pea15	Phospho-protein enriched in astrocytes 15
PI3K	Phosphatidyl Inositol-3 kinase
Pim2	Pim-2 oncogene
PKC	Protein kinase c
PMSF	Phenyl methyl sulphonyl flouride
pNA	para-Nitroaniline
Puma	p53 upregulated modulator of apoptosis
rasGRP	RAS guanyl-releasing protein 1
RIN	RNA Integrity Number
RIPA	Radio-Immuno-Precipitation Assay
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SCS	Static Cold Storage
SOS	Son of Sevenless homolog
TBARS	Thiobarbituric acid reactive substances
TLM	Two Layer Method
TNFα	Tumour necrosis factor
TRAF	Tumour necrosis factor receptor-associated factor
UW	University of Wisconsin

Chapter 1 Review of literature

1.1 Diabetes

1.1.1 Background

Polyuric states have been described over 3500 years ago in Egyptian papyrus writings[1]. The involvement of the pancreas in the pathogenesis of diabetes was first suspected by Oskar Minkowski and Josef von Mering in 1889[2]. In 1921, Frederick Banting and Charles Best working at Toronto University discovered insulin in extracts of pancreas and used them for treating the first patient with diabetic ketosis[3, 4]. Even today, treatment with insulin remains the cornerstone of treatment for Type 1 diabetes.

Diabetes mellitus is a global problem. There are an estimated 2.35 million people with diabetes in England[5]. Around 5 per cent of total National Health Service spend (and up to 10 per cent of hospital in-patient spend) is used for the care of people with diabetes[6]. Type 1 diabetes constitutes 10% of all diabetes patients but consumes a disproportionate portion of these resources. Type 1 diabetes is caused by a total lack of endogenous insulin production. It is characterised by the progressive destruction of β cells in the islets of Langerhans which is visualised as lymphocyte infiltration of the islets (insulitis). The destruction is considered to be an autoimmune response triggered by a combination of genetic and environmental factors[7].

1.1.2 Clinical features

The diagnosis of diabetes is based on the presence of clinical symptoms such as polyuria, polydipsia, loss of weight and the documentation of elevated blood glucose levels[8]. Patients with type 1 diabetes are at risk of both acute and chronic complications. Acute complications are diabetic keto-acidosis and acute hypoglycaemic episodes. Both are potentially fatal, and the latter greatly limits the everyday life of patients.

Chronic complications can involve any organ in the body. Nephropathy can lead to end stage renal failure, retinopathy and cataracts lead to blindness, peripheral neuropathy and peripheral vascular disease cause non-healing ulcers, foot infections and gangrene. Vascular involvement also causes increased risk of cerebrovascular accidents and coronary artery disease.

1.1.3 Treatment

Insulin therapy is the main treatment modality available for type 1 diabetes. It effectively controls hyperglycaemia and can delay the development of chronic complications. Glycaemic control is however difficult to achieve in some patients with high insulin sensitivity [9]. Intensive insulin therapy using an insulin pump improves glycaemic control but increases the risk of life-threatening hypoglycaemia[10]. Transplantation of the whole pancreas leads to normalised blood glucose levels, delays the progression of chronic organ damage and improves the quality of life[11]. It is however a major undertaking and the

procedure is still associated with significant operative morbidity[12]. Islet transplantation can be an alternative therapeutic modality in selected patients with type 1 diabetes with low insulin requirements and high incidence of hypoglycaemia related complications[13].

1.2 Pancreatic Islet Transplantation

Pancreatic islet transplantation involves the transplantation of islets of Langerhans. A human pancreas contains over a million islets[14]. Each islet contains cell populations producing all hormones involved in glucose homeostasis (insulin, glucagon and somatostatin). Early attempts at clinical islet transplantation were rarely successful at achieving insulin independence[15, 16]. Islet transplantation was finally recognised as an achievable goal when the Edmonton group reported their success in seven type 1 diabetes patients who remained insulin independent a year after islet transplantation[17]. This event has spurred an enormous amount of clinical and experimental research in this field.

1.2.1 Pancreatic islet transplantation- The procedure

Islet transplantation as currently practised is a complicated procedure carried out in specialised units. The entire procedure includes several important phases (Figure 1).

Pancreas recovery and transport for islet transplantation

Recovery of donor pancreas for islet transplantation is technically similar to recovery for whole organ transplant. Careful handling of the pancreas, minimal warm phase dissection and quick cooling of the pancreas after aortic cross clamping are essential for good quality grafts [18]. Once retrieved, the pancreas is transported at 4°C in University of Wisconsin (UW) solution to the islet isolation centre. This is static cold storage and is the simplest form of preservation. The use of oxygenated perfluorocarbon solution along with UW solution (Two-layer-method) has been shown in experimental studies to improve the islet yield[19]. However results from clinical studies have been inconclusive[20, 21].

Islet isolation

This is a multi-step process and includes pancreas digestion using collagenase, de-activation of the enzyme after completion of digestion and separation of the islet fraction from exocrine tissue[22].

The pancreas is dissected in a cold chamber, the pancreatic duct is identified and cannulated. Cold enzyme solution is injected into the duct to distend the pancreas. Digestion is started in a closed chamber called the 'Ricordi chamber' at 37°C. Mechanical agitation of the chamber aids the digestion process. Intermittent sampling of the digest is carried out to confirm the completion of digestion. Once digestion is complete, the enzyme is rapidly inactivated by adding large volumes of cold buffer solution with albumin. The digest is collected by centrifugation and immediately purified by continuous density gradient

separation. The islet rich fractions are collected, washed and stored in an islet storage medium. The yield and viability of the isolated islets is confirmed. Islets vary in size (25μm to 500μm) and absolute count of islets does not give a true measure of islet yield. Islet yield is instead reported in terms of islet equivalents (IEQ) which takes into account the relative numbers of islets in each size category. This gives a better estimate of the actual islet mass that is recovered from a donor pancreas or transplanted into a patient.

Recipient procedure

The transplantation procedure takes place in a radiology intervention suite. The portal vein of the recipient is cannulated under local anaesthesia. The islet suspension is infused by gravity into the portal vein with intermittent monitoring of portal pressures. Once transfusion is complete, the track is sealed to minimise bleeding complications. Insulin therapy may be required in the immediate post-operative period until the graft starts functioning. Post-transplant immunosuppression is based on rapamycin, low dose tacrolimus and dacluzimab[17].
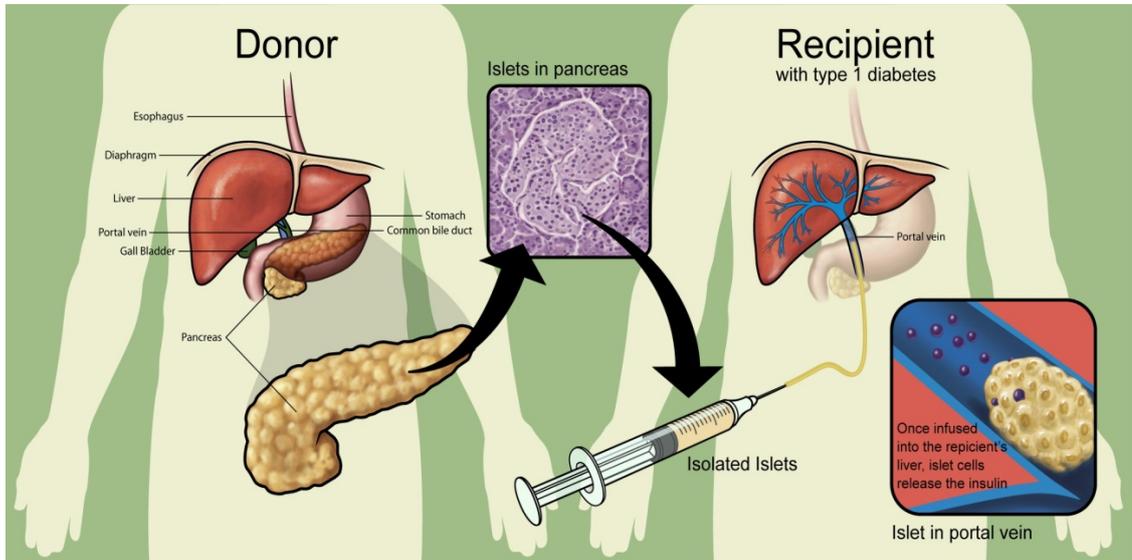
Figure 1 Schematic diagram of the steps involved in pancreatic islet transplantation.

Picture downloaded from
http://commons.wikimedia.org/wiki/File:Islet_transplantation_PLoS_Medicine.jpg [23].
Pancreas from the donor is retrieved usually as part of a multi-organ recovery
procedure. The pancreas is transported to an islet isolation facility where it is processed
to isolate islets of Langerhans. The islets are then transplanted into the recipient by
injecting into the portal vein. Islets are deposited in the portal vein radicals where they
develop neo-vascularisation over a period of days. Insulin production from the islets
occurs in response to glucose levels in the portal venous blood.

1.2.2. Complications of islet transplantation

Post-procedural complications are usually related to portal cannulation. They may present as bleeding from the cannulation site, portal vein thrombosis and portal hypertension due to multiple transplantation procedures[24, 25]. Delayed graft function and primary graft failure may occur due to poor quality of islets, insufficient number of transplanted islets or the early destruction of islets by inflammatory and immune mechanisms. The latter termed the instant blood-mediated inflammatory reaction (IBMIR) can lead to the death of transplanted islets within hours of the transplantation procedure[26]. Tissue factor (also called thrombokinase or factor III) and collagen on the surface of damaged islets initiate a strong innate immune response in the recipient's portal circulation. IBMIR is characterised by platelet and complement activation, fibrin deposition around the islets and leukocyte infiltration[27]. It can lead up to 50% loss of transplanted islets.

1.2.3 Outcome after islet transplantation

Since the year 2000 over 500 islet transplantations have been performed in the United States, Europe and Asia[28]. The success of islet transplantation depends on a sufficient mass of viable islets being transplanted. Clinical data suggests that a dose of 9000-12000 islet equivalents/kg body weight is required to achieve insulin independence[28, 29]. This would normally require transplantation of islets from 2-3 donor pancreases. Adherence to this guideline

routinely achieves insulin independence in over 80% of recipients at one year. Follow up data of 44 patients published in 2005 has shown that only 10% of patients remain insulin independent after five years[30]. Patients who return to insulin therapy have improved glycaemic stability and better awareness of hypoglycaemia. Hence islet transplantation can be a valuable therapeutic option for selected type 1 diabetic patients with high risk of severe hypoglycaemia episodes [28].

1.2.4 Expanding islet transplantation

The availability of donor pancreases is the key to expanding islet transplantation. Two to three donor pancreases are needed for the successful treatment of each patient [31, 32]. This demand for organs can lead to direct competition with whole organ pancreas transplant programs in many centres. Donor pancreas availability for islet transplantation programs can be greatly improved by utilising pancreases from marginal donors such as non-heart-beating-donors (NHBD) which are not routinely used for whole organ pancreas transplantation.

1.3 Non-heart-beating-donation

'Non-heart-beating-donation' (NHBD) or 'donation after cardiac death' (DCD) implies that the donor had sustained irreversible cardiac arrest before effective cooling of the organs has been achieved[33]. Organs from these donors

experience a period of warm ischaemia i.e. the tissues are starved of oxygen and nutrients while being at normal body temperatures. Historically, non-heart-beating-donors were the only type of donors available for organ donation. The development of the criteria for diagnosis of brain death in 1968 contributed to the legalisation of the use of brain-dead donors for organ donation[34]. This led to an immediate decline in the use of organs from non-heart-beating-donors for transplantation. Lengthening waiting lists and the increasing disparity between the demand and supply of donor organs has renewed interest in non-heart-beating-donors as a means of expanding the donor pool[35, 36]. Recent years have seen a gradual increase in the utilisation of these donors both in the United Kingdom[37] and the United States[38]. Non-heart-beating-donation remains the major form of cadaveric organ donation in countries like Japan.

1.3.1 Classification of non-heart-beating-donors

Non-heart-beating-donors are classified based on the circumstances of the terminal cardiac arrest (Table 1). The Maastricht classification provides an indication of the amount of warm ischaemic damage the organs have suffered[33]. Category I donors are rarely used as the duration of warm ischaemia is unknown though some centres in Spain have reported acceptable results from these donors[35]. Category II donors are utilised in a few dedicated centres around the world. Category III & IV donors are controlled donors and used in most transplant centres.

Non-heart-beating-donors now account for nearly 25% of all organ donors in the United Kingdom[39]. Kidneys were the most common organs utilised from these donors. Results of these kidney transplants depend on the non-heart-beating-donor category. While category III & IV non-heart-beating-donor kidneys have outcomes similar to heart-beating-donor grafts, category II grafts have an increased risk of delayed graft function and early graft failure. However their medium term and long term outcome is similar to heart-beating-donor grafts[40, 41]. Use of category III non-heart-beating-donor for liver transplantation is being increasingly reported with satisfactory results[42]. Their use in whole pancreas transplants is more limited due to concerns about increased post-operative morbidity.

Table 1 Maastricht classification of categories of non-heart-beating-donors[33]

Non-heart-beating-donors are classified based on the place where the terminal cardiac event takes place. Category 1 and 2 are termed uncontrolled non-heart-beating-donors as cardiac arrest is unexpected and takes place away from immediate medical attention. This increases the risk of significant warm ischaemia. Categories 3 and 4 are termed controlled non-heart-beating-donors as the terminal cardiac event occurs close to medical attention and may even be planned in patients with serious non-recoverable brain injury.

Donor category	*Description of terminal event*	*Location in hospital where terminal event occurs*	*Current status with regards to the feasibility of organ recovery*
I	Dead on arrival	Accident and emergency	Not yet accessible
II	Unsuccessful resuscitation	Accident and emergency, regular ward	Accessible in some dedicated centres only
III	Awaiting cardiac arrest	Intensive care, regular ward	Accessible, high percentage of success
IV	Cardiac arrest during or after diagnosis of brain death	Intensive care, operating theatre	Switch immediately to non-heart-beating-donor organ recovery protocol

1.3.2 Non-heart-beating-donors for pancreatic islet transplantation

Non-heart-beating-donors are being studied as possible source of organs for islet transplantation [43, 44]. Islet yield and function from non-heart-beating-donor pancreases with short warm ischaemia times is as good as heart-beating-donor pancreases[45]. Studies have however shown that islet yield is decreased in pancreases damaged by warm ischaemia longer than 30 minutes[46]. Reducing the duration of warm ischaemia is the most direct way to improve islet yield from these organs. Prolonged warm ischaemia is inevitable in category II non-heart-beating-donor organs. Improved preservation of these pancreases is necessary for them to be routinely utilised for islet transplantation. Non-heart-beating-donor pancreases are being used for clinical islet transplantation in Japan. Their reported technique involves the placement of an intra-aortic balloon catheter before cardiac arrest. This enables rapid cooling of the intra-abdominal organs immediately after cardiac arrest. Warm ischaemia duration of just 4 minutes has been reported with this technique [47]. It is unlikely that such pre-mortem interventions to improve organ quality will be acceptable in all countries.

1.3.3 Organ damage in the non-heart-beating-donor

Warm ischaemia is the hallmark of the non-heart-beating-donor[33]. This impacts graft quality and post-transplant graft function by several mechanisms. Lack of oxygen prevents aerobic respiration leading to the depletion of

intracellular reserves of ATP[48]. This causes cell membrane dysfunction and an influx of sodium ions and water and cellular swelling. Anaerobic respiration leads to lactate accumulation and acidosis [49],[48]. Depletion of glucose stores during prolonged ischaemia blocks glycolytic energy production. Reinstitution of blood supply to the ischaemic organ after transplantation leads to the production of large quantities of cytokines and oxygen free radicals which cause graft damage. The combination of initial ischaemia followed by reperfusion plays a major role in the dysfunction of the transplanted organ or tissues[50].

1.4 Ischaemia reperfusion injury

1.4.1 Background

Ischaemia-reperfusion injury (IRI) refers to the damage caused to cells or tissues when blood supply returns after a period of hypoxia. Ischaemia-reperfusion injury is seen in multiple clinical situations. It was first described by Cerra and co-workers[51] who reported sub-endothelial haemorrhagic necrosis in the myocardial pedicles associated with warm ischemia in a canine model. They demonstrated that the extent of necrosis was proportional to the duration of warm ischaemia. Ischaemia-reperfusion injury has been identified as a major factor affecting post-transplant outcome in solid organ transplantation [52]. The severity of this phenomenon is much more significant in the setting of non-heart-beating-donation.

1.4.2 Pathophysiology of ischaemia reperfusion injury

IRI is triggered by the onset of ischaemia and arrest of aerobic respiration in an organ or tissue [53] (Figure 2). This results in a decrease in ATP levels, accumulation of acidic metabolites and decrease in intracellular pH [54]. Cellular swelling develops due to dysfunction of the energy-dependent sodium potassium pump. This is reflected as endothelial cell swelling which compromises the capillary lumen[55]. Acute ischaemia also causes activation of endothelium resulting in increased permeability and increased expression of adhesion molecules. The severity of these changes depends on the duration of ischaemia[56] and the temperature at which the tissue suffers ischemia[57]. Small amounts of reactive oxygen species are produced during the ischaemia phase.

The reperfusion component of IRI is initiated when the ischaemic organ is perfused by blood and re-warmed to physiological temperatures. Reperfusion flushes the accumulated toxic metabolites from the ischaemic tissues into the systemic circulation. This causes a haemodynamic response in the form of tachycardia and hypotension [54]. Reperfusion leads to platelet and neutrophil adhesion to the activated endothelium. Platelet activation releases inflammatory cytokines [54]. Reperfusion also stimulates the production of large amounts of reactive oxygen species in the mitochondria. They activate the toll like receptor (TLR) pathway[58]. TLR signalling leads to the up-regulation of multiple inflammatory genes and pathways[59]. Reactive oxygen species cause cell

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membrane damage, lipid peroxidation[60], mitochondrial damage and activate mitochondrial pathway of apoptosis[61].

1.4.3 Outcome of ischaemia reperfusion injury

IRI initiates multiple pathways involved in cellular injury, cell death, and cellular repair[62]. The options available to a cell undergoing ischaemia reperfusion injury are necrotic cell death or apoptotic cell death or cellular repair and survival[63] (Figure 3). All three processes have been found to occur concomitantly in experimental and clinical studies of ischaemia reperfusion injury[64, 65]. The net result depends on the balance between the pro-death and the pro-survival processes. Several factors influence this delicate balance in the setting of non-heart-beating-donor organs. Factors such as the mode of death[66], presence or absence of ischaemic preconditioning, durations of warm ischaemia and cold ischaemia[67] have the potential to affect the outcome. Extensive damage after ischaemia reperfusion injury presents clinically as primary non-function or delayed graft function.

Figure 2 Pathophysiology of ischaemia-reperfusion

The intracellular changes during ischaemia reperfusion are depicted. Ischaemia leads to the arrest of normal aerobic respiration. This leads to a decrease in the cellular ATP level. ATP depletion leads to failure of the sodium potassium pump and cellular swelling. Small amounts of reactive oxygen species are produced during the ischaemia phase. The reperfusion component of IRI is initiated when the ischaemic organ is perfused by blood and re-warmed to physiological temperatures. Reperfusion stimulates cytokine production and production of large amounts of reactive oxygen species. Reactive oxygen species have multiple roles in reperfusion injury. They also cause cell membrane damage, lipid peroxidation, mitochondrial damage and activate mitochondrial pathway of apoptosis.
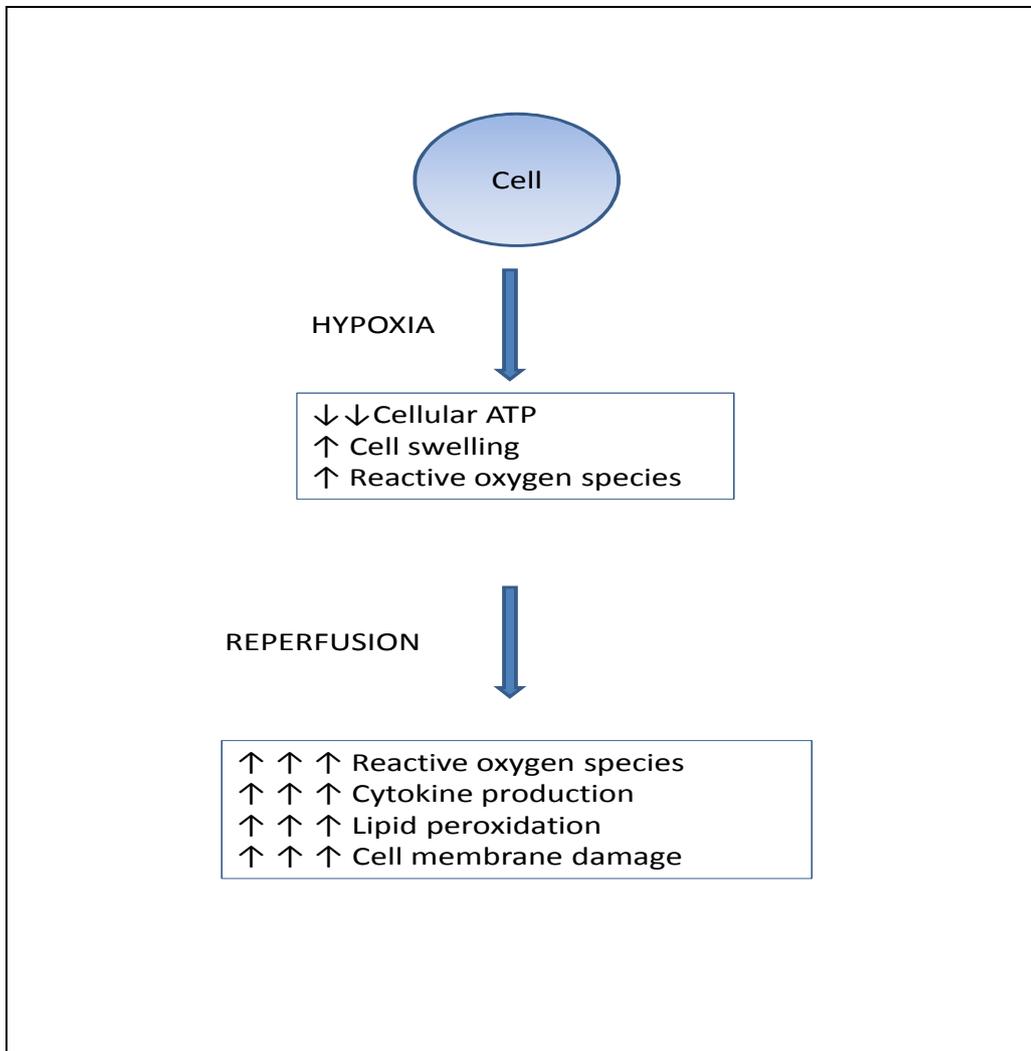
Figure 3 Outcomes after ischaemia-reperfusion injury

Ischaemia reperfusion injury initiates multiple pathways involved in cellular injury, cell death, and cellular repair. The options available to a cell undergoing ischaemia reperfusion injury are necrotic cell death or apoptotic cell death or cell survival. All three processes have been found to occur in experimental and clinical studies of ischaemia reperfusion injury. The net result depends on the balance between the pro-death and the pro-survival processes. Several factors such as the severity and duration of injury, pre-injury cellular state influence this balance.
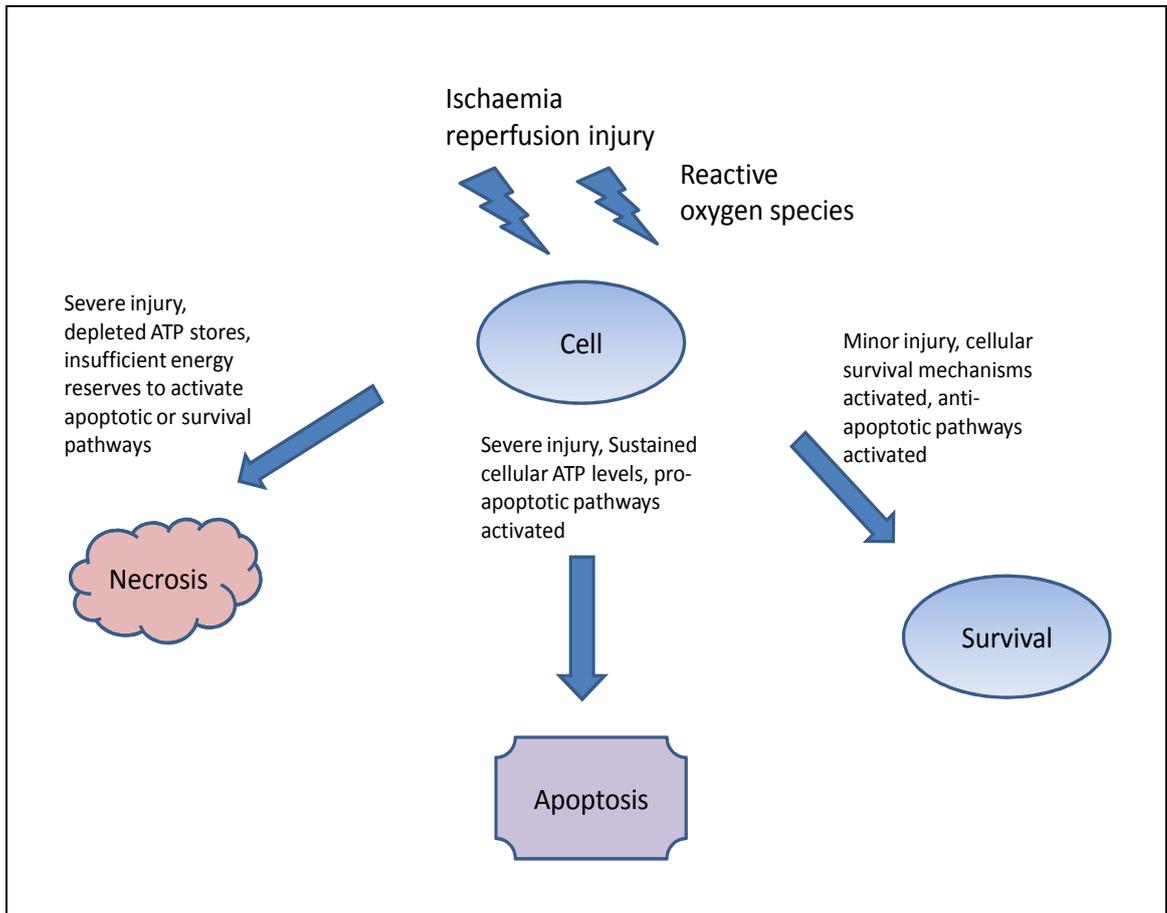
1.5 Apoptosis

1.5.1 Background

Apoptosis refers to a form of programmed cell death. The term 'apoptosis' was first suggested by Kerr and co-workers in 1972. who described the morphological appearances of cells undergoing this form of death[68]. Apoptotic cells are characterised by cytoplasmic and nuclear condensation with blebbing of the plasma membranes. The apoptotic cell shrinks and eventually breaks up into multiple membrane bound particles called apoptotic bodies containing intact organelles and nuclear material. These bodies are rapidly recognized and engulfed by phagocytes. The process occurs without the induction of inflammation and does not lead to scarring.

Apoptosis forms an important mechanism that directs the development and homeostasis in normal cell populations. It is considered to play an important role in an organ's response to ischaemia and reperfusion. It is an energy consuming process and can only proceed when energy rich substrates are available. In their absence, cell death occurs by necrosis. Apoptosis has been found to be a key determinant of the immediate post-transplant function after islet transplantation [69].

The cellular machinery contains both pro-apoptotic and anti-apoptotic factors. The ultimate fate of the cell i.e. apoptotic death or survival depends on the balance of these two sets of factors. The major pro-apoptotic proteins are the caspases and the pro-apoptotic members of the B cell lymphoma-2 (Bcl-2)

family. Caspases are a family of cysteine proteases that are closely involved in the initiation and execution of apoptosis[70, 71]. They are characterised by their very specific site of action- cleaving their substrates after the aspartic acid residues. Caspases have been classified into three groups based on their function. Group 1 are the inflammatory caspases (caspase 1). Group 2 are the effector caspases (Caspase 3, 7) and group 3 are the initiator caspases (caspase 2,8,9,10). Caspases are produced within the cell in a pro-enzyme form and undergo cleavage in suitable environments to form active units. The Bcl-2 family includes several proteins involved in the regulation of cellular apoptosis[72]. The family includes pro-apoptotic proteins such as the Bcl-2 associated X protein (Bax)[73], Bcl-2 associated agonist of cell death (Bad) and anti-apoptotic proteins such as Bcl-2, Bcl-2 related protein A1 (Bcl2A1 or BFL-1) and B-cell lymphoma-2 like 1 (Bcl2L1 or Bcl-xl). They affect their respective functions by altering the permeability of the mitochondrial membrane. The third group of proteins which includes the BH3 interacting domain death agonist (Bid) are also involved in the apoptotic process [74].

1.6.4 Pathways of apoptosis

The pathways of apoptosis have been largely conserved throughout the animal kingdom [75]. Apoptosis is activated by a cascade of reactions that lead to the proteolytic activation of the effector caspases. Initiation of apoptosis occurs

primarily through two major pathways- extrinsic also called the death receptor mediated pathway and the intrinsic or mitochondrial pathway (Figure 4).

Death receptor or extrinsic pathway

This pathway is activated by the binding of ligands to a family of transmembrane receptors. Binding of the ligands activates the cytoplasmic domains of these receptors to form a death-inducing signalling complex (DISC). DISC binds initiator caspases such as caspase 8 and caspase 10 leading to their auto-activation. These activate downstream effector caspases (caspase 3) [76].

Mitochondrial or intrinsic pathway

Cellular stresses lead to the activation of pro-apoptotic Bcl-2 proteins such as Bad, Bax which are normally present in the cytoplasm [76]. Activation leads to their localisation to the outer membrane of the mitochondria. They bind and inhibit the protective function of Bcl-2 and Bcl2L1 leading to increased mitochondrial membrane permeability. This results in the leakage of cytochrome C from the inter-membrane space into the cytoplasm. Cytoplasmic cytochrome C binds with the apoptotic protease activator factor -1 (Apaf-1) to form the apoptosome. The apoptosome recruits caspase 9 which undergoes auto-activation in its vicinity. Caspase 9 activates caspase 3 leading to apoptosis.

BID is a protein normally present in an inactive form in the cytoplasm. Activated Caspase 8 formed through the death receptor pathway converts it to truncated Bid (tBid). tBid moves to the mitochondrial membrane and activates Bax and Bad. It thus serves as the connecting link between the death receptor and

mitochondrial pathways of apoptosis. Bcl-2 like protein 11 (Bcl2L11 or Bim) and the p53 up-regulated modulator of apoptosis (Puma) are two Bid-like proteins involved in the activation of Bax [77].

Execution of apoptosis

Effector caspases execute terminal processes involved in apoptotic cell death [71, 75]. They lyse a wide variety of cellular substrates including cytoskeletal proteins, regulatory proteins and nuclear proteins. Activated caspase 3 is essential for the characteristic morphological features of apoptosis such as the formation of apoptotic bodies, DNA fragmentation and nuclear condensation.

Figure 4 Outline of the steps involved in apoptosis (adapted from MacFarlane et al[78])

Apoptosis is initiated by a multitude of noxious stimuli. In the extrinsic or mitochondrial pathway, cell death stimulus directly acts on the mitochondria increasing outer membrane permeability and leakage of cytochrome c from the inter-membrane space into the cytoplasm. Cytoplasmic cytochrome c forms a complex with Apaf and caspase 9 to form the apoptosome. Apoptosome activates the effector caspase 3. Apoptosis can also be initiated through activation of the TNF/Fas receptor complex present on the cell membrane. Receptor activation by TNFα and Fas ligand (FasL) leads to receptor trimerisation and formation of the death inducing signalling complex (DISC). DISC activates caspase 8. Caspase 8 can activate the effector caspase 3. Activated caspase 3 acts on multiple cellular components to initiate the morphological features of apoptosis. Bcl2 family of proteins are usually present on the mitochondrial surface. Pro-apoptotic Bcl2 members (Bax) increase outer membrane permeability which is opposed by anti-apoptotic members such as Bcl2. Net membrane permeability depends on the relative activities of these two types of proteins. Activated caspase 8 also lyses cytoplasmic Bid to form tBid. tBid moves to the mitochondrial outer membrane to increase membrane permeability and induce leakage of cytochrome c. Bid hence acts as the connecting link between the intrinsic and extrinsic pathways of apoptosis.
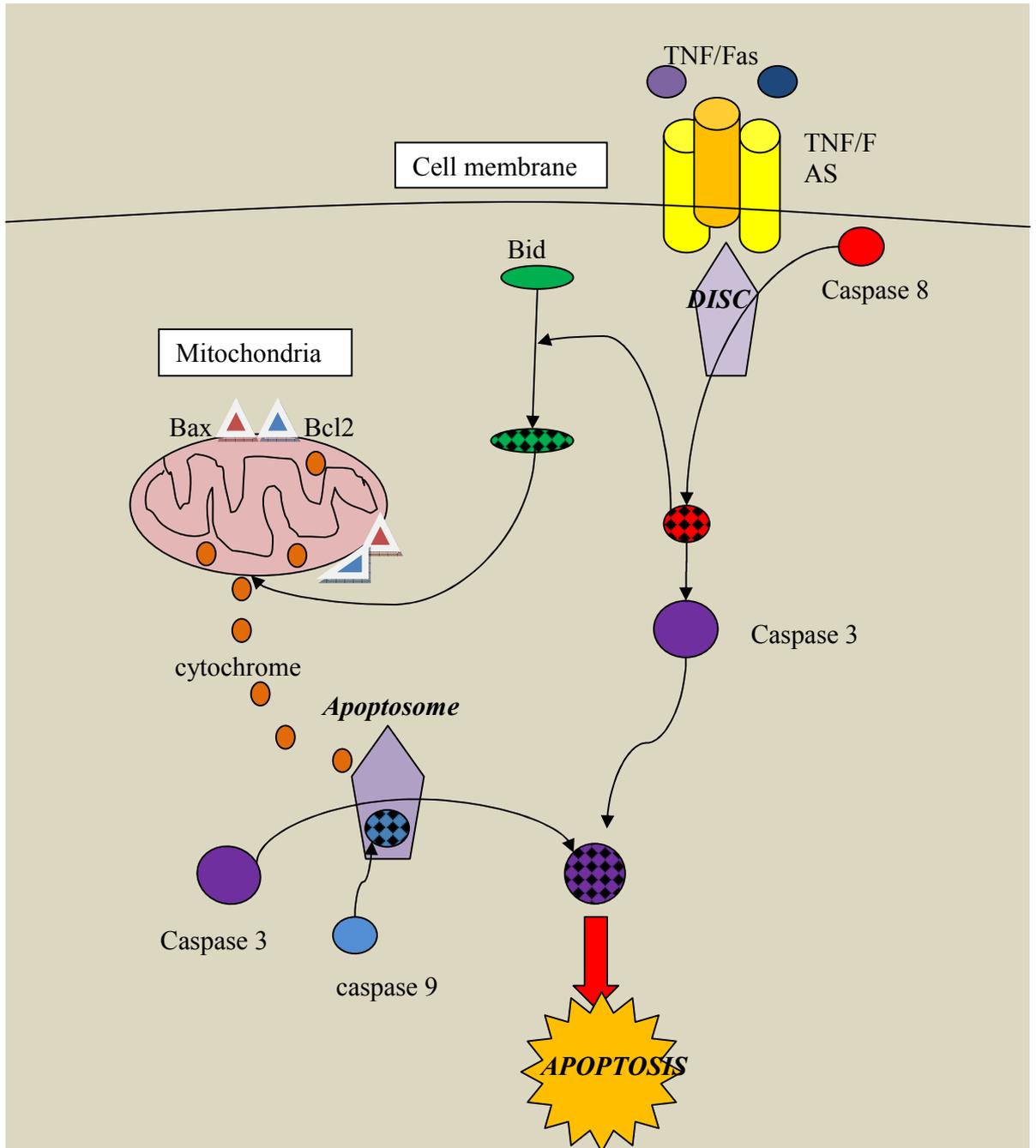
Figure 4 Outline of the steps involved in apoptosis

1.6.5 Anti-apoptotic mechanisms

Proteins of the Bcl-2 family have prominent roles in inhibiting the mitochondrial pathway of apoptosis[72]. Bcl-2, Bcl2L1 and Bcl2A1[73, 79] are normally present on the mitochondrial surface. They interfere with the action of Bax and Bad which increase mitochondrial membrane permeability. Several other intracellular proteins act to counteract apoptosis. Baculoviral IAP repeat-containing factors (BIRC2, BIRC3) bind to tumour necrosis factor receptor-associated factors (TRAF1 and TRAF2) and   block the death receptor pathway of apoptosis activation[80]. CASP8 and FADD-like apoptosis regulator (CFLAR) has been reported to block the death receptor pathway of apoptosis by inhibiting the Fas-associated death domain and caspase 8 activation. Phospho-protein enriched in astrocytes 15 (PEA15)[81], pim-2 oncogene (PIM2)[82] are other genes encoding proteins involved in the inhibition of apoptosis. PEA15 blocks TNFα induced apoptosis by inhibiting caspase 8 activation by DISC. PIM2 blocks the function of the pro-apoptotic protein BAD.

Haemoxygenase-1 (HO-1 or hsp32) is an inducible enzyme which is synthesised in large quantities during periods of cellular stress. This enzyme catalyses the breakdown of haeme into biliverdin, carbon monoxide and ferrous iron. Carbon monoxide is postulated to inhibit apoptosis by multiple mechanisms [83].

Heat shock proteins are a group of proteins that are produced in large quantities during periods of cellular stress. They function as intracellular chaperones preventing the accumulation of misfolded proteins. They inhibit apoptosis

through the death receptor and mitochondrial pathways [84, 85]. They have been shown to block TNFα receptor mediated activation of apoptosis and disrupt apoptosome formation. Several signalling cascades involved in the regulation of apoptosis are also subject to modulation by heat shock proteins. Clusterin [86] and Crystallin α have chaperone like activity and inhibit mitochondrial apoptosis[87].

1.7 Oxidative stress and cellular anti-oxidant mechanisms

Reactive oxygen species (ROS) are free radicals such as hydroxyl and superoxide ions or oxidants such as hydrogen peroxide. Minute quantities of reactive oxygen species are routinely produced during aerobic metabolism in all cells. Their activity is normally kept under check by a variety of anti-oxidative pathways present in the normal cellular milieu. Small amounts of reactive oxygen species are formed in the mitochondria during the ischaemia phase[88]. Reperfusion accelerates the production of reactive oxygen species through the xanthine oxidase pathway and from damaged mitochondria[89]. The uncontrolled production of ROS and their secondary products such as the peroxynitrite anion leads to membrane damage, lipid peroxidation and cell death [90]. Inactivation of ROS is an energy dependent process and requires ATP. During ischemia, ATP reserves drop precipitously which makes the cell ill-prepared to deal with the large amounts of reactive oxygen species that are formed during the reperfusion phase.

Anti-oxidant enzyme systems such as catalase, peroxidase and super-oxide dismutase directly inactivate ROS. Catalase and peroxidase breakdown hydrogen peroxide to form water and molecular oxygen. Superoxide dismutase is an enzyme which catalyses the breakdown of the superoxide ion into molecular oxygen and hydrogen peroxide.

Reduced glutathione is an important anti-oxidant mechanism. It is a tripeptide which is normally present in abundant quantities in the cytoplasm. Synthesis of glutathione occurs in two ATP dependent steps by the enzymes glutathione-cysteine ligase (rate-limiting step) and glutathione synthetase[91]. It acts as a substrate for the enzyme Glutathione-s-transferase transferring a hydrogen ion to ROS. The oxidised glutathione then binds with another oxidised glutathione to form glutathione disulphide. Reduced glutathione is regenerated by the activity of the enzyme glutathione reductase.

Uncoupling protein 2 (UCP2) is an enzyme complex present in the mitochondrial inner membrane of all cells. It has been suggested that it uncouples electron transport from ATP production. Several studies have suggested a role for this protein in the neutralisation of mitochondrial ROS[92, 93].

Sestrins are a group of highly conserved proteins which act by regenerating over oxidized peroxiredoxins that deoxidize reactive oxygen species[94]. This leads to a decrease in intracellular ROS and confers resistance to oxidative stress.

Several transcription factors are induced by ROS. These include the Nuclear factor Kappa B (NF-κB) pathway, Mitogen activated protein kinase (MAPK) pathways and Nuclear Factor 2 (NRF2) pathway. The latter is a transcription

factor that is normally localised to the cytoplasm. During periods of oxidative stress it translocates to the nucleus where it binds with other co-factors and activates the transcription of multiple cytoprotective genes. These genes are involved in increasing the anti-oxidant capacity of the cells and include those involved in glutathione synthesis and regeneration, aldehyde dehydrogenases, quinine oxido-reductases and haeme-oxygenase 1 [95-98].

1.8 Pathways involved in ischaemia reperfusion injury

Ischaemia reperfusion injury initiates multiple inter-linked biological pathways. These affect the inflammatory, immune and repair responses of cells. Toledo-Pereyral and co-workers in 2004 identified three phases of ischaemia reperfusion injury with respect to the involved molecular pathways [62]. Phase I, which occurs seconds to minutes after the injury is characterized by leukocyte - endothelial interactions and is dependent on the activation of phospholipases, intracellular calcium and lipid molecules. Phase II, which occurs minutes to hours after I/R injury is associated with the active transcription of inflammatory cytokines such as TNFα and IL-1β. Initiation of multiple signalling pathways leads to kinase activation, increased transcription and progression of the inflammatory process. Phase III, which occurs several hours to days after ischaemia reperfusion is associated with the production of anti-inflammatory cytokines, adhesion molecules and growth factors.

The second phase of ischaemia reperfusion is the most complex and critical with simultaneous activation of both pro-survival and pro-death pathways. Innate

immune responses are initiated by the activation of the cellular 'trinity of sensors' i.e. Toll-like receptor (TLR) family which are distributed in the cell membrane, Nod-like receptors (NLR) family and the RIG-like receptor (RLR) family which are cytoplasmic in distribution[99]. Activation of these receptors by bacterial and viral products, cellular necrotic debris and ROS leads to activation of pro-inflammatory genes and NF-κB activation. TLR activation is relevant in the setting of ischaemia reperfusion injury[100, 101]. Activation of pro-survival pathways such as the Extracellular signal-regulated kinases (ERK/MAPK), Protein kinase B (Akt/PKB) and NF-κB leads to the expression of genes involved in anti-apoptosis, anti-oxidant, cell repair processes. Activation of pro-death pathways such as p38/MAPK and cJun N-terminal kinase (JNK/MAPK) pathways leads to expression of genes involved in apoptotic and necrotic cell death. The net result depends on the cell state, its energy status, nature of the initiating stress and the balance between pro-death and pro-survival pathways.

1.8.2 Mitogen Activated Protein Kinase pathway

Mitogen activated protein kinases (MAPK) are a series of conserved metabolic pathways involved in the control of differentiation, proliferation and cell death (Figure 5). MAPK pathways are characterised by the presence of at least three protein kinases in series which ultimately leads to the phosphorylation of a multi-functional MAPK [102]. The setup allows for signal amplification and additional pathway regulation. Based on the initiating factors and the ultimate targets, three pathways have been well-defined. These include the extracellular-signal

regulated kinases 1 and 2 pathways (ERK/ MAPK), the P38 MAPK pathway and the c-Jun N-terminal kinase /Stress activated protein kinase pathway (SAPK/JNK pathway). Additional pathways like the ERK5 pathway have been described.

The ERK/MAPK pathway is primarily activated by growth factors binding to cell surface receptors. Receptor ligation leads to a series of events involving the proteins Growth factor receptor-bound protein 2 (GRB2) and son of sevenless homolog (SOS) leading to the activation of the G protein, *Ras*. Activated *Ras* binds and activates the serine threonine specific kinase, *Raf* which is the Mitogen activated protein kinase kinase kinase (MAP3K) for this pathway. Activation of *Raf* also occurs through the diacyl glycerol (DAG) pathway *via* protein kinase c (PKC) and the *ras* guanyl -releasing protein 1 (*ras*GRP) [103, 104]. *Raf* phosphorylates the pathway specific mitogen-activated protein kinase kinases (MAP2K) which are MAP2K1 and MAP2K2. These phosphorylate the pathway specific MAPKs (ERK1 or ERK2). Activated ERK activates several transcription factors. It activates the p90 ribosomal s6 kinase (p90RSK) which is involved in translation. p90RSK also inhibits the pro-apoptotic Bcl-2 protein BAD through phosphorylation[105]. The ERK pathway is involved in cell proliferation and differentiation.

The p38 MAPK pathway is activated by cellular stress factors such as heat shock, reactive oxygen species, lipopolysaccharide and by the inflammatory cytokines tumour necrosis factor alpha (TNFα) and Interleukin 1 beta (IL1β) [106]. They act through a variety of protein kinases to activate multiple MAP3Ks

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such as *Tao* 1/2, Tpl2, MEKK4, MEKK1. These activate the specific MAP2Ks, MAP2K3 and MAP2K6. The MAPK for this pathway is the p38 protein. Activated p38 induces a variety of transcription factors and inflammatory mediators. The net result is the progression of the inflammatory process and induction of apoptosis.

The cJun N-terminal kinases (JNK) pathway is activated by a variety of environmental stresses and inflammatory cytokines. Stress signals are delivered to this pathway by GTPases such as *Rac* and *Rho*. These activate a member of the mixed lineage kinases (MLK) that phosphorylate and activate the MAP2Ks for this pathway, MAP2K4 or MAP2K7. These activates JNK proteins 1-3 which translocate to the nucleus and regulate the activity of multiple transcription factors.

The activation of these pathways is kept under control by the dual specificity phosphatases (DUSP) and protein tyrosine phosphatases (Ptpn). These pathways are also controlled by activity the protein phosphatases and activated *Akt*/PKB.

| ERK/MAPK | cJUN /MAPK | P38 MAPK |

STIMULUS

Growth factors, protein kinase c

Heat shock, ROS, TNF, IL -1, DNA damage

MAP3K → *Raf* / Tpl2 → MLK3 → TAK/TAO1

MAP2K → MAP2K 1/2 → MAP2K 4/7 → MAP2K 3/6

MAPK → ERK1/2 → JNK 1 -3 → p38

DOWNSTREAM TARGETS

Proliferation differentiation survival

Proliferation differentiation apoptosis
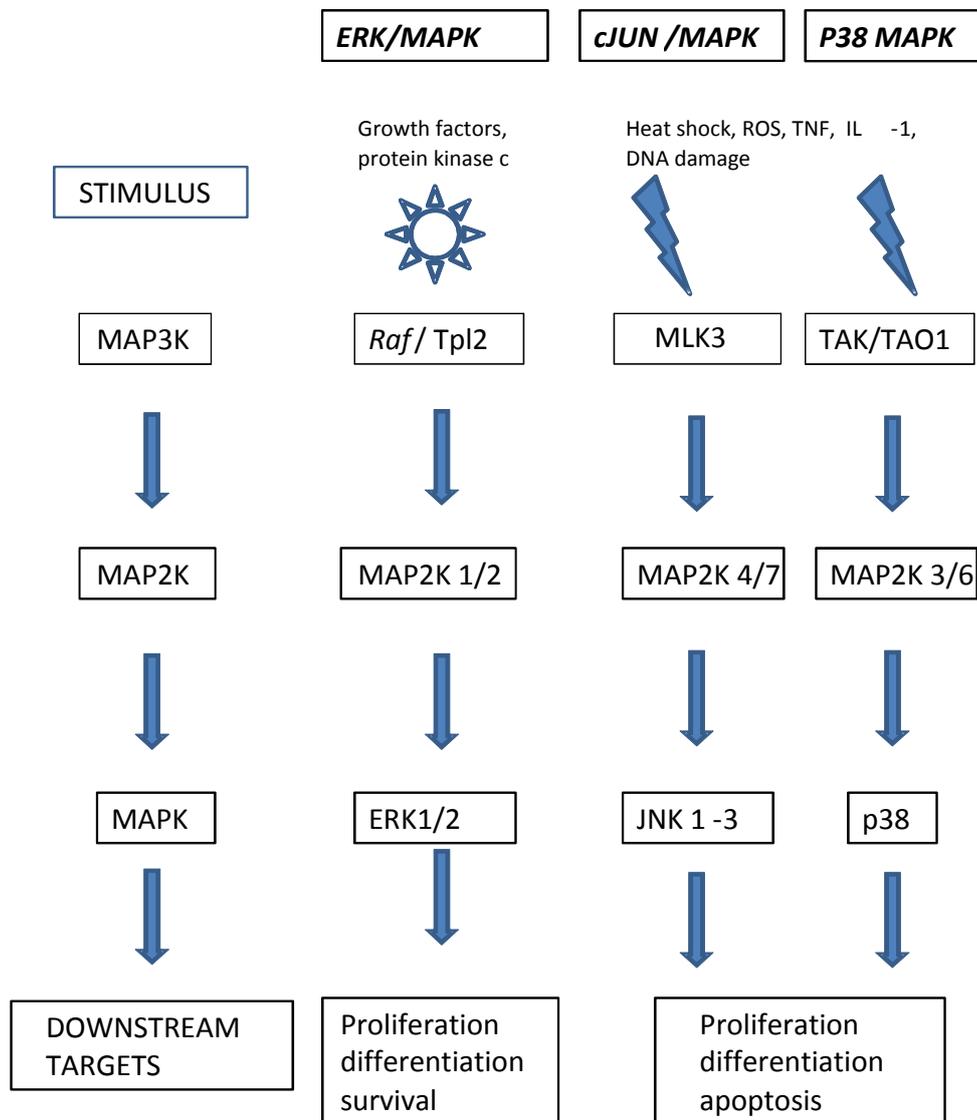
Figure 5 Mitogen Activated Protein Kinase (MAPK) pathways

MAPK pathways contain a series of at least three protein kinases terminating at a multifunctional protein kinase (MAPK). The outline of the three major pathways is depicted along with their initiating stimuli and downstream role in cell survival.

1.8.3 Phosphatidyl Inositol-3 kinase/ Protein kinase B pathway

Phosphatidyl Inositol-3 kinases (PI3K) are signal transduction enzymes that are involved in cell pathways promoting cell survival and proliferation [107]. Class 1A PI3K phosphorylates phosphatidyl inositol and produces phosphatidyl inositol (3,4,5)-trisphosphate (PIP3). PIP3 is a key second messenger in the cell. It activates downstream serine-threonine protein kinases such as Protein kinase B (Akt/PKB) and protein kinase C (PKC). Class 1A PI3K enzyme is a heterodimer and consists of a catalytic subunit and a regulatory subunit. The regulatory subunit at high concentrations has an inhibitory effect on the activity of the enzyme by blocking the active site[108, 109].

Protein Kinase B (Akt/PKB) is a critical regulator of cell survival and proliferation[110]. A variety of growth factors and insulin activate Akt/PKB through the PI3Kinases. Cellular stress has also been reported to directly activate Akt/PKB pathway through heat shock proteins[111]. The activation of Akt is tightly regulated by the quantity of the second messenger Phosphatidyl inositol triphosphate (PIP3). Levels of PIP3 can be reduced by the activity of enzyme phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN). Akt itself can be deactivated by the action of protein phosphatases[112].

Functions of Akt/PKB pathway

Akt/PKB contributes to several cellular functions including inhibition of apoptosis and cell survival, nutrient metabolism, cell growth, transcriptional regulation. Its

mechanism of action is through the phosphorylation of serine or threonine residues in various proteins.

Akt inhibits apoptosis by multiple pathways. It phosphorylates the pro-apoptotic Bcl-2protein BAD and induces its dissociation from the mitochondrial membrane[113]. Akt mediated phosphorylation of caspase 9 has been reported to inhibit the catalytic activity of caspase 9. The forkhead group of transcription factors have been identified as another target of Akt phosphorylation. These transcription factors induce the transcription of pro-apoptotic genes including the Fas ligand (FasL) involved in death receptor pathway of apoptosis. Activated Akt prevents the nuclear localisation of these proteins and thus blocks their activity[114].

Akt acts on several enzymes involved in carbohydrate metabolism. It phosphorylates and inactivates the enzyme glycogen synthetase kinase 3. Akt phosphorylation has also been reported to activate the transcription of glucose transporter (GLUT 1) and the glycolytic enzyme Phospho-fructo-2-kinase. The net effect is to increase the uptake of glucose from the extracellular fluid and utilising it for the glycolytic pathway for generation of ATP[110]. AKT activation activates the NF-κB pathway by multiple mechanisms.

1.8.4 Nuclear Factor- Kappa B (NF-κB) pathway

Nuclear factor- kappa B (NF-κB) are a set of transcription factors which are involved in the cellular response to stress. They were first identified as proteins which bind to the intronic enhancer region of the κ chain in activated B lymphocytes[115]. These enhancer sequences are now known to be ubiquitous in distribution. These factors act to modify the expression of multiple target genes associated with the inflammatory response.

Five different NF-κB elements (NF-κB 1, NF-κB 2, RelA, RelB, cRel) are known [116]. These are usually present in the cytoplasm as homo or hetero-dimers. They are localised to the cytoplasm by being complexed to proteins called the Inhibitors of Kappa B (IκB). There are three types of IκB termed IκB -alpha, IκB -beta and IκB -epsilon. Activation of the NF-κB pathway involves the breakdown of these complexes. This leads to the translocation of the NF-κB elements into the nucleus where they interact with other transcription factors and modify transcription.

Hypoxia, reactive oxygen species and cytokines such as TNFα and IL-1β activate the NF-κB pathway through trans-membrane receptor complexes. The latter include the tumour necrosis factor receptor (TNFR) super-family, IL-1β receptor and TLR family[116, 117]. Activation of these receptor complexes leads to a cascade of events involving several adapter proteins. Tumour necrosis factor receptor associated factors (TRAF) family of proteins are important transducers of signals from these receptor complexes[118]. These ultimately

activate protein kinases termed the inhibitors of kappa kinase (IKK) complex. Phosphorylated Akt activates the NF-κB pathway through direct activation of IKK and through phosphorylation of the MAP3kinase, Tpl2[119, 120]. Three Inhibitors of Kappa kinases (IKK) have been described. These include two catalytic sub-units (α, β) and a regulatory subunit IKKγ. The latter is also called the NF-κB essential modifier (NEMO). The IKK complexes phosphorylate the IκB proteins which releases them from the NF-κB protein subunits. The released IκB proteins undergo proteosomal degradation leaving the NF-κB proteins to translocate to the nucleus. Other modes of both IKK dependent and IKK independent activation of the NF-κB pathway in response to cellular stress have been described.

Functions of the NF-κB pathway

NF-κB pathway has been reported to have multiple roles in effecting the cellular response to stress. It has a primarily pro-inflammatory function. The list of target genes for NF-κB runs into hundreds in published literature[121-123]. It induces the transcription of genes related to cytokines, cell surface receptors, pro-apoptotic and anti-apoptotic proteins, adhesion molecules and anti-oxidants. It is primarily considered to be anti-apoptotic, pro-survival though some studies have also reported a pro-apoptotic, pro-death role. The ultimate effect of the NF-κB pathway depends on the activity of other stress induced pathways and the cellular state[124].

NF-κB has been shown to inhibit TNFα induced apoptosis[125]. Activation of the NF-κB pathway leads to the transcription of factors involved in anti-apoptosis.

These include bfl-1/a1, Bcl-XL, Caspase 8/FADD like apoptosis regulator (CFLAR), TRAF 2 and inhibitor of apoptosis proteins (BIRC2 and BIRC3, XIAP). Activation of the NF-κB pathway has been found to control the accumulation of reactive oxygen species in response to TNFα. This is achieved primarily by the increased expression of ferritin heavy chain (FHC) and mitochondrial form of superoxide dismutase[126, 127]. NF-κB activation also inhibits the JNK pathway[128]. Activation of the JNK cascade by TNFα is a recognised inducer of apoptotic cell death. It has been reported that activation of the NF-κB pathway blocks the JNK pathway at multiple steps. Gadd45β, a protein up-regulated by the NF-κB pathway is a prominent blocker of the JNK pathway[128, 129].

Regulation of the NF-κB pathway

NF-κB pathway is regulated by several feedback loops. One mechanism of interest is the increased transcription of the IκBε gene in response to NF-κB activation. The IκBε protein enters the IκB complexes to decrease NF-κB protein activation thus acting as a feedback mechanism. The extent of IκBε transcription is hence an indicator of NF-κB pathway activity in the cell[130].

1.9 Microarrays- a primer

The full complement of genetic material present in an organism is called the genome. While a genome contains thousands of genes, only a fraction of these genes are expressed at any point in time. The set of genes expressed at any point in time is called the transcriptome. Measurement of the level of expression of these genes in a given clinical or experimental condition provides important

information regarding the response of the cell or tissue to these conditions at a molecular level. Technologies such as the polymerase chain reaction (PCR) can provide information regarding one or a handful of genes of interest. However it is now known that expression of any single gene can be modified by multiple factors due to the extensive redundancy that exists in the cellular response. Better understanding of the cellular state can be obtained by analysing the expression of all the genes in a genome. This gives information regarding thousands of genes in a single experiment. This information, whilst complex, can be used to study the pattern of global gene expression and provide information regarding the biological pathways activated or suppressed. Microarrays are tools for analyzing expression of large number of genes in a single experiment[131].

1.9.1 Microarray techniques and analysis

The technique of DNA microarray analysis is based on the principle of competitive binding of mRNA to complementary sequences attached to a glass slide or synthetic membrane. The procedure begins with extraction of RNA from cells or tissue. The RNA is labelled with a fluorescent dye. The RNA is incubated on a glass slide containing thousands of spots-each containing oligo-nucleotide sequences (a short fragment of a single-stranded DNA that is typically 5 to 50 nucleotides long) specific for different genes. After overnight incubation, the slides are washed to remove excess unbound RNA. The slide is then analysed by computerised laser equipment which detects the intensity of

fluorescence in each of the spots and converts it into a numerical value which is a measure of the amount of mRNA specific to that gene in the RNA sample[132]. These data are normalised by correcting for experimental conditions. After further statistical analysis, the level of gene expression is presented as fold change, false discovery rate or P value.

The data available from a microarray experiment usually consist of a data matrix which includes the expression levels of thousands of genes in each experimental condition. Analysis of gene expression data can be carried out in several ways. The simplest way to analyse these data is to compare the level of expression of specific genes which have been identified as relevant to the nature of the investigation. This has limited utility and does not utilise the full potential of microarrays. Comparing expression of multiple genes involved in selected relevant biological processes can give a better idea regarding the pattern of gene expression under various experimental conditions. This is best carried out by an ontological approach where the genes are grouped based on their putative function. The Gene Ontology (GO) project (accessible at http://www.geneontology.org/) is a worldwide collaborative effort intended to standardise the terminology used by the scientific community to describe biological processes. Structured and controlled vocabularies (ontologies) have been developed that describe gene products in terms of their associated biological processes, cellular components and molecular functions in a species-independent manner[133]. The format of Gene Ontology is in the form of a directed acyclic graph, which is a hierarchical structure similar to a tree. Each of

the three ontologies (biological process, cellular component and molecular function) has been built with multiple levels of abstraction. Hence each general biological process has multiple child processes each of which can in turn have multiple specific child processes. Each of the processes has an assigned alphanumeric code, the 'GO term' which can ensure consistency in analysing, presenting and circulating information.

Pathway analysis and pictorial visualisation of the gene expression data is another powerful tool that utilises the wealth of data that is provided by microarrays. Here specific well studied biological pathways are represented with the expression of data of various involved genes highlighted in different colours to mark over and under-expressed genes.

1.9.2 Utility of gene micro arrays in biology

DNA micro arrays have proven their utility in a multitude of biological research areas[134]. These include measurement of gene expression to relate it to cellular physiology, detection of bio-markers for disease, detection of markers of disease prognosis, pathogen detection and evaluation of defects at the transcriptional level of various genetic disorders.

1.9.3 Limitations of gene microarrays

Microarray technology is expensive and needs specialised equipment and trained personnel. The risk of random errors entering the analysis is high due to the vast amount of data produced by microarrays. Strict statistical validation of expression data using novel statistical methods should therefore be carried out

before further analysis. Variation in gene expression could be due to multiple confounding factors especially in complicated experiments. Comparison of results from different microarray experiments is difficult due to the wide variability in the experimental protocols, and the actual microarray techniques. Attempts are now being made to standardise the form of data presentation and analysis and establish public databases to store microarray data[135]. Microarrays only provide a guide to the relative levels of gene expression. Ideally the accuracy of microarray data should be confirmed by quantitative polymerase chain reaction (PCR) analysis of selected genes from the same RNA sample. Finally, microarrays look at the first step in the 'central dogma of life' *i.e.* gene transcription. The effect of any physiological stimulus on the cell depends on multiple processes downstream of transcription. Gene microarrays provide no information regarding actual protein expression levels in the cells.

1.10 Ischaemia reperfusion injury in pancreas and islet transplantation

IRI is a well known complication after pancreas transplantation[136] and pancreatic islet transplantation[137]. It presents as graft pancreatitis after pancreas transplantation and immediate blood mediated inflammatory response (IBMIR) after islet transplantation. Both lead to graft dysfunction and graft loss. It has been reported that the exocrine component of the pancreas is much more susceptible to IRI as compared to the endocrine component [138]. Exocrine

injury after pancreatic ischaemia reperfusion leads to extravasation of enzymes into the systemic circulation. These enzymes can cause auto-digestion and progression of tissue injury. Petersson and co-workers in 1998 [139] investigated reperfusion injury in cold preserved porcine pancreases. They reported that protease activation starts during the cold ischemia phase and increases several fold during the early reperfusion phase. This was decreased by the use of free-radical scavengers suggesting a role for reactive oxygen species in protease activation. Garcia-gil and co-workers in 2006 reported a significant increase in the lipid peroxidation during the reperfusion phase after pancreas preservation[89]. Mayer and co-workers in 1999 [140] identified significant microcirculatory disturbance in pancreas with prolonged cold ischaemia. Cold ischemia time of 16 hours significantly reduced functional capillary density, erythrocyte velocity, and leukocyte-endothelium interaction when compared to a cold ischemia time of 1.5 hours.

Ischaemia reperfusion injury in islet transplantation originates before the transplant procedure at the time of donor death. Induction of brain death is an activator of inflammation [141]. Contreras and co-workers in 2003 reported decreased islet yield and increased cytokine activation in pancreases retrieved from brain dead rats[142]. The severity of ischaemia reperfusion injury depends on the duration of warm ischaemia and subsequent cold ischaemia. Pileggi and co-workers in 2009 studied the effect of increasing duration of cold ischaemia on the yield and viability of rat islets. They reported that pancreases exposed to long cold ischaemia times yielded lower islet numbers and showed reduced

cellular viability. Islets from pancreases with prolonged cold ischaemia time also displayed increased activation of the p38/MAPK and JNK/MAPK pathways and lower levels of pro-survival factors[143]. The process of re-warming of the pancreas and the mechanical stresses involved in islet isolation lead to activation of multiple inflammatory pathways. Noguchi in 2007 reported activation of JNK pathway during islet isolation [144]. Production of reactive oxygen species during the islet isolation phase has been reported [145]. Armann and co-workers in 2007 investigated the production of ROS in human pancreatic islets. They reported that the basal levels of ROS in human islets were higher than in islets isolated from rat pancreases. High ROS levels were also associated with increased lipid peroxidation as evidenced by islet malondialdehyde levels. Apoptosis has been identified as an important determinant of post transplant islet function[137]. Blockage of apoptotic mediators and increase in anti-apoptotic mediators has been shown to improve islet function[69, 146]. Similarly inhibition of the p38MAPK pathway during pancreas preservation has been shown to improve post-transplant islet function[147].

Improvement in the outcomes of islet transplantation can only be achieved by ameliorating the extent of post-transplant early islet loss due to ischaemia reperfusion injury[14]. It is hoped that improved quality of pancreas preservation which can provide an opportunity for the commencement of reparative processes during the cold ischaemia period may improve the viability and function of these grafts.

1.11 Techniques of preservation of non-heart-beating-donor pancreas

1.11.1 Static cold storage

Static cold storage in UW solution is the preservation method currently used for transporting human pancreases. It is cheap and does not need specialised equipment or staff. In this technique, the pancreas is initially cooled by perfusion with cold UW solution through its arterial system. The pancreas is then placed in a sterile sealed bag containing cold UW solution and stored in ice. The low temperature slows metabolism and minimises continuing ischaemic damage in the tissues.

Preservation of heart-beating-donor pancreases by static cold storage is sufficient to maintain tissue viability. Improved preservation techniques are required for non-heart-beating-donor pancreases that have sustained warm ischaemia damage. Some new methods of organ preservation are already in clinical use while others are still at the experimental stage.

1.11.2 Two Layer Method with oxygenated perfluorocarbon

Perfluorocarbons are biologically inert compounds with the capacity to dissolve 20-25 times their own weight of oxygen. They have a low oxygen-binding capacity and can release oxygen easily at low oxygen partial pressures[148].

The use of perfluorocarbons in pancreas preservation was first reported in 1988 and it became known as the 'two layer method' (TLM)[149]. Here the preservation container contained a bottom layer of perfluorocarbon which was continuously oxygenated with pure oxygen and a top layer of cold UW solution. The pancreas was suspended at the interface of the two layers with at least two-thirds of the organ immersed in the perfluorocarbon layer[150] (Figure 6). Its superiority over static cold storage has been demonstrated in experimental models of pancreas[19] and small bowel transplantation[151]. The two layer method has been shown to improve oxygen delivery[152], increase tissue ATP levels in the graft[153], decrease cellular apoptosis[154] and reduce ischaemia-reperfusion injury[155] when compared to static cold storage. Kakinoki and co-workers in 2005 [19] compared the islet yield and *in vivo* function of islets from non-heart-beating-donor rat pancreas preserved for 24 hrs by UW solution or two layer method. They reported better success rates of islet grafts from the two layer method group (80% vs. 0%). However several reports have queried the feasibility of adequate oxygenation of large animal pancreas by simple diffusion[156]. In a study of pig pancreas preservation, the two layer method did not improve islet yield compared to static cold storage[157]. Kin and co-workers in 2006 [20] reported a large scale clinical trial comparing the two layer method with static cold storage for preservation of pancreases from heart-beating donors. They reported no differences in tissue ATP content, islet yield, *in vitro* function or transplant success rates between the two preservation groups.

Another study reported that use of the two layer method did not improve the ischaemia tolerance of pancreatic grafts retrieved from elderly donors [158].

1.11.3 Hypothermic machine perfusion

Hypothermic machine perfusion involves the continued perfusion of the organ with preservation solution. The purported benefits of machine perfusion include better flush out of the organ, maintained patency of the vascular bed, provision of nutrients and removal of metabolites from the organ[159]. This technique was initially reported in clinical kidney transplantation in the early 1970's[160]. Increasing use of non-heart-beating-donor kidneys has revived interest in this technique of preservation[161]. Machine perfusion is now widely accepted as the better preservation technique for non-heart-beating-donor kidneys[162] (Figure 7). Machine perfused non-heart-beating-donor kidneys have been shown to have shorter periods of delayed graft function[163] and better outcomes than static cold storage kidneys [164, 165]. A recent multicentre randomised controlled trial compared static cold preservation and hypothermic machine perfusion in deceased donor kidney transplantation. This has confirmed the beneficial effect of machine perfusion in decreasing the duration of delayed graft function[166]. Several groups have also reported the utility of machine perfusion parameters in assessment of graft viability[167, 168].

Machine perfusion for the preservation of the pancreas for whole-organ or islet transplantation has been studied in experimental models (Table 2). However these studies have involved pancreases from heart-beating-donor models and

have not provided conclusive data. Most of these studies used plasma based perfusion fluids which are costly, have batch to batch variation in composition and have the theoretical risk of the transmission of blood borne infections. The high perfusion pressures used also lead to a high incidence of pancreatic oedema because the pancreas is a low-flow organ and lacks a well defined capsule that can limit the development of oedema.

More recent studies using this modality of organ preservation have provided promising results. Leeser and co-workers in 2004 [169] reported that human pancreases preserved with low-pressure machine perfusion had higher islet yield and better *in vitro* function compared to static cold storage. Taylor and co-workers in 2008 [170] investigated the effect of 24 hours of low-pressure machine perfusion of porcine pancreases on islet yield and function. They reported higher islet yields in the machine perfusion group. They also reported more uniform digestion and fewer entrapped islets in the machine perfusion group. They postulated that the oedema which is a constant feature in machine perfused pancreases may aid pancreas digestion.
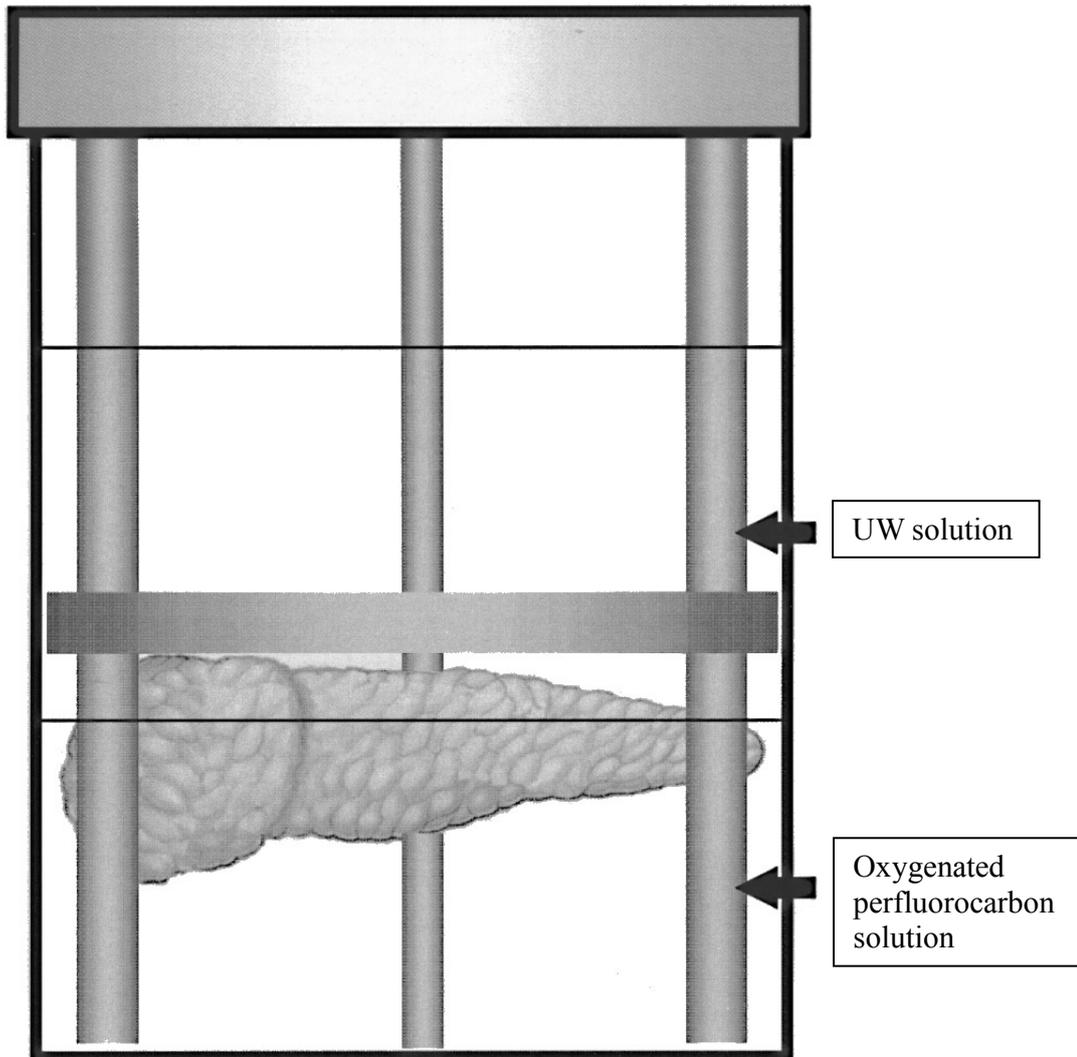
Figure 6 Schematic representation of the 'two-layer method' of pancreas preservation (from Noguchi *et al*[150])
The two layer method involves the pancreas floating at the interface of two fluids in a sterile container. The bottom layer is perfluorocarbon solution which is continuously oxygenated by bubbling pure oxygen into the solution . The top layer is University of Wisconsin solution . The whole container is maintained at 4°C by keeping it in ice. The pancreas is suspended at the PFC-UW interface. Oxygen from the perfluorocarbon is hypothesised to diffuse into the pancreas and provide oxygen during the cold ischaemia period.

Figure 7 Lifeport™ Renal hypothermic machine perfusion system used by transplant units for the preservation of non-heart-beating-donor kidneys

Table 2 Summary of previous published reports investigating the potential role of machine perfusion as a method of pancreas preservation

Author	Description of study	Details of animal model & machine perfusion model	Conclusion
Tersigni *et al.*[171]	Comparison of two perfusion fluids varying in electrolyte composition and osmolarity	Canine pancreas transplantation, Cryoprecipitate based fluids, pulsatile perfusion of 30-35 mmHg.	Pancreases perfused with high osmolarity fluid had better post-transplant function
De Gruyl *et al.*[172]	Comparison of machine perfusion with static cold storage	Canine pancreas transplantation, Pulsatile machine perfusion with cryo-precipitated plasma	No difference in graft outcome between machine perfusion and static cold storage groups
Florack *et al.*[173]	Machine preservation compared with static storage for varying time periods	Canine segmental pancreas auto-transplantation, Silica gel filtered plasma, perfusion at 30mmHg	Static cold storage better than machine perfusion

Author	Description of study	Details of animal model & machine perfusion model	Conclusion
Toledo-Pereyra *et al.*[174]	Machine perfusion for 24 and 48 hours compared with fresh pancreases	Canine islet auto-transplantation, Cryo-precipitate based pulsatile perfusion at 20-25 mmHg.	Good success with 24 hour grafts, poor outcome with 48 hour grafts
Kenmochi *et al.* [175]	Non-heart-beating-donor pancreases preserved with machine perfusion for one hour	Canine segmental pancreas transplantation, Cryo-precipitated plasma, pulsatile perfusion for one hour, 50 mmHg	Tissue flow rate predicts super-oxide dismutase levels in the pancreas and post-transplant function
Leeser *et al.*[169]	Pancreases preserved by machine perfusion or static cold storage	Human pancreas- *in vitro* study, Pulsatile perfusion with UW solution, 30-35mmHg pressure	Perfused pancreases had higher islet yields and *in vitro* function when compared to static storage.
Taylor *et al.* [170]	Porcine pancreases preserved for 24 hours by machine perfusion or static cold storage	Porcine pancreas-*in vitro* study, Pulsatile perfusion with KPS-1 solution, 10mmHg pressure	Perfused pancreas had higher islet yields. They also had more uniform digestion and fewer entrapped islets.

1.11.4 Venous oxygen persufflation

This method comprises the gaseous oxygenation of the graft through the venous side of the vascular system. The oxygen reaches all parts of the graft through the capillaries and escapes through small incisions or punctures made on the graft surface. During this process, the graft is placed in organ preservation solution maintained at 4°C. The technique of venous oxygen persufflation was described in early 1970's by Isselhard and co-workers [176, 177]. Rolles and co-workers in 1984 [178] studied the effectiveness of oxygen persufflation in the preservation of canine kidneys after 30 and 60 minutes of warm ischemia. They reported good post transplant function in grafts with 30 minutes warm ischemia and 48 hours preservation with oxygen persufflation. Persufflation with air was not as effective as pure oxygen and inert gases were completely ineffective in conferring any benefit. In a pilot clinical trial, the same group [179] reported data from 10 pairs of non-heart-beating-donor kidneys, one of each pair was preserved by oxygen persufflation and the other by static cold storage. Two grafts from the persufflation group were lost compared to one graft from the static group. Kidneys in the persufflation group had a shorter period of delayed graft function and lower creatinine levels. However, the numbers were too small to reach statistical significance.

There is a now a large amount of experimental data supporting the role of venous oxygen persufflation in improving preservation of non-heart-beating-donor kidneys, livers[180] and heart[181]. The importance of using anti-oxidants

such as super-oxide dismutase or taurine during this method of preservation is well documented [182, 183]. Multiple mechanisms have been postulated to explain the beneficial effect of oxygen persufflation. Minor and co-workers in 2000 investigated this preservation technique in fatty livers of rats. They reported decreased hepatocyte damage, decreased Kupffer cell activation and better preservation of mitochondria and endothelial cells with oxygen persufflation[184]. Other researchers have reported reduced apoptosis[185] and increased intra-cellular ATP levels [186] with persufflation. Saad and co-workers in 2001 compared the post-transplant outcome of porcine livers exposed to 60 minutes of warm ischemia and preserved with static cold storage or oxygen persufflation. They reported 100% 5-day survival of pigs transplanted with persufflated grafts, while no pigs receiving the static storage grafts survived [180]. Treckmann and co-workers in 2006 compared post-transplant graft outcomes of porcine kidneys exposed to 60, 90 and 120 minutes of warm ischemia. Kidneys in each warm ischaemia group were preserved by static cold stored or by oxygen persufflation. They reported 100% graft survival beyond one week in the 60 minutes warm ischaemia + oxygen persufflation group while all other groups had early recipient deaths or high post-transplant creatinine levels [187]. Treckmann and co-workers in 2008 also reported a pilot clinical study in which five marginal livers rejected by three other centres were preserved for a short period (1-3 hours) with venous oxygen persufflation. All five livers were transplanted. Pre and post persufflation biopsies showed higher tissue ATP levels after persufflation. All grafts had immediate function and were functioning

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at two years. Bleeding from the puncture sites was a problem in two patients (peri-operative bleeding in one, subcapsular haematoma in one). One patient had hepatic artery thrombosis which was successfully thrombectomised. The authors concluded that venous oxygen persufflation in the clinical liver transplant setting was feasible and that it improved aerobic metabolism and post-transplant graft function[188].

The utility of venous oxygen persufflation to preserve pancreas grafts has not been investigated. While the chief concern with this technique in solid organ transplantation was the risk of gas embolism after transplantation this is not an issue in islet transplantation. It is possible that the dissection of tissue planes that is caused by the persufflation may aid pancreas digestion. The anatomy of the pancreatic venous drainage involves multiple small venules draining into the splenic vein and proximal superior mesenteric vein. The latter two join to form the portal vein. Oxygen persufflation through the portal vein should hence oxygenate the pancreas, spleen and the duodenum.

1.12 Summary of literature review

Pancreatic islet transplantation is a therapeutic option in carefully selected patients with type 1 diabetes. Successful islet transplantation depends on the transplantation of sufficient number of viable islets and minimising early islet loss. Non-heart-beating-donors are not routinely used for islet transplantation. Static cold storage is not optimum for the preservation of pancreases damaged

by warm ischaemia. The two layer method of preservation has shown promising results in the experimental setting, but its advantage over static cold storage in the clinical setting is still unclear. Hypothermic machine perfusion and venous oxygen persufflation both have theoretical benefits over static cold storage and the two layer method. Their role in preservation of pancreases in the non-heart-beating-donor setting has not been investigated in any detail. This project aims to bridge that gap.

1.13 Research Hypothesis

The hypothesis tested in the present study is that 'pancreatic damage caused by warm ischaemia can be ameliorated by improved methods of pancreas preservation'.

1.14 Research aims

The main aims of the study defined for testing the research hypothesis were as follows.

- Develop a model of non-heart-beating-donor rat pancreas retrieval and preservation.

- Compare the yield of islets isolated from non-heart-beating-donor rat pancreases preserved with static cold storage, hypothermic machine perfusion, and portal venous oxygen persufflation.

- Compare the severity of reperfusion injury after *in vitro* warm reperfusion of non-heart-beating-donor rat pancreases preserved by static cold storage and portal venous oxygen persufflation.

- Compare the gene expression profile of non-heart-beating-donor rat pancreases preserved by static cold storage and portal venous oxygen persufflation after *in vitro* warm reperfusion.

Chapter 2   Materials and methods

2.1 Experimental design

The study was designed in two phases (Figure 8).

Phase I of the study investigated the relative benefits of hypothermic machine perfusion, portal venous oxygen persufflation and two-layer method in improving islet yield from non-heart-beating-donor rat pancreases as compared to static cold storage. An additional group of pancreases with no warm ischaemia also underwent islet isolation and assessment to act as a positive control.

Phase II of the study compared the severity of reperfusion injury in non-heart-beating-donor rat pancreases preserved by static cold storage or portal venous oxygen persufflation followed by *in vitro* warm oxygenated reperfusion. Pancreatic tissue removed from freshly killed rats was used as a control group. RNA extracted from the pancreases after *in vitro* reperfusion was used for microarray analysis. The differential expression of genes in each preservation group when compared with control pancreatic tissue was used to identify differential activation of pathways pertaining to ischaemia reperfusion.
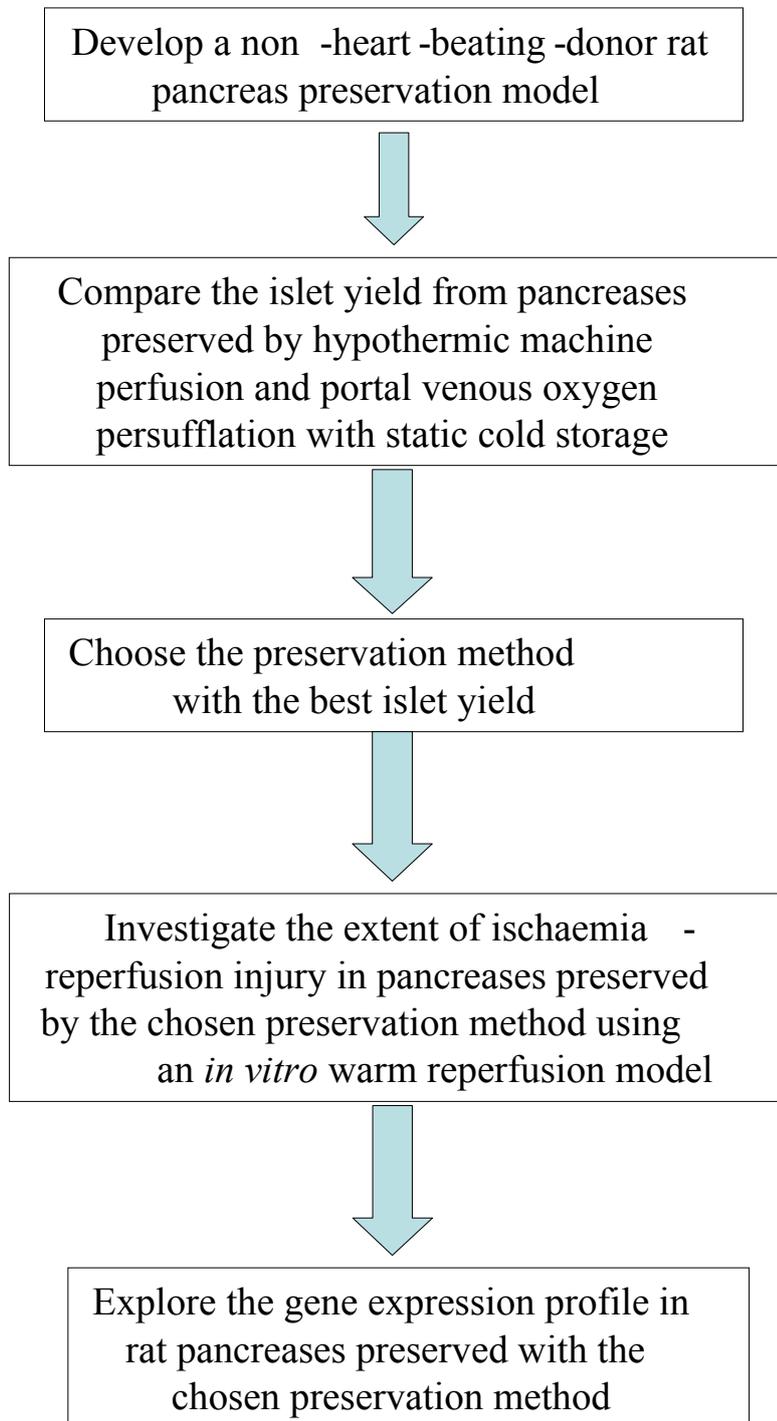
Figure 8 Flow-chart depicting the overall plan of study

2.2 Materials

General laboratory glassware and plastic-ware including pipettes, pipette tips, tubes, tissue culture flasks, multi-well plates, slides, cover slips, were supplied by Bibby Sterilin, unless otherwise stated.

Chemicals, solvents, reagents were of Analytical Reagent (AR) grade. All chemicals were obtained from Sigma Aldrich (St.Louis, Mo) unless specified. Water was purified through a Milli Q ion exchange system (Millipore UK) for use in the preparation of aqueous solutions. Full list of reagents and equipment used is detailed in appendix 1

All tissue culture ware and consumables were sterilised by autoclaving at 121°C for 15 minutes. Reagents for islet culture media were already sterile and used directly or were filter-sterilised using a 0.2micron filter prior to use. University of Wisconsin solution was stored at 4°C. Pancreas recovery, preservation and digestion for islet isolation was carried out in clean but not sterile conditions.

2.3 Methods

2.3.1 Preparation of reagents and media

Wash medium for washing steps during islet isolation

To make 500 ml of wash solution, 50ml of 10X concentrated HBSS solution was added to 450ml of autoclaved de-ionised water. To this solution, 0.5g of anhydrous glucose, 175mg of sodium bicarbonate and 2.5g of bovine serum albumin were added and gently mixed. The pH was adjusted to 7.4.

Collagenase solution for pancreas digestion

10mg of Collagenase P was weighed out carefully and transferred to a 20ml universal container. 10ml of HBSS solution (without bovine serum albumin) was added and mixed gently. The solution was prepared fresh prior to each pancreas digestion. The prepared solution was kept on ice and used immediately.

Molar glucose solution for static glucose stimulated insulin secretion assay

7.2g of anhydrous glucose was weighed and transferred to a 50ml centrifuge tube. 40ml of autoclaved de-ionised water was added. The tube was warmed in a water bath to 37°C and mixed to ensure complete dissolution. The solution was sterilised by filtering through a 0.2μm syringe filter into a sterile centrifuge tube. The stock solution was stored at 4°C.

Islet culture medium

To make 500ml of sterile culture medium, 430ml of Dulbecco's Modified Eagle medium was transferred aseptically to an autoclaved glass bottle. 50 ml of foetal calf serum, 5.5ml of 1M glucose, 10 ml of Penicillin-streptomycin solution, 5ml of Amphotericin B were added. The medium was prepared under an aseptic hood. It was stored at 4°C.

Islet incubation medium for static glucose stimulated insulin secretion assay

Glucose free medium was prepared aseptically by adding 2.5g of bovine serum albumin, 5ml of 1M Hepes, and L-Glutamine (final concentration of 2mM) to 500ml of Glucose free Dulbecco's Modified Eagle Medium. The medium was stored at 4°C.

High glucose incubation medium (medium with 40mM glucose) was prepared by adding 830 microlitre of sterile 1M glucose solution to 20 ml of glucose free medium. Low glucose incubation medium (medium with 3mM glucose) was prepared by adding 4ml of high glucose medium to 50 ml of glucose free medium.

Dithizone solution for staining islets

20mg of dithizone powder was weighed and transferred to a 20ml universal container. 2ml of dimethyl sulphoxide was pipetted into the container and mixed

well. 10ml of wash medium was added and shaken vigorously. The mixture was filtered through a 20 micron syringe filter. The filtrate was stored at 4°C for up to one week.

Trypan blue solution for islet viability assessment

20 mg of trypan blue powder transferred to a 20ml universal container. 5ml of wash medium was added and mixed by shaking. The solution was stored at 4°C.

Fluorescein diacetate /Propidium iodide solutions for islet viability assessment

Fluorescein diacetate (FD) stock solution was prepared by adding 5mg of Fluorescein diacetate to 5ml of pure acetone and mixed well. This was stored in a universal container away from light. Propidium iodide (PI) stock solution was prepared by adding 1ml of PI solution (1mg/ml) to 9ml of wash solution. The solution was stored away from light. Working solution for staining islets was prepared immediately before use. 1ml of the stock PI solution was transferred to a 1.5ml microcentrifuge tube. Ten microlitres of the stock FD solution was added and mixed well.

Krebs- Henseleit solution for in vitro warm reperfusion experiment

450ml of autoclaved de-ionised water at room temperature was transferred to a 1 litre sterile flask. 4.8 g of the reagent was weighed and added to the water

while stirring. To the solution 0.186g of calcium chloride dehydrate was added and stirred until dissolved. 1.05g of sodium bicarbonate was added and stirred until dissolved. 50ml of autoclaved water is added to the final solution and the pH adjusted to 7.4 with 1N hydrochloric acid or 1N Sodium hydroxide solutions. The final composition was NaCl (118 mM), KCl (4.9mM), KH_2PO_4 (1.2 mM), $MgSO_4.7H2O$ (1.2 mM), Glucose (11.1 mM), $NaHCO_3$ (25mM), $CaCl_2$ (2.5 mM). It was used immediately.

Radio-Immuno-Precipitation Assay buffer for homogenisation of pancreatic tissue

180 ml of sterile de-ionised water was transferred to an autoclaved glass bottle. The following were added in sequence with continuous stirring : Sodium chloride: 1755 mg, NP-40 : 2 ml, Sodium deoxycholate: 2 g, Sodium deoxy sulphate: 0.2g, 1.5 M Tris HCL (ph=7.6) : 3.3 ml. The solution was made to 200ml by adding 15ml sterile de-ionised water. The solution was stored at 4°C.

Phenyl Methyl Sulphonyl fluoride

PMSF is unstable in aqueous solution. Hence a 100mM stock solution was prepared by adding 175 mg of PMSF to 10 ml of Isopropanol). The solution was stored in 0.5ml aliquots at -20°C. For homogenisation of pancreatic tissue, 0.1ml of 100mM stock solution was added to 10 ml of cold buffer just before its use for homogenisation.

2.3.2 Choice of buffers in various experiments

Several different buffered solutions and media were used at various stages of the study. The choice of buffer for a particular step depended primarily on the literature review. Buffer solutions that have been repeatedly used for a specific purpose in the pancreas and islet literature were used in these experiments.

University of Wisconsin solution (Viaspan™) was used for aortic flushing and preservation of the pancreas. This is a organ preservation solution with ionic composition similar to the intra-cellular fluid. This is the current standard of care in clinical pancreas preservation.

Ringer's lactate solution has composition similar to extracellular fluid. It is cheap and easily prepared in the lab. It was used for the irrigation of the pancreas during the retrieval procedure.

Hanks' buffered salt solution has been widely used for experimental and clinical islet isolation[189]. It is relatively cheap and was available as a concentrated solution that could be made up to the correct strength with sterile water.

Dulbecco's Modified Eagle medium is a cell culture medium enriched with amino acids and vitamins. They provide essential nutrients needed for cells and tissues to remain viable. DMEM with additives was used for islet culture. It was also used for the *in vitro* glucose stimulated insulin secretion assay as the glucose free version was commercially available. This enabled the preparation of DMEM with high and low glucose concentrations to be used for the assay.

Krebs-Henseleit solution has been extensively reported in the literature for use in *in vitro* reperfusion experiments. Hence this was used in the oxygenated reperfusion experiments [190-192].

2.3.2 Techniques of pancreas preservation

Static cold storage of pancreas

The prepared pancreas was stored in a closed chamber containing ice-cold UW solution. The chamber was placed in a cold bath containing crushed ice.

Two layer method

50ml of Perfluorocarbon solution was oxygenated by bubbling pure oxygen through it for at least 30 minutes prior to start of preservation. The retrieved pancreas was placed in the PFC. 100ml of cold UW solution was layered over the pancreas. The pancreas remains at the interface of the PFC and UW solution layers. Oxygenation of the PFC was continued during the preservation period.

Hypothermic Machine perfusion

UW solution was used as a perfusion fluid for machine perfusion. The pancreas was placed in a chamber containing the preservation solution. This chamber was placed in crushed ice to maintain a temperature of around 4°C (Figure 9).

The perfusion circuit was powered by a peristaltic pump (Watson Marlow 323). A 0.2μm filter was included in the circuit to trap and prevent particulate material from entering the pancreatic circulation. With the help of a Y connector the

circuit at the pancreas end was connected through high pressure tubing to a digital pressure monitoring device. After initial trials, the perfusion flow rate was fixed at 3rpm which gave an average flow rate of 0.27ml/min. This gave an approximate flow rate within the pancreas of 0.03ml/gram/min (taking the average weight of the retrieved pancreas-duodenum-spleen as 9 grams). This provided a perfusion pressure between 10-15mmHg.

Portal venous oxygen persufflation

The pancreas was placed in a chamber containing ice-cold UW solution. This chamber was placed in crushed ice to maintain a temperature of around 4°C. Medical grade oxygen at low pressure was filtered through a 0.2µm syringe filter and connected to the portal vein cannula for controlled low pressure persufflation of the pancreas. The persufflation pressure was constantly monitored by a digital pressure monitoring device using a Y connector. The initial oxygen flow rate was set to obtain a persufflation pressure between 10-15 mmHg (Figure 10).

At the start of persufflation the oxygen entering the portal circulation was seen to gradually distend the capillaries on the surface of the pancreas, duodenum and spleen.

Using a hypodermic needle, punctures 1mm deep were made on the surface of the spleen to release the gas bubbles and partially decompress the circulation. The oxygen flow was then adjusted to maintain the desired persufflation pressure.

Significant leaks were identified at the start of persufflation and repaired by suture ligation. If leaks could not be repaired satisfactorily the pancreas was discarded.
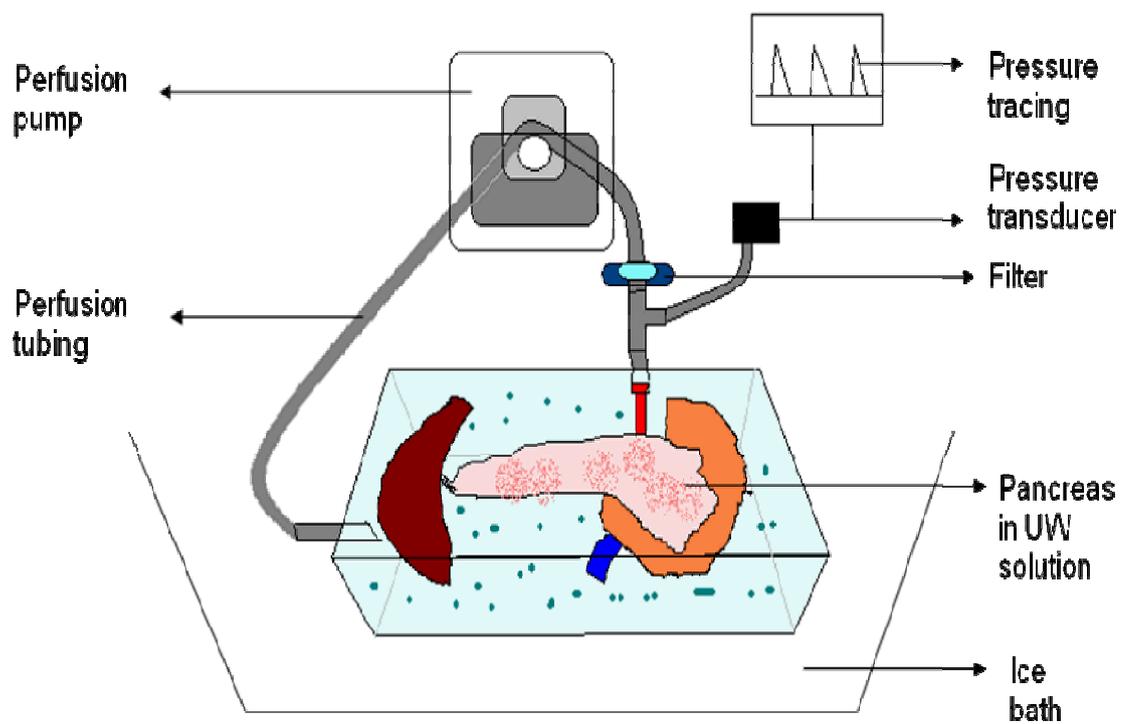
Figure 9 Schematic representation of the hypothermic machine perfusion circuit used for rat pancreas preservation
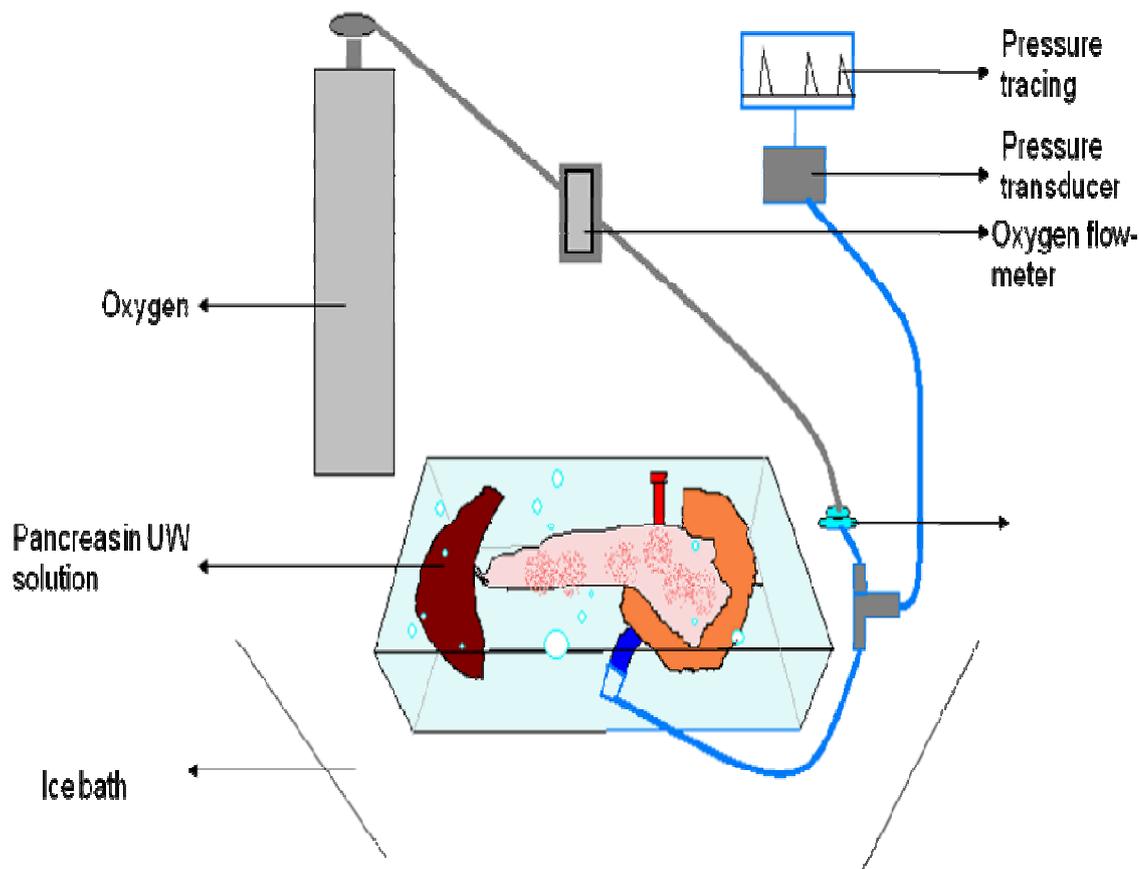
Figure 10 Schematic diagram of the circuit used for rat pancreas portal venous oxygen persufflation

Chapter 3 Method development & optimization

3.1 Development of the animal model

3.1.1 Background

Research in pancreas preservation using hypothermic machine perfusion and the two layer method has been carried out in the rat model[57, 193], canine model[174, 175, 194] and the pig model[195]. Surgical techniques to retrieve the rat pancreas have been previously described[196]. These reports of the procedure have involved retrieval from anaesthetised rats under controlled circumstances. In view of limitations due to Home Office license requirements; this method could not be adapted directly for our purpose. Our group has gained experience of retrieving kidneys from schedule I killed non-heart-beating-donor rats. We aimed to extend these principles to retrieving rat pancreas.

The aim of the following experiments was to develop a reproducible model of non-heart-beating-donor rat pancreas retrieval. This included standardization of the durations of warm ischaemia and the total retrieval procedure. The procedure should result in a pancreas with an intact vascular supply suitable for preservation by static cold storage, hypothermic machine perfusion or portal venous oxygen persufflation. The technique aimed to simulate the temperature changes (initial warm ischaemia, rapid cooling by aortic flush, and maintenance

of cooling during the cold dissection phase) in a standard non-heart-beating-donor donor during the retrieval process.

3.1.2 Evolution of pancreas preservation models

Male Wistar rats were used as pancreas donors. The weight range of the rats was limited to 350-400g as dissection and safe retrieval of the pancreas was difficult in smaller rats. Rats larger than 450g were avoided as excessive intra-abdominal fat complicated pancreas retrieval.

In-situ preservation model

The animal was killed by a Schedule 1 method (knock on the head or cervical distraction). After the pre-defined warm ischaemia time, a laparotomy was carried out. The abdominal aorta was cannulated and flushed with cold University of Wisconsin solution and vented through the intra-thoracic IVC. The whole animal was then submerged in a tub containing cold preservation solution and machine perfusion was instituted through the aorta.

This was technically simple and caused no trauma to the pancreas. However the problem of frequent blockage of the perfusion circuit filter with debris was difficult to manage. Prolonged perfusion lead to excessive distension and rupture of the small bowl leading to faecal contamination. This model also involved the use of large volumes of expensive preservation fluid for the immersion of the entire animal.

Visceral block model

The animal was killed by a Schedule 1 method. After the pre-defined warm ischaemia period, laparotomy was carried out and abdominal aorta was flushed with University of Wisconsin solution. The pancreas, proximal small bowel, kidneys, liver and stomach was retrieved along with the abdominal aorta, portal vein and inferior vena cava (Figure 11). This was then immersed in cold preservation solution and preserved.

This model had the advantage of being technically simple and less traumatic for the pancreas itself. However the problem with excessive small bowel distension persisted. Leakage of preservation fluid from small arteries was inevitable. Perfusion/ persufflation of multiple organs along with the pancreas made measurement of the actual perfusion/ persufflation pressure in the pancreas inaccurate.

Isolated pancreas model

In view of these problems, an isolated pancreas+ duodenum+ spleen model was developed. This was an adaptation of a previously published method of rat pancreas recovery with minimal circulatory impairment [196]. This model has several advantages. Changes in vascular resistance during preservation would primarily be due to changes in the pancreatic vascular bed itself. This model best approximated the clinical situation where the pancreas is retrieved along with the duodenum and spleen. The model provided reliable access to both the arterial (aorta) and venous (portal vein) sides of the pancreas for machine perfusion and gaseous persufflation respectively (Figure 14).

The problems with the isolated pancreas model were essentially technical. It involved prolonged dissection which increased the risk of rewarming. Capsular tears and leaks from the vascular system were common due to the fragile nature of the rat pancreas, leading to wastage of organs.
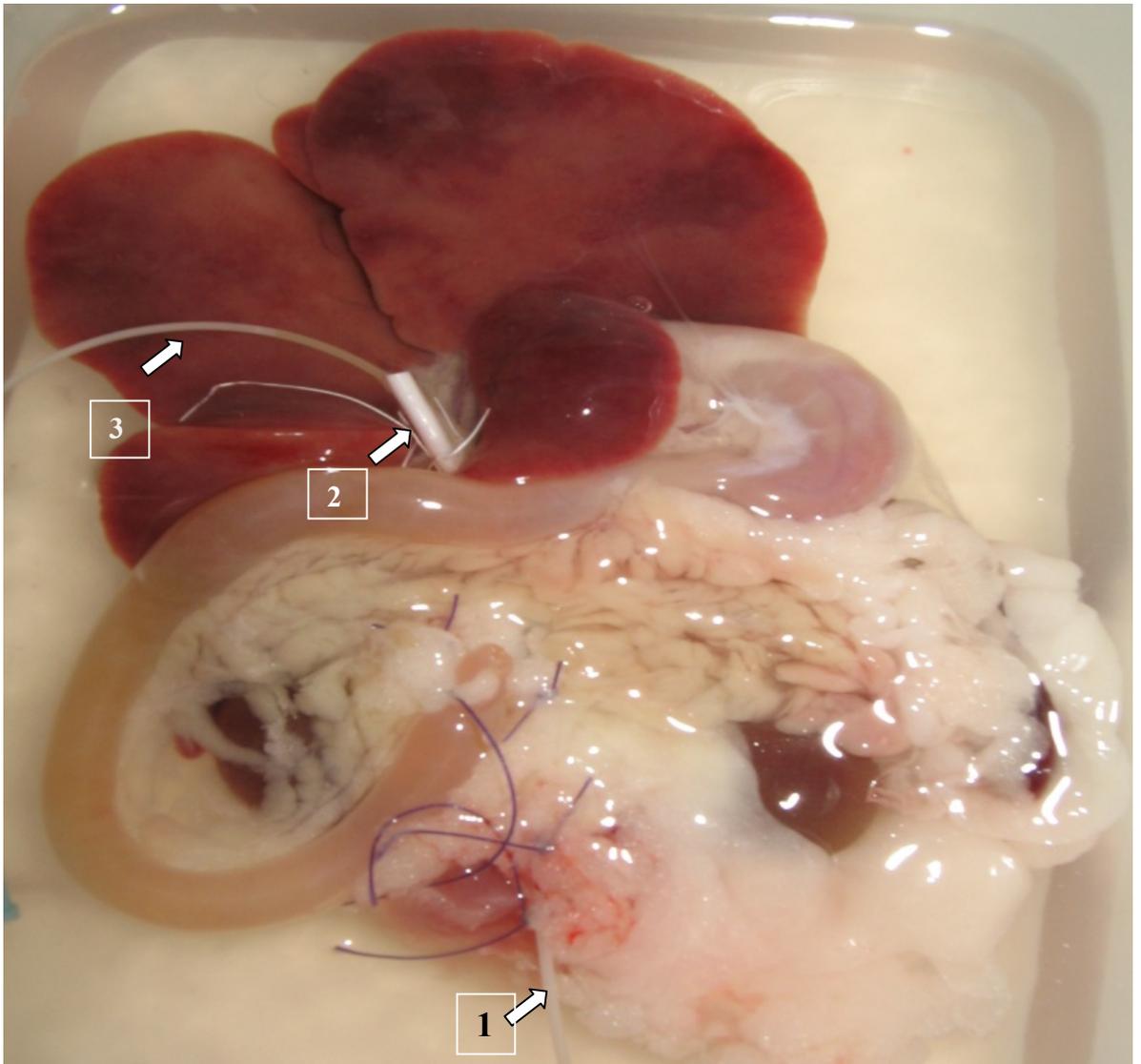
Figure 11 Visceral block model of pancreas retrieval

The visceral block model of pancreas retrieval involved removal of the liver, stomach, duodenum and proximal jejunum, pancreas and spleen. together. The photograph shows the prepared block with the aortic(1) and portal vein (2) cannulas. A silastic catheter in the bile duct(3) is also seen.
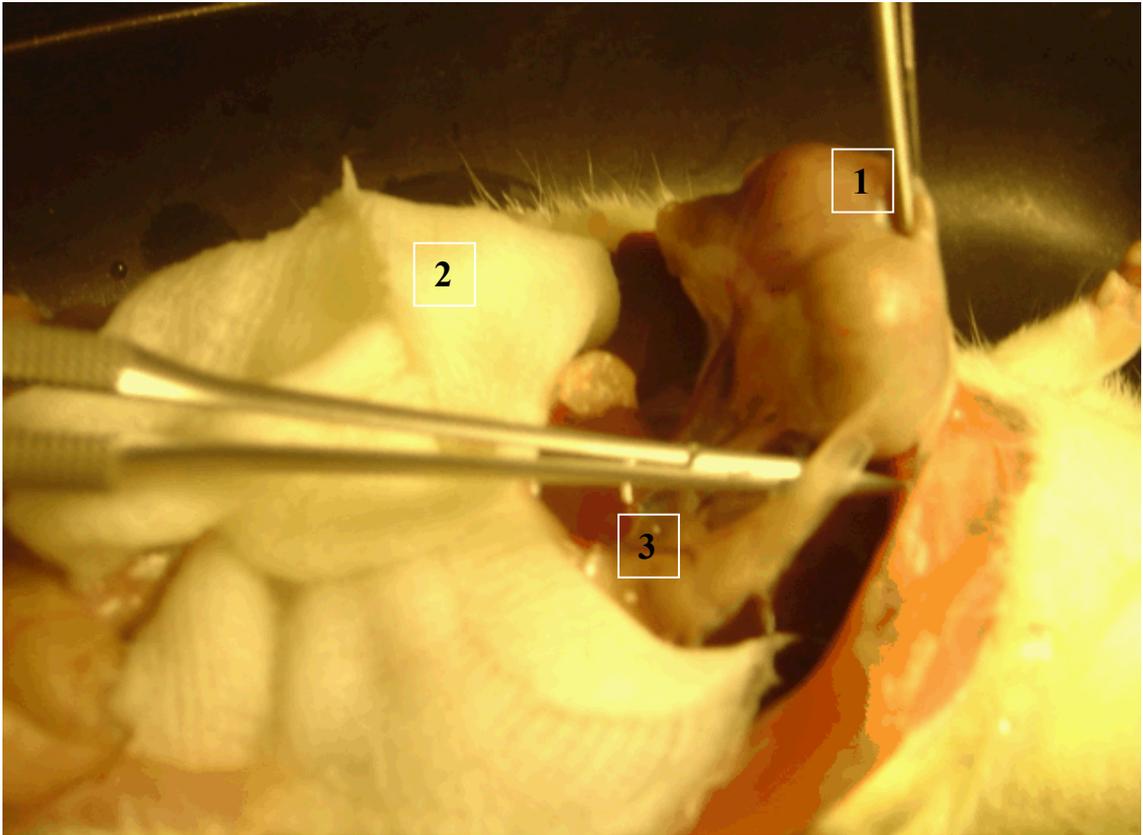
Figure 12 Technique of Non-heart-beating-donor rat pancreas retrieval-initial dissection

The initial dissection of mobilising the stomach (1) by dividing its peritoneal attachments is shown. Gauze swabs soaked in warm Ringer's lactate solution (2) are used to cover the pancreas (3) during the dissection phase to maintain its temperature close to 37°C to simulate warm ischaemia.
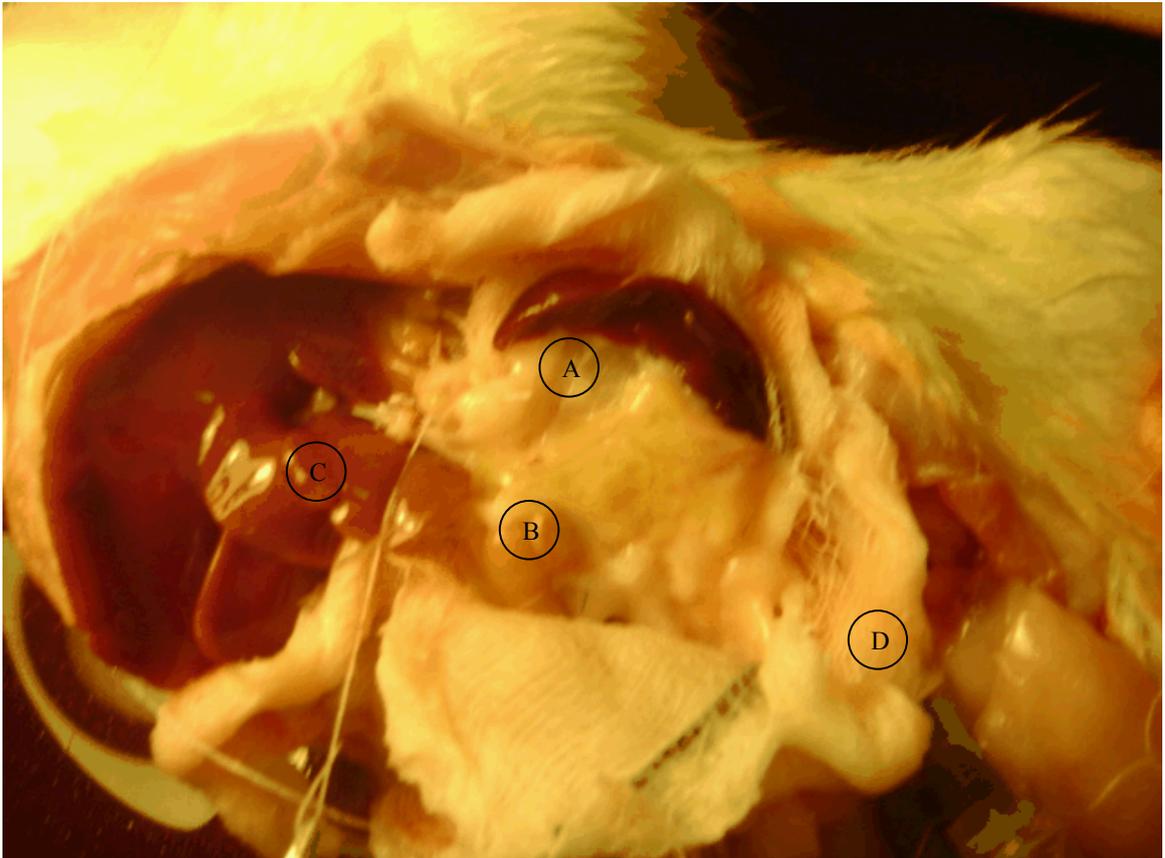
Figure 13 Technique of non-heart-beating-donor rat pancreas retrieval- in situ aortic flush with cold university of Wisconsin solution

The pancreas and spleen (A) has been fully exposed by removing the stomach and ligating the proximal duodenum (B). The common bile duct has been cannulated by a fine silastic cannula (C). The aortic cannula (D) is connected to a perfusion pump and is flushed with cold University of Wisconsin solution. The pale pancreas following cold flush is clearly seen.
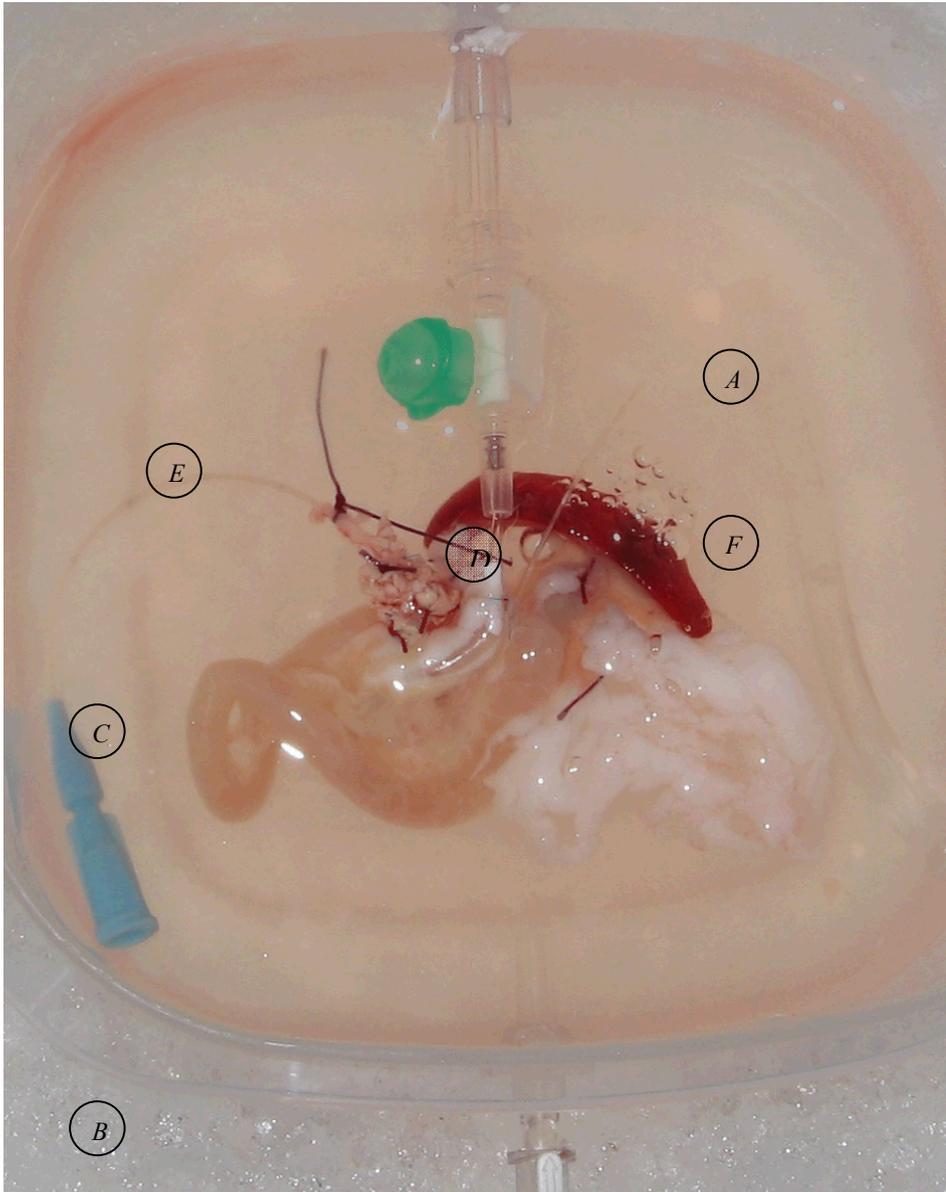
Figure 14 The retrieved non-heart-beating-donor rat pancreas with adjoining duodenum and spleen in the preservation chamber

The prepared rat pancreas is placed in the preservation chamber (A) containing cold university of Wisconsin solution. The chamber is placed in a box containing crushed ice (B) to maintain the temperature at 4°C. The aortic cannula (C), portal vein catheter (D) and bile duct catheter (E) are seen. The pancreas is being persufflated through the portal vein cannula. Gas bubbles escaping through the spleen surface can be seen (F).

3.1.3 Technique of isolated pancreas retrieval

The retrieval process lasted about 65 minutes. The procedure was divided into four phases.

The first phase (warm ischaemia period) lasted 35 minutes. This started with cardiac arrest and lasted until start of aortic flush with cold University of Wisconsin solution. During this period, the peritoneal cavity and organs were kept warm by regular irrigation with warm Ringer's lactate solution (37°C) and the pancreas, duodenum and spleen were kept covered with swabs soaked in warm saline. Five minutes before completion of this phase, 10,000 units of streptokinase dissolved in 1ml of Ringer's lactate solution was injected into the aortic cannula. Streptokinase stimulates thrombolysis. This was used in the model as an initial flush to lyse clots formed during the warm ischaemia period and improve the quality of perfusion. The benefit of using thrombolytic agents in non-heart-beating-donor has been proven [197]. The dose used in the study was calculated by using data from non-heart-beating-donor kidney preservation.

The second phase lasted 5 minutes. This started with aortic flush with cold UW solution and simultaneous external cooling of the pancreas by flooding the peritoneal cavity with ice cold Ringer's lactate solution. Twenty millilitres of UW solution was injected into the aorta at a rate of 200ml/hr using a syringe pump.

The third phase lasted 15 minutes. During this period dissection of the pancreas was completed. Aortic perfusion with cold UW solution continued at a slower

rate of 20ml/hr during this phase. Swabs soaked in cold Ringer's lactate solution were used to keep the pancreas cold during this period.

The fourth phase lasted 5-10 minutes. The pancreas was transferred to a petridish. It was gently flushed with cold UW solution to identify any leaks. Any identified leaks were sutured. The pancreas was flushed finally with 2ml UW solution containing 1000U of superoxide dismutase. Super-oxide dismutase is a free-radical scavenger. Its benefit in preventing free-radical injury in livers preserved by venous oxygen persufflation has been reported in experimental studies[198]. We included it as a step in all preservation arms to ensure uniformity. The dose used was calculated from the dose used in published studies of liver preservation [180].

3.1.4 Procedure of non-heart-beating-donor rat pancreas retrieval

- The animal was killed by a Schedule I method (blow on the head and cervical distraction). It was placed on the dissecting table supine with its tail end towards the operator.

- 70% ethanol was sprayed on the abdomen and thorax. A midline vertical incision was made with transverse extensions at the level of the costal margins to improve exposure.

- Dissection was started by separating the colon and caecum from the pancreas and the duodenum. The middle colic and right colic pedicles were ligated and cut.

- The proximal jejunum was mobilised by dividing the mesenteric vessels after ligation.

- The root of mesentery with the SMA and the SMV was suture ligated and divided.

- Mobilisation was continued by dividing the gastro-splenic ligament and greater omentum. The short gastric and the left gastric pedicles were ligated and cut close to the fundus (Figure 12).

- The stomach was divided at the pyloro-duodenal junction after closing the duodenum with a heavy suture. The stomach was removed from the abdomen by dividing at the gastro-oesophageal junction to fully expose the pancreas.

- The animal was turned 90° i.e. head towards the surgeon's left.

- The duodenum was retracted to the left of the animal to expose the common bile duct and portal vein at the hepatic hilum.

- A fine cut was made in the duct with micro-scissors distally (towards liver) and a fine cannula was passed through the incision towards the pancreas and secured with fine ties.

- The portal vein at hilum was cut partly to insert a short & wide silastic cannula towards the pancreas. Care was taken not to occlude the junction of splenic vein into the portal vein. The cannula was fixed in position with ties.

- The portal structures were sutured ligated with prolene suture to secure the cannula in position and also ligate the hepatic artery which would otherwise leak during machine perfusion.

- The infra-renal aorta was dissected and cannulated.

- The supra-coeliac aorta at the diaphragm was looped and ligated.

- After completion of warm ischaemia period, 10000 U of streptokinase in 1ml normal saline was injected into the aortic cannula.

- This was followed after 2minutes with cold UW flush (20 ml) through the aortic cannula. The flush was done using a syringe driver pump initially set to deliver UW solution at a rate of 100ml/hr for the first 10ml and then reduced to 20ml/hr for the next 10 ml.

- Good perfusion was confirmed with the efflux of blood through the portal vein cannula and the pancreas and duodenum becoming pale (Figure 13).

- Simultaneous external cooling of the pancreas and duodenum was started by irrigation with cold saline and swabs soaked in ice-cold saline.

- The portal structures were divided close to the liver and above the ligatures for the bile duct and portal vein to separate the pancreas block from the liver.

- The pancreas was immediately rinsed in a petri-dish containing ice-cold Ringer's lactate solution and transferred to another petridish containing cold UW solution.

- Using 5ml of cold UW solution to flush the aortic cannula, the pancreas was examined for leaks which if present were ligated.

- Super-oxide dismutase (1000units in 2ml of University of Wisconsin) was flushed through the aortic cannula as the final step.

- The weight of the pancreas was recorded.

3.1.5 Temperature control during pancreas retrieval

The period of warm ischemia was simulated by keeping the pancreas moist and warm at 37°C during the initial 30 minutes of the dissection. This was then changed to external cooling from the time of the aortic flush.

To evaluate the temperature control during the entire dissection period, continuous temperature monitoring was carried out in three animals. Three temperature probes were placed in the duodenal lumen, spleen and in the neck close to the carotid vessels immediately after laparotomy. One probe was left outside to record the ambient lab temperature. The temperature readings in the four probes were recorded continuously. This showed a gradual fall in the pancreas temperature to 30°C during the warm phase followed by a sharp fall during aortic flush to around 8°C. The temperature remained around 10°C -15°C during the remaining duration of the retrieval procedure (Figure 15).
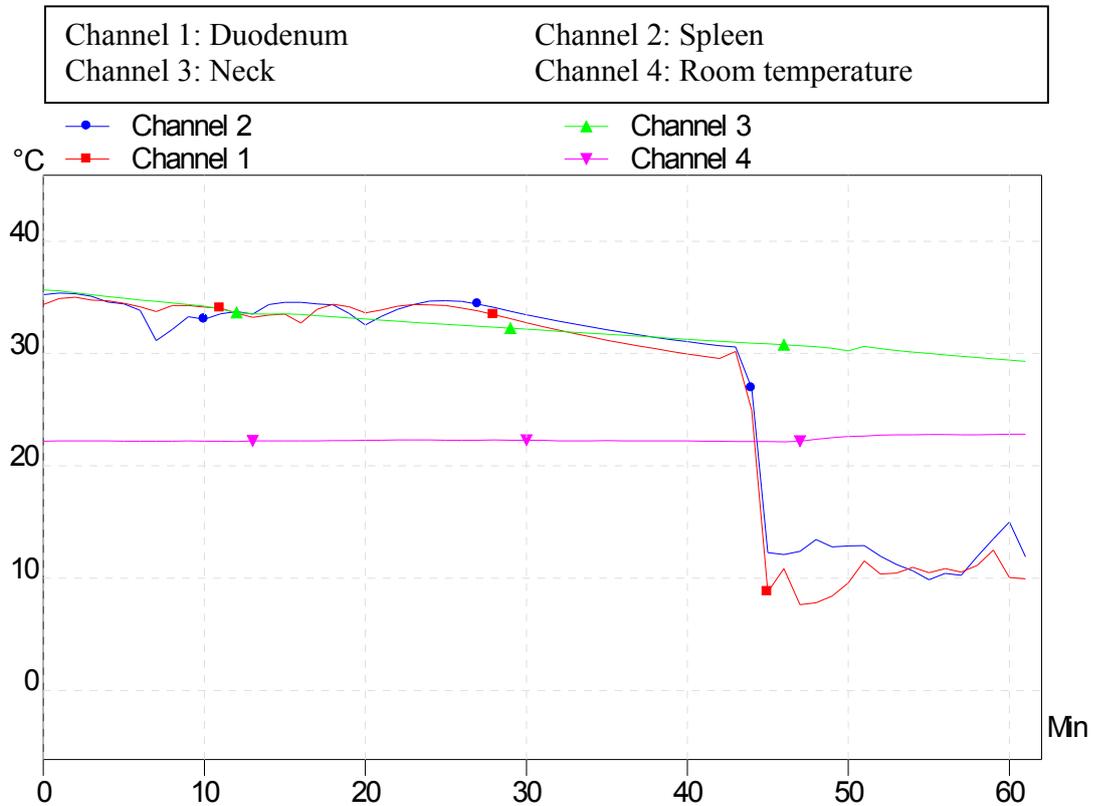
Figure 15 Line chart demonstrating the trend in temperature recordings in four probes during one rat pancreas retrieval procedure

Three temperature probes were placed in the rat immediately after laparotomy. Probes 1 and 2 were placed in the duodenum and in the spleen respectively. Probe 3 was placed in the neck to monitor temperature changes in the rat away from the operative zone. Probe 4 was left exposed to measure ambient lab temperature. Temperature recordings were made automatically at 2 minute intervals throughout the retrieval procedure. Recordings were started 10 minutes before start of dissection and stopped when the pancreas was removed from the rat abdomen (end of phase 3 of the retrieval)

3.2 Batch testing of collagenase

Collagenase is a proteolytic enzyme that is used extensively for islet isolation from pancreas. Batch to batch variability is a well known problem with the use of this enzyme. Gradual decline in the potency of the enzyme during storage has also been reported. Variation in potency may affect the completeness of digestion of pancreases.

In an attempt to overcome this problem, periodic assessment of collagenase activity was done to adjust the duration of pancreas digestion.

Collagenase P was stored at 4°C in a sealed container. The collagenase activity was assessed at approximately 2 monthly intervals. Freshly retrieved rat pancreas was distended with 10 ml of collagenase solution in HBSS (1mg/ml). The distended pancreas was bluntly divided into two parts and incubated in universal containers in a water bath at 37°C. After 30 minutes of incubation, the tubes were gently shaken to disrupt the pancreas. Intermittent sampling of digest from the two tubes was done every 4 minutes. The sampling was done alternately so that one tube was sampled every 2 minutes. In between sampling the tubes were incubated in the water bath. The samples were immediately stained with dithizone. The time point when free islets were present in the digest was noted. This was used as the guide for the optimum duration of pancreas digestion. In practice the actual time was 2-3 minutes longer than the time identified from these experiments. The duration of incubation of the pancreas

for optimum digestion increased from 40 minutes immediately after purchase of

the collagenase preparation to 52 minutes towards the end of its shelf-life.

3.3 Effect of increasing warm ischaemia time on total islet yield and viability

3.3.1 Background

Increasing warm ischaemia times are associated with decreasing islet yield and poor islet viability[199]. Previous studies have reported that warm ischaemia periods of 30 minutes or more are associated with a significant decrease in islet yield[46]. This experiment was planned to study the effect of increasing warm ischaemia duration on islet yield. The aim of these experiments was to identify a warm ischaemia duration that would lead to 50% decrease in islet yield. This would provide a useful point from where to compare various preservation techniques for their ability to improve islet yield.

3.3.2 Methods

Male Wistar rats were used as pancreas donors. The animal was killed by a schedule I method. Laparotomy was carried out after various durations of warm ischaemia (0, 15, 30, 45 or 60 minutes). The pancreas and the common bile duct were identified. The common bile duct was cannulated and 10mg collagenase (1mg/ml) was injected into the common bile duct. The distended pancreas was bluntly dissected out and transferred to a universal container. The pancreas digestion technique is described in detail elsewhere (section 4.2.2). The crude islet yield was calculated by counting dithizone stained islets in the digest. Density gradient separation was not used for this experiment. Islets from

the digest were hand-picked under the microscope and cultured overnight at 37°C. Islet viability was assessed with trypan blue exclusion staining. The crude islet count and viability were compared using the analysis of variance (ANOVA) test.

3.3.3 Results

The crude islet counts ranged from 140 to 1600 islets per pancreas. Pancreases with no warm ischaemia produced the largest number of islets. There was a significant decrease in crude islet yield with increasing warm ischemia period (ANOVA, $p<0.001$). A warm ischaemia of 30 minutes lead to a 65% fall in crude islet count (Figure 16). Viability was lower following longer periods of warm ischaemia; however the difference was not significant (ANOVA, $p=0.163$). Islets isolated from pancreases with 60 minutes warm ischemia were misshapen and irregular as compared to islets from pancreases with shorter warm ischaemia times (Figure 16).

3.3.4 Discussion

The experiment showed that increasing warm ischaemia time decreased the crude islet count. The decrease was most significant with warm ischaemia periods of more than 15 minutes. Very few islets were obtained after 60 minutes of warm ischaemia. The decrease in islet viability with longer ischaemia times did not reach levels of statistical significance. Hand picking islets from the digest for culture would have introduced a confounding factor in viability assessment. Hand-picking depends on the identification of islets based on morphology. It is

possible that non-viable islets (which have deformed morphology) may have been picked less often.

Based on these data it was decided to develop a non-heart-beating-donor rat pancreas model with warm ischaemia duration of 30 minutes. This experiment also demonstrated the inherent problem with crude islet counts and hand-picking of islets. Density gradient purification of pancreatic digest was used for the rest of the islet experiments to obtain good numbers of representative islets to enable viability and *in vitro* function testing.
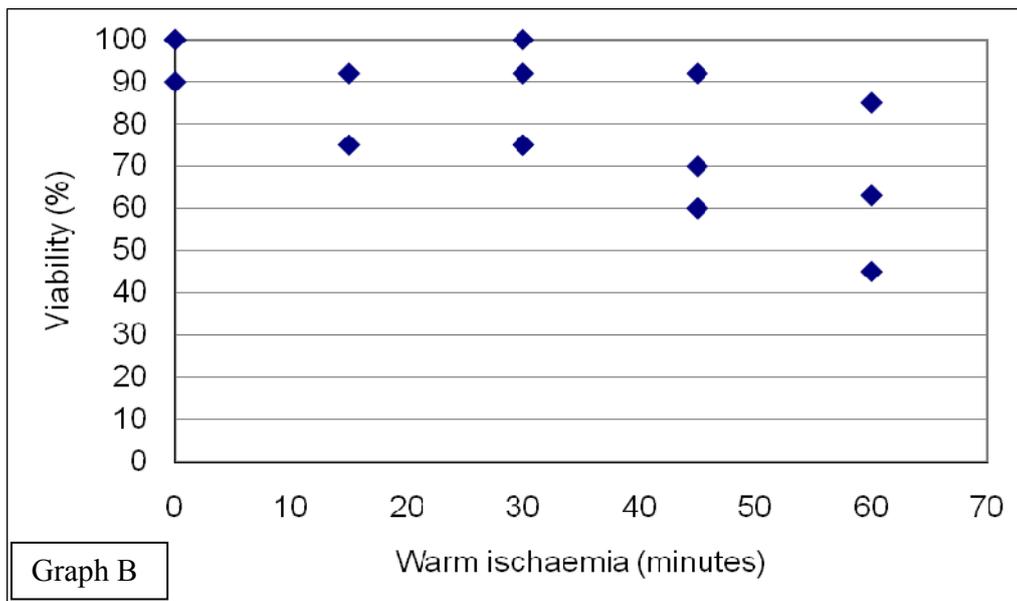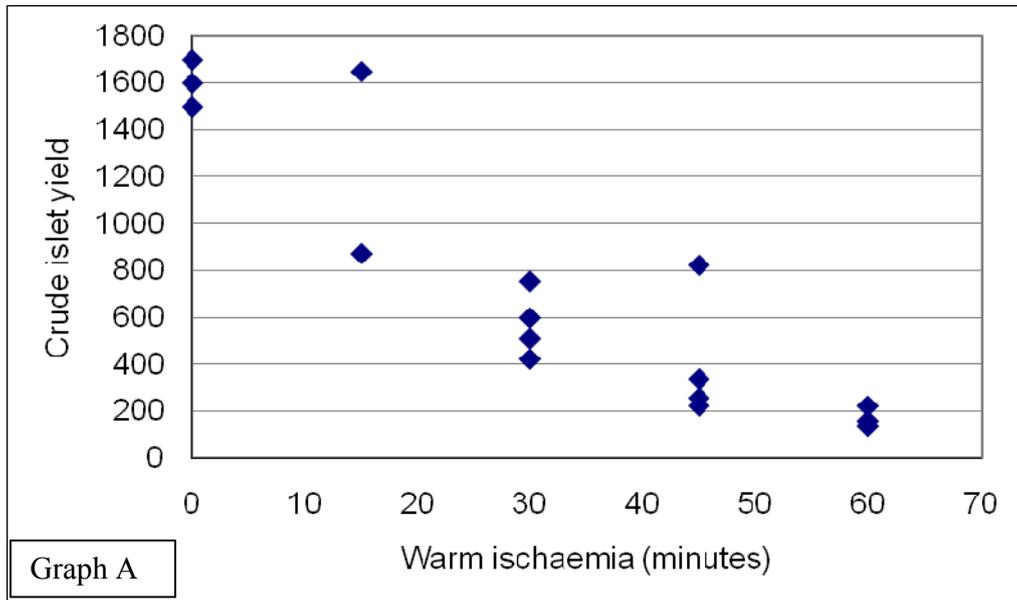
Figure 16 Effect of increasing warm ischaemia duration on islet yield and islet viability

Each point of the graph denotes islet isolated from a single rat pancreas. Graph A shows a linear decrease in crude islet yield with increasing warm ischaemia. Warm ischaemia of 30 minutes produced a 65% decrease in crude islet yield. Graph B shows a decrease in islet viability with increasing warm ischaemia times. The decrease in viability was however not consistent (discussed in text)

3.4 Optimisation of Static Glucose Stimulated Insulin Secretion assay

3.4.1 Background

Numerous techniques exist for the assessment of islet function. The most accurate method is their ability to correct hyperglycaemia following transplantation into immuno-deficient diabetic mice. Routine utilisation of this technique is expensive and time-consuming. The test is irrelevant in the clinical setting as results from these experiments will be available long after the islets had been transplanted. *In vitro* methods of testing for islet function have been based on the ability of functional islets to secrete insulin in response to glycaemic stress, measurement of oxygen consumption of the islets[200], ADP/ATP ratio[201] and the levels of stimulated and basal ROS levels in an islet isolate[145].

The Static Glucose Stimulated Insulin Secretion test was used to assess the *in vitro*

function of the islets in our study. It was based on the principle that viable functioning islets produce insulin when exposed to glucose[202]. The amount of insulin produced depends on the concentration of glucose in the islet medium. Thus by exposing aliquots of islets to serial high and low glucose concentrations it was expected that viable islets will produce more insulin in high glucose containing medium. Non-viable islets on the other hand do not show a differential response in insulin production to different glucose concentrations. The ratio of insulin secreted at high glucose to the insulin at low glucose is the

stimulation index[28, 203]. Studies have shown that the results from GSIS assays have poor correlation with post-transplant function. Documentation of a biphasic response of islets to a glucose challenge with a return to baseline was suggested as an essential criteria for suitability for transplantation[204]. Stimulation indices of clinically transplanted islets ranges from 2-4[28].

3.4.2 Initial protocol of static glucose stimulated insulin secretion test

Dulbecco's Modified Eagle Medium with low or high Glucose (2.5mM or 20mM) concentrations was used as the incubation media in initial trials. Purified islets in islet culture medium were transferred to a petridish. A 12 chamber culture plate was pre-filled with 2ml of low glucose incubation medium. 10 islets were transferred to each well. The contents of each well were mixed by gentle pipetting using a sterile Pasteur's pipette. The islets were allowed to settle for 5 minutes. The top 1.5ml of the incubation medium was removed and replaced by fresh low glucose incubation medium twice. The islets were incubated in the wells for one hour. At the end of this period, the contents of each well were again gently mixed and the supernatant pipetted off after 5 minutes. The procedure was repeated except that either high or low glucose containing incubation medium was added to alternate wells. Three repeats of each isolate under high and low glucose incubation were done. The islets were placed in a shaker set at 50 rpm in a walk-in incubator at 37°C. At the end of two hours the contents of each well were transferred to 1.5ml micro-centrifuge tubes and

centrifuged at 17500g for 2 minutes. The supernatant was collected in labelled micro-centrifuge tubes and stored at -80°C for future analysis.

This technique was used for the initial 16 isolates. These included 7 control, 3 machine perfusion, 3 cold storage and 3 oxygen persufflation pancreases. This technique however caused a wide variation in the insulin levels and stimulation index of individual sets (Figure 17).

In view of the poor consistency of results the technique was reviewed to identify areas of improvement. Three major areas were identified and corrective modifications were made to the procedure.

1. Picking islets under the microscope using a pipette was time-consuming. It was difficult the keep track of the individual sizes of the islets in individual wells. The number of islets left in each well after the washes were found to be highly variable. It was felt that the process of repeated pipetting to pick islets was traumatic to the islets.

We attempted to improve the consistency by using the same set of islets for serial low and high glucose incubations. However this led to very low insulin secretions in the high glucose phase. Viability staining of islets after 5 hours in the incubation medium showed that the majority of islets were non-viable.

The technique was modified so that 50% of the islets isolated from each pancreas were used for the assay after overnight culture. The islets were washed in low glucose medium twice by centrifugation at 100g for 1 minute. The pellet was uniformly suspended in 2ml of low glucose medium and 200μl of the

suspension was used in each well instead of picking up individual islets. This

provided more consistency in the number of islets in each well.
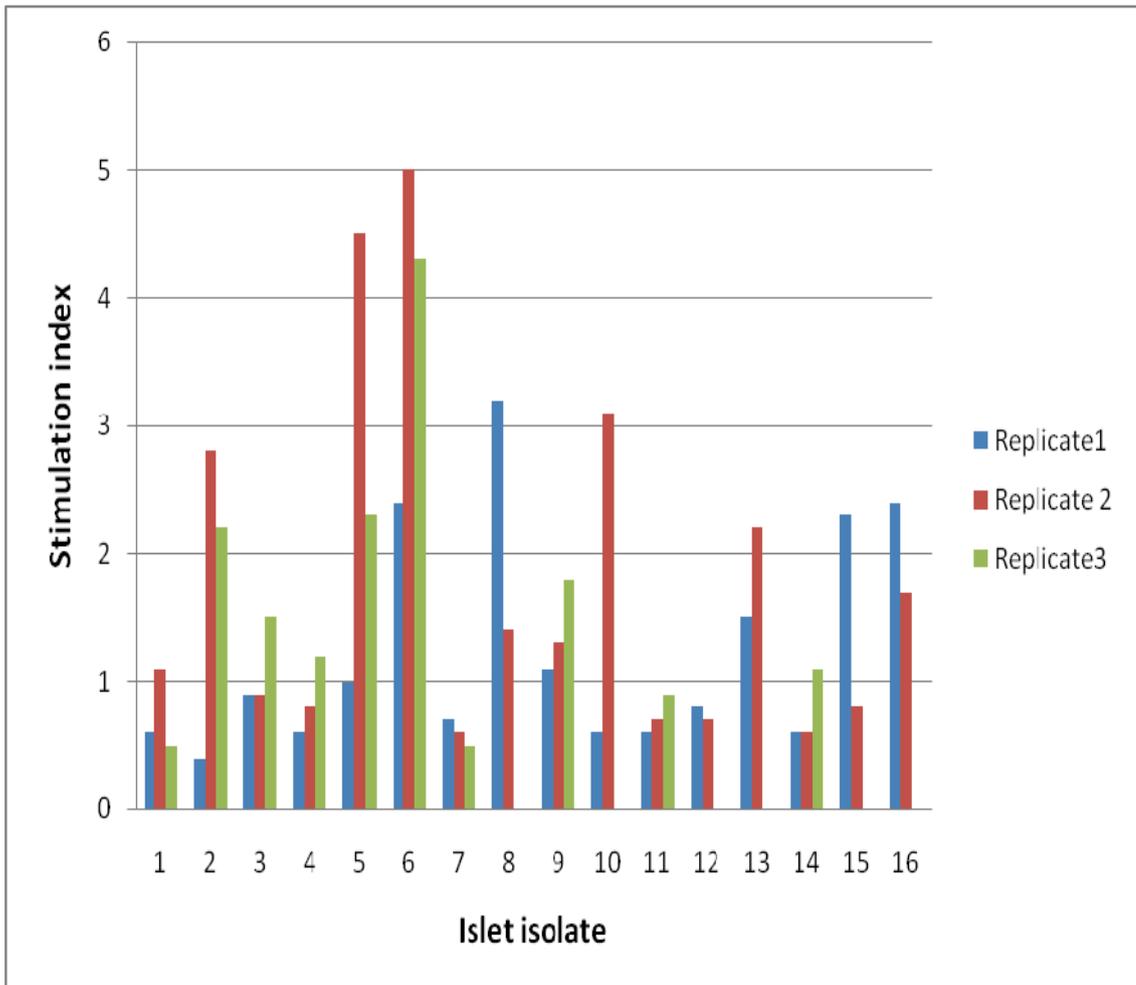
Figure 17 Bar graph showing the variation in the stimulation indices of the replicates in an initial series of 16 static glucose stimulated insulin secretion assays

Bar chart summarizing the results of the initial glucose stimulated insulin secretion test. Sixteen isolates were assessed. The individual replicates for each isolate are represented by the different coloured bars in each isolate on the horizontal axis. The stimulation index is shown on the vertical axis. There was wide variation in the stimulation indices of the replicates for each isolate.

2. The use of the walk-in incubator led to excessive evaporation of the medium from the wells. This was evident as condensation on the inside of the cover. This had the potential of changing the concentration of various components in the incubation fluid. This was evident by the change of the colour of the ph indicator in the medium to a very bright pink. 10mM Hepes buffer was added to the incubation medium to counteract the change in pH on incubation. Attempts at incubating the plates in a closed water bath did not completely correct these problems and was prone to mishaps. The most consistent results were obtained by placing the incubation plate in a table top incubator containing 5% CO_2 along with intermittent manual shaking of the plate.

3. The insulin production remained low even in the control islets using the above mentioned medium. Following further review of the literature, additives were added to the incubation medium to improve the insulin secretion from the islets. Addition of 0.5% Bovine serum albumin and 2mM Glutamine improved insulin secretion.

3.4.3 Final protocol for static glucose stimulated insulin secretion assay

Incubation medium (Glucose free DMEM supplemented with 0.5% Bovine serum albumin, 10mM Hepes, 2mM Glutamine) was used for the stimulation studies. High glucose medium (with 40 mM glucose) and low glucose medium (with 3 mM glucose) were prepared from the sterile incubation medium and warmed to 37°C in a water bath.

Islets after overnight incubation were washed twice in low glucose medium by centrifugation (100g for 1 minute). The islet pellet was re-suspended in 2 ml of low glucose medium. 200µl of islet suspension were added to 800 µl of low glucose medium placed in six wells in a 24 well plate and mixed by gentle aspiration. The islets were incubated at 37°C for one hour. After the incubation period, the islets were washed thrice with low glucose medium. 500µl of the supernatant medium was aspirated from each well and replaced by the same volume of either low or high glucose medium (3 wells each) and mixed with gentle aspiration. This gave a final glucose concentration of 3mM and 21.5mM in the low and high glucose medium wells. The islets were re-incubated at 37°C for 3 hours. The plate was gently shaken every 30 minutes. At the end of the incubation period the contents of each well were transferred to labelled tubes taking care to collect all the islets from the wells. The tubes were centrifuged at 17500g for 2 minutes. The supernatant was collected in labelled tubes and stored at -80°C. Insulin concentration in the fluid was assayed using

commercially available kit (Mercodia High range Rat Insulin Elisa kit) according to the manufacturer's instructions.

3.4.4 Evaluation of intra-replicate covariance using the modified static glucose stimulated insulin secretion assay

There was significant reduction in the covariance between individual sets of GSIS after the above protocol modifications (Figure 18). This was more evident for the non-heart-beating-pancreases compared to the control pancreases. This is expected as islets isolated from non-heart-beating-donor pancreases would have endured greater stress in the form of warm and cold ischaemia compared to the control pancreas islets. That makes them more susceptible to poor function in challenging environments. Improvement of the quality of the incubation medium with additives and decreased handling of the islets contributed to more consistent islet function.
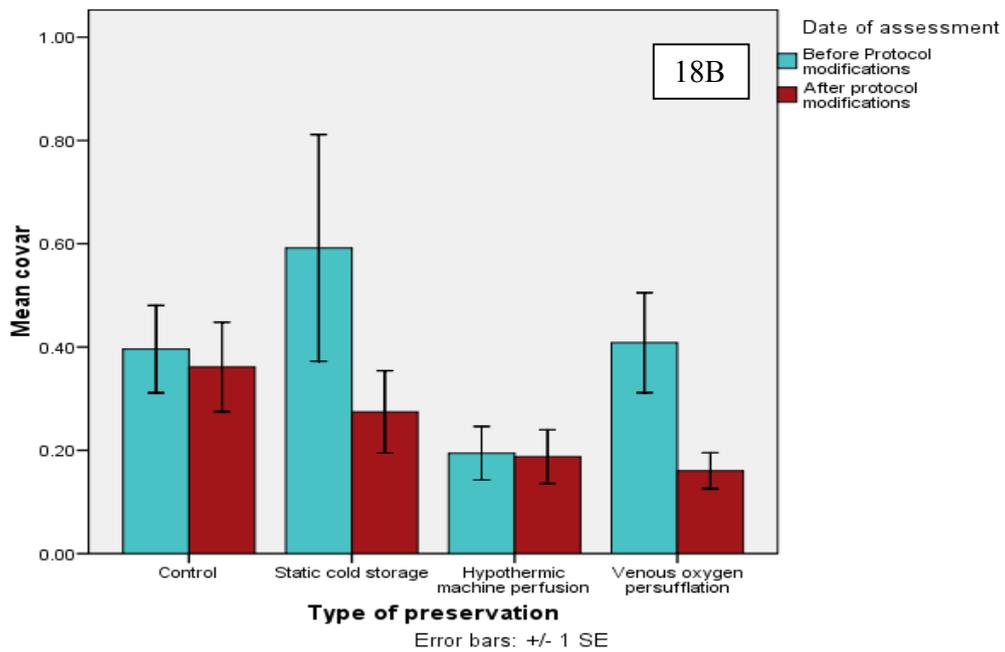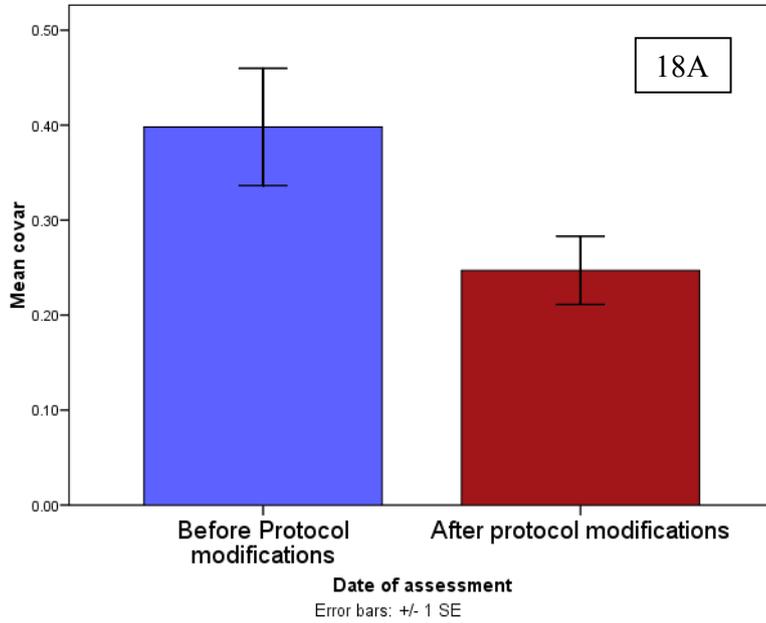
Figure 18 Bar charts comparing the covariance of stimulation indices of replicates for islet isolates assessed before and after protocol modifications of the glucose stimulated insulin secretion assay.

Figure 18A: Overall comparison, Figure18 B: Differential effect in various preservation groups

3.5 Determination of activated Caspase 3 levels in islet extracts and pancreatic tissue as a measure of the extent of apoptosis

3.5.1 Background

Caspases (Cysteine-requiring aspartate proteases) are a family of proteases that mediate cell death by apoptosis. Caspase 3 is an effector caspase and is a critical enzyme involved in apoptosis.

Rationale for methodology

The caspase 3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by activated caspase 3, resulting in the release of the p- nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405 nm. The concentration of pNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA controls. Samples with selective caspase-3 inhibitor are included in the assay protocol to identify non-specific cleavage of the substrate that produces false positive results.

Basic assay protocol

The assay buffer, lysis buffer, substrate and inhibitor and caspase 3 enzyme were prepared using the assay kit according to manufacturer's instructions.

The micro-plate assay method was used for all experiments (Table 3). All assays were carried out in duplicate. Test samples or caspase 3 positive controls were added to wells. Caspase 3 inhibitor was added to selected wells to identify false positive results. The contents in each well were made up to 90µl with assay buffer. 10µl of caspase 3 substrate was added and the plate was

incubated overnight at 37°C. A p-nitroaniline (pNA) calibration curve was prepared according to manufacturer's instructions. The plate was then read in a micro-plate spectrophotometer at 405nm.

Table 3 Reaction scheme for the caspase 3 assay using the micro-plate assay method

	Cell lysate	Caspase 3 5 µg/ml	1x Assay buffer	Caspase 3 inhibitor Ac-DEVD-CHO 200 µM	Caspase 3 substrate Ac-DEVD-pNA 2 mM
Reagent blank	----	----	90 µl	----	10 µl
Non-induced cells	5 µl	----	85 µl	----	10 µl
Non-induced cells + inhibitor	5 µl	----	75 µl	10 µl	10 µl
Induced cells	5 µl	----	85 µl	----	10 µl
Induced cells + inhibitor	5 µl	----	75 µl	10 µl	10 µl
Caspase 3 positive control	----	5 µl	85 µl	----	10 µl
Caspase 3 positive control + inhibitor	----	5 µl	75 µl	10 µl	10 µl

Standard calibration curve and calculation of test results

The standard calibration curve was prepared by using increasing concentrations of pNA (10µM to 200µM). 100µl of each dilution was added to duplicate wells in a 96 well microtitre plate. 100µl of assay buffer is added as a blank. The absorbance was read at 405nm. A calibration curve was prepared by plotting the mean absorbances against the amount of pNA (in µmol) in each well (Figure 19). The amount of pNA released by individual test samples was calculated on the basis of the calibration curve using curve fitting software (CurveExpert™).

The caspase 3 activity was then calculated in terms of µmol pNA released per minute per ml of cell extract.

Activated Caspase 3 activity = pNA x D/ T x V

pNA=p-Nitroaniline in µmoles present in the well at the end of incubation period

V=volume of test sample used in the well (in ml)

D= dilution factor of the test sample

T= reaction time in minutes

The protein content in the cell extract/ tissue extracts was assayed. The Caspase 3 activity in terms of µmoles of pNA released per min per mg of tissue protein extract was then calculated.
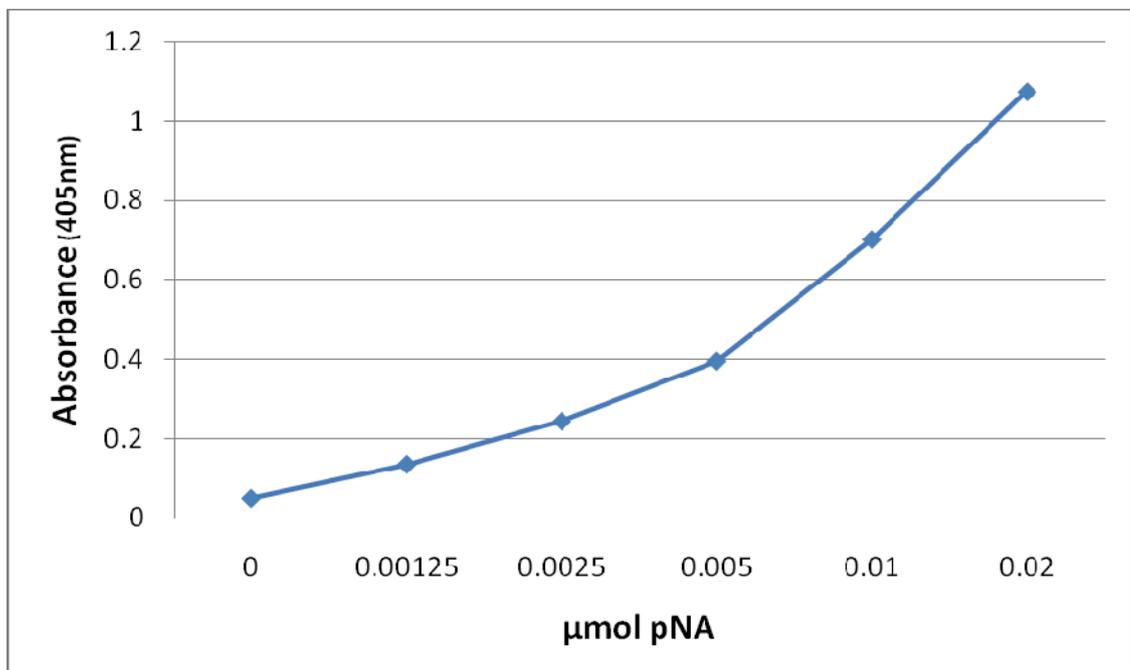
Figure 19 Sample calibration curve for µmoles of p-nitroaniline in each well plotted against the absorbance at 405nm

3.5.2 Determination of apoptosis in human peripheral blood mononuclear cells

Initial work to standardize utilization of the kit was done using lymphocytes separated from blood of human volunteers (informed consent and University of Sunderland ethics committee approval obtained). These experiments were used to confirm that sensitivity of the assay could be increased by increasing the volume of the test sample and by increasing the duration of incubation.

Preparation of peripheral blood mononuclear cells (PBMC)

5 ml of blood was collected and mixed with 100 units of preservative free heparin to prevent clotting. The blood was diluted 1:1 with RPMI and carefully underlaid with 10 ml of Nycoprep™ (1.077g/L). This was centrifuged at 400g for 25 minutes. An interface layer of PBMC developed between the Nycoprep™ and plasma columns. This layer was carefully removed using a Pasteur pipette. This was added to phosphate buffered saline (PBS) and centrifuged for 10 minutes at 400g. The sedimented PBMC were collected and transferred to fresh PBS for culture.

Induction of apoptosis

PBMC were incubated in 5 ml phosphate buffered saline at 37°C in an environment of 5% carbon dioxide. After overnight incubation, PBMC were washed twice with fresh PBS by centrifugation at 400g for 5 minutes. The washed pellet was resuspended in 1 ml of cold PBS and sonicated for 15

seconds. The contents were centrifuged at 5000g for 5 minutes at 4°C. The supernatant was collected and used for caspase 3 assay.

Caspase 3 assay

Samples of the PBMC extract were assayed in duplicate. To confirm the dose-effect relationship, 10µl and 25µl of the extract was used in duplicate wells. To assess the incubation time-effect relationship on the caspase 3 activity in the wells the plate was read twice, once after 4 hours of incubation and then again after overnight (16 hours) incubation. Measurable caspase 3 activity was seen in the PBMC extract. A proportional relationship was noted between the caspase 3 activity in the extract as measured by the absorbance and the volume of extract in each well. There was also an increased caspase 3 activity after overnight incubation (Figure 20).
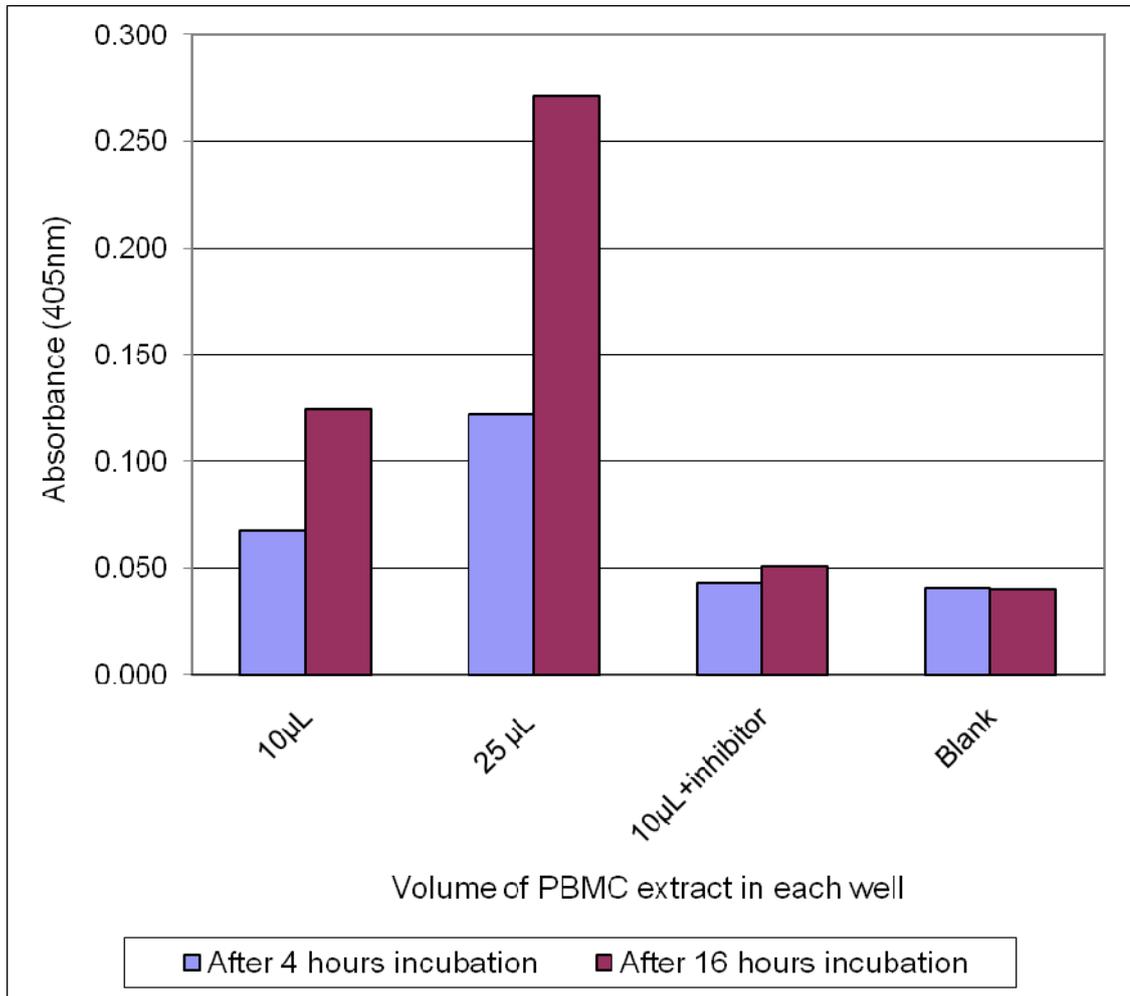
Figure 20 Effect of volume of PBMC extract and duration of incubation on the measured caspase 3 activity
Extract from apoptotic PBMC was prepared as described in the text. Two reaction volumes of the extract (10µl and 25 µl) were used to confirm dose-reaction relationship. The plate was read twice (after 4 hours and 16 hours of incubation) to confirm incubation time-reaction relationship.

3.5.3 Determination of activated caspase 3 levels in rat pancreatic digest

Background

Pancreatic islets isolated from non-heart-beating-donor rat pancreases are likely to have undergone significant stress. We attempted to estimate the extent of caspase 3 activation in aliquots of islet samples from control pancreases and from non-heart-beating-donor pancreases preserved by various preservation techniques. Estimation of caspase levels in islet isolates has been reported as a means of investigating apoptosis [154].

Rationale for use of pancreatic digest instead of islet isolates for caspase 3 assay

In an initial series of experiments an aliquot of the islet isolate was lysed in the lysis buffer and used for the caspase-3 assay. However only 100-200IEQ of islets were available for the assay after their use for viability testing and glucose stimulated insulin secretion assay. The levels of expected caspase 3 in the samples were below the sensitivity of the colorimetric assay and no increased absorbance at 405nm was seen over the blank controls. To overcome this problem, whole pancreas digest at the end of the washing step and before density gradient separation was used for the assay. It was assumed that a similar level of apoptotic change would be noted in both islet and exocrine components of the pancreatic digest. Briefly at the end of the washing step the digest was suspended in 5 ml of wash medium. 0.5ml of the suspension was

transferred to a microcentrifuge tube while the rest of the digest proceeded to islet purification. The microcentrifuge tube was centrifuged at 5000g for 2 minutes. The supernatant was removed and the tube was snap frozen by immersing it in liquid nitrogen. The tubes were stored at -80°C until analysis.

Protocols for preparation of cell extract

Two techniques were evaluated for lysis of the pancreatic digest. In the first method (freeze-thaw technique), the pellet was suspended in 100µl of lysis buffer. It was then snap frozen by immersing in liquid nitrogen and then thawed in a water bath at 37°C. The micro-centrifuge tube was vigorously agitated for 5 seconds. The steps were repeated twice. At the end of three cycles the tube was centrifuged at 4°C and 15,000g for 5 minutes. The supernatant was collected and transferred to labelled tubes and stored at -80°C for analysis. In the second method (sonification technique), the pellet was suspended in 100µl of lysis buffer. It was subjected to sonication for three periods of 5 seconds each with 10 second cooling down interval in an ice bath. The tube was then centrifuged at 15,000g for 5 minutes. The supernatant was transferred to labelled tubes and stored at -80°C for analysis.

The two methods were compared for three different pancreatic digest samples. For each pancreas, aliquots were processed by both methods. The protein concentration and caspase 3 activity in the extracts were assayed. Protein concentration in the extracts was calculated using the Invitrogen Quant-iT protein assay kit. Caspase 3 activity was assayed using the Caspase 3

colorimetric assay kit. The manufacturer's instructions were modified and 20μl of extract was used for each well with a concomitant reduction in the volume of assay buffer per well. The plate was incubated overnight and analysed in a micro-titre plate reader at 405nm.

Sonication protocol gave better results for both total protein concentration and activated caspase 3 activity for all three samples (Figure 21&Figure 22). The sonication technique was hence utilized for pancreatic digest caspase assays.
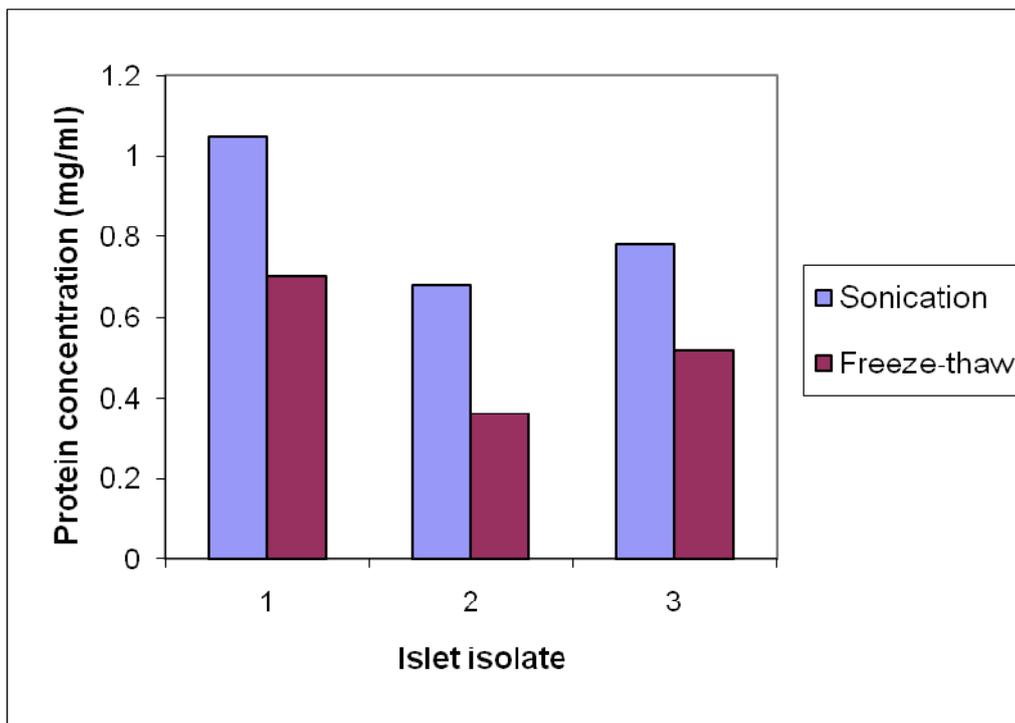
Figure 21 Bar chart comparing the sonication and freeze-thaw technique for tissue lysis from three rat pancreatic digest samples- total protein concentration

Duplicate aliquots of the three rat pancreas digest samples (1,2,3) were subjected to either sonication or freeze-thaw methods of tissue lysis (protocol in text). The concentration of protein in the supernatant after centrifugation of the extract was assayed using the Qubit Quant-iT protein assay kit. Data is presented as protein concentration in mg/ml of extract. The sonication technique provided better tissue lysis and higher total protein concentration for all three samples.
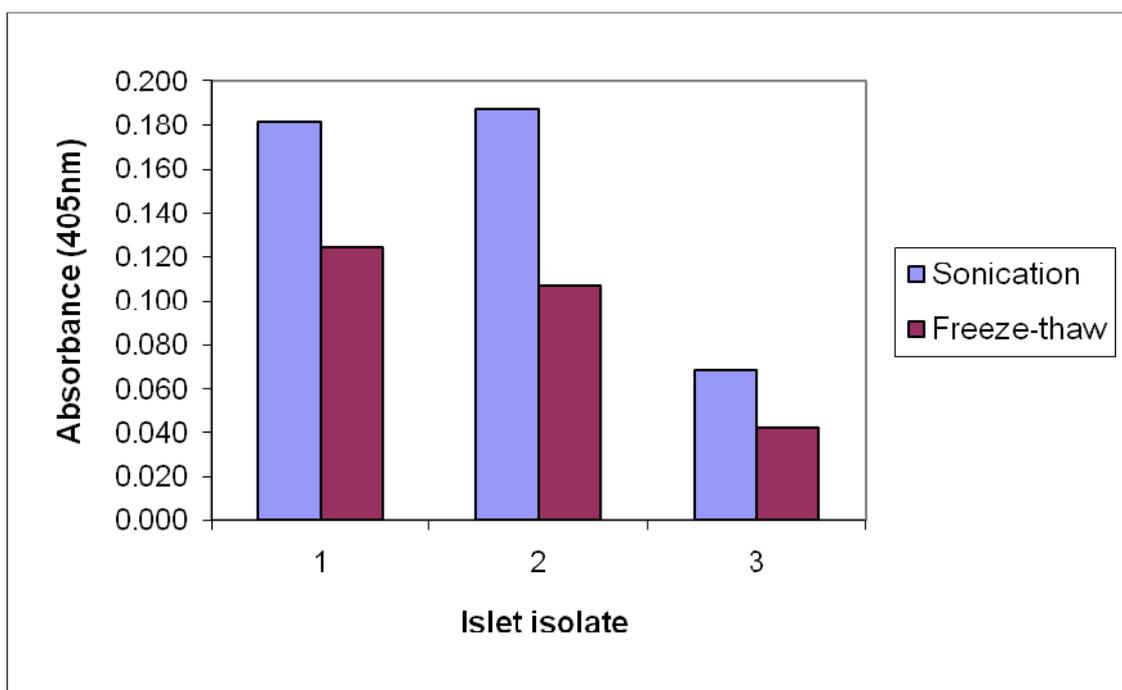
Figure 22 Bar chart comparing the sonication and freeze-thaw techniques for tissue lysis from three rat pancreatic digest samples- Caspase 3 assay

Duplicate aliquots of the three rat pancreas digest samples (1,2,3) were subjected to either sonication or freeze-thaw methods of tissue lysis (protocol in text). The concentration of activated caspase 3 in the supernatant after centrifugation of the extract was assayed using the Caspase-3 colorimetric assay. The caspase 3 level is represented as absorbance at 405 nm. The sonication technique provided better tissue lysis and higher absorbance for all three samples.

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3.5.4 Activated Caspase 3 levels in rat pancreas tissue extracts after warm oxygenated perfusion

Background

A comparison of the degree of apoptotic activation in non-heart-beating-donor pancreases which underwent prolonged cold ischaemia and oxygenated warm reperfusion *in vitro* was attempted.

Pancreatic tissue is a rich source of proteolytic enzymes that are released during exocrine tissue injury. These enzymes can break down cellular proteins and enzymes during homogenisation. There is therefore a risk of spuriously low results from caspase 3 assay of the pancreas digests due to proteolysis. Use of pan-protease inhibitors during sample preparation will affect the cysteine protease activity of caspase 3 making its estimation using a substrate based assay inaccurate. The following measures were taken to limit the extent of proteolysis during the homogenization phase. The entire storage and homogenization process was completed at low temperatures to inhibit enzyme activity. Radio-Immuno-Precipitation Assay (RIPA) buffer supplemented with phenyl methyl sulfonyl fluoride (PMSF) (1mM) was used as the vehicle during homogenisation. RIPA buffer is commonly used for the lysis of mammalian cells and tissues. It does not contain any protease inhibitors of its own. Pancreatic enzymes are serine proteases. Phenyl methyl sulfonyl fluoride irreversibly inhibits serine proteases such as trypsin and chymotrypsin but does not inhibit

cysteine proteases such as caspase 3. Hence it was chosen as the protease inhibitor during homogenisation.

Preparation of tissue extracts

RIPA buffer with 1 mM PMSF (0.1ml of 100mM stock solution added to every 10 ml of RIPA buffer just before homogenization) was used. The frozen biopsy (approximately 0.5-1 gm) was transferred into a universal container containing 3ml of the prepared RIPA buffer. The contents were transferred to a clean glass tube and homogenized with a motorised glass-Teflon homogeniser in an ice bath. The extract was then transferred to micro centrifuge tubes and centrifuged at 12000g for 5 minutes. The supernatant was collected in labelled micro centrifuge tubes and stored at -80°C.

Caspase 3 assay procedure

Caspase 3 assay using the tissue extracts was initially attempted according to the manufacturer's instructions. Using the 96 microtitre plate assay and 5µl of tissue extract per well, the absorbance at 405nm after overnight incubation was very low. To increase the sensitivity, the volume of tissue extract per well was increased along with a decrease in the volume of assay buffer per well. This increased the sensitivity of the test. However the problem with this was the high background non-specific absorbance in some specimens. In an attempt to account for this variable, the tests were run in triplicates with a negative control for every test sample. This involved adding a caspase 3 specific inhibitor that is included in the kit to one well containing the test samples. The absorbance was read at 405nm after overnight incubation. The true absorbance for each sample

was calculated by subtracting the excess absorbance in the test well with caspase 3 inhibitor from the mean absorbance in the test wells (Figure 23). Caspase 3 levels were calculated in terms of µmol pNA released after overnight incubation per mg of protein in the extract.
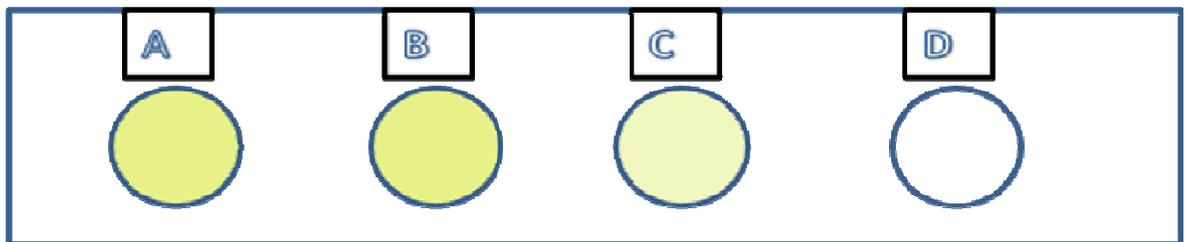
A: Test sample
B: Test sample
C: Test sample with specific caspase 3 inhibitor
D: Blank (assay buffer only)
True absorbance of sample – (aA+aB)/2 – (aC-aD) where aA, aB, aC, aD are the absorbances at 405nm of wells A, B,C and D respectively.

Figure 23 Schematic representation of the method for calculating true absorbance of study samples with high non-specific absorbance

3.5 Extraction of pancreatic RNA for microarray analysis

The quality of information obtained from microarray analysis depends to a large extent on the quality of RNA used [205]. RNA quantity can be adversely affected by degradation or by contamination with genomic DNA, proteins and solvents used during the extraction process. RNA extraction from pancreas is specifically complicated by the high concentration of ribonuclease enzyme present in pancreatic tissue[206, 207]. Background noise from degraded RNA adversely affects the ability of RNA microarrays to identify differential expression. TRIzol reagent was used for the extraction of pancreatic RNA in our study. This extraction is based on the Guanidinium thiocyanate-phenol-chloroform extraction method that was developed by Chomczynski and co-workers in 1987 [208].

3.5.1 RNA extraction protocol

TRIzol reagent was used for immediate RNA isolation according to manufacturer's instructions. Briefly the steps in RNA extraction were as follows.

Homogenisation: The pancreas biopsy was immediately chopped into small pieces (2mm) and placed in a universal container containing approximately 10 times the amount of TRIzol (approximately 5ml). The contents were then homogenised in a glass and Teflon electrical homogeniser which had been cleaned with RNAzap and rinsed with autoclaved water. The homogenised mixture was allowed to stand at room temperature for 5 minutes. The mixture was then transferred to six sterile 1.5ml microcentrifuge tubes. These were

centrifuged at 12000g for 10 minutes to precipitate insoluble remnants. The supernatant was transferred to fresh tubes.

Phase separation: To each tube, 100 µl of 1-bromo-2 chloropropane was added. The mixture was shaken vigorously for 15 seconds and incubated at room temperature for 2-3 min. The samples were centrifuged for 15 minutes at 12000g. The top aqueous phase was collected and transferred to fresh tubes.

RNA precipitation : To the aqueous phase in each tube, 500 µl of isopropanol was added. After gently mixing the contents by inverting the capped tube 4-5 times, it was allowed to stand for 10 minutes. The samples were then centrifuged at 12000g for 10 minutes. This precipitates the RNA at the bottom and sides of the microcentrifuge tube. The isopropanol was pipetted out.

RNA purification: To each tube, 1ml of 75% ethanol was added and mixed by gentle inversion of the capped tube. The tubes were then centrifuged at 7500g for 5minutes. The ethanol was then pipetted off leaving the RNA pellet dry at the bottom of the tube.

RNA storage: The pellet was air-dried. It was then dissolved in 100µl (per microcentrifuge tube) of RNA secure. This was then stored at -80°C.

3.5.2 Assessment of quality of RNA

Assessment and further purification of RNA was carried out by Dr Noel Carter. RNA samples from each pancreas were run on an agarose gel. Three samples from each preservation group with the best electrophoretic pattern were identified. These samples were re-precipitated using 3.5N lithium chloride

according to manufacturer's instructions and resuspended in RNAsecure. Samples were run on a gel again to confirm RNA integrity. The samples were quantified using a Qubit assay. The selected samples within each preservation group and control group were mixed in equimolar ratios for a final concentration of 1µg/ml. 20µl aliquots of the three pooled samples were supplied to Dr Heiko Peters, Department of Human Genetics, Centre for Life, Newcastle university for microarray analysis. Quality of RNA was assessed in terms of rRNA ratio (28s/18s) and RNA integrity number (RIN) using Agilent 2100 bioanalyser. The rRNA ratio was 1.1 for the three samples. The RIN was 6.2 for the control and the portal venous oxygen persufflation groups and 6.6 for the static cold storage group (Figure 47).

3.5.4 Modifications to RNA extraction method

Samples of RNA extracted during the initial part of the study using the manufacturer's instructions were significantly degraded. Following literature review [207, 209], protocol modifications were made. The amount of pancreas tissue used for extraction was decreased. Approximately 0.2g of tissue was homogenised with 5ml of the reagent. The tissue sample was snap frozen in liquid nitrogen to minimise RNA degradation while being taken to the homogeniser station.

These modifications improved the quality of RNA extracted from samples in the later part of the study. Li and co-workers in 2009[210] attempting to improve the quality of pancreatic RNA extracted using TRIzol reagent arrived at similar

conclusions. Despite our modifications, the 28s/18s ratio of the samples was 1.1 and the RNA integrity number (RIN) of the analysed samples ranged between 6.1-6.6. This was sub-optimal as the recommended 28s/18s ratio and RIN levels for microarray analysis are greater than 1.63 and 7.8 respectively [205].

Figure 24 in opposite page.

The two methods of RNA assessment used in our experiments were the rRNA ratio and the RNA integrity number. The gel images of the three RNA samples are shown on the right side of the picture. The gel shows the prominent 18S and 28S bands representing rRNA in the sample. The electropherogram on the left is a graphical representation of the gel. The numerical representation of RNA quality in terms of rRNA ratio (28s/18s) and the RNA integrity number is depicted at the lower end of the figure. Sample key (Control group: Sample 4; Static cold storage: Sample 5; Portal venous oxygen persufflation: Sample 6)
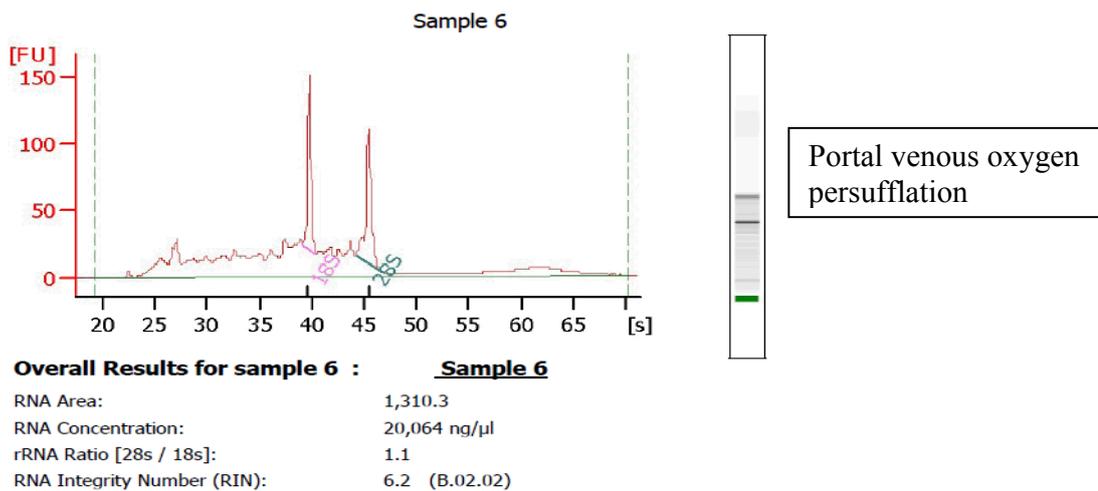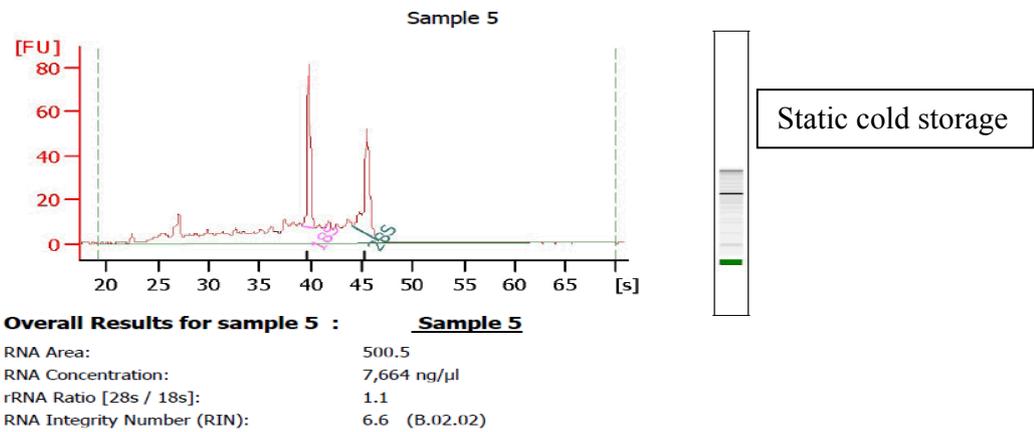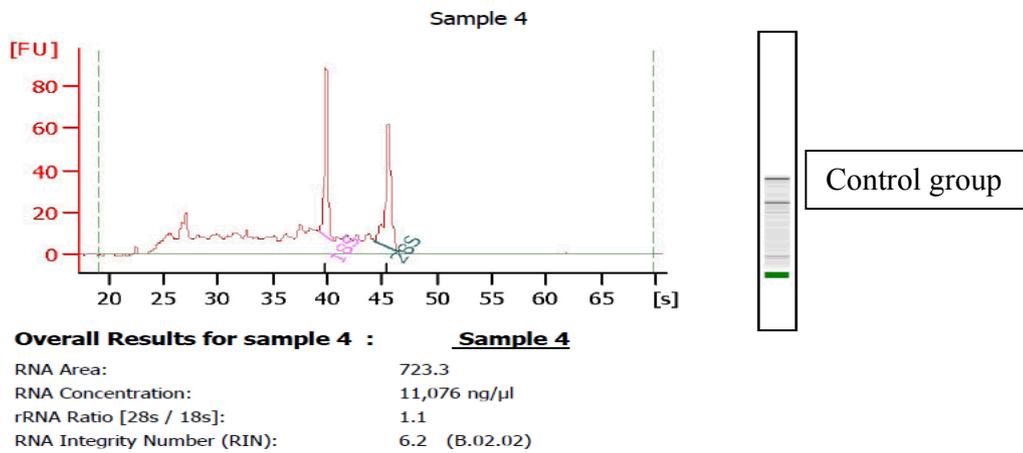
Figure 24 Assessment of RNA quality for microarray analysis

3.6 Discussion

This chapter describes some of the optimisation work carried out for this study. Developing the rat model of pancreas preservation was by far the most time-consuming part of the study. After multiple attempts with *en bloc* models, the isolated pancreas model was adapted. The retrieval procedure as described could be repeated consistently. Technical problems leading to organ wastage and some degree of rewarming injury during the prolonged dissection process were some of the problems identified. The temperature during the third phase of the dissection stayed around 10-15°C, which can be considered to be sub-optimal. Hence the effective warm ischaemia time experienced by the pancreas could have been longer than 35 minutes.

The work described previously, investigating the effect of warm ischaemia time on islet yield, highlighted the problems with hand-picking of islets for assessment. Representative samples of the islet yield cannot be obtained by hand-picking and hence the density gradient separation technique was used for further islet work (Chapter 4). Numerous protocols for glucose stimulated insulin secretion assay are described in the literature. The consistency of the final protocol used in this study was shown to be better than the initial protocol. However the biphasic function of the islets was not evaluated. The cellular insulin levels in the islets were also not estimated.

Assessment of apoptosis can be carried out in multiple ways. Various techniques exploit the characteristic features of apoptosis such as the ladder

pattern of DNA breakdown on DNA electrophoresis, tdT annexin assay to identify specific breakpoints of DNA strands, assays of apoptotic enzymes such as caspases and the expression of genes involved in apoptosis. The caspase 3 assay was used in the study. It is well described and inexpensive. It could be used with both tissue and cell extracts and hence could be used for both pancreatic tissue and islet cell extracts. The same tissue extracts could also be used for assessment of lipid peroxidation in our experiment. The chief drawback of the chosen assay was that it was not sensitive enough for the requirements of our study. The optimisation steps described in this chapter improved its sensitivity. The assay also looked at a single step in the complicated process of apoptosis. All these increase the risk of false positive and false negative results. Hence levels of caspase 3 measured in the test samples would only be estimates of the extent of apoptosis.

RNA microarrays are being increasingly used as a screening tool to identify relevant underlying biological processes in experimental studies. Portal venous oxygen persufflation is a novel preservation technique which has been investigated in the setting of experimental and clinical organ preservation. The effects of portal venous oxygen persufflation on global gene expression in the pancreas have not been previously reported.

The control RNA for the study was obtained from pancreases of freshly killed rats. This should provide the gene expression set in a metabolically active pancreas. Since it is not possible to completely flush the control pancreases before RNA extraction, this tissue would contain leukocytes which have a

different gene expression signature to pancreatic tissue. This makes it an imperfect control. Both preservation groups were compared to the same control group independently to identify differential expression of genes and pathways separately.

Chapter 4 Comparison of islet yield from non-heart-beating-pancreases with different methods of preservation

4.1 Background

Successful islet transplantation is predicted by the mass of viable islets or β cells transplanted in the recipient[211]. Studies have shown that approximately 9,000-12,000 islet equivalents per kg body weight of the recipient are required to achieve insulin independence[28, 29]. Pancreases retrieved from donors with significant periods of warm ischaemia have poor islet yields[46]. The currently used methods of pancreas preservation are static cold storage and two layer method. Static cold storage is not ideal for these organs. Experimental studies of two-layer method have been promising though the benefit in the clinical setting has been unclear[20, 150, 158]. The relative benefits of hypothermic machine perfusion and portal venous oxygen persufflation in preserving pancreases that have suffered significant warm ischaemia was investigated in this chapter.

Early attempts to isolate islets involved meticulous dissection of islets from the pancreases. Lacy and Kostianovsky were the first to describe large scale isolation of islets from pancreases of rats and mice[212]. Their technique involved initial disruption of pancreatic parenchyma by intraductal injection of Hanks' buffered salt solution (HBSS). The pancreas was then finely chopped and incubated in Krebs-Henseleit solution containing collagenase. Intra-ductal injection of collagenase solution and digestion at 37° C in a shaking water bath

was described by Noel and co-workers in 1982 [213]. The method used in our experiments was based on the static digestion technique described by Gray and co-workers in 1984 [214].

Several methods to purify the islet fraction from the pancreatic digest have been described. Methods such as hand-picking under a low power microscope are still utilised when a small number of pure islets are required. Density based separation methods are the most commonly utilised to separate large numbers of relatively pure islets. They exploit the fact that digested exocrine tissue has a higher density compared to islets. Sucrose, Percoll™, Ficol™, Histopaque™ have all been used to prepare density gradients for islet purification. Both continuous and discontinuous density gradients have been used. Discontinuous gradients are commonly used for rat or mouse pancreas isolation[202].

Assessment of islet yield includes the counting of islets, calculation of islet equivalents, viability and estimation of *in vitro* and *in vivo* function[203]. Dithizone (Diphenylthiocarbazine) intensely stains the proinsulin/insulin complexes of β cells giving a deep red colour. It is a helpful guide in identifying islets from exocrine tissue (Figures 25, 26).

Islet Equivalent is the term used to estimate the actual volume of the islet. This measurement has better correlation with post-transplant outcome when compared to islet count. To calculate islet equivalents each islet is considered to be 150μm in diameter. Islets of different sizes are converted to this standard size and expressed as IEQ [203]. Viability of islets is an essential criterion for assessing the quality of islet yield. Exclusion staining with propidium iodide[215]

or trypan blue are commonly used techniques to assess viability. Cell membranes of live cells are impermeable to these dyes. Uptake of these stains by islets indicates ineffective cell membrane function and cell death[216]. Morphology of isolated islets is a strong predictor of islet function and post-transplantation success. Studies have shown that healthy and functional islets are larger, more globular and have a regular outline. Small, irregular and fragmented islets reflect damage and predict poor post transplant function[217, 218]. Islet index or islet isolation index is calculated from the ratio of islet equivalents to the islet number. It is a measure of the average size of the islets and is a guide to the degree of fragmentation of the islets. Islet isolation index less than 1 indicates extensive fragmentation. Studies have shown better results with islets with isolation index greater than 1.0[31, 217, 218]. However it has also been suggested that smaller islets have a better chance of surviving the hypoxic conditions after transplantation. These studies have claimed that larger islets develop a central core of hypoxic cell death leading to graft failure [219].

The most accurate method to assess islet function is their ability to correct hyperglycaemia following transplantation into immuno-deficient diabetic mice. However routine utilisation of this technique is expensive and time-consuming. The test is also irrelevant in the clinical setting as results from these experiments will be available long after the islets had been transplanted. *In vitro* methods of testing islet function are based on the ability of islets to secrete insulin in response to glycaemic stress[202, 220]. Static glucose stimulated insulin secretion test is the simplest and most commonly used test to assess *in vitro*

islet function. This is based on the principle that viable islets secrete more insulin in high glucose environments. The ratio of insulin produced at high glucose environment and low glucose environment (basal insulin production) is called stimulation index. The stimulation index of viable islets is usually greater than one [203].
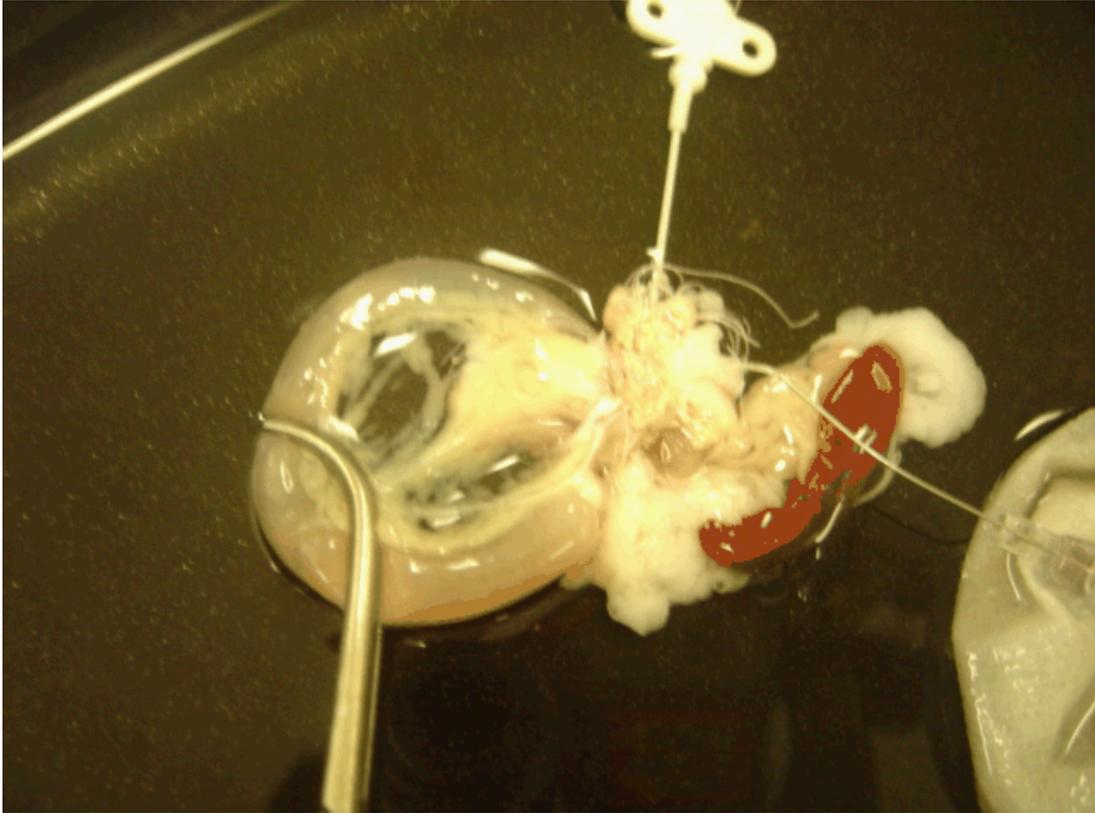
Figure 25 Injection of collagenase into the common bile duct
The duodenum at the insertion of the bile duct is clamped. This ensures retrograde filling of the pancreatic duct and pancreas distension.
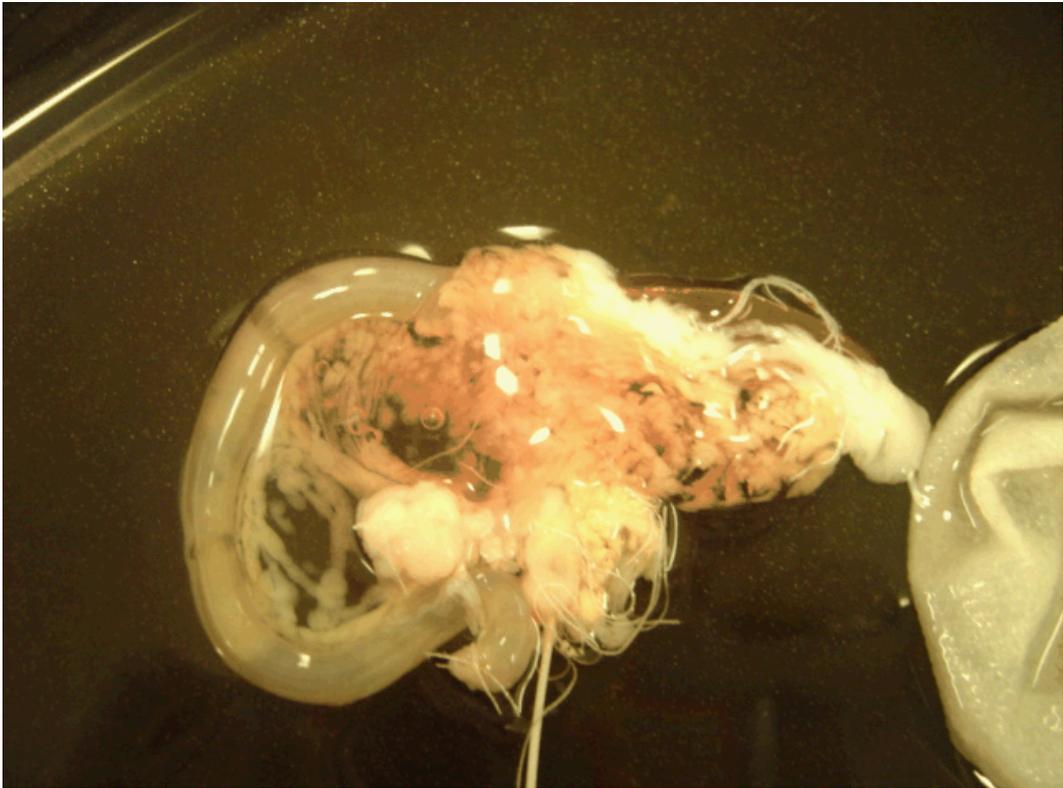
Figure 26 Distension of pancreas with collagenase solution

The pancreas was distended with collagenase solution by injecting it into the pancreatic duct through the common bile duct cannula. The distended pancreas is pink in colour due to the use of Hanks' buffered salt solution to prepare the collagenase solution. Phenol red used as the indicator in the solution gives it the pink colour.
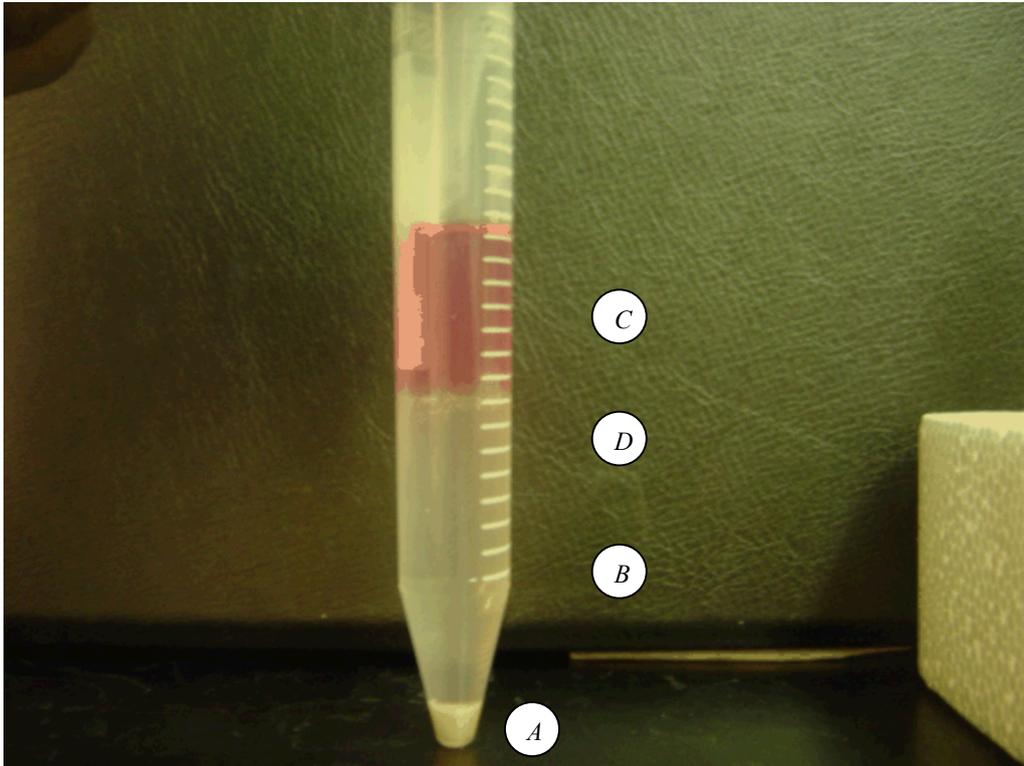
Figure 27 Completion of discontinuous density gradient islet purification step
The density gradients and separated components of the pancreatic digest are clearly seen. The exocrine tissue has settled at the bottom of the tube (A). Above this is the high density Histopaque column (B). The low density Hanks' buffered salt solution forms the top layer(C). Purified islets can be seen at the interface between the low and the high density columns (D).
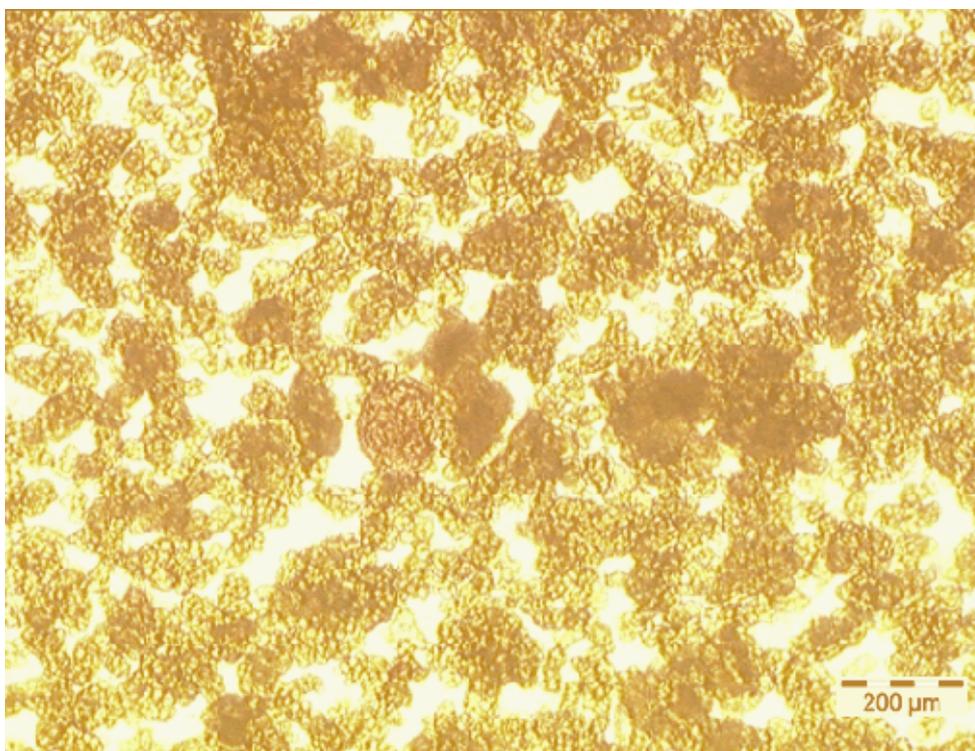
Figure 28 Pancreatic digest stained with dithizone to demonstrate the staining characteristics of exocrine tissue and islets

An aliquot of the pancreatic digest has been stained with dithizone solution and examined under the low power microscope. The large amount of exocrine tissue which is not stained by dithizone is yellow in colour. The single islet in the centre of the field stains red.
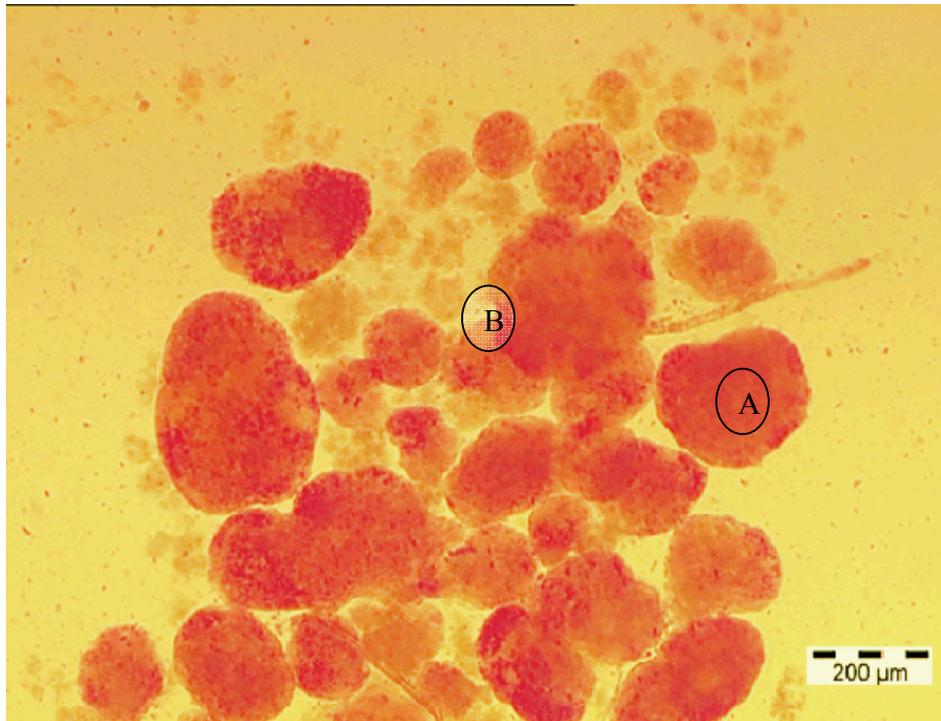
Figure 29 Purified islets stained with dithizone
Purified islets after overnight culture were stained with dithizone and examined under a low power microscope. Islets are identified as globular structures with characteristic red staining with dithizone (A). Clumps of irregular exocrine tissue are also seen in the picture (B). These do not stain with dithizone.
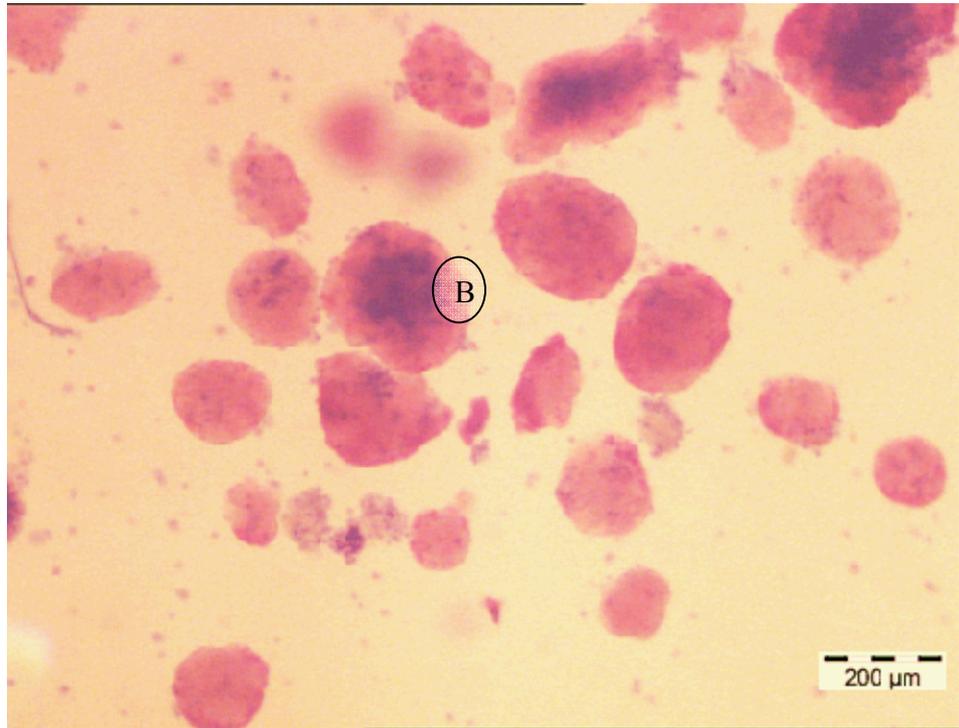
Figure 30 Viability testing of islets-Staining with trypan blue and dithizone
The islets after overnight culture were stained with dithizone and trypan blue and examined under a low power microscope. Dithizone stains all islets red in colour. Trypan blue stains dead cells blue in colour. These are predominantly viable islets (A) with only a few blue staining cells. Larger islets show a central area of staining with trypan blue (B). This represents core necrosis of islets due to ischaemia.
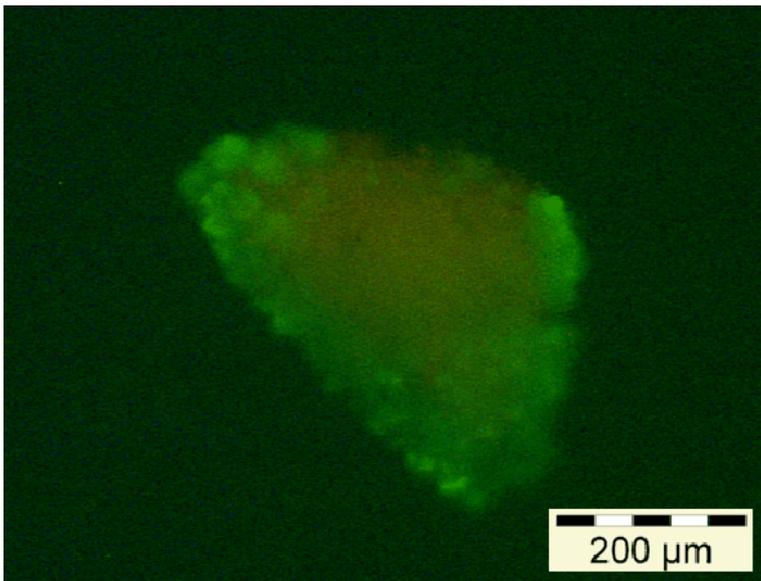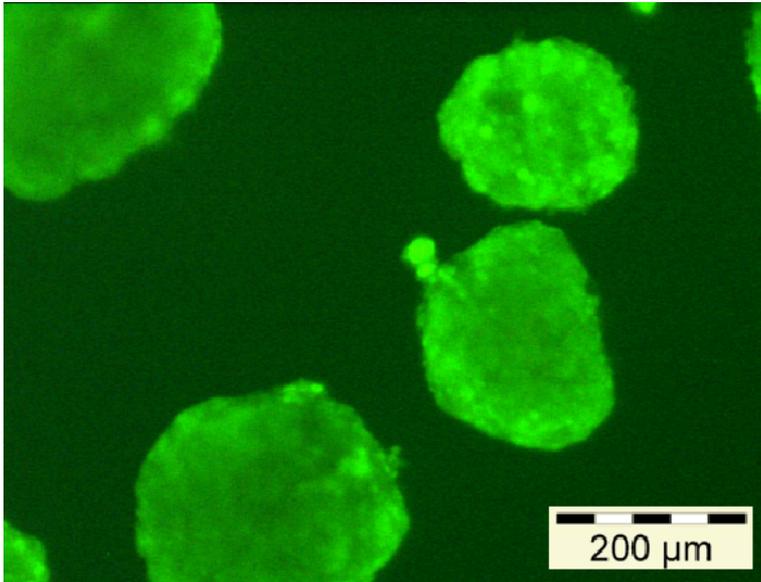
Figure 31 Viability testing of islets-Staining with Fluorescein diacetate and Propidium iodide

Islets after overnight culture were stained with Fluorescein diacetate and propidium iodide and examined under blue light using a fluorescent microscope. The bright green fluorescence of islet clusters is clearly seen in the top image. The bottom image shows a single islet with red fluorescence in the central part of the islet suggestive of dead cells.

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4.2 Methods

4.2.1 Study plan and positive control group

The study plan is depicted in figure 31. The null hypothesis formulated for this phase of the study was that 'change in technique of pancreas preservation does not alter the islet equivalent yield and the stimulation index of the islets isolated from the pancreas'.   Retrieval of non-heart-beating-donor rat pancreas was described previously (section 3.3.1 The duration of warm ischaemia was set at 35 minutes as previous experiments had shown that a warm ischaemia of 30 minutes lead to a greater than 50% reduction in crude islet yield. The techniques of static cold storage, two-layer method, hypothermic machine perfusion and portal venous oxygen persufflation have been previously described (section 2.3.2). The perfusion pressure and persufflation pressure was continuously recorded during the preservation period. At the end of the preservation period the pancreas was weighed and the change in weight after preservation was calculated. The pancreas then underwent islet isolation.

Pancreases retrieved from rats without significant warm ischaemia served as positive controls. Here the rat was killed by a schedule I method and an immediate laparotomy was carried out. The animal was exsanguinated by dividing the inferior vena cava. The bile duct was identified and cannulated. Cold collagenase solution (10mg of collagenase P in 10 ml of HBSS) was injected into the pancreatic duct after clamping the insertion of the bile duct into the duodenum (Figures 25, 26). The distended pancreas was bluntly excised and

processed for islet isolation. Assessment of islet yield in terms of islet count, viability and *in vitro* function were carried out.
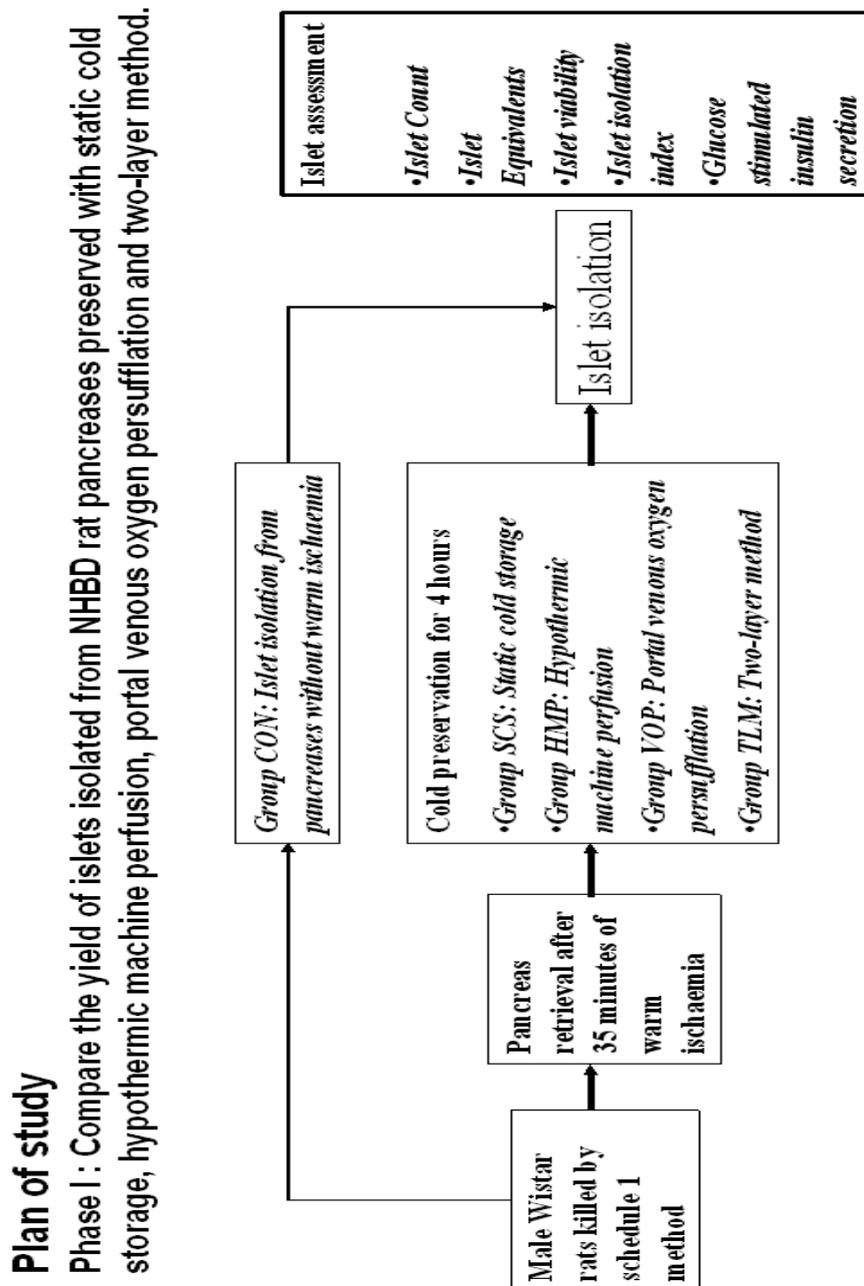
Figure 32 Flow chart depicting the plan of study (Phase I)

4.2.2 Procedure of rat pancreas islet isolation

The pancreas was transferred to a petridish containing cold University of Wisconsin solution. The duodenum was clamped at the point of insertion of the common bile duct (CBD). 10ml of 1mg/ml Collagenase P solution (in ice cold Hanks' buffered salt solution) was injected through the CBD cannula slowly. Care was taken to ensure that the collagenase solution was not leaking out or into the duodenum. The uniformly distended pancreas was bluntly separated from the duodenum taking care not to tear the duodenum or the pancreatic capsule. Pancreas was separated from the spleen and placed in a sterile 20ml centrifuge tube. It was incubated in a static water bath at 37°C for 50 minutes. After completion of the incubation period the tube was shaken for 10 seconds to homogenize the pancreas.

The digest was washed thrice with the wash solution by centrifugation at 300g for one minute. The digest was then passed through a 400 micron sieve to remove the undigested pancreatic tissue, lymph nodes and fibro-fatty tissue. The filtrate was centrifuged at 300g for 1 minute with brakes off. The precipitate was suspended in 20 ml of sterile Histopaque 1.083. The digest-Histopaque suspension was divided into four aliquots and carefully layered over 2 ml of Histopaque 1.083 in each of four centrifuge tubes. 4ml of Hanks' buffered salt solution was layered over the digest-Histopaque suspension in each centrifuge tube using a Pasteur pipette.

The tubes were centrifuged at 800g for 10 minutes with brakes off in a refrigerated centrifuge (4°C). Islets concentrate at the Histopaque-HBSS interface (Figure 27). This was aspirated with a Pasteur pipette and transferred into sterile centrifuge tubes. This was washed twice with wash solution by centrifugation at 300g for 1 minute. The precipitate was suspended in 10 ml of sterile culture medium previously warmed to 37°C and transferred to a gas-permeable culture flask. The flask was incubated overnight at 37°C in an incubator with 5% carbon dioxide.

Discussion

Discontinuous density gradient separation is the preferred method of purification of islets from the rat pancreas. This is based on the differential density of digested exocrine tissue and separated islets. Exocrine tissue has a higher density and hence is precipitated. By using suitable density medium the islets form a column separate from the precipitated exocrine tissue. Various studies have reported the use of sucrose, percoll, iodine based medium of various densities to purify the islets. After several attempts, the best results were obtained by using Histopaque which is an iodine contrast medium. This is available in various density preparations. Histopaque 1.083 provided the best separation of islets from the digest with minimal loss of islets in the exocrine precipitate.

Several measures were found to improve the purification process. The Histopaque was warmed to room temperature before use. The bottom column of pure Histopaque improved the delineation of the exocrine tissue pellet at the end

of purification. Dividing the digest into 4 aliquots minimized the clumping of all the islets together in too small a space at the interface. The centrifuge was pre-cooled. The centrifuge was used with the breaks off to prevent disruption of the density columns due to rapid deceleration. After separation, the islet rich interfaces were collected and washed thrice with wash medium as Histopaque has been reported to be toxic to the islets on prolonged contact.

4.2.3 Staining islets with dithizone and islet count

All counts and size estimations were done by a single observer who was not blinded to the type of pancreas preservation.

1 ml of islet suspension was transferred to a 10 ml universal container. An equal volume of prepared dithizone solution was added to the suspension and mixed by shaking gently. The mixture was incubated at room temperature for 5 minutes. The islets were rinsed twice with the wash solution. They were suspended in 2 ml of the wash solution and transferred to multiple wells of a 96 well micro-titre plate using a Pasteur pipette. Islets were counted under low power microscope (4X objective). Islets stain bright red while exocrine tissue does not stain (Figures 28,29)

4.2.4 Calculation of islet equivalents (IEQ) and islet isolation index

Islet suspension was stained with dithizone as described above. The number of islets and the size of each islet were noted. Size of each islet was estimated using an eye-piece graticule fixed in the microscope. The graticule provided a scale in the visual field of the microscope where 1mm is divided into 100 sub-

divisions by 99 lines. The diameter of each islet was measured using this scale in two perpendicular directions. The mean of the two observations was documented as the islet diameter. The islet yield in IEQ was then calculated using the following formula[203].

Islet equivalents (IEQ) = (n50-100* / 6) + (n100-150 /1.5) + (n150-200 X 1.6) + (n200-250 X 3.5) + (n250-300 X 6.3) + (n300-350 X 10.4) + (n350-400 X 15.8) + (n>400 X 22.8)
* n50-100 is the number of islets with diameter from 50μ to 100μ and so on

The islet isolation index is a measure of the extent of fragmentation of the islets. It was calculated for each isolate by dividing the islet yield in Islet equivalents by the actual islet count. Islet isolation index was low (<1) in isolates where the islets are highly fragmented. Well preserved islets will have an index greater than one.

4.2.5 Assessment of islet viability

Viability assessment by propidium iodide staining

25-50 islets suspended in 0.5 ml of culture medium were transferred to a 1 ml microcentrifuge tube. An equal volume of the fluorescein diacetate/propidium iodide working solution was added. The tube was gently inverted several times to ensure mixing and incubated at room temperature for one minute. Supernatant fluid was aspirated and the islets were washed twice by sedimentation with wash solution. They were transferred to a custom made glass slide with central depression and examined under blue light in a

fluorescent microscope. Non-viable islets stained red while viable islets were stained fluorescent green (Figure 31).

Islet viability testing by trypan blue staining

25-50 islets suspended in 0.5 ml of culture medium were transferred to a 1 ml micro-centrifuge tube. 100 µl of 0.4% trypan blue solution was added to the islet suspension and mixed by inverting the tube. After incubation at room temperature for five minutes the islets were washed twice by sedimentation using the medium solution. The suspension was transferred to a custom-made slide and examined under low power microscope. Dead islets were blue and viable islets were cream coloured. Pale islets with central blue staining suggested viable with central necrosis. Pale islets with peripheral blue staining suggested viable islet with mantle of non-viable exocrine tissue. Simultaneous counterstaining with dithizone helped differentiation of viable islets from viable clumps of exocrine tissue (Figure 30).

4.2.6 Assessment of islet *in vitro* function

The static glucose stimulated insulin secretion test was used in the study to assess the *in vitro* function of islets. The rationale for the use of this assay and the optimisation of the assay procedure was previously detailed. Briefly, islets after overnight incubation were washed twice in low glucose medium. Aliquots of the islet suspension were transferred to six wells containing low glucose medium. The islets were incubated at 37°C for one hour. The islets were rinsed

with fresh low glucose medium. To each well, either low or high glucose medium (3 wells each) was added and mixed with gentle aspiration. The islets were re-incubated at 37°C for 3 hours. At the end of the incubation period the contents of each well were transferred to labelled tubes taking care to collect all the islets from the wells. The tubes were centrifuged at 17500g for 3 minutes. The supernatant was collected in separate labelled tubes and stored at -80°C. Insulin concentration in fluid was assayed using Mercodia High range Rat Insulin Elisa kit according to manufacturer's instructions. Stimulation index was calculated as previously described.

4.2.7 Statistical analysis

Minitab™ was used to calculate the sample size required to reject the null hypothesis. A sample size of 10 animals per treatment group was required to reject the hypothesis with a power of 80% at a 5% level of significance. Comparison of islet yield and insulin stimulation index between the various groups was done using analysis of variance (ANOVA). The relation between perfusion or persufflation pressures and islet yield and *in vitro* function was analysed by Pearson's correlation coefficient.

4.3 Results

4.3.1 Laboratory animal usage and number of successful isolations

For the control arm, 15 rats killed by schedule I method were used. Islet isolation was not satisfactory for one pancreas and it was excluded from further analysis. Fourteen pancreases underwent successful islet isolation and assessment. Fifty eight non-heart-beating-donor pancreases were retrieved after standardisation of the pancreas retrieval procedure. All these pancreases were exposed to 35 minutes of warm ischemia. The pancreases were then preserved by any of the four preservation techniques. Problems in preservation or islet isolation led to 11 pancreases being excluded from the further analysis (table 4). The reasons for not using 11 pancreases were as follows.

1. Leaks identified during back-table preparation which could not be satisfactorily repaired (Total=5, HMP=3, PVOP=2)

2, Poor distension of pancreas with collagenase injection due to leakage (Total=3, SCS=2, PVOP=1

3. Loss of significant portion of pancreatic digest during the purification process (Total=2, SCS=1, PVOP=1)

Hand-picking of islets was used to collect islets for viability assessment in the initial phase of the project. This was changed to discontinuous density gradient separation once the technique was standardised. A total of fifty one pancreases (control group=12, study groups=39) underwent density gradient purification (Table 4).

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Table 4 Table showing the number of animals and the number of successive islet isolations in various preservation groups.

	Control group pancreases	*Non-heart beating pancreases*				
		Total	*SCS*	*HMP*	*PVOP*	*TLM*
Total number of rats used	15	58	20	16	17	5
Number of pancreases discarded	1	11	4	3	4	0
Number of successful preservation- islet isolations	14	47	16	13	13	5
Isolates which underwent density gradient separation	**12**	**39**	**12**	**11**	**11**	**5**

SCS: Static cold storage, HMP: Hypothermic machine perfusion, PVOP: Portal venous oxygen persufflation,
TLM: Two layer method

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4.3.2 Quality of pancreas distension

Distension with intra-ductal collagenase injection was good and uniform in the control pancreases. The quality of pancreas distension was poor in non-heart-beating-donor pancreases preserved by static cold storage. It was non-uniform with small parts in the tail remaining un-distended. Resistance to intra-ductal injection was high and it was difficult to inject the entire 10ml of collagenase solution into the pancreas. The quality of distension was better in the machine perfused and oxygen persufflated pancreases with lower resistance to collagenase injection. 10 ml of the collagenase solution could be injected without difficulty.

4.3.3 Comparison of islet yield parameters in control group pancreases and non-heart-beating-donor group pancreases

Pancreases which underwent islet isolation immediately without any warm ischaemia yielded more islets. Control group islets also had improved viability and higher islet isolation index as compared to non-heart-beating-donor pancreases (Table 5).

Table 5 Comparison of islet parameters between islets isolated from control group and all non-heart-beating-donor group rat pancreases

Islet assessment parameter	Control group pancreas (n=12)		Non-heart-beating-donor pancreas (n=39)		Mann Whitney U test statistic	p value
	Median	IQR	Median	IQR		
Crude islet count	1200.0	830.0	920.0	445.0	213.0	0.047
Crude islet equivalents	2360.7	949.0	1708.0	746.0	231.0	0.093
Pure islet count	400.0	275.0	200.0	130.0	197.5	0.024
Pure islet equivalents	1042.5	639.0	481.7	411.0	167.0	0.005
Islet isolation index	2.8	2.3	2.4	1.0	107.0	0.012
Islet viability (%)	89.7	8.9	76.0	10.3	47.0	0.0001

IQR: Inter quartile range

4.3.4 Comparison of islet yield parameters within the non-heart-beating-donor groups

Islet count and islet equivalents

Post-purification data was available for 39 non-heart-beating-donor pancreas isolates. Static cold storage group had the lowest crude islet count and islet equivalents. Two layer method and Portal venous oxygen persufflation groups had better islet yields There was no significant difference in islet count or IEQ between the four groups (ANOVA p=0.863 and 0.403). Portal venous oxygen persufflation group had the highest purified islet count and equivalents. Differences in purified islet equivalents was statistically significant (ANOVA, p=0.018) though there was no statistical difference in purified islet counts (ANOVA, p=0.121) (Figures 33,34)

Islet isolation index & islet morphology

The islet isolation index was lowest for static cold storage group. The difference in the index between the four preservation groups was not statistically significant (p=0.080). Within the non-heart-beating-donor preservation groups islet morphology showed prominent differences. Islets isolated from static cold storage group were small, irregular and fragmented. This was much improved in the portal venous oxygen persufflation group and the two-layer method groups where the islets were more rounded and larger (figures 35.37).

Islet viability

Islet viability based on trypan blue exclusion staining and propidium iodide staining gave comparable results with good correlation (Pearson's correlation coefficient= 0.757) Portal venous oxygen persufflation and two-layer method

group had improved viability as compared to machine perfusion and static cold storage. The difference was not statistically significant (ANOVA, p=0.151) (Figure 36).
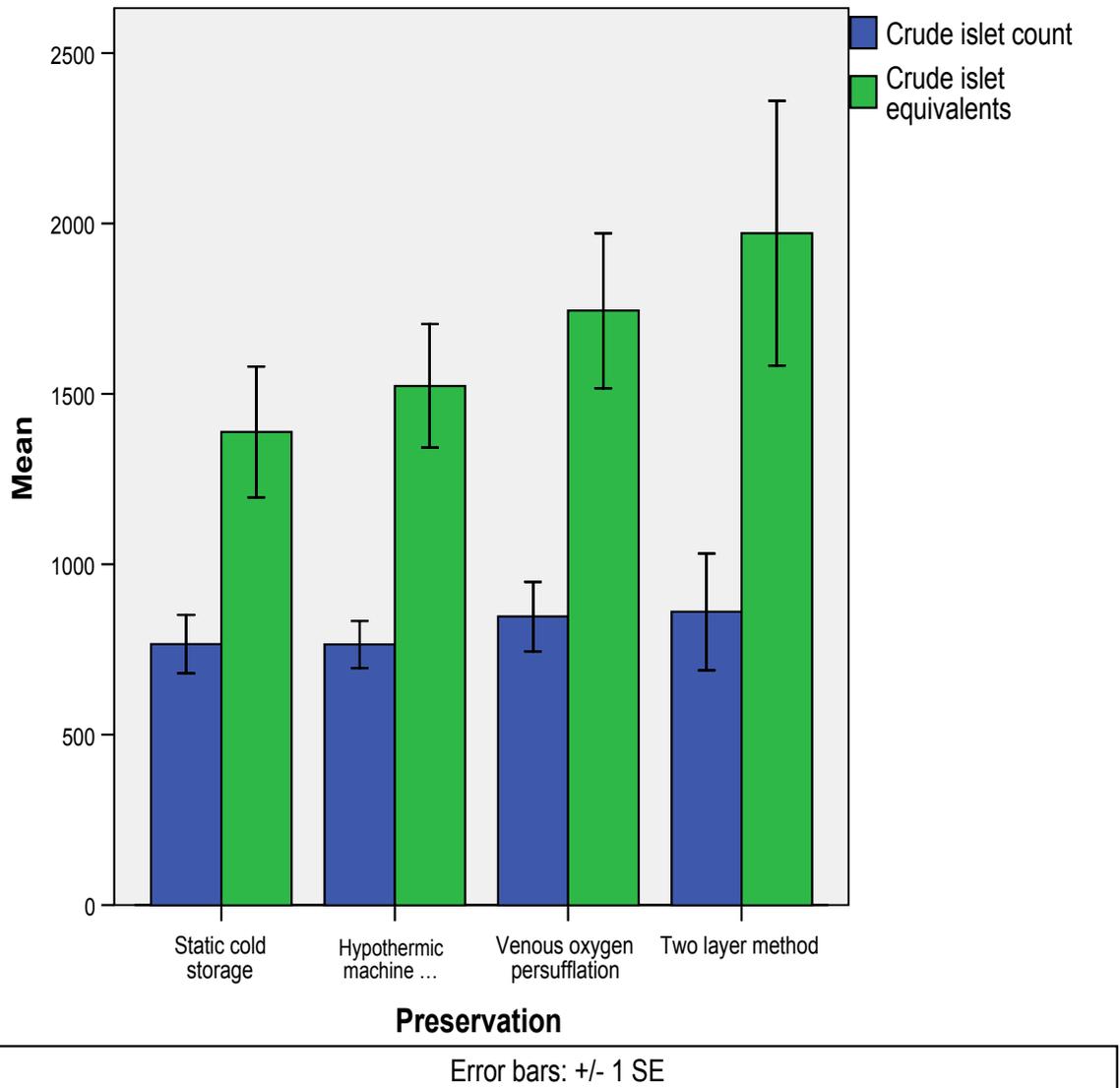
Figure 33 Bar graph comparing crude islet count & islet equivalents within the four non-heart-beating-preservation groups.

Crude (pre-density gradient purification) islet count and islet equivalent yield recovered per non-heart-beating-donor rat pancreas preserved by the static cold storage (n=16), hypothermic machine perfusion (n=13), portal venous oxygen persufflation (n=13) or two layer method (n=5) were compared. Bars represent mean while the error bar represents one standard error of mean. The differences in both crude islet count and islet equivalents were not statistically significant (ANOVA).
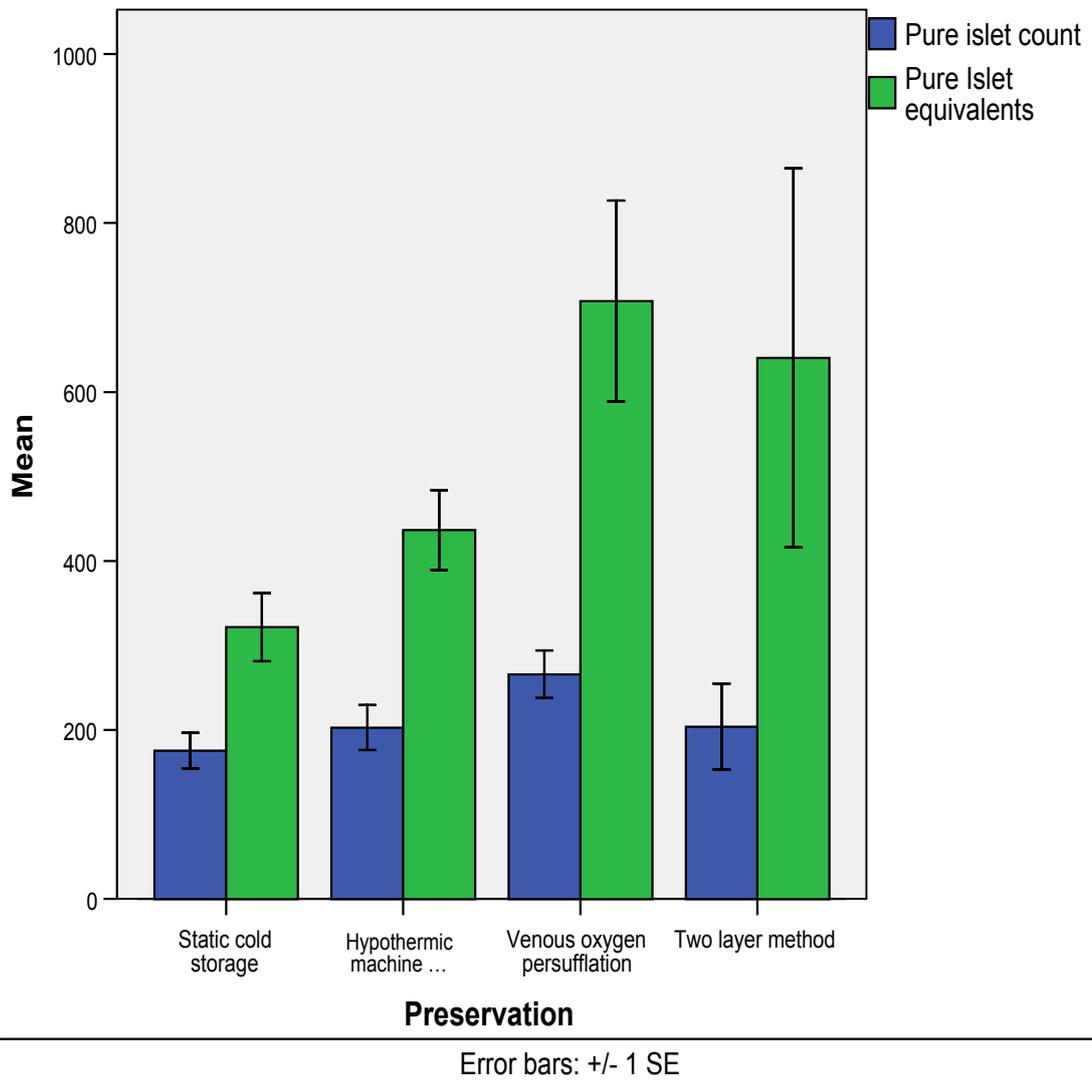
Figure 34 Bar graph comparing the purified islet count and islet equivalents within the four non-heart-beating-donor pancreas preservation groups.

Post-density gradient purification islet count and islet equivalent yield recovered per non-heart-beating-donor rat pancreas preserved by the static cold storage (n=12), hypothermic machine perfusion (n=11), portal venous oxygen persufflation (n=11) or two layer method (n=5) were compared. Bars represent mean while the error bar represents one standard error of mean. The differences in purified islet equivalents was statistically significant (ANOVA; p=0.018).
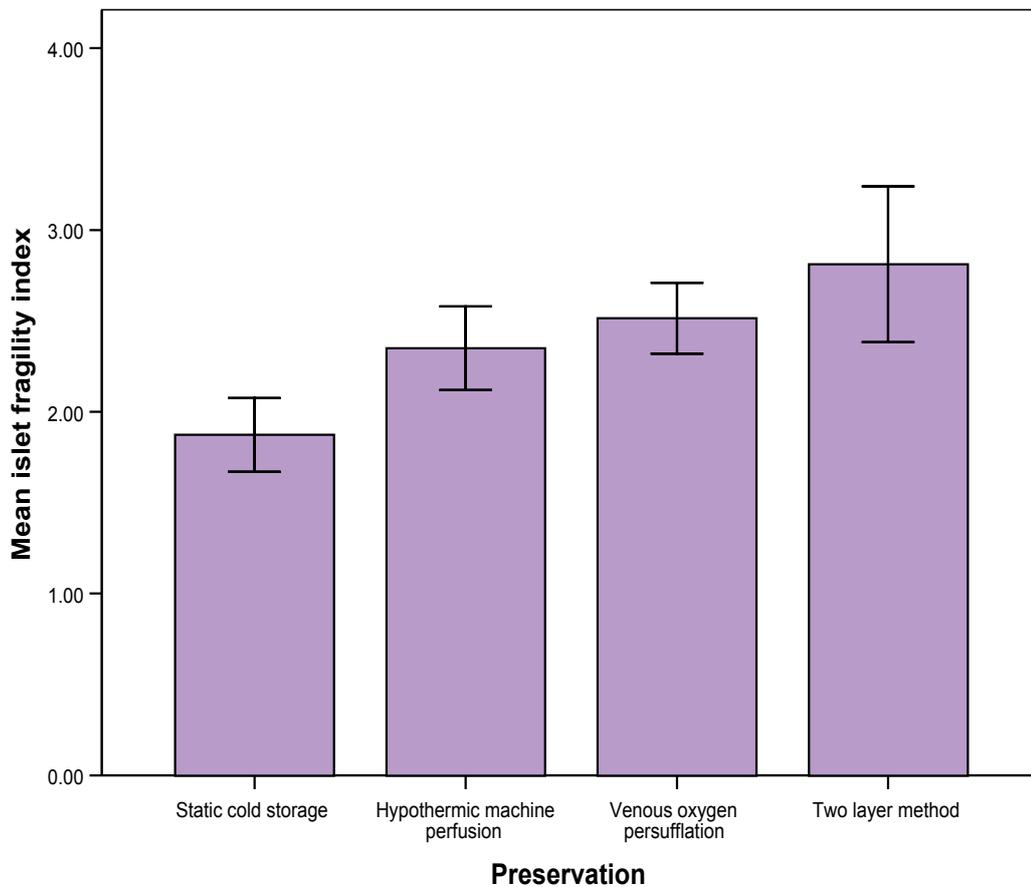
Figure 35 Bar graph comparing the isolation index of islets within the four non-heart-beating-donor rat pancreas groups.

Islet isolation index for islets recovered from non-heart-beating-donor rat pancreas preserved by the static cold storage (n=12), hypothermic machine perfusion (n=11), portal venous oxygen persufflation (n=11) or two layer method (n=5) were compared. Bars represent mean while the error bar represents one standard error of mean. The isolation index is a marker of islet fragmentation during the isolation process. Within the four groups static cold storage had the lowest isolation index. The differences in isolation indices between the four groups was not statistically significant (ANOVA; p=0.080).
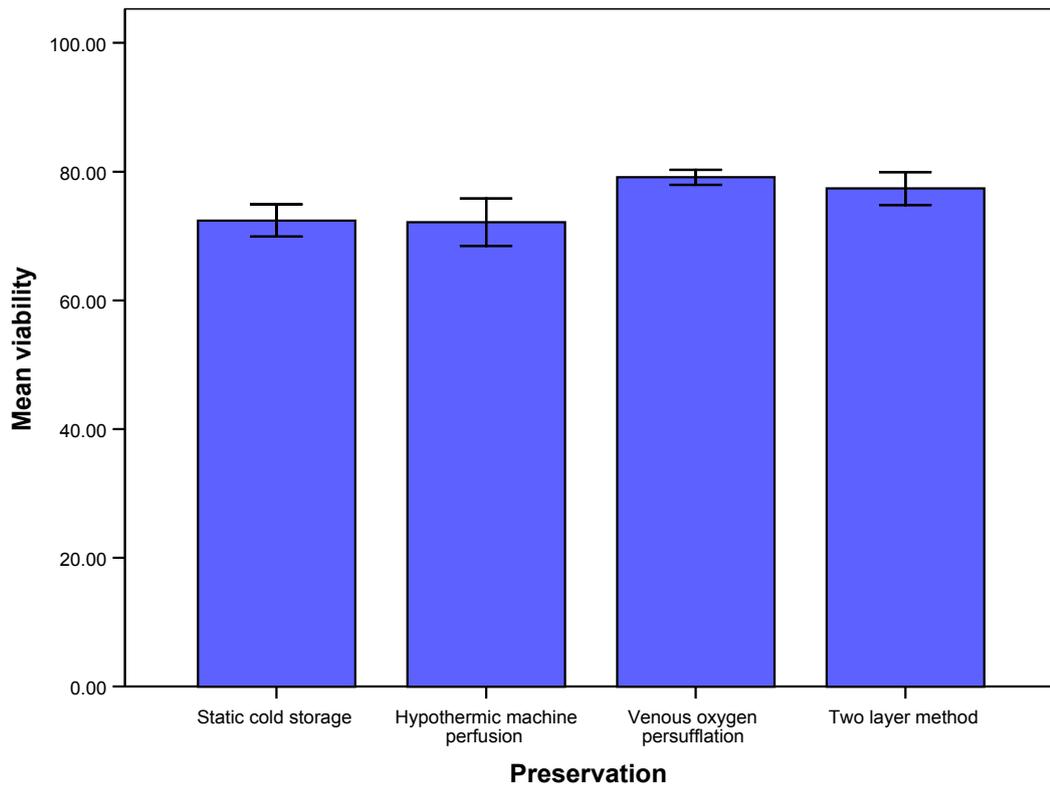
Figure 36 Bar graph comparing the viability of islets retrieved from four non-heart-beating-donor rat pancreases preservation groups.

Viability of islets recovered from non-heart-beating-donor rat pancreas preserved by the static cold storage (n=12), hypothermic machine perfusion (n=11), portal venous oxygen persufflation (n=11) or two layer method (n=5) was compared. Bars represent mean while the error bar represents one standard error of mean. The viability represented in the graph as a percentage was the average of the viabilities measured by trypan blue staining and propidium iodide staining of two separate aliquots of islets from each isolate. Within the four groups static cold storage and hypothermic machine perfusion had the lowest viability. The differences in viabilities between the four preservation groups was not statistically significant (ANOVA; p=0.151).
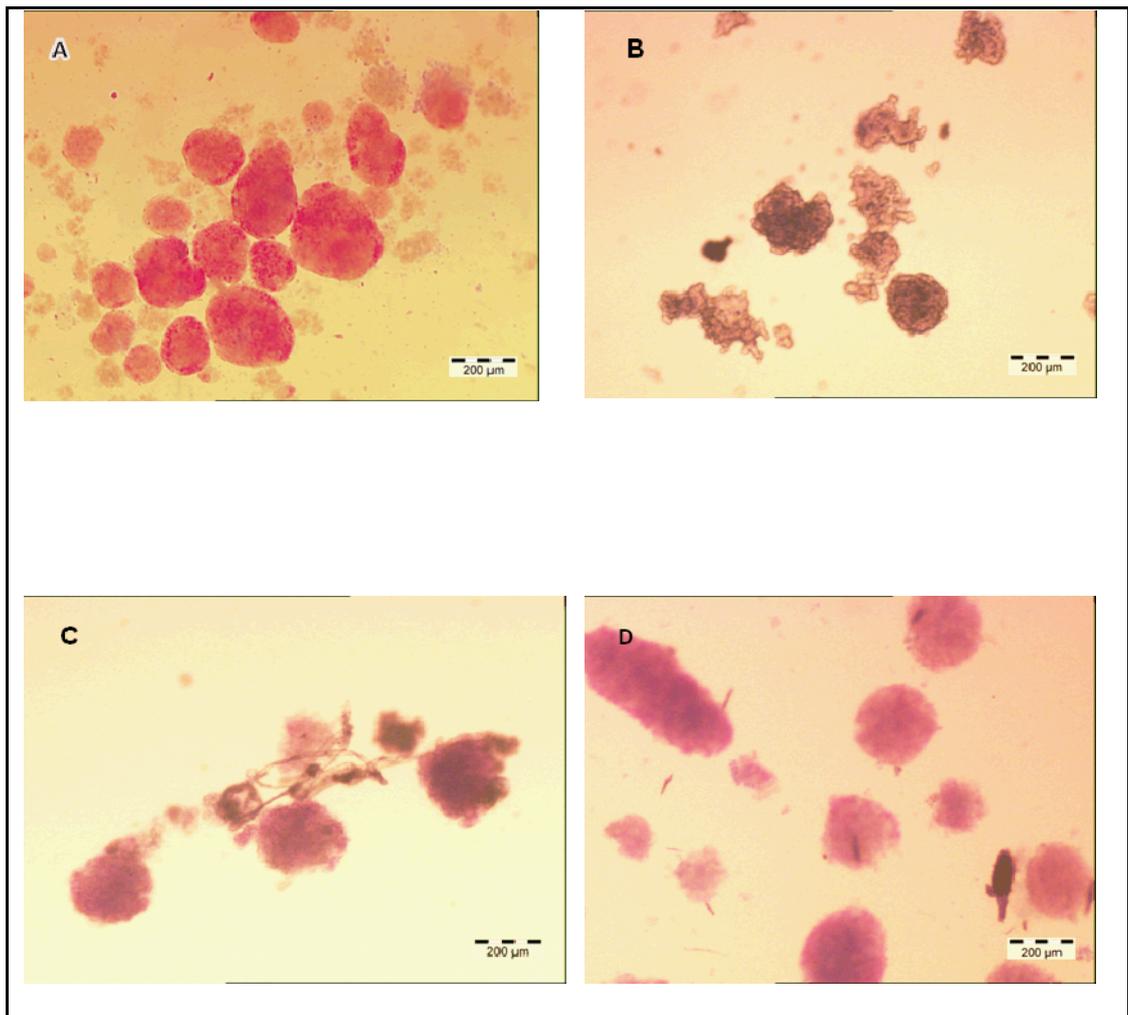
Figure 37 Morphology of islets retrieved from positive control and non-heart-beating-donor pancreases

The islets were double stained with dithizone and trypan blue. Islets isolated from positive control pancreas (A) are globular with smooth margins and minimal staining with trypan blue. Islets from static cold storage pancreas (B) are small, irregular and frequently fragmented. They also have increased staining with trypan blue suggestive of poor viability. Islets isolated from hypothermic machine perfusion (C) and portal venous oxygen persufflation (D) are less fragmented as compared to static cold storage group. The margins are not smooth and there is evidence of trypan blue staining of the larger islets. This is more prominent in the islets from pancreases preserved by hypothermic machine perfusion (C).

4.3.5 Comparison of *in vitro* function by static glucose stimulated insulin secretion test

The mean stimulation index for the control group pancreases was 2.99. This was significantly higher than the stimulation indices for the non-heart-beating-donor pancreas groups irrespective of the type of preservation (ANOVA, p=0.035). Within the non-heart-beating-donor preservation groups, static cold storage group had the lowest mean stimulation index. There was no significant difference in the stimulation indices of the three non-heart-beating-donor preservation groups (ANOVA, p=0.311). The percentage of isolates from each preservation group which had a stimulation index greater than 1.0 was evaluated. Static cold storage group had the lowest percentage of islet isolates with stimulation index lesser than 1.0 (Figures 38,39).
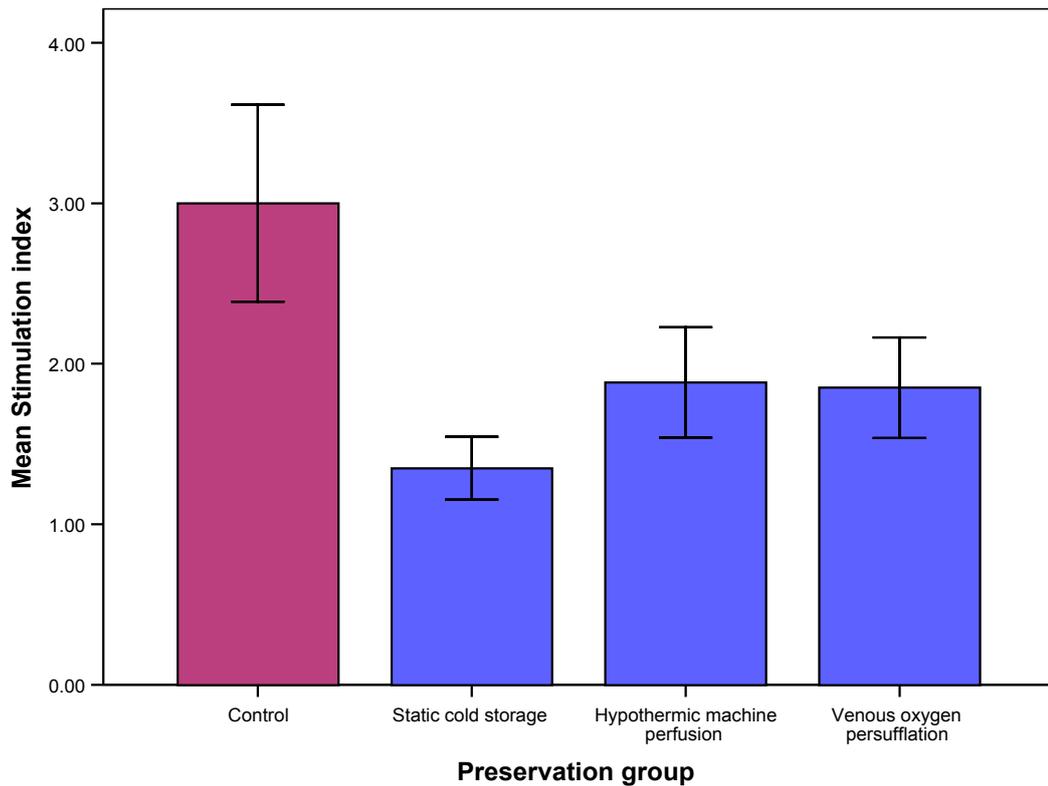
Figure 38 Bar chart comparing the stimulation indices of islets isolated from positive control and non-heart-beating-donor rat pancreases

The bars represent the mean stimulation index for the control/ preservation group. Error bar represents one standard error of mean. The stimulation index of the positive control group pancreases was significantly higher than the non-heart-beating-donor pancreases (ANOVA, p=0.035). Within the three preservation groups static cold storage had the lowest stimulation index. The stimulation indices of hypothermic machine perfusion and portal venous oxygen persufflation were similar. The difference in stimulation indices in the three preservation groups were not statically significant (ANOVA, p=0.311)
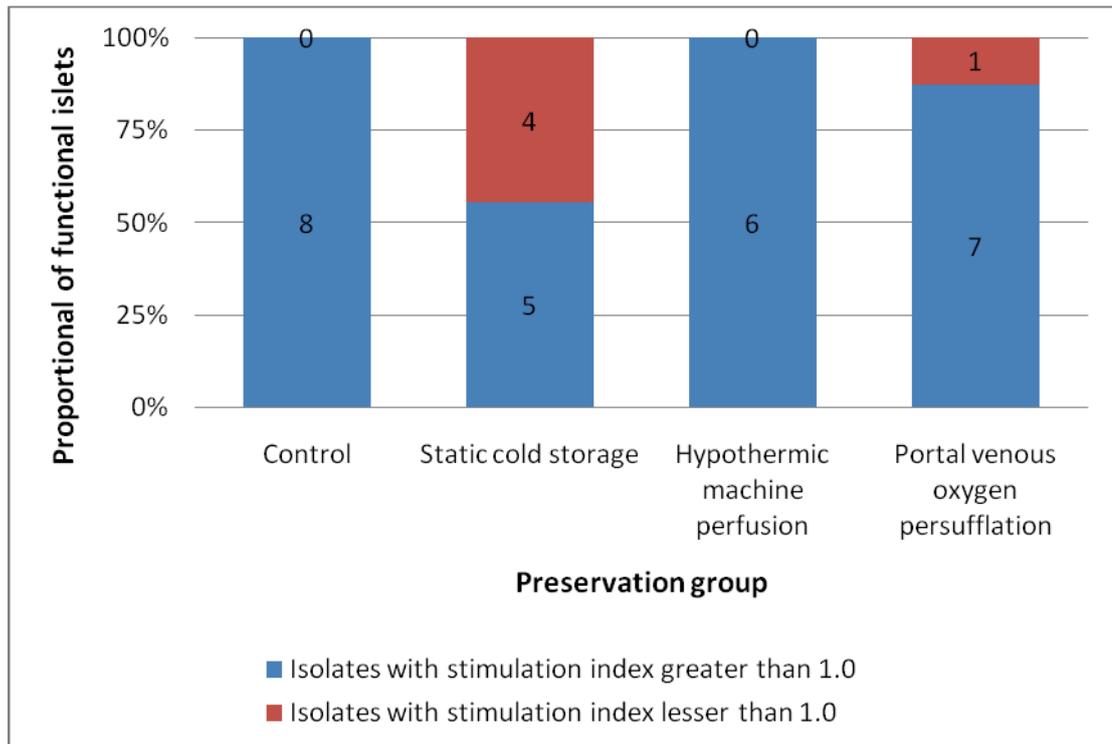
Figure 39 Bar chart comparing the percentage of islet isolates with confirmed *in vitro* function in various preservation groups

Islet isolates with a mean stimulation index greater than one by static glucose stimulated insulin secretion test were considered to have confirmed in vitro function. The graph depicts the comparative percentage of isolates from the positive control and non-heart-beating-donor pancreas preservation groups that have a stimulation index greater than 1. The actual number of isolates assessed for each group is shown in the graph. Islets from positive control group and hypothermic machine perfusion group were 100% functional. 88% of islets isolates from portal venous oxygen persufflation group were functional. Only 55% of islet isolates from static cold storage were functional. The differences were not statistically significant

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4.3.6 Perfusion parameters during hypothermic machine perfusion and their correlation with islet yield

Eleven non-heart-beating-donor rat pancreases underwent hypothermic machine perfusion followed by islet isolation and assessment. The median duration of perfusion was 252 min (Inter-quartile range, 240 -285 min). The mean starting pressure was 16.2 mmHg (13 mmHg-18 mmHg). The perfusion pressure decreased during the perfusion period in 7 pancreases (63.6%). Averaged perfusion pressure during the entire perfusion period was 14.3mmHg. The pre-preservation weight of the pancreas (including duodenum and spleen) was 8.5g (8.0-9.3). All pancreases increased in weight at the end of machine perfusion. The median percentage weight gain at the end of the perfusion period was 14.3% (7.8-17.9%).

Relation between pancreas perfusion parameters and islet yield

There was significant positive correlation between the initial perfusion pressure and crude islet count (p=0.028). There was no correlation between the perfusion pressure and purified islet yield. There was a significant negative correlation between the averaged perfusion pressure and the viability of isolated islets (p=0.026). Pancreases with perfusion pressure greater than 15mmHg had decreased viability (78% vs. 60%, p=0.012).

4.3.7 Persufflation parameters during portal venous oxygen persufflation of non-heart-beating-donor pancreases

Eleven pancreases underwent portal venous oxygen persufflation for a median of 250 minutes (240-300 minutes). There was no consistent change in the weight of the pancreas following persufflation (median weight loss of

0.3%). The initial persufflation pressure was 15.2mmHg (7.3-16.8mmHg). Averaged persufflation pressure during the entire persufflation period was 12.3mmHg. A decrease in persufflation pressure was noted in all pancreases. There was no correlation between persufflation parameters and islet isolation variables.

4.4 Discussion

The yield and *in vitro* function of islets from pancreas damaged by 35 minutes of warm ischaemia was significantly worse than control group pancreases. Within the non-heart-beating-donor preservation groups, the purified islet equivalent yield was highest in pancreases preserved by venous oxygen persufflation and was similar to the yield from two layer method. This was significantly higher than the purified IEQ from static cold storage. Islets from persufflation group also had higher viability, lesser fragmentation and better morphology when compared to static cold storage and machine perfusion groups. The difference in these parameters was not statistically significant.

The technique of islet isolation and purification itself can influence islet numbers and viability as damaged islets are more likely to be lost during islet isolation. The islet yield obtained from damaged pancreases may hence exclude the poor quality islets lost in the process. This can explain the significant differences in islet numbers between various preservation groups but broadly insignificant differences in other islet parameters.

The *in vitro* function test did not show any significant difference in the stimulation indices of the three preservation groups. There was an however a tendency for islets isolated from static cold storage pancreases to have stimulation index less than 1.0.

The decrease in islet yield after significant duration of warm ischaemia is well described in the literature[46]. Static cold storage had the worst islet parameters amongst all the preservation techniques studied. This demonstrates the potential room for improvement that exists in non-heart-beating-donor pancreas preservation. Improved distension of the pancreas with intraductal collagenase after preservation with portal venous oxygen persufflation and two layer method was seen. This could partly explain improved islet yield from each pancreas because a greater portion of the pancreas is exposed to collagenase and is available for digestion. Improvement in islet yield with these methods could also be due to the improved oxygenation during the cold ischaemia period. Both methods would be expected to act in similar ways i.e. by improving the energy status of the grafts and providing an opportunity for tissue repair. The evidence of preserved islet architecture seen with these preservation techniques also suggest improved quality of the isolated islets.

There was no significant difference in the stimulation indices for the three preservation groups. It is possible that the significant warm ischaemic damage has lead to a period of islet dysfunction that could improve over a period of time. This is very common in clinical practice and delayed graft function is a universal occurrence in non-heart-beating-donor kidney transplantation. Hence glucose stimulated insulin secretion after overnight

incubation may not be a reliable test to compare function in non-heart-beating-donor islets. Another explanation is that the assay as being performed is not sensitive enough to detect small difference in function between the groups. Techniques such as islet perifusion to confirm a biphasic response[220] and islet oxygen consumption rate[221] are more sensitive methods to assess islet response to glycaemic stress.

Analysis of perfusion pressure data in the hypothermic machine perfusion group showed that higher perfusion pressures correlated with poorer viability of islets. The machine perfusion circuit used in our experiment was volume controlled. High perfusion pressure indicates increased intravascular resistance and could be a marker of severe micro-vascular injury. A pilot study to investigate the feasibility of hypothermic machine perfusion of porcine pancreases was carried out (Appendix 1). It was found that the perfusion parameters correlated with the levels of activated caspase 3 levels in the pancreatic tissues. This once again suggests that organs with high intravascular resistance are pre-damaged. It corroborates the data from the rat study suggesting a possible role of this preservation technique in identifying grafts that are not suitable for islet isolation.

The portal vein was used as the inflow for oxygen persufflation in the experiments. This enables oxygen to reach the entire vascular network in the pancreas, spleen and the duodenum. Small punctures on the surface of the spleen were made to decompress the system and allow for the oxygen to escape. There is a theoretical risk of oxygen bypassing the venules of the pancreas and duodenum by directly escaping through the spleen. However because the punctures are very superficial, there would still be a pressure

gradient within the vascular system that would improve diffusion of oxygen to the pancreas. While persufflation studies of liver and kidneys have involved decompression through punctures directly on the surface of the preserved organ we avoided it in the pancreas for risk of creating leaks into the pancreatic duct system which might compromise ductal collagenase injection.

The objective of this phase of the study was to investigate whether islet yield from non-heart-beating-donor rat pancreas could be improved by alternate means of preservation. We have shown that portal venous oxygen persufflation improves islet yield in terms of islet numbers and equivalents. Islets from this preservation group also had improved viability, morphology, decreased fragmentation and improved *in vitro* function as compared to static cold storage. Both portal venous oxygen persufflation and two layer method improve tissue oxygenation. The advantage of portal venous oxygen persufflation is that it does not depend entirely on the ability of oxygen to diffuse into the pancreas. Studies have shown that two-layer method oxygenates only the outer part of the pancreas leaving the core parenchyma ischaemic[156]. While this may not be important in the rat pancreas which is very thin, it would be particularly relevant for large animal and human pancreases. Hypothermic machine perfusion also improved islet parameters as compared to static cold storage in our study. The study did not identify significant differences between these two groups, but there was a trend for improved preservation with persufflation. In view of these results we feel that portal venous oxygen persufflation has greater potential for clinical application and have chosen this technique for further investigation.

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Chapter 5 Comparison of the severity of reperfusion injury using *in vitro* warm oxygenated reperfusion of non-heart-beating-donor rat pancreas

5.1 Background

Phase I of the study (Chapter 4) showed that portal venous oxygen persufflation provided the best chance of improved pancreas preservation and islet yield. It resulted in a significant increase in purified islet equivalent yield. Islets from pancreases preserved by portal venous oxygen persufflation also had better viability, morphology and *in vitro* functionality as compared to static cold storage.

The mode of action of portal venous oxygen persufflation has been investigated in the area of liver preservation. Reports have suggested that portal venous oxygen persufflation leads to increased cellular ATP levels[186, 222] and decreases the extent of apoptosis in the graft[185]. Pancreas preservation is complicated because the exocrine component of the pancreas produces multiple lytic enzymes. Warm and cold ischaemia and subsequent reperfusion can lead to extravasation of these enzymes[223]. The extravasated enzymes can cause breakdown of cellular components and exacerbate tissue injury. This is one of the causes of post transplant graft pancreatitis in whole organ pancreas transplantation. Reperfusion injury is also relevant in islet transplantation. Increased levels of amylase in preservation fluid around a pancreas graft has been reported to correlate with low islet yield and poor *in vitro* islet function[224]. It can also occur during the

digestion of pancreas, islet purification and after transplantation. Reperfusion injury is an important component of the IBMIR which is responsible for the loss of transplanted islets that occurs within hours of islet transplantation. Measures to reduce the severity of reperfusion injury in the non-heart-beating-donor pancreas can improve outcomes after both islet and whole organ pancreas transplantation. This phase of the study was aimed at evaluating the effect of portal venous oxygen persufflation on the severity of reperfusion injury in the non-heart-beating-donor rat pancreas compared to static cold storage.

5.2 Methods

5.2.1 Overview of the study

The plan of the study is depicted in the flow chart (Figure 40). The study included two experimental arms and one control arm. Male Wistar rats (350-400g) were used for the study. The retrieval of the pancreas and the techniques of pancreas preservation have been described previously (Sections 3.1 & 2.3.2). In the experimental arm, 14 non-heart-beating-donor rat pancreases were preserved by static cold storage (n=6) or portal venous oxygen persufflation (n=8). The duration of warm ischaemia was 35 minutes. The duration of cold preservation was extended to 16 hours in an attempt to induce a severe ischaemic injury. The pancreases then underwent oxygenated warm reperfusion for 60 minutes. In the control arm, four animals were killed by cervical dislocation and underwent an immediate laparotomy. They were rapidly exsanguinated and pancreatic tissue was collected. Tissue was processed for caspase 3 activity, TBARS assay and RNA extraction.

Figure 40 Flow chart depicting the plan of study for Phase 2 (opposite page)

Comparison of the severity of reperfusion injury after oxygenated warm reperfusion of non-heart-beating-donor pancreases preserved with static cold storage and portal venous oxygen persufflation. Rat pancreases retrieved after 35 minutes of warm ischaemia were preserved by either static cold storage or portal venous oxygen persufflation for 16 hours. After cold preservation they were re-warmed and subjected to 60 minutes of oxygenated warm reperfusion using Krebs-Henseleit buffer through the aortic cannula at 37°C. Portal venous effluent was collected at timed intervals from the portal vein catheter. These samples were analyzed for exocrine enzymes (amylase, lipase), glycerol, lactate, pyruvate and nitrates. After completion of reperfusion, biopsies of the pancreas were collected and processed for estimation of malondialdehyde levels (lipid peroxidation), activated caspase 3 levels (apoptosis). RNA was extracted immediately from pancreas biopsies and stored at -80°C for microarray analysis. A control arm (not shown) was also included. Here pancreas biopsies were taken from freshly killed rats and were immediately processed for malondialdehyde and activated caspase 3 level estimation and for RNA extraction.
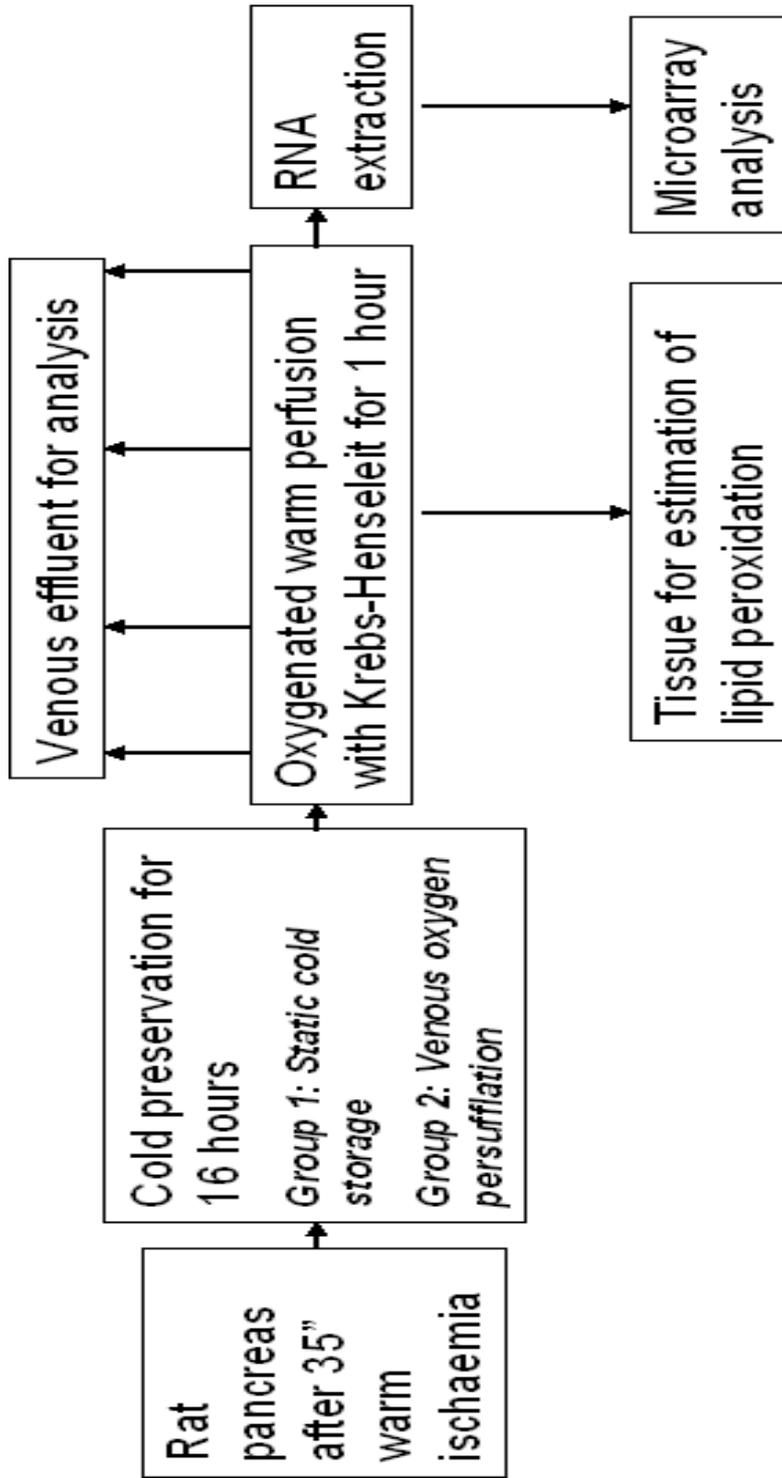
5.2.2 *In vitro* warm oxygenated reperfusion of rat pancreas

Preparation of the perfusion medium

The perfusion fluid used for the reperfusion experiments was Krebs-Henseleit solution. 500ml of the solution was made fresh for each experiment using the commercially available powder and sterile de-ionised water (section 2.3.1). The pH of the solution was adjusted to 7.4±0.1. The fluid was warmed to 37°C in a water bath and maintained at that temperature. The mouth of the sterile beaker containing the solution was covered with aluminium foil. The solution was oxygenated with a mixture of oxygen and carbon dioxide (95% and 5% respectively) bubbled into the perfusion fluid for at least 15 minutes before the start of reperfusion and continued throughout the reperfusion period. This ensured that the partial pressure of dissolved oxygen in the solution was at least 450-500mmHg. The pH of the solution was monitored and maintained at 7.4±0.1 by addition of small quantities of 0.1N Hydrochloric acid or 0.1N Sodium hydroxide.

Procedure of warm oxygenated reperfusion

After 16 hours of static cold storage or venous oxygen persufflation, the pancreas was allowed to re-warm to room temperature in UW solution over 20 minutes. This is to simulate the gradual re-warming of the organ during implantation[225]. It was then placed in the perfusion chamber containing approximately 50ml of warm oxygenated Krebs-Henseleit solution. The aortic cannula was connected to the reperfusion circuit. A bubble trap was used in the inflow to prevent air bubbles from entering the pancreas. Re-perfusion was started at the slowest rate (3 cycles per minute) and gradually increased

over the next 4-5 minutes to achieve an initial perfusion pressure of 60±2 mmHg. The perfusion pressure of 60mmHg was chosen because it is closer to the physiological blood pressure (80-90mmHg) and can thus ensure uniform perfusion and oxygenation of the tissues. This flow rate was then fixed for the entire re-perfusion duration. The perfusion pressure was continuously monitored and recorded as a surrogate measure for the vascular resistance during the reperfusion period. The pancreas was re-perfused for a maximum duration of 60 minutes. Venous effluent was collected directly from the portal vein catheter in labelled cryovials (1ml each) at pre-determined time points (5, 20, 40 and 60 minutes) and stored at -20°C for later analysis. The remaining venous effluent drained into the perfusion chamber. This was transferred intermittently using a clean 20 ml syringe back into the main reservoir.

At the end of warm reperfusion, pancreas was biopsied with scissors. One biopsy was held with a forceps and kept immersed in a flask containing liquid nitrogen until the bubbling had stopped. The biopsy was then transferred into a labelled cryovial and stored at -80°C. Another biopsy was immediately processed for RNA extraction.

5.2.3 Measurement of amylase and lipase levels

Amylase and lipase are enzymes produced in the pancreatic acini. They are only released into the blood circulation by injured exocrine tissue. Levels of these enzymes in the portal venous effluent give a measure of tissue injury during reperfusion. Effluent samples were collected at specific time periods and stored at -20°C.They were analysed for amylase and lipase levels using

a commercially available radioimmunoassay kit. These assays were kindly carried out by Dr Peaston, Department of Biochemistry, Freeman Hospital, Newcastle upon Tyne.

5.2.4 Measurement of fluid glycerol, lactate and pyruvate levels

Portal venous effluent samples collected at timed intervals during the reperfusion period were stored at -20°C until analysis. These were batched analysed using the CMA600 Micro dialysis analyser according to manufacturer's instructions. All three assays were based on the ultimate production of the red-violet coloured quinoneimine. The rate of formation of this product was measured photometrically at 530 nm and was proportional to the substrate concentration. Detailed reactions involved in these assays are included in appendix 2 of this thesis.

5.2.4 Estimation of fluid Nitrite levels

Estimation of nitrite levels in the fluid was used as a marker of nitric oxide production during reperfusion. Griess reagent kit for Nitrite determination was used with minor modifications to manufacturer's instructions. Briefly, Griess reagent was freshly prepared according to manufacturer's instructions. In a microtitre plate, 20 µl of Griess reagent was added to wells containing 280 µl of the test fluid samples in duplicate. The plate was incubated at room temperature for 30 minutes. The plate was then read at 548nm in a spectrophotometric plate reader. A calibration curve was prepared by using sodium nitrite solutions at concentrations ranging from 1-100µM. Nitrite

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concentrations in the test samples were then calculated using the standard calibration curve.

5.2.5 Preparation of tissue extracts

Lytic enzymes (trypsin, chymotrypsin, DNA and RNA nucleases) released from the pancreas can breakdown cellular proteins, enzymes and nucleic acids. The extent of proteolysis can be limited by storing the specimens at -80°C, homogenisation on ice and addition of protease inhibitors to the homogenisation buffer. RIPA buffer with PMSF was used for homogenisation of the pancreatic tissues. The rationale and actual technique of homogenisation has been described in the method development chapter (section 3.5.4).

5.2.6 Estimation of thio-barbituric acid reactive substances (TBARS) in tissue extracts

The protocol used was modified from the original protocol by Okhawa and co-workers in 1979 [226] (available at http://www.mccullylab.org.tbarsassay , retrieved on 25 June 2009).

Malondialdehyde (MDA) standards were prepared by diluting pure 1,1,3,3 Tetra-methoxy propane (6M) with 1.15% potassium chloride solution. Initially a 100 mM stock solution of malondialdehyde was prepared. The stock solution was further diluted to make a 100 µM solution. This was used to make 50µM,20µM,10µM, 7.5µM, 5µM, 4µM, 2µM, 1µM and 0.5µM concentrations using 1.15% potassium chloride solution.

For the assay, 500µl of standards (0.5µM to 50µM) in duplicate were added to labelled 15ml centrifuge tubes. 500µl aliquots of tissue extract were also

added in duplicates to labelled tubes. 0.2 ml of 8.1% sodium docyl sulphate and 1.5ml of 20% acetic acid were added in sequence to all samples and standards with vigorous mixing after each step. The pH was adjusted to 3.5 with 10 M sodium hydroxide. 1.5 ml of 0.8% aqueous thio-barbituric acid was added to the mixture. The volume was made up to 4 ml by adding 250 µl of distilled water. The tubes were capped and placed in standing position in a boiling water bath for 60 minutes. The tubes were then cooled in a cold water bath. To each tube 3 ml of N-butanol/pyridine (15:1 volume by volume) was added. The tubes were shaken to mix well and then centrifuged at 800g for 10 minutes. The upper organic layer from each tube was carefully removed and transferred to numbered glass cuvettes. The absorbance of the supernatant was read at 532 nm in a spectrophotometer. Thio-barbituric acid reactive substances (TBARS in µM) in the test samples were calculated using the following formula.

$$TBARS = At/As \text{ x } Cs \text{ x } 14 \textit{ where,}$$
At was absorbance of test sample
As was absorbance of the standard closest to the range of results
Cs was the MDA concentration with absorbance of As
14 was for the dilution used (when 500 uL of test tissue extract was assayed in a final total volume of 7 mL)

The protein content in the tissue extracts was assayed using the Quant-iT protein assay kit according to manufacturer's instructions. The extent of lipid peroxidation was reported as µmoles of TBARS per mg protein.

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5.2.7 Microarray analysis

RNA was extracted from pancreatic biopsies taken at the completion of warm reperfusion. The technique of RNA extraction is described in chapter 3. Analysis of microarray data is described in chapter 6. The expression of genes associated with apoptosis, oxidative stress and anti-oxidant mechanisms are presented here.

5.2.8 Sample size estimation

Sample size calculations were not carried out for this phase of the study as it was primarily exploratory.

5.3 Results

Persufflation pressure data was not recorded in one pancreas due to technical problems. In the remaining seven pancreases, the mean starting persufflation pressure was 14.8 mmHg. The general trend was a decrease in the persufflation pressure over the duration of preservation. The average persufflation pressure decreased by 25% in the first 3 hours of persufflation.

5.3.1 Reperfusion parameters

The mean initial flow rate was 50cycles/minute (4.5 ml/min) in both preservation groups. This resulted in an initial perfusion pressure of 60.5mmHg for the cold storage group and 59.6mmHg for the oxygen persufflation group. The pressure gradually decreased to 50.1mmHg and 46.6mmHg respectively after one hour of re-perfusion. The pressures were not significantly different between the two study groups (Figure 41).

Re-perfusion for 60 minutes was completed in 12 pancreases. Re-perfusion was stopped in two cases at 45 and 52 minutes respectively due to leakage of duodenal contents in one pancreas and tipping over of the perfusion chamber in another. In both cases the pancreas was immediately biopsied and tissue processed for analysis.

In three cases (all preserved with portal venous oxygen persufflation) visible duodenal peristalsis was documented during reperfusion. This started at 15, 20 & 15 minutes after the start of reperfusion and continued throughout the reperfusion period. The increased intra-duodenal pressure due to peristalsis in one pancreas led to release of duodenal contents (video clips in attached

CD). There was no visible peristalsis seen in any pancreases from the static cold storage group.
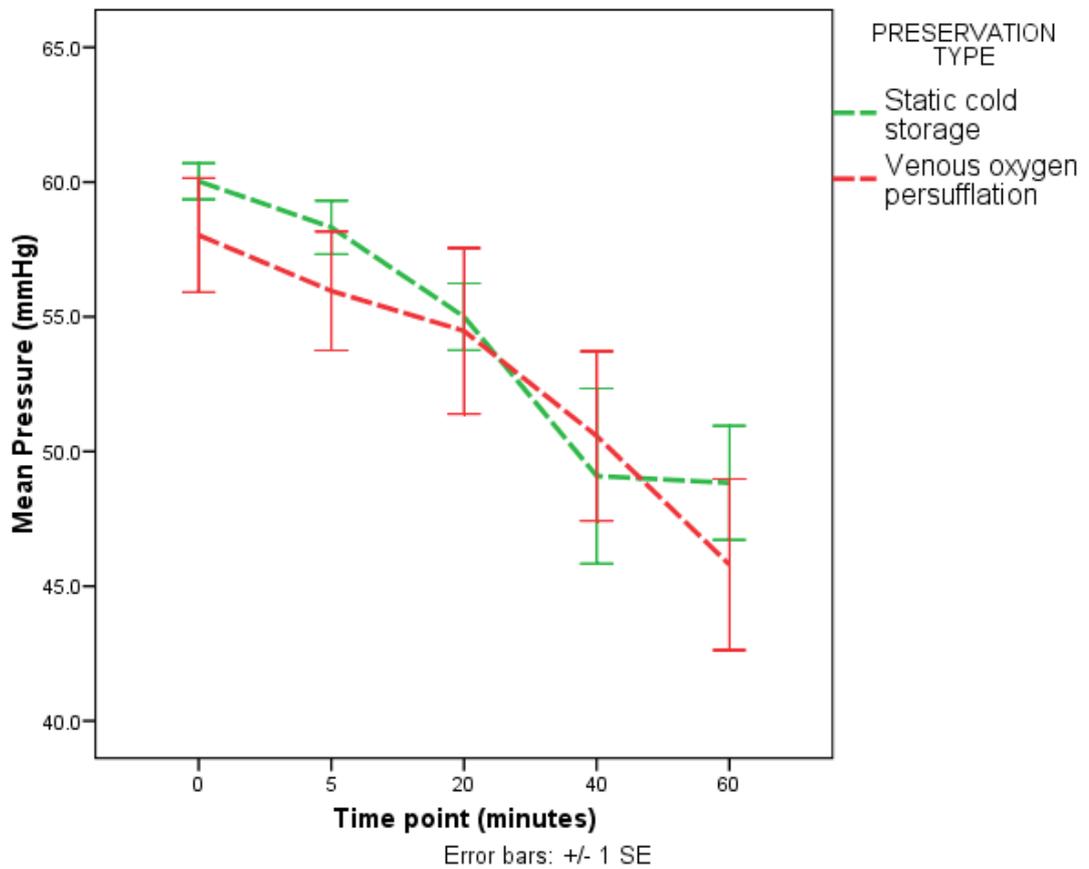
Figure 41 Mean perfusion pressures in the two preservation groups during warm re-perfusion

Line chart showing the comparative perfusion pressures during the reperfusion period. The truncated Y axis shows the perfusion pressure in mm of mercury. The X axis shows the time points during the reperfusion. The data points indicate the mean pressure in the preservation group at each time point. The error bars represent one standard error of mean. There was no significant difference in the perfusion pressures between the two study groups.

5.3.2 Analysis of portal venous effluent

There was a steady increase in the concentrations of amylase and lipase in the portal venous effluent over the duration of reperfusion. The increase was not uniform throughout the preservation period. It was mainly restricted to the time interval between 20 - 40 minutes for amylase and between 40-60 minutes for lipase. This trend was similar in both preservation groups. There was no significant difference in the enzyme concentrations in pancreases preserved by static cold storage or venous oxygen persufflation at any of the time points (Figures 42,43).

There was a serial increase in the glycerol levels in the effluent during warm reperfusion. The glycerol levels in the portal venous oxygen persufflation group were higher than the static cold storage group though the difference was not statistically significant (Figure 44). The lactate:pyruvate ratio was higher in the venous oxygen persufflation group after 5 minutes but the difference was not statistically different (Mann Whitney U, $p=0.093$). There was an initial dip in the ratio at 20 minutes which was more prominent in the venous oxygen persufflation group followed by a progressive increase in the lactate pyruvate ratio during the remaining duration of reperfusion (Figure 45).

The fluid nitrite levels could not be assayed as the Griess reagent kit was not sensitive enough for the low nitrite concentrations in the effluent samples.
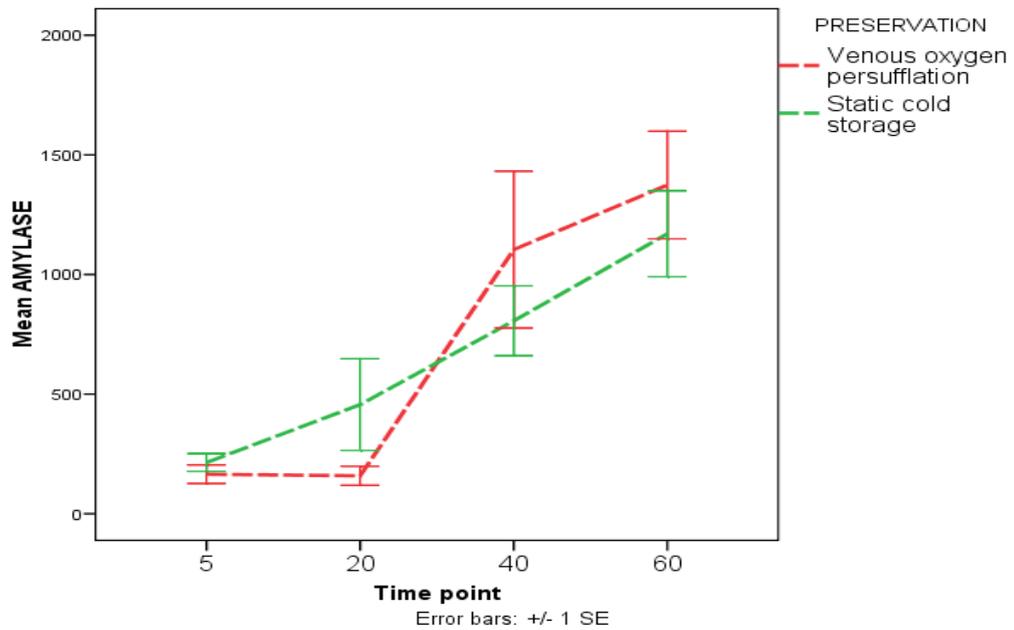
Figure 42 Effluent amylase concentrations during warm re-perfusion

The amylase concentration in the portal effluent collected at each time point during warm reperfusion was compared between static cold storage and venous oxygen persufflation groups. The time point during reperfusion is denoted on the X axis. The amylase concentration in international units is denoted on the Y axis. The data points denote the mean amylase concentration. The error bars denote one standard error of mean. A serial increase in amylase levels throughout the reperfusion was noted (5-10 fold, median increase 6.7 fold). The increase was maximal between 20 and 40 minutes of reperfusion. There was no statistically significant difference in amylase concentrations between the two preservation groups at any time point.
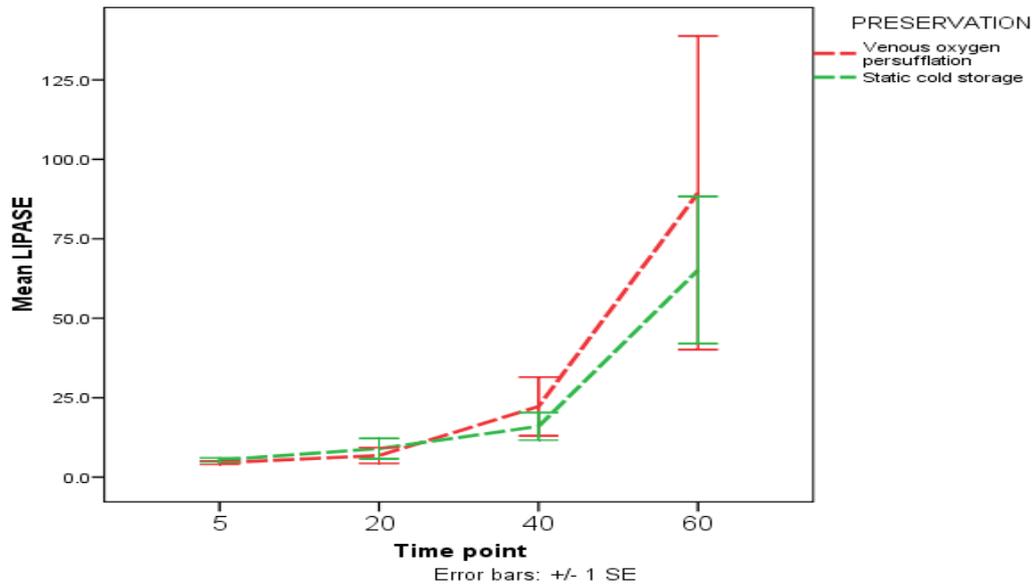
Figure 43 Effluent lipase concentration during warm re-perfusion

The lipase concentration in the portal effluent collected at each time point during warm reperfusion was compared between static cold storage and venous oxygen persufflation groups. The time point during reperfusion is denoted on the X axis. The lipase concentration in international units is denoted on the Y axis. The data points denote the mean lipase concentration. The error bars denote one standard error of mean. A serial increase in lipase levels throughout the reperfusion was noted (5-75 fold, median increase 9.8 fold) The increase was maximal between 40 and 60 minutes of reperfusion. There was no statistically significant difference in lipase concentrations between the two preservation groups at any time point.
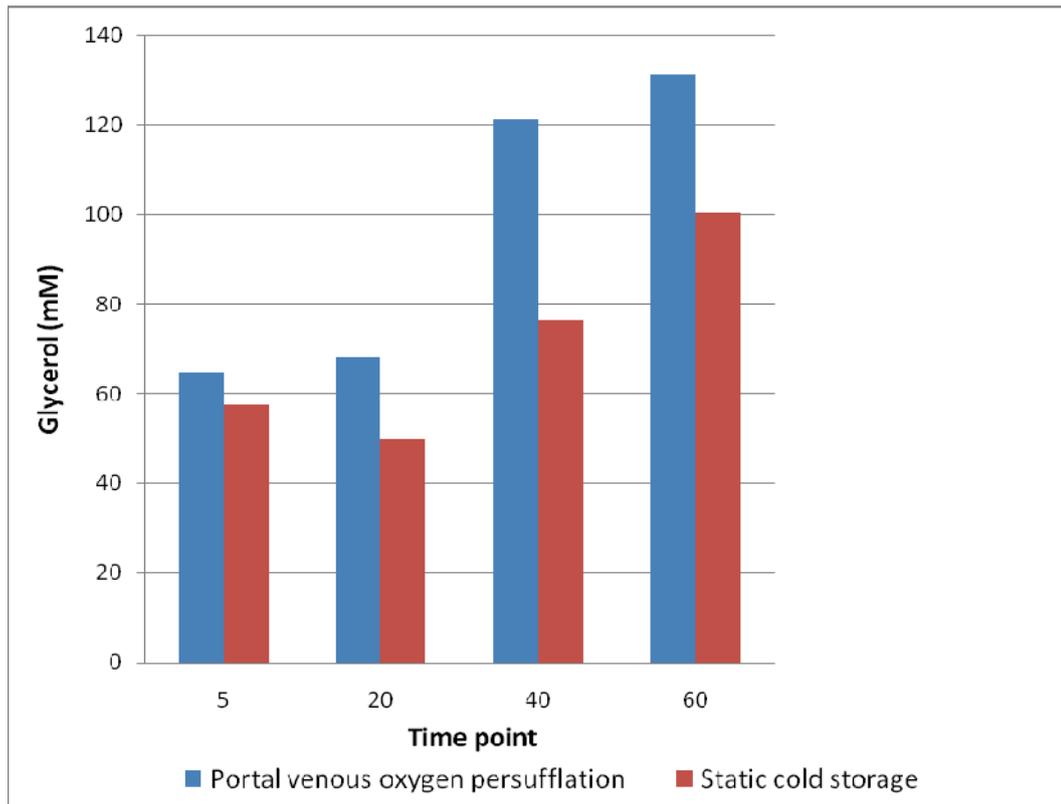
Figure 44 Glycerol concentration in the venous effluent during warm re-perfusion
The glycerol concentration in the portal effluent at each time point during warm reperfusion was compared between the static cold storage and the venous oxygen persufflation groups. The glycerol concentration is denoted on the Y-axis. The time points during reperfusion was denoted on the X axis. The bars represent the mean glycerol concentration. There was a gradual increase in the glycerol levels during the reperfusion period. The increase was more prominent between 20 and 40 minutes. There was a tendency towards increased levels in the portal venous oxygen persufflation group. The difference was not statistically significant.
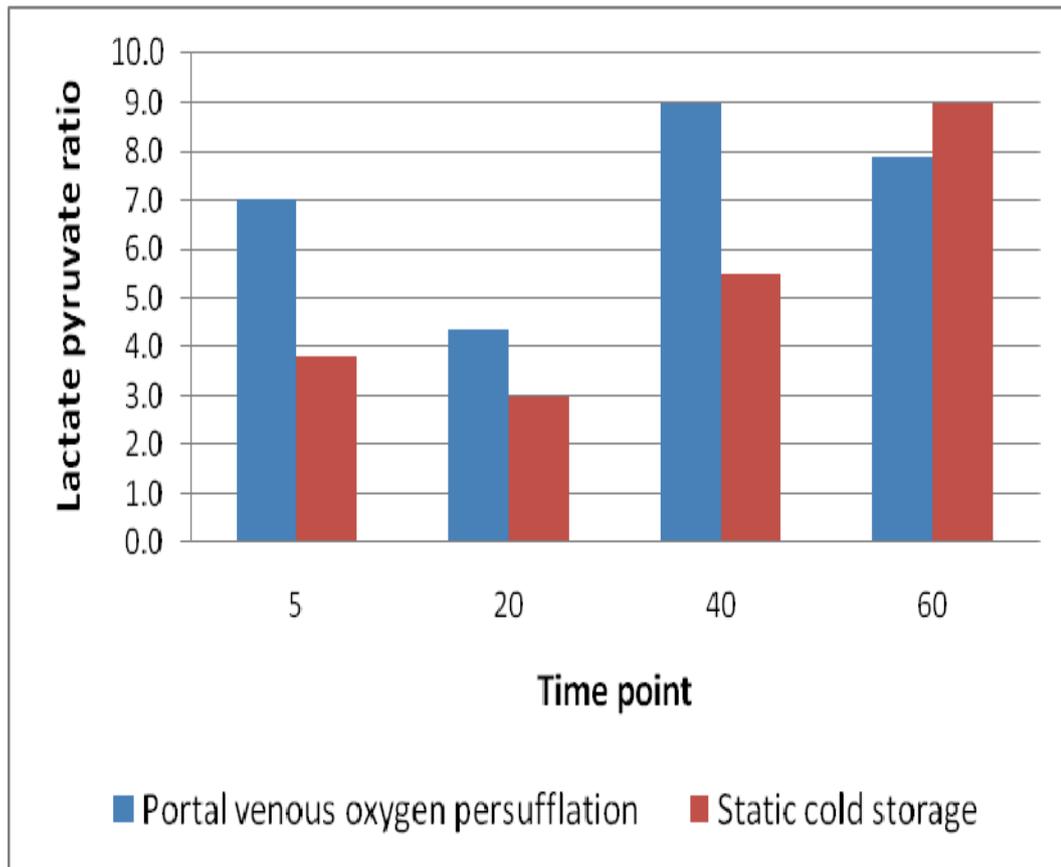
Figure 45 Lactate:pyruvate ratio in the venous effluent during warm re-perfusion
The lactate pyruvate ratio in the portal effluent at each time point during warm reperfusion was compared between the static cold storage and the venous oxygen persufflation groups. Both lactate and pyruvate levels were presented as μM. The ratio is denoted on the Y-axis. The time points during reperfusion were denoted on the X axis. The bars represent the mean lactate pyruvate ratio. There was a tendency towards increased levels in the portal venous oxygen persufflation group. The difference was not statistically significant. The lactate:pyruvate ratio in blood at rest is about 10. Levels greater than 20 indicate an oxygen debt i.e. anaerobic respiration[48].

5.3.3 Estimation of lipid peroxidation

Thio-barbituric acid reactive substances (TBARS) in the tissue extracts were determined to estimate the extent of lipid peroxidation (Figure 46). The levels of TBARS in the non-heart-beating-donor pancreas group were significantly higher than the control group pancreas. (19.6 nmoles/mg protein in NHBD group and 6.7 nmoles/mg protein in control group, T test, p=0.008). Within the two non-heart-beating-donor preservation groups, levels in the portal venous oxygen persufflation group were higher. The difference was not statistically significant (17.2 nmoles/mg protein in static cold storage group and 22.3 nmoles/mg protein in the portal venous oxygen persufflation group. (t test, p=0.206).

5.3.4 Estimation of activated caspase 3

The activated caspase 3 levels in the tissue extracts were measured using a modification of the manufacturer's protocol as previously discussed (section 3.5.4). It was not significantly different between the control group and NHBD group (0.215 µmoles pNA/mg protein and 0.109 µmoles pNA/mg protein respectively; t test, p=0.379). There was no significant difference in caspase 3 activity between the two preservation groups (Figure 47).
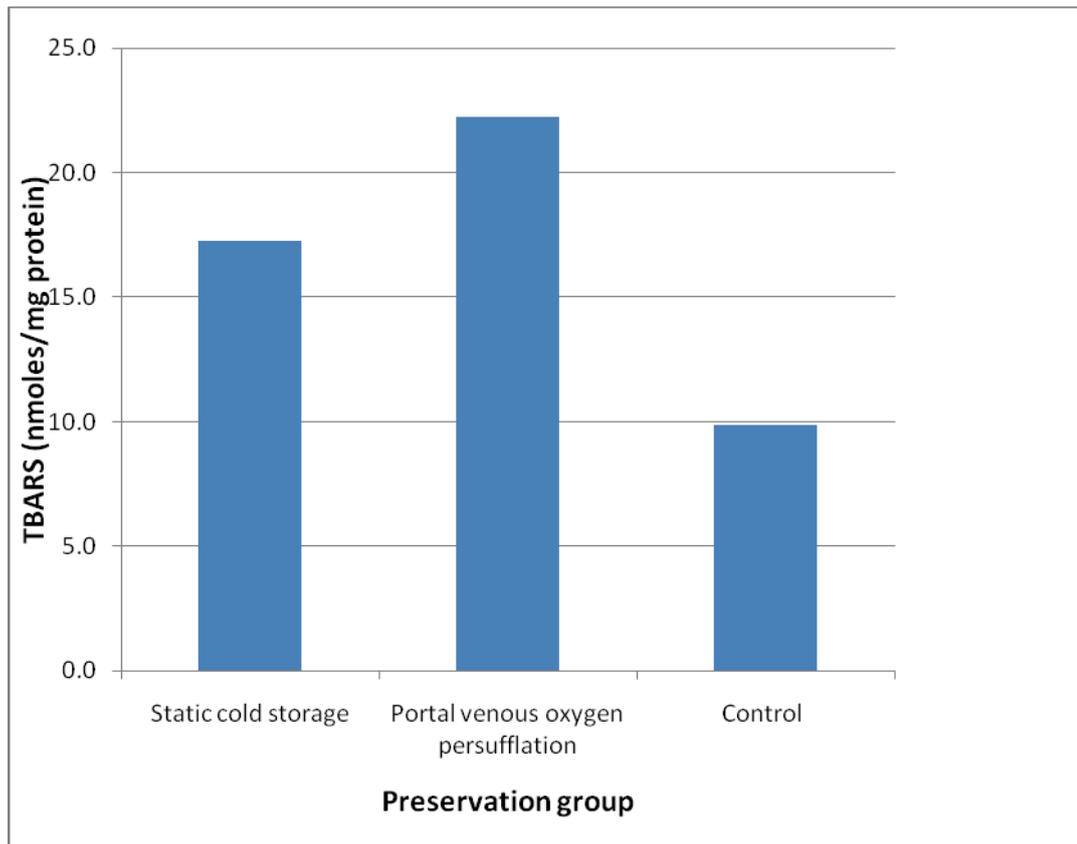
Figure 46 Comparison of TBARS in tissue extracts of control and two preservation groups.
Bar graph showing the thio-barbituric acid reactive substances (TBARS) levels in tissue extracts from pancreases preserved by the static cold storage (n=6), portal venous oxygen persufflation group (n=8) and control pancreas group (n=4).
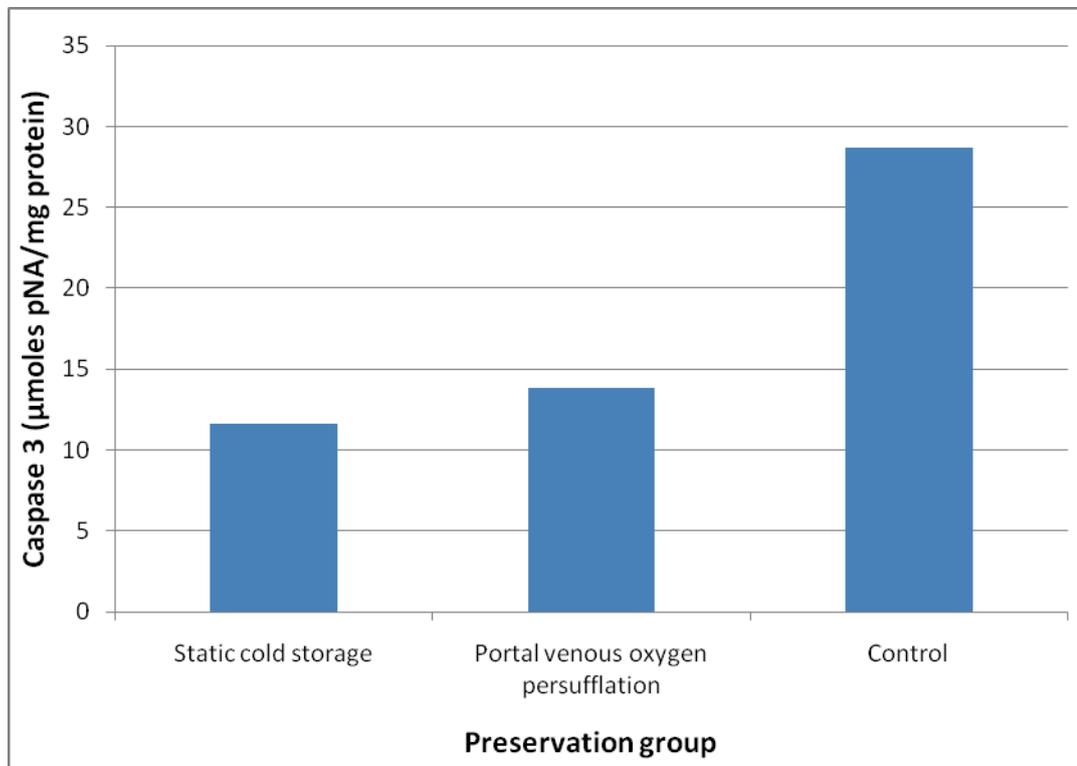
Figure 47 Comparison of activated caspase 3 levels in the tissue extracts of control group and two preservation groups.

Activated caspase 3 was measured as the amount of p-nitroaniline (pNA) released from the sample tissue extract after overnight incubation with the peptide substrate Ac-DEVD-pNA. The assay procedure was modified as described in section 3.5.4 to account for the non-specific background absorbance. Bar graph showing the mean pNA levels in tissue extracts from the static cold storage (n=6), portal venous oxygen persufflation group (n=8) and control pancreas group (n=4).

5.3.8 Apoptosis and anti-apoptosis- Microarray data

General description of apoptotic pathways is given in chapter 1. The following MAPP diagram represents the interactions between the various gene products involved in apoptosis (Figure 50).

Apoptosis can be initiated through the death receptor pathway or the mitochondrial pathway. Apoptosis by the death receptor pathway is initiated by the binding of ligands such as TNFα to the cell surface receptors of the tumor necrosis factor receptor family. This leads to receptor aggregation and recruitment of procaspase 8 and the adapter molecule FADD to form the death inducing signalling

complex (DISC). This complex leads to the cleavage and activation of caspase 8. This activates the effector caspases 3,7 and 2. It also cleaves Bid to tBid. Bid is a pro-apoptotic protein residing in the cytoplasm which on cleavage moves to the mitochondria to activate the mitochondrial pathway of apoptosis. The mitochondrial pathway is activated by intracellular stress signals. These activate the pro-apoptotic bcl2 proteins such as Bax to increase the permeability of the outer membrane of the mitochondria. This leads to the leakage of cytochrome c from the inter-membrane space into the cytoplasm. The leaked cytochrome c forms the apoptosome in combination with Apaf1 and Caspase 9. This complex cleaves the effector caspases 3 and 7. Action of the effector caspases on a multitude of intracellular proteins leads to cell death and the characteristic features of apoptosis. Anti-apoptotic proteins are primarily the bcl2 proteins that stabilize the mitochondrial outer membrane and prevent cytochrome c leakage, inhibitors of apoptosis proteins which inhibit the caspases, CFLAR (FLIP) which regulates the

activation of caspase 8 by the death receptor complex. Activation of the TNFA receptor family can also lead to activation of the NF-κB pathway and the JNK/MAPK pathways. Both these pathways lead to the increased transcription of inflammatory cytokines. NF-κB has a cyto-protective, anti-apoptotic function by inducing the production of multiple anti-apoptotic proteins. JNK/MAPK has a pro-apoptotic

Differential expression in pancreas preservation groups

Initiators of apoptosis such as Tumour necrosis factor alpha (TNFα) and proteins involved in the transduction of apoptotic signals from TNFR and TLR were differentially expressed in both preservation groups when compared to control group. Caspase 8 was over-expressed in the portal venous oxygen persufflation group while Caspase 1 was under-expressed in the static cold storage group. Death receptor pathway was significantly over-represented in the portal venous oxygen persufflation group. Genes coding for the pro-apoptotic BH3 proteins, BID and PUMA were differentially expressed in static cold storage. Bid was under-expressed and PUMA was over-expressed (fold change 3.4). Other pro-apoptotic Bcl-2proteins involved in the mitochondrial pathway of apoptosis were not differentially expressed in either preservation group (Figures 48,50).

The effector pathway of apoptosis including terminal caspases was not differentially expressed in either preservation group in comparison to the control group. Inter-group comparison revealed a 2.0 fold increased expression of caspase 3 in the portal venous oxygen persufflation group as compared to the static cold storage group.

Figure 48 Bar diagram comparing the fold change of differentially expressed genes involved in the initiation and transduction of apoptosis.

Figure in opposite page. The vertical axis indicates the symbols of relevant differentially expressed genes. The fold change is denoted on the horizontal axis. Both over-expression (to the right) and under-expression (to the left) are depicted. By definition, fold changes between -1.0 and +1.0 cannot exist. The cut off for significant differential expression used in the analysis was 2.0. Hence genes with fold change changes between -2.0 and -1.0 or between +1.0 and +2.0 are not shown. If a gene is differentially expressed in one group only, then it is represented with the correct fold change for that group and an arbitrary fold change value set at 0.2 for the second group.

The genes shown are inducers of apoptosis (TNFα, IL1β), components of the TNF receptor complex and proteins involved in transduction of apoptotic signals through the death receptor pathway (TNFRSF1A and TNFRSF21, TANK, RIPK1, RELA, MYD88, IRAK2), caspase proteins (CASP1, CASP8) and BH3 domain protein linking the death receptor and mitochondrial pathways of apoptosis (BID)

Figure 48: Legend in opposite page
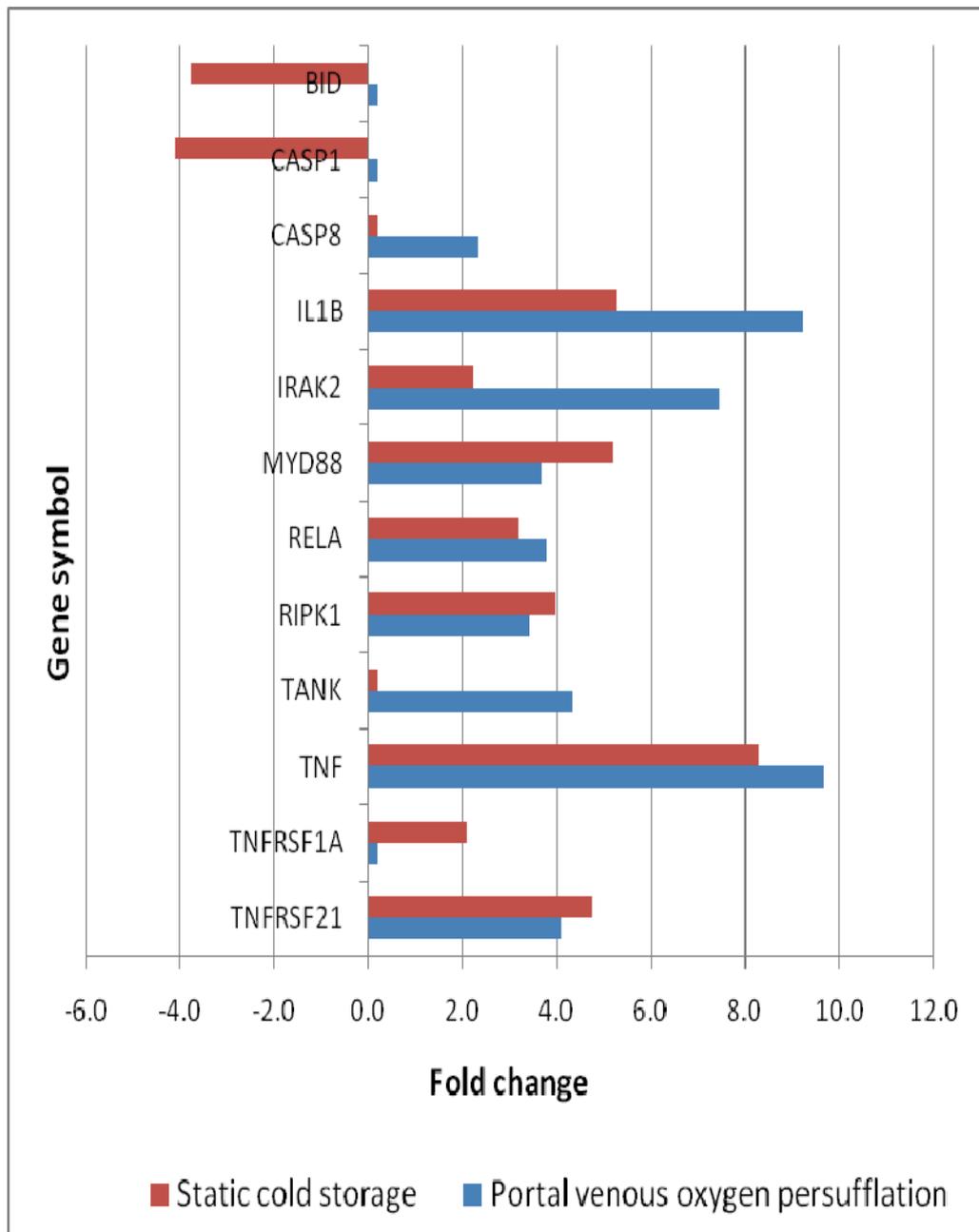
Genes coding for 20 proteins with anti- apoptotic properties were differentially expressed in the two preservation groups when compared to control group (17 in PVOP group and 16 in SCS group).

This was more prominent in the portal venous oxygen persufflation group. Inter-group comparison (PVOP vs SCS, fold change ≥2.0) showed that nine genes were significantly over-expressed in the portal venous oxygen persufflation group as compared to the static cold storage group. These included Bcl2a1, inhibitor of apoptosis proteins (BIRC2, BIRC3), PIM2 and PEA15. Three genes were significantly over-expressed in the static cold storage group when compared to the portal venous oxygen persufflation group. These included the enzyme mitochondrial superoxide dismutase (SOD2) (Figure 49,50).

Figure 49 Bar-chart comparing the differential expression of genes encoding proteins with anti-apoptotic functions between the two preservation groups.

Figure in next page. The vertical axis indicates the symbols of relevant differentially expressed genes. The fold change is denoted on the horizontal axis. Both over-expression (to the right) and under-expression (to the left) are depicted. By definition, fold changes between -1.0 and +1.0 cannot exist. The cut off for significant differential expression was 2.0. Hence genes with fold change changes between -2.0 and -1.0 or between +1.0 and +2.0 are not shown. If a gene is differentially expressed in one group only, then it is represented with the correct fold change for that group and an arbitrary fold change value set at 0.2 for the second group.

Genes differentially expressed included chaperone proteins such as heat shock proteins (HSPB1, HSPB1, DNAjB9), haeme-oxygenase 1 (HMOX), inhibitors of apoptosis (BIRC2, BIRC3), anti-apoptotic Bcl2 proteins (Bcl2a1), inhibitor of death receptor pathway (CFLAR, PEA15), inhibitors of pro-apoptotic BAD (PIM2) and the mitochondrial form of super-oxide dismutase. There was increased expression of anti-apoptosis related genes in the portal venous oxygen persufflation group.

Figure 49 : legend in opposite page
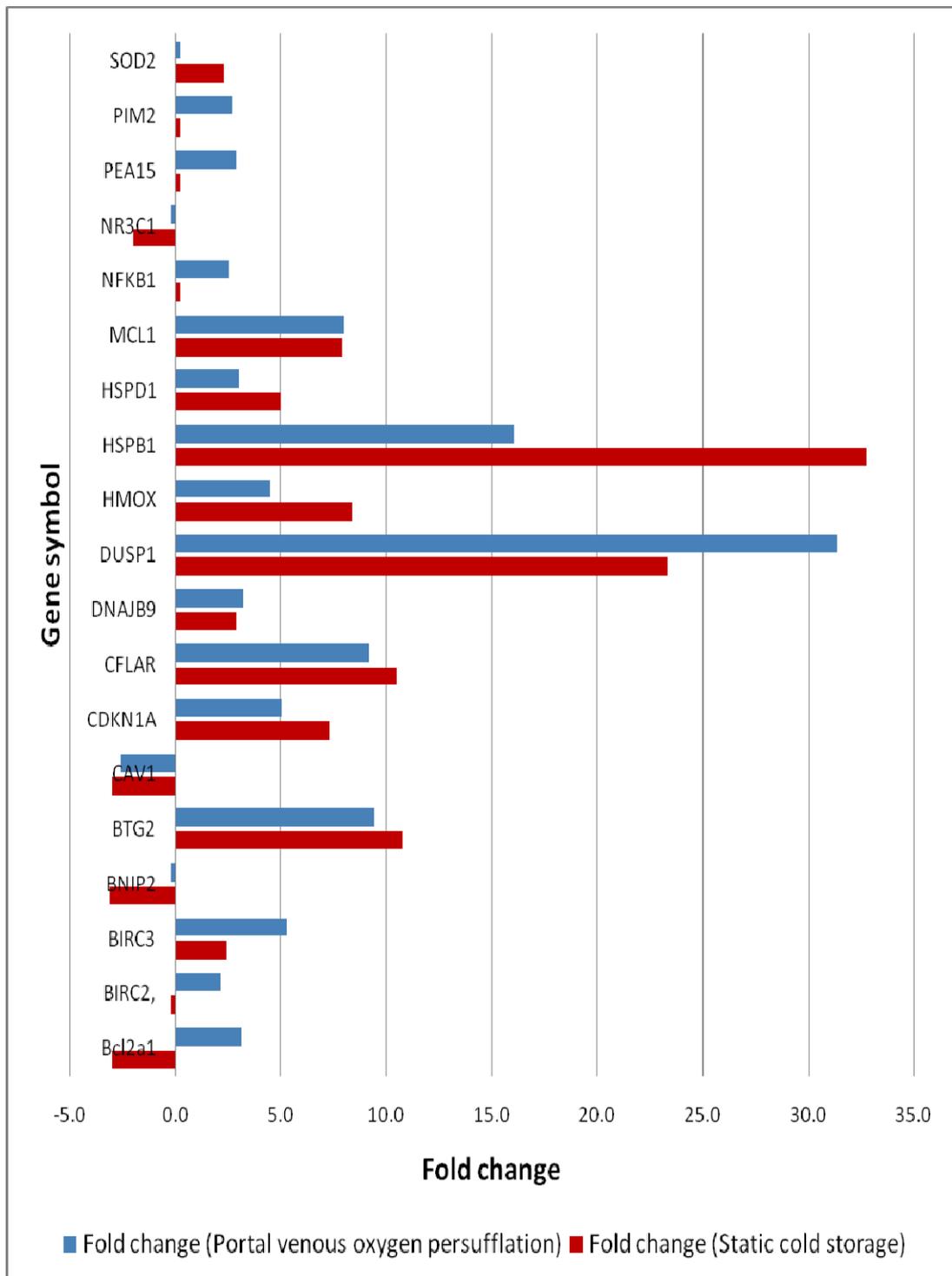
Figure 50 Pathway diagram of apoptosis demonstrating the differential expression of genes in the static cold storage (SCS) and portal venous oxygen persufflation (PVOP) groups

Figure in opposite page.
MAPP demonstrating apoptotic and anti-apoptotic mechanisms. This MAPP was modified from the version available at GenMAPP version 2.1. Genes are denoted by rectangles with arrows showing the relation between individual genes. Red lines connecting genes indicate pro-apoptotic action, blue lines indicate anti-apoptotic mechanisms. The rectangle for each gene is split into two parts to show the nature of differential expression of that particular gene for each of the two preservation groups. The colour codes for over-expression and under-expression for each preservation group are noted in the diagram legend.

Apoptosis Mechanisms

5.3.9 Oxidative stress and anti-oxidant mechanisms- Microarray data

Genes whose expression is known to be modified by oxidative stress were differentially expressed in both preservation groups. Twenty genes were differentially expressed in the Portal venous oxygen persufflation preservation group as compared to 14 genes in the static cold storage group. Inter-group comparison identified six genes which were differentially expressed between the two preservation groups. Five of these genes were over expressed in the portal venous oxygen persufflation group while one gene was over-expressed in the static cold storage group (mitochondrial super-oxide dismutase). Genes over expressed in the portal venous oxygen persufflation group included pro-inflammatory genes (VCAM and CCL5) and genes coding for proteins with anti-oxidant functions *i.e.* NAD(P)H dehydrogenase (NQO1), NRF2 and NF-κB1 (Figures 51 & 52).
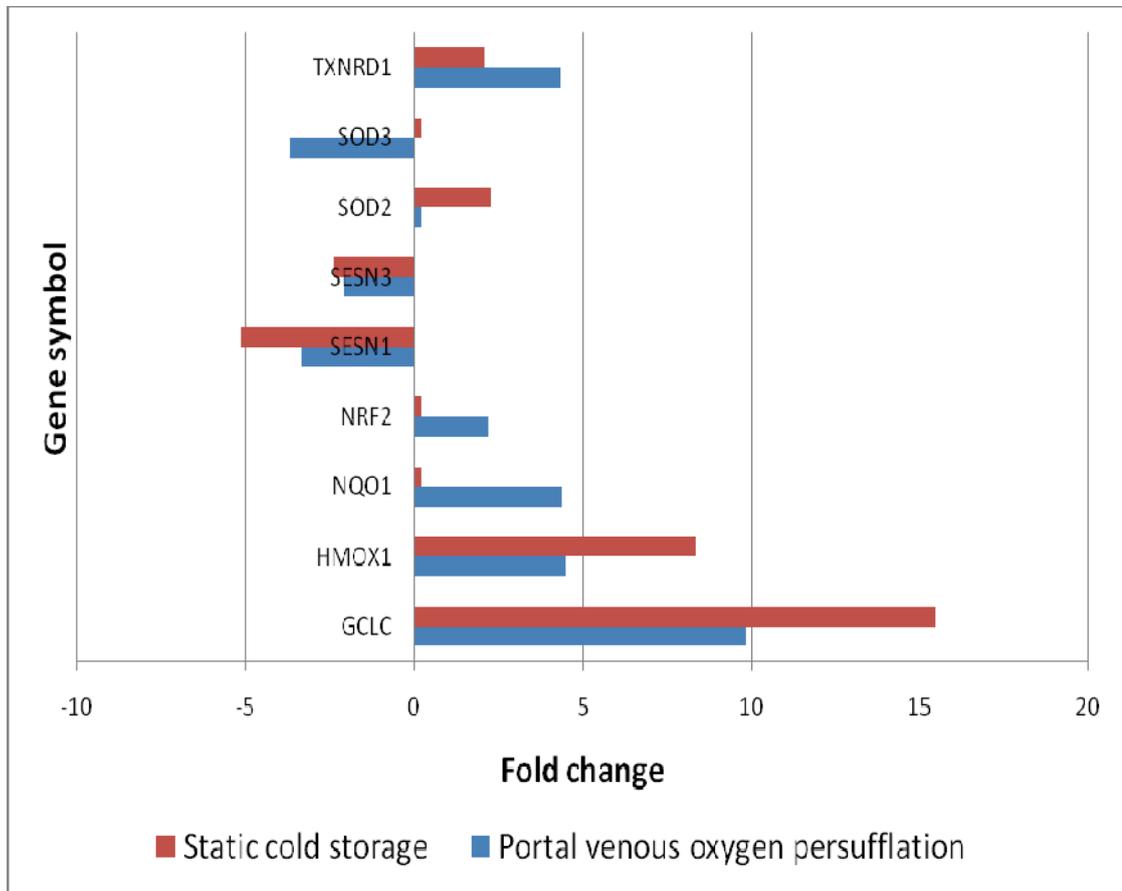
Figure 51 Bar-chart comparing the differential expression of genes coding proteins with anti-oxidant roles in the two preservation groups

Bar chart comparing differential expression of genes involved in the cellular response to oxidative stress between the two preservation groups. Nuclear factor 2 is a transcription factor that induces the expression of several proteins with anti-oxidant functions such as thioredoxin (TXNRD1), glutathione-cysteine ligase (GCLC) and NAD(P)H dehydrogenase. They were all preferentially over-expressed in the portal venous oxygen persufflation group. Haemoxygenase 1 (HMOX1) was over-expressed in both groups. Mitochondrial superoxide dismutase was over-expressed exclusively in the static cold storage group while the extracellular form of super-oxide dismutase (SOD3) was under-expressed in portal venous oxygen persufflation group alone. Sestrin1 and 3 which have a role in regeneration of oxidized peroxiredoxins were suppressed in both groups.
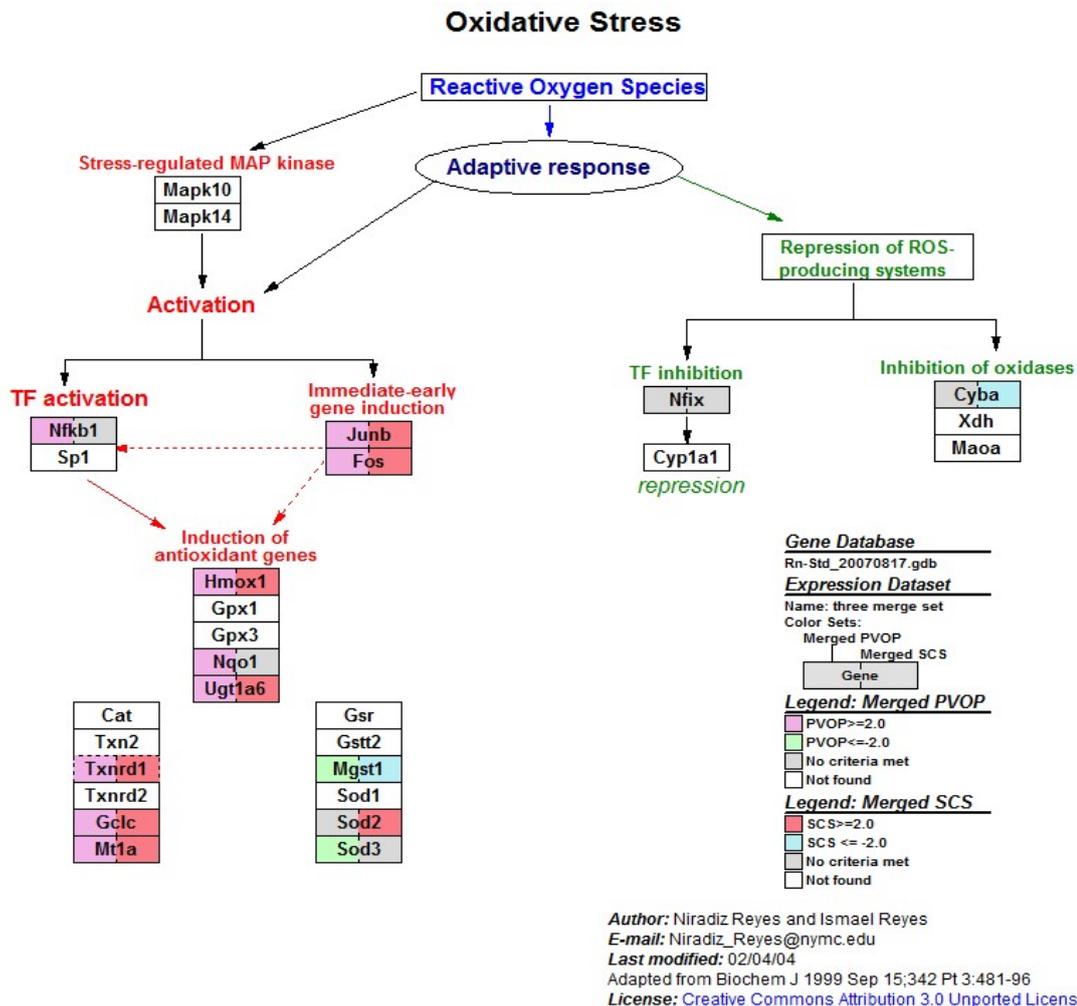
Figure 52 MAPP diagram depicting the cellular response to oxidative stress and the differential expression of involved genes in the two preservation groups

MAPP demonstrating cellular antioxidant mechanisms. This MAPP was modified from the version available at GenMAPP version 2.1. Genes are denoted by rectangles with arrows showing the relation between individual genes. The rectangle for each gene is split into two parts to show the nature of differential expression of that particular gene for each of the two preservation groups. The colour codes for over-expression and under-expression for each preservation group are noted in the diagram legend. Oxidative stress occurs due to an excess formation of reactive oxygen species. Reactive oxygen species activate the JNK/MAPK pathways and also stimulate the production of immediate early genes such as cFos and Junb. Multiple transcription factors including NRF2 and NF-KB are activated leading to the production of anti-oxidant proteins such as enzymes involved in glutathione metabolism, super-oxide dismutase, thioredoxin. Reactive oxygen species causes repression of multiple genes including oxidases such as xanthine oxidase which are involved in ROS generation and transcription factors such as Nuclear factor 1 (NFIX) and cytochrome p450 system[227].

5.4 Discussion

These experiments have shown no improvement in the markers of reperfusion injury with portal venous oxygen persufflation of the non-heart-beating-donor rat pancreas compared to static cold storage.

In vitro warm reperfusion is a well established technique to assess reperfusion injury in cells, tissues and organs. The technique of *in vitro* reperfusion used in the study has been described previously for the liver, kidneys and pancreas [184, 190, 222, 228]. The technique provides preliminary data regarding the severity of reperfusion injury in the graft without the need for actual transplantation. It also has the advantage of not utilizing blood products or protein solutions. Krebs-Henseleit buffer is widely used in this technique[190]. It contains glucose as a source of energy and bicarbonate as the buffer. Krebs-Henseleit buffer was used without addition of colloids in these experiments. Some authors have however advocated the addition of albumin to the buffer as a means of improving the quality of warm reperfusion[229]. Maintenance of perfusate pH within the physiological range, close monitoring of temperature and adequate oxygenation of the perfusate are other important aspects that have been reported to affect experimental results. The perfusate temperature was maintained at 37°C and the pH was monitored and maintained close to 7.4. A mixture of oxygen and carbon dioxide (95% and 5%) was used to provide oxygenation. This has been shown to provide sufficient oxygen levels in the perfusate. The carbon dioxide improves the buffering capacity of bicarbonate in

the Krebs-Henseleit solution. The perfusion flow rate is another key factor in this technique. The rate of perfusion should be enough to provide sufficient oxygen to the tissues and low enough to avoid baro-trauma. Studies using warm reperfusion of the isolated rat liver have suggested a flow rate of 3.0ml/gram tissue/minute[190]. The average weight of the rat pancreas with duodenum and spleen as used in our experiment was 9 grams. This would indicate an ideal flow rate of 27 ml/minute. Such high flow rates could be not achieved during initial trials. This could be due to the small capacity of the pancreatic vascular bed in comparison to the liver. The injury to the microvasculature from warm and cold ischaemia would also be expected to increase vascular resistance in the pancreas. Other researchers have used isolated pancreas perfusion at lower flow rates[230]. The perfusion flow rate was hence adjusted to simulate physiological perfusion pressures. It is possible that the resultant flow rate (4.5 ml/minute) in the circuit was not sufficient to oxygenate the pancreas. The experimental setup therefore had the potential to drive the warm ischaemia injury during the reperfusion phase.

Studies have shown that the exocrine component of the pancreas is more susceptible to reperfusion injury than the endocrine component [138]. Pancreatic exocrine enzymes are released into the circulation following acinar damage as in pancreatitis and pancreatic reperfusion injury[231]. We measured the levels of amylase and lipase in the portal effluent as a marker of pancreatic exocrine injury. Cell membrane damage is an important component of ischaemia and reperfusion injury. This is caused during both warm and cold ischaemia due to

depletion of ATP reserves and during reperfusion due to production of reactive oxygen species. Glycerol is a component of cell membranes. Increased serum and interstitial glycerol levels have been reported to predict cell membrane injury[232, 233]. Studies have shown increased interstitial levels of glycerol during cold ischaemia[233]. The impact of reperfusion on interstitial glycerol levels depends on several factors. In cases where the ischaemic injury is small and reperfusion is satisfactory there is a prompt decrease in the interstitial glycerol concentrations suggestive of an arrest of further cell membrane damage. In cases where ischaemia is prolonged, glycerol concentrations remain high due to severe ongoing reperfusion injury[233].

The lactate:pyruvate ratio has been reported as a surrogate marker for oxygen debt[48]. The normal lactate:pyruvate ratio in the blood is approximately 10. In instances of anaerobic respiration and lactic acidosis the ratio can go up to 40[48]. Studies of experimental and clinical liver transplantation have identified characteristic changes in this ratio during the phases of donor retrieval, cold ischaemia, re-warming and reperfusion [234-236]. The ratio is low during cold ischaemia with low levels of both lactate and pyruvate, increases during anoxic re-warming and decreases sharply after reperfusion with a decrease in lactate and increase in pyruvate concentrations as aerobic respiration ensues. Increased lactate:pyruvate ratio following reperfusion indicates 'no-reflow' *i.e.*, insufficient delivery of oxygen or disruption of cellular apparatus for aerobic respiration.

Analysis of timed effluent samples in our study showed interesting trends. The levels of exocrine enzymes and glycerol were similar in both groups at 5minutes and at 20 minute time points. There was a decrease in the lactate:pyruvate ratio between the two time points. The later was more prominent in the portal venous oxygen persufflation group. After 20 minutes the levels of exocrine enzymes, glycerol and lactate:pyruvate ratio increased in both preservation groups.

There could be several factors contributing to these findings. The prolonged warm and cold ischaemia duration mean that all pancreases irrespective of the type of preservation have endured extensive preservation injury. Sub-optimal oxygenation during the reperfusion phase due to insufficient perfusion or no-reflow phenomenon can cause continued ischaemic damage. This could explain the continued release of exocrine enzymes and glycerol seen throughout the reperfusion period.

The reperfusion phase was preceded by a period of gradual re-warming of the pancreas to room temperature over 15 minutes. The pancreas is anoxic during this period. The higher lactate:pyruvate ratio seen in the portal venous oxygen persufflation group at the start of reperfusion indicates a speedier restoration of metabolic activity and anaerobic glycolysis and the build up of an oxygen debt. The prompt decrease in the lactate:pyruvate ratio during the first 20 minutes of reperfusion could also indicate better clearance of the oxygen debt by the resumption of aerobic respiration. The low initial lactate:pyruvate ratio and minimal change in the initial 20 minutes of reperfusion seen in the static cold storage group indicates a more delayed return to active metabolism. It is

interesting to note that the ratio in both groups was less than 10 at all time points.

An additional explanation for the prominent increase of exocrine enzymes and glycerol in effluent concentrations after 20 minutes may reside in the design of the experiment itself. The warmed, oxygenated Krebs-Henseleit solution was pumped through the pancreas from a separate reservoir. Venous effluent collected in the pancreas chamber during reperfusion. Intermittently when the chamber was full, the effluent was transferred back into the Krebs-Henseleit reservoir. This could have contributed to the steep increase in effluent enzyme and cellular injury marker levels after 20 minutes of reperfusion. To avoid this confounding factor, we suggest that future studies with reperfusion circuits should be either continuously recycling or entirely non-recycling.

The TBARS assay was used to estimate the extent of lipid peroxidation. It is based on the reactivity of thio-barbituric acid (TBA) toward malondialdehyde which is a product of oxidative lipid degradation. TBA reacts with malondialdehyde to yield a fluorescent red adduct. TBARS assay is not specific for lipid peroxidation and other proteins, nucleic acids and bilirubin can produce an MDA like adduct. MDA is also not produced stochiometrically during lipid peroxidation and hence is not quantitatively related to the extent of lipid peroxidation [237]. The advantage of the assay is its ease of use and low cost. Hence a number of studies have used it as a measure of lipid peroxidation [89, 238, 239]. Since the assay was used here to compare lipid peroxidation between groups and not as an absolute measure of lipid peroxidation we felt

that it was reasonable to use it. As expected, tissue from control pancreas had significantly lower levels of TBARS as compared to the non-heart-beating-donor pancreas samples. There was a tendency for higher TBARS levels in the oxygen persufflation group though the difference was not statistically significant. One pancreas from the persufflation group had TBARS levels nearly three times higher than the median level in that group (sample G, TBARS=67.8 nmoles/mg). Review of this pancreas identified no specific issues during persufflation or warm reperfusion. Duodenal peristalsis was identified in this pancreas during warm reperfusion and there was no corroborating evidence of excessive reperfusion injury such as elevated glycerol or caspase 3 levels.

Caspase 3 activation is the definitive step in apoptosis and can occur through both the death receptor and mitochondrial pathways. Its presence indicates cells that have irreversibly entered the apoptotic pathway. The levels of caspase 3 activation in the control and preservation groups were low, needing modification of the assay methods to increase its sensitivity. Surprisingly, we have found increased levels of caspase 3 activity in the control pancreases as compared to the preservation groups. One possibility is that the levels seen in the control group are the normal level of apoptotic activity seen in the pancreas. Apoptosis and measurable caspase 3 levels have been reported in healthy liver tissue[240, 241]. It is expected that metabolically active pancreatic tissue would have some basal caspase 3 activity. The damage secondary to warm and cold ischemia in the study group pancreases should activate both the apoptotic and necrotic pathways of cell death. The apparent low levels seen in the reperfused

pancreases could be due to breakdown of caspase 3 by autolytic enzymes activated during this period.

We believe that the phenomenon of duodenal peristalsis observed in some of the pancreases during reperfusion is a significant finding. Peristalsis is an energy dependent process. It indicates availability of energy rich phosphates such as ATP in the tissues. It also signifies viable duodenal smooth muscle and maintenance of the necessary inter-myocyte communications to produce coordinated contractions. Several studies have reported onset of duodenal peristalsis after reperfusion in experimental pancreato-duodenal transplantation[242]. Development of peristalsis has also been considered as a marker of viability of small bowel in studies of isolated small bowel perfusion[243]. The fact that all the three pancreases showing peristalsis belonged to the persufflation group further strengthens the idea that these organs are more metabolically active.

To summarise, these experiments were planned to evaluate the efficacy of prolonged portal venous oxygen persufflation in ameliorating the severity of reperfusion injury in the non-heart-beating-donor rat pancreas. Our results did not show significant improvement in markers of reperfusion injury after portal venous oxygen persufflation. Though there were indications to suggest that pancreases in this preservation group were more metabolically active, the evidence is not conclusive. It is possible that the preservation injury due to the prolonged cold ischaemia may have been too extreme. The technique of oxygenated reperfusion used may have been sub-optimal. Finally, reperfusion

injury in the clinical setting progresses over a period of hours to days. It is possible that the effects of improved preservation may not be evident within the first 60 minutes of warm reperfusion.

Reperfusion injury activates cellular responses through a multitude of intracellular signalling pathways. While the activation of these pathways occurs during cold ischaemia and early reperfusion, their effects in the form of biochemical, physiological or morphological changes take longer to be apparent. Investigating the gene expression profile early in reperfusion can provide a better understanding of the effect of portal venous oxygen persufflation on the organs' response to ischemia reperfusion.

These data suggest that the genes related to the death receptor pathway of apoptosis were over-expressed in the portal venous oxygen persufflation group. This included the caspases 3 and 8 and Bid. This suggests an increased propensity for the portal venous oxygen persufflation group pancreas to undergo apoptosis. Though this might suggest a worse outcome for this preservation group, it is not necessarily so. Ischaemia and reperfusion will induce severe cellular and mitochondrial injury in both preservation groups. Mitochondrial injury with mitochondrial permeability transition has been reported to be an important step during both apoptosis and necrosis[244]. Cells undergo apoptosis only when sufficient ATP reserves are available. Progression of apoptosis cannot occur in ATP depleted cells which undergo death by necrosis. Necrotic death has the distinct disadvantage of exacerbating the inflammatory process leading to continued recruitment of pro-cell death pathways. In an interesting study,

Zhao *et al.* investigated the effect of artemisinin, a known inducer of cellular apoptosis in experimental acute pancreatitis[245]. They found that administration of this drug increased apoptosis, decreased necrosis and improved the markers of inflammation such as serum amylase, histological grading and serum cytokine levels. Similar conclusions have been reached by other studies [246, 247]. In a study by Lemasters and co-workers in 2003 [248] the nature of hepatocyte death after ischaemia reperfusion was investigated. They found that 4 hours of culture in anoxic medium followed by two hours of oxygenated reperfusion caused necrosis in 60% of cells. Provision of fructose for 20 minutes prior to start of reperfusion and during the reperfusion period lead to increased glycolysis, increased ATP, increased apoptosis and decreased necrosis. The authors concluded that both apoptosis and necrosis occur through mitochondrial permeability transition. Induction of glycolysis increases the energy status of tissues and redirects cellular outcome from necrosis to apoptosis.

Increased expression of anti-apoptotic proteins was seen in both study groups. This was more prominent in the portal venous oxygen persufflation group indicating a more robust cellular response to ischaemia reperfusion injury.

To summarise, reperfusion in both groups resulted in a significant up regulation of the processes of transcription and inflammatory cytokine response. We postulate that the increased expression of apoptotic and anti-apoptotic genes in the portal venous oxygen persufflation group reflects an improved energy status in the graft which helps it to circumvent cell death by necrosis.

Chapter 6 Microarray analysis of RNA extracted from non-heart-beating-donor rat pancreas

6.1 Background

Numerous molecular pathways are activated after ischaemia reperfusion as the cells attempt to limit the extent of injury and survive. Multiple genes are over or under-expressed during this period. DNA microarrays are useful tools to study this complex cellular response.

Flamant and co-workers in 2009 investigated the effect of hypoxia alone on inflammatory cytokine expression in cell lines. They identified increased levels of the alpha subunit of hypoxia inducible factor (HIFα) after 16 hours of hypoxia along with increased transcription of HIF- induced genes. There was decreased binding of NF-κB in hypoxic cells. This study investigated only the hypoxia part of IRI and did not investigate the gene expression after re-oxygenation[249]. Supavekin and co-workers in 2003[250] studied the changes in gene expression in early renal ischemia reperfusion injury with special emphasis on apoptotic pathways. Over-expression of pro-apoptotic and anti-apoptotic genes was identified by three hours post reperfusion. Both death receptor pathway and mitochondrial pathway were activated. However, evidence of apoptosis in the form of positive TUNEL test and DNA laddering was identified only 12 and 24 hours post-reperfusion. Aravindan and co-workers in 2006 [251] investigated gene expression after ischaemia reperfusion in rat kidneys. They identified up

regulation of over 70 genes related to apoptosis. Stegall and co-workers in 2002 investigated global gene expression in transplanted hearts after 3, 5 and 7 days post-transplant. They reported over-expression of genes related to MHC class 2 antigens, adhesion molecules and inflammatory cytokines. Under-expressed genes were primarily related to metabolism such as monoamine oxidase, mitochondrial proteins and cytochrome oxidase subunits [252]. Drognitz and co-workers in 2006 [253] investigated gene expression with microarrays in a rat model of pancreas transplantation . They compared the global gene expression in pancreases preserved by different preservation solutions and different cold ischaemia times. They identified a total of 49 genes that were consistently up regulated (more than threefold) in all three study groups. These genes included transcription factors, cytoskeletal factors, heat-shock proteins and molecules involved in inflammation, signal transduction, and translation. Further analysis of up-regulated pathways was not carried out in this study. Benz and co-workers in 2002 [254] used microarrays to study temporal changes in gene expression in pancreas during preservation and reperfusion. They found that the expression of genes was maximal during the reperfusion phase. Up regulated genes included those coding for chaperone proteins, transcription factors and inflammatory cytokines.

Significant up-regulation of inflammatory pathways occurs in grafts from non-heart-beating-donors. Factors that decrease up-regulation of pro-death pathways and increase the expression of pro-survival pathways will have a positive impact on graft survival and function. Microarrays are a useful screening

tool to investigate such factors. Jassem and co-workers in 2009 [255] investigated the effect of the technique of ischaemic preconditioning during donor liver retrieval on the expression of cell death and survival pathways after liver transplantation using microarray technology. They identified 60 genes involved in cell death, inflammation and immune response and stress which were over-expressed in the ischaemia reperfusion injury group. Ischaemic preconditioning led to decreased up-regulation of pro–cell death and pro-inflammatory genes and increased expression of anti–cell death and anti-inflammatory genes.

Several researchers have investigated the mechanism of improved organ preservation with venous oxygen persufflation[222, 256, 257]. Improved ATP synthesis during persufflation has been considered the main reason for improved function in these grafts. Transplantation of non-heart-beating-donor grafts induces a prominent and prolonged ischaemia reperfusion injury. Improved survival of these grafts can only be expected if preservation techniques precipitate a modification of the gene expression profile through induction of pro-survival pathways over pro-death pathways. To date there is no published literature investigating the effect of venous oxygen persufflation on global gene expression. This information, when available, will improve our understanding of the mechanism of graft dysfunction and graft protection. Identification of specific pathways involved in the injury and recovery of non-heart-beating-donor pancreases will also provide an opportunity to selectively modify these pathways.

The aim of the study was to compare gene expression patterns following *in vitro* reperfusion in rat pancreas exposed to warm ischaemia after static cold storage and portal venous oxygen persufflation.

6.2 Methods

6.2.1 RNA samples for microarray analysis

Microarray analysis was performed by Dr Liming Wang from the Centre for Life, University of Newcastle using the Affymetrix Rat Genome 230 2.0 Array. Three pooled RNA samples were analysed. These were RNA from control group pancreases and the two study groups i.e. non-heart-beating-donor pancreases preserved by static cold storage or portal venous oxygen persufflation. Gene expression data in each preservation group were presented as fold change (increased or decreased expression) of each identified gene in comparison to the control group pancreas. Three separate data matrices comparing static cold storage to control, portal venous oxygen persufflation to control and static cold storage (SCS) to portal venous oxygen persufflation (PVOP) were obtained for further analysis. All subsequent analysis and discussion relates to the differential expression of genes in each preservation group when compared to the control group (PVOP vs control and SCS vs control). Instances where differential gene expression between the two preservation groups (PVOP vs SCS) is presented will be specified.

6.2.2 Gene Ontology analysis of microarray data

Genes with a fold change of greater than 2 (mRNA concentration for the gene was more than double or less than half the specific mRNA concentration of control group pancreas) were included in the analysis. Initial analysis to identify over-represented Gene Ontology (GO) terms was carried out using DAVID bioinformatics database (available at http://david.abcc.ncifcrf.gov/tools.jsp)[258]. For each preservation group, the Affymetrix gene identifiers of the differentially expressed genes were uploaded. Highly represented GO terms for the uploaded genes were identified. Terms with a gene count between 5 and 50 and with an EASE score (modified Fischer's test used in DAVID) less than 0.01 were identified. Duplicate terms and redundant parent terms were removed. The top five highly represented terms in each Ontology are presented.

6.2.3 Pathway analysis using microarray data

Data were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com)[259]. Briefly the dataset containing the gene identifiers and the expression values was uploaded to the server. The Functional Analysis identified the biological functions and/or diseases that were most significant to the data set. Fischer's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. Canonical pathways analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical

pathways that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured by the ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway. The Fischer's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. A p value less than 0.05 was considered statistically significant.

The biological processes investigated were apoptosis, anti-apoptosis and anti-oxidant mechanisms. The canonical pathways investigated were the Akt/PKB pathway, NF-κB pathway and the MAPK pathways. Validated Map Annotator and Pathway Profiler (MAPP) sets available as free downloads from GenMAPP version 2.0[260] were used for the visualisation of the pathways of interest. MAPP builder functionality available in GenMAPP program was used to develop MAPP set for NF-κB pathway. The relative expression (in terms of fold change) of pertinent genes in the two preservation groups was extracted from the dataset for each of the selected functions and pathways. Gene expression data were presented as bar charts demonstrating both over-expression and under-expression of genes.

6.3 Results

6.3.1 Pattern of differential gene expression

2441 gene transcripts were identified as differentially expressed with a fold change of at least 2.0 in the static cold storage group when compared with control group. Similarly 2060 differentially expressed genes were identified in the portal venous oxygen persufflation group when compared to control group. There was significant overlap in the expression of genes between the two preservation groups. Of 3238 genes that were differentially expressed by at least 2 fold in either group, 1263 genes were common to both groups (39%). The direction of differential expression was the same for 1231 genes (over-expressed or under-expressed in both preservation groups) while 32 genes were differentially expressed in opposite directions (over-expressed in one group and under-expressed in the other group. Both preservation groups had genes that were commonly under-expressed or over-expressed (Figures 53, 54).

1129 genes were differentially over-expressed in the static cold storage group. These were genes encoding chaperone proteins (heat shock proteins), transcription factors (ATF3, Fos, EGR1) and genes involved in cytokine response to cellular injury (Cxcl10,Cxcl1). 1312 genes were differentially under-expressed. These genes were primarily those involved in the cell's immune response to antigens and cell surface components (Tables 6, 7).

1266 genes were over-expressed in the portal venous oxygen persufflation group. These were genes involved in stress response, cellular cytokine

response and transcription factors. 794 genes were under-expressed. These genes were primarily those involved in haemopoesis and lipid metabolism (Tables 8, 9).
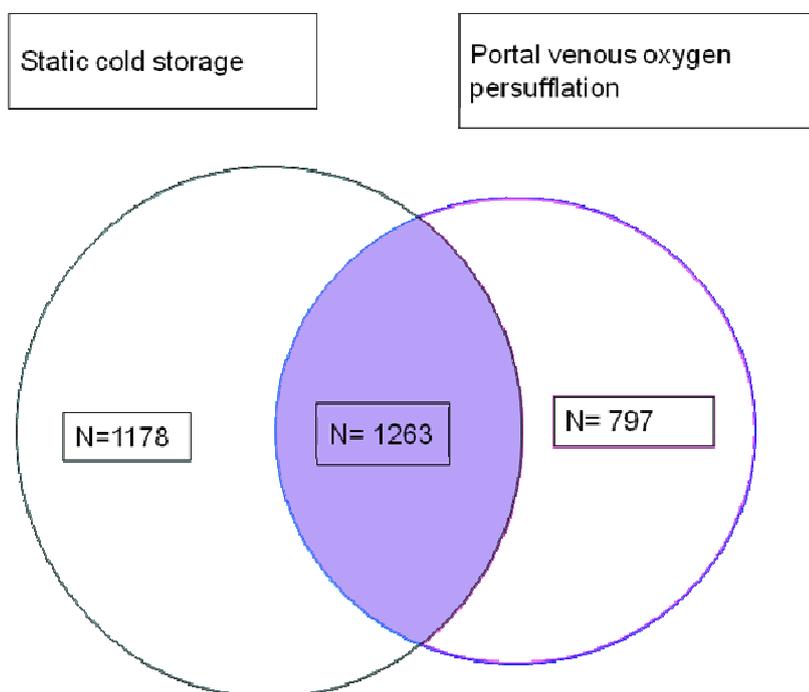
Figure 53 Venn diagram showing the number of common and unique differentially expressed genes in the two preservation groups-all genes

The number of differentially expressed genes in the two preservation groups is shown. 2441 genes were identified as differentially expressed in static cold storage group with a fold change of 2.0 or more (blue circle). 2060 genes were differentially expressed with a fold change of 2.0 or more in the portal venous oxygen persufflation group (pink circle). Of these, 1263 genes were common to both preservation groups. The direction of differential expression was the same for 1231 genes (over-expressed or under-expressed in both preservation groups) while 32 genes were differentially expressed in opposite directions (over-expressed in one group and under-expressed in the other group).

Static cold storage

Portal venous oxygen persufflation

N=331   N= 798   N= 468

Over-expressed genes

Static cold storage

Portal venous oxygen persufflation

N=879   N= 433   N= 361

Under-expressed genes
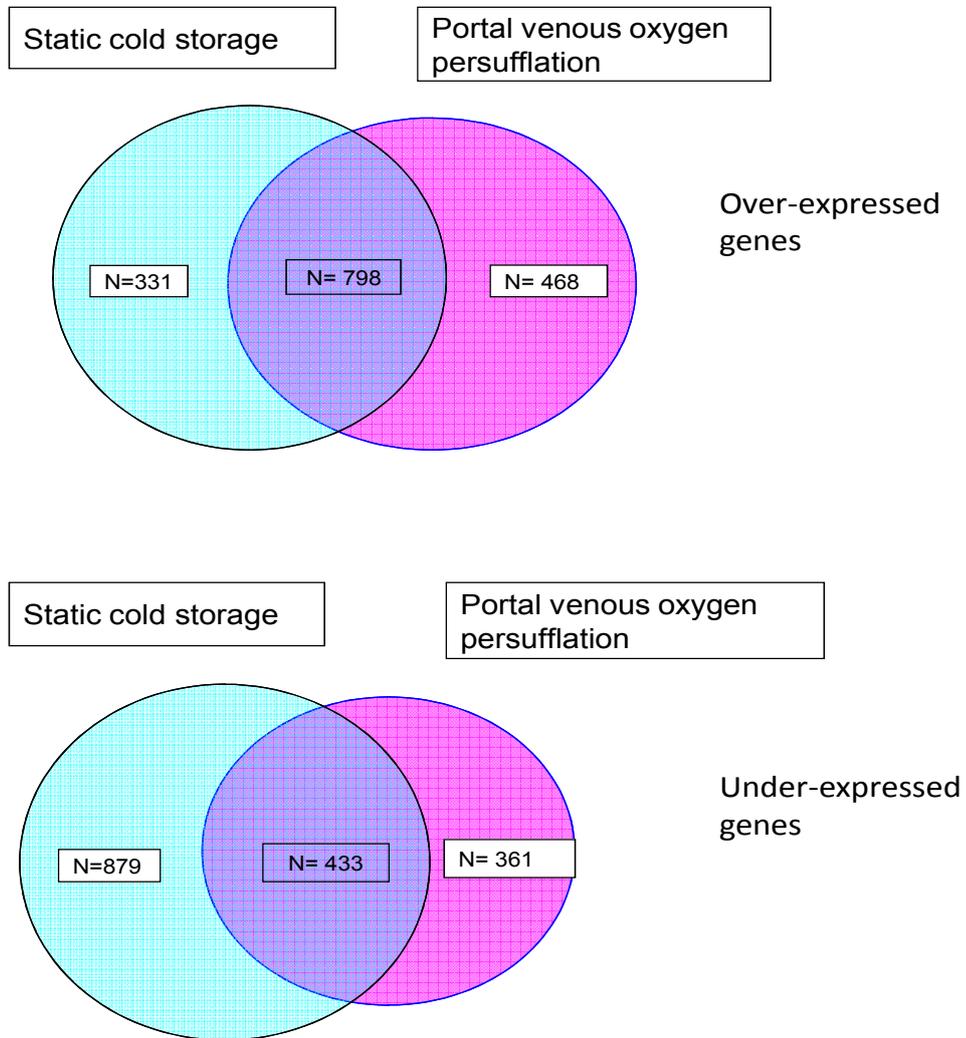
Figure 54 Venn diagrams showing the number of common and unique differentially expressed genes in the two preservation groups- direction of differential expression
The number of differentially expressed genes in the two preservation groups is shown. The top Venn diagram shows the genes over-expressed in the static cold storage group. Similar numbers of genes were over-expressed in both preservation groups with a large number of common genes. The bottom Venn diagram shows the genes under-expressed in static cold storage group. Under-expressed genes were more common in the static cold storage group. Over twice as many genes were uniquely under-expressed in the static cold storage group as compared to the portal venous oxygen persufflation group.

Table 6 List of ten most over-expressed genes identified in the static cold storage group

The gene title, the biological function of its encoded protein is shown. The Entrez gene identifier[261] for each gene was extracted from http://www.ncbi.nlm.nih.gov/gene. The fold increase in expression as compared to the control group pancreas is shown. These were genes encoding chaperone proteins, transcription factors and genes involved in cytokine response to cellular injury.

Gene title	Function	Entrez gene	Fold change
Heat shock protein 1B	Chaperone protein	294254	3156.5
FBJ osteosarcoma oncogene	Transcription factor	314322	1235.8
Activating transcription factor 3	Transcription factor	25389	1058.5
Chemokine (C-X-C motif) ligand 10	Chemokine	245920	485.5
Heat shock protein 1A	Chaperone protein	24472	449.8
Chemokine (C-X-C motif) ligand 1	Chemokine	81503	326.6
Protein phosphatase 1, regulatory subunit 15A	Enzyme	171071	243.4
Interleukin 6	Cytokine	24498	226.3
Early growth response 1	Transcription factor	24330	162.9
Zinc finger protein 36	RNA binding protein	79426	162.6

Table 7 List of ten most under-expressed genes in the static cold storage group

The gene title and biological function of its encoded protein are shown. The Entrez gene identifier[261] for each gene was extracted from http://www.ncbi.nlm.nih.gov/gene. The fold decrease in expression as compared to the control group pancreas is shown. These genes were primarily those involved in the cell's immune response to antigens, cell surface components.

Gene title	*Function*	*Entrez gene*	*Fold change*
Immunoglobulin heavy chain (alpha polypeptide)	Immunoglobulin subunit	314487	289.3
Carbonic anhydrase 3	Enzyme	54232	246.2
Rho, GDP dissociation inhibitor (GDI) beta	Rho protein signal transduction	362456	152.6
Cd53 molecule	Cell membrane protein	24251	91.6
Beta globin minor gene	Haemoglobin subunit	LOC 100134871	67.6
Similar to T-cell receptor alpha chain precursor V and C regions (TRA29)	Cell surface receptor	290071	59.6
Coronin, actin binding protein 1A	Cytoskeletal protein	155151	53.7
Membrane-spanning 4-domains, subfamily A, member 6B	Cell surface receptor	293749	50.0
T-cell receptor beta chain	Cell surface receptor	24820	48.3
Immunoglobulin joining chain	Immunoglobulin subunit	360922	46.1

Table 8 List of top ten most over-expressed genes in the portal venous oxygen persufflation group

The gene title and biological function of its encoded protein is shown. The Entrez gene identifier [261] for each gene was extracted from http://www.ncbi.nlm.nih.gov/gene. The fold increase in expression as compared to the control group pancreas is shown. These were genes involved in stress response, cellular cytokine response and transcription factors.

Gene title	Function	Entrez gene	Fold change
Heat shock protein 1B	Chaperone protein	294254	2139.7
FBJ osteosarcoma oncogene	Transcription factor	314322	1283.6
Activating transcription factor 3	Transcription factor	25389	935.4
Chemokine (C-X-C motif) ligand 1	Chemokine	81503	500.6
Heat shock protein 1A	Chaperone protein	24472	355.5
Interleukin 6	Cytokine	24498	246.6
Chemokine (C-X-C motif) ligand 2	Chemokine	114105	223.5
Protein phosphatase 1, regulatory (inhibitor) subunit 15A	Enzyme subunit	171071	189.3
SERTA domain containing 1	Transcription regulator	361526	184.8
Early growth response 1	Transcription factor	24330	151.5

Table 9 List of top ten most under-expressed genes in the portal venous oxygen persufflation group

The gene title and biological function of its encoded protein is shown. The Entrez gene identifier[261] for each gene was extracted from http://www.ncbi.nlm.nih.gov/gene. The fold decrease in expression as compared to the control group pancreas is shown. These genes were primarily those involved in haemopoesis and lipid metabolism.

Gene title	Function	Entrez gene	Fold change
Haemoglobin alpha, adult chain 2	Subunit of haemoglobin	25632	675.7
Carbonic anhydrase 3	Enzyme	54232	202.4
Haemoglobin, beta	Subunit of haemoglobin	24440	104.2
Aminolevulinate, delta-, synthase 2	Enzyme (haeme synthesis)	25748	31.8
Adiponectin, C1Q and collagen domain containing	Protein hormone related to fat catabolism	246253	22.9
Complement factor B	Complement factor	294257	13.2
Deoxyguanosine kinase	Mitochondrial enzyme	297389	8.9
Malic enzyme 1, NADP(+)-dependent	Enzyme	24552	8.6
N-acylsphingosine amidohydrolase 1	Enzyme	84431	8.4
Retinol binding protein 4, plasma	Carrier protein	25703	7.4

6.3.2 Analysis of the gene ontology of differentially expressed genes

Gene ontology analysis of the datasets of the two preservation groups showed multiple similarities. The five top represented terms in the three ontologies for both preservation groups are presented (Tables 10, 11). Common biological processes identified in both preservation groups included cytokine production, cellular response to stress and negative regulation of apoptosis. Genes related to antigen processing and presentation were over-represented in the static cold storage group. Interestingly, the majority of these genes were under-expressed in the static cold storage group as compared to control group. The NF-κB cascade was significantly over-represented in the portal venous oxygen persufflation group. Analysis of the cellular component terms showed that genes coding for cell surface components and major histocompatibility complex proteins were over-represented in both groups. Transporter associated with antigen presentation (TAP) complex proteins were significantly over-represented in the static cold storage group alone. Major molecular function terms over-represented were related to transcription, protein kinase and cytokine/chemokine activity in both groups. GO analysis of the differentially expressed genes between the two preservation groups identified significant over-representation of GO terms related to antigen transport and presentation.

Table 10 Over-represented Gene Ontology terms in the static cold storage group

Results of the gene set for static cold storage group is shown. Rat230_2 array was used as the background while the Affymetrix identifiers of the differentially expressed genes were entered as the gene list. GO terms containing 5-50 genes with an EASE score less than 0.01 were selected. The top five distinct terms in each ontology are presented in the table. The actual number of genes associated with each term is included. The actual p value, fold enrichment and false discovery rate (FDR) is included.

Ontology	GO term description	GO Term	Gene Count	P value	Fold Enrichment	FDR
Biological process	Cytokine production	GO:0001816	28	5.04E-07	2.815835289	9.57E-04
	Antigen processing and presentation	GO:0019882	22	9.56E-06	2.839300583	0.018174
	Negative regulation of transferase activity	GO:0051348	23	1.10E-05	2.740024339	0.020821
	Regulation of immune response	GO:0050776	29	1.20E-05	2.388966641	0.02275
	Chemotaxis	GO:0006935	30	1.20E-05	2.346529407	0.022876
Cellular component	TAP complex	GO:0042825	9	2.24E-05	5.825789923	0.034499
	Cell surface	GO:0009986	41	6.46E-05	1.895693546	0.099309
	Multivesicular body	GO:0005771	7	5.92E-04	5.437403928	0.90596
	Adherens junction	GO:0005912	20	0.001462	2.157699972	2.22448
	Caveola	GO:0005901	15	0.00269	2.377873438	4.05578
Molecular function	Chemokine receptor binding	GO:0042379	15	3.65E-05	3.421980242	0.064966
	Peptide antigen-transporting ATPase activity	GO:0015433	9	5.25E-05	5.369876688	0.093406
	Transcription repressor activity	GO:0016564	42	2.61E-04	1.770502821	0.464423
	Unfolded protein binding	GO:0051082	22	0.001819	2.031461287	3.191087
	Protein kinase inhibitor activity	GO:0004860	12	0.002006	2.820541291	3.514

Table 11 Over-represented Gene Ontology terms in the portal venous oxygen persufflation group

The DAVID genomic database was used to identify Gene ontology terms over-represented in the gene subsets. Results of the gene set for static cold storage group is shown. Rat230_2 array was used as the background while the Affymetrix identifiers of the differentially expressed genes were entered as the gene list. GO terms containing 5-50 genes with an EASE score less than 0.01 were selected. The top 5 distinct terms win each ontology are presented in the table. The actual number of genes associated with each term is included. The actual p value, fold enrichment and false discovery rate (FDR) is included.

Category	GO description	GO Term	Count	P value	Fold Enrichment	FDR
Biological processes	I-kappaB kinase/NF-kappaB cascade	GO:0007249	31	1.24E-06	2.5880	0.0024
	Cytokine production	GO:0001816	24	4.78E-06	2.8363	0.0091
	Response to biotic stimulus	GO:0009607	41	1.34E-05	2.0388	0.0254
	Response to unfolded protein	GO:0006986	22	1.85E-05	2.7805	0.0351
	Negative regulation of apoptosis	GO:0043066	47	2.48E-05	1.8841	0.0472
Cellular component	Adherens junction	GO:0005912	21	7.07E-05	2.6319	0.1086
	MHC protein complex	GO:0042611	13	1.23E-04	3.5548	0.1886
	Cell junction	GO:0030054	35	2.32E-04	1.9141	0.3553
	Basement membrane	GO:0005604	15	0.0014	2.5539	2.1964
	Cell-matrix junction	GO:0030055	13	0.0023	2.6661	3.4945
Molecular function	Transcription repressor activity	GO:0016564	44	7.34E-07	2.2029	0.0013
	Chemokine receptor binding	GO:0042379	13	1.41E-04	3.5223	0.2503
	Cytokine activity	GO:0005125	38	2.76E-04	1.8424	0.4913
	Protein kinase inhibitor activity	GO:0004860	11	0.0019	3.0707	3.3864
	Unfolded protein binding	GO:0051082	19	0.0034	2.0837	5.8835

6.3.3 Identification of over-represented canonical pathways

Ingenuity pathway analysis identified sixty canonical pathways that were significantly over-expressed in the static cold storage group. Thirty-six pathways were significantly over-expressed in the portal venous oxygen persufflation group. Pathways involved in the cellular response to stress and inflammation and activation of transcription were over-represented in both preservation groups (Table 12).

The over-representation of the pathways of interest was compared between the two preservation groups. There was significant over-representation of all pathways in the static cold storage group. The PI3K/Akt signalling and NF-κB signalling pathways only were significantly over-represented in the portal venous oxygen persufflation group (Table 13).

Canonical Pathway	Static cold storage		Portal venous oxygen persufflation	
	p value	Ratio	p value	Ratio
Glucocorticoid Receptor Signalling	0.0001	0.19	0.024	0.15
Acute Phase Response Signalling	0.0006	0.22	0.0014	0.18
NRF2-mediated Oxidative Stress Response	0.0015	0.20	0.0021	0.17
IL-6 Signalling	0.0018	0.25	0.0013	0.23
Hypoxia Signalling in the Cardiovascular System	0.0155	0.23	0.0026	0.24
IL-10 Signalling	0.0019	0.24	0.003	0.26

Table 12 Over-represented canonical pathways in the two preservation groups

Table shows the top 5 over-represented canonical pathways related to ischaemia reperfusion injury in both the preservation groups when each was compared to the control group pancreases. Canonical pathway analysis was carried out using Ingenuity Pathways Analysis library of canonical pathways. The significance of the association between the data set and the canonical pathway was measured by the ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway. The Fischer's exact test was used to calculate a p-value. A p value less than 0.05 was considered statistically significant.

There was over-representation of common pathways in both preservation groups. Glucocorticoid receptor signalling pathway is related to the binding of cortisol to the glucocorticoid receptor. Nuclear translocation of the ligand-receptor complex leads to increased transcription. Acute phase response signalling is the mechanism of the cell's response to stress. This includes transcription of cytokines and chaperone proteins, and activation of the JAK/STAT pathway. Nuclear Factor 2 is activated by oxidative stress and induces the transcription of genes with anti-oxidant function. Hypoxia signalling is related to the activation of the hypoxia inducible factor and the resultant transcription of proteins such as heat shock proteins, enzymes of the ubiquitin system and the NF-κB pathway. Interleukins 6 is involved in the local and systemic inflammatory response to ischaemia reperfusion injury. IL10 is an anti-inflammatory cytokine and inhibits synthesis of pro-inflammatory cytokines.

Table 13 Over-representation of the pathways of interest in two preservation groups

Ingenuity Canonical Pathways	Static cold storage		Portal venous oxygen persufflation	
	p value	Ratio	p value	Ratio
ERK/MAPK Signalling	0.0042	0.18	0.2101	0.10
NF-κB Signalling	0.0159	0.18	0.0334	0.15
p38 MAPK Signalling	0.0200	0.21	0.1604	0.14
PI3K/Akt Signalling	0.0478	0.15	0.0235	0.15

Table shows the canonical pathways of interest in both the preservation groups when each was compared to the control group pancreases. Canonical pathways analysis was carried out using Ingenuity Pathways Analysis library of canonical pathways. The significance of the association between the data set and the canonical pathway was measured by the ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway. The Fischer's exact test was used to calculate a p-value. A p value less than 0.05 was considered statistically significant.

There was significant over-representation of all four pathways in the static cold storage group. Only PI3K/Akt signalling and NF-κB signalling were over-represented in the portal venous oxygen persufflation group. Over-representation indicates that a significant number of genes related to the pathway were differentially expressed by a fold change of at least 2.0 in comparison to the control group pancreas. It does not take into account whether the genes are over-expressed or under-expressed. Over-representation of a pathway can indicate either significant over-expression or suppression of that pathway in comparison to the control.

6.3.6 PI3K/Akt/NF-κB pathway

The PI3K/Akt and NF-κB pathways are described in chapter 1. The following MAPP diagram (Figure 55) details the interactions and links between the two pathways. Briefly,activation of growth factor receptors by growth factors or cellular stress leads to the activation of the phosphatidyl inositol-3 kinases (PI3K). PI3K phosphorylate the inositol ring of phosphatidyl inositol to produce phosphatidyl inositol (3,4,5)-trisphosphate (PIP3). PIP3 activates downstream serine-threonine protein kinases such as Protein kinase B (Akt/PKB) through the kinase PDK1. Activation of Akt/PKB is tightly regulated by the quantity of the second messenger phosphatidyl inositol triphosphate.(PIP3). Akt itself can be dephosphorylated by the action of protein phosphatases. Akt contributes to several cellular functions including inhibition of apoptosis and cell survival, nutrient metabolism, cell growth, transcriptional regulation. Akt inhibits apoptosis by the phosphorylated inhibition of BAD and caspase 9. It phosphorylates and inactivates the enzyme glycogen synthetase kinase 3 and increases transcription of glucose transporter (GLUT 1) and the glycolytic enzyme phospho-fructo-2-kinase. Akt activates the NF-κB pathway through direct activation of IKK and through phosphorylation of the MAP3K, Tpl2. Activation of the NF-κB pathway also occurs through trans-membrane receptor complexes (TNF receptor superfamily, IL-1 receptor and the TLR family). Hypoxia, reactive oxygen species and cytokines such as TNFα and IL-1β activate the NF-κB pathway through these receptors. NF-κB elements are usually present in the cytoplasm as homo or heterodimers. They are localised to the cytoplasm by

being complexed to proteins called the Inhibitors of Kappa B (IκB). Activation of the NF-κB pathway involves the breakdown of these complexes by proteosomal degradation of the IκB subunits and translocation of the NF-κB elements into the nucleus. This is catalyzed by the action of protein kinases called the inhibitors of kappa kinase (IKK) complex. In the nucleus NF-κB elements induce the transcription of genes related to cytokines, cell surface receptors, pro-apoptotic and anti-apoptotic proteins, adhesion molecules and anti-oxidants. NF-κB pathway is regulated by several feedback loops including increased transcription of the IκBε gene in response to NF-κB activation.

Differential expression of pathways in two preservation groups

Differential expression of the catalytic and regulatory subunits of the PI3K enzyme was demonstrated in the two preservation groups. Static cold storage was associated with decreased expression of the catalytic subunit and increased expression of the regulatory subunit. Portal venous oxygen persufflation was associated with a decreased expression of the regulatory subunit without differential expression of the catalytic subunit as compared to control. This indicates a net over-expression of PI3K enzyme activity. There was no differential expression of the Akt isoforms in either groups. The expression of the catalytic subunit of protein phosphatase 2 was elevated in the static cold storage.

The levels of IKKβ were over-expressed in the portal venous oxygen persufflation group (2.3 fold) but not in the static cold storage group. There was increased expression of three NF-κB proteins in the portal venous oxygen

persufflation group and two in the static cold storage group. IκBε was over-expressed in the portal venous oxygen persufflation group (4.5 fold) suggestive of increased NF-κB pathway activation.

Several genes downstream of the Akt/NF-κB pathway were differentially expressed. Genes coding for enzymes involved in energy metabolism were over-expressed in the portal venous oxygen persufflation group. These included the glucose transporter GLUT-1(2.3 fold), hexokinase 2 (4.9 fold) and phospho-fructokinase platelet isoform (6.1 fold). Increased expression of anti-apoptotic genes such as Bcl2A1, CFLAR, BIRC2, BIRC3 was noted preferentially in the portal venous oxygen persufflation group.

Figure 55 MAPP delineating the PI3Kinase-Akt-NF-KB pathway and the differential expression of genes related to this pathway in the two preservation groups.

This MAPP was developed by this researcher (Mettu Reddy) for the purpose of this research project using the MAPPbuilder facility available at GenMAPP version 2.1. The final version of the MAPP is available for download at http://www.wikipathways.org/index.php/Pathway:WP1491.

Genes are denoted by rectangles with arrows showing the relation between individual genes. Rat genes (Rattus norvegicus) were identified in the MAPP set by their Entrez identities. When multiple isoforms of a single gene were encountered, the specific Affy id was used. The rectangle for each gene is split into two parts to show the nature of differential expression of that particular gene for each of the two preservation groups. The colour codes for over-expression and under-expression for each preservation group are noted in the diagram legend.

PI3K_AKT_NFKB pathway

Detailed legend in previous page

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6.3.7 Mitogen Activated Protein Kinase pathways

General description of the mitogen activated protein kinase (MAPK) pathways is given in chapter 1. The following MAPP diagram (figure 57) details the different components of these pathways.

The ERK/MAPK pathway is primarily activated by growth factors through cell surface receptors. Binding of growth factors to the receptors leads to the activation of the G protein Ras. Activated Ras binds and activates the MAP3K (Raf). Activation of Raf also occurs through the diacyl glycerol pathway via Protein kinase C and the calcium signalling pathway through rasGRP proteins. Raf phophorylates MAP2K1 and MAP2K2. These phosphorylate the MAPKs for this pathway, ERK1 or ERK2. ERK activates several transcription factors. It activates the p90 ribosomal s6 kinase which is involved in translation. p90RSK also inhibits the pro-apoptotic Bcl2 protein BAD through phosphorylation. ERK pathway is mainly involved in cell proliferation and differentiation, cell division.

The p38 MAPK pathway is activated by cellular stress factors such as heat shock, reactive oxygen species, lipopolysaccharide and inflammatory cytokines. These act through a variety of protein kinases to activates multiple MAP3Ks. These in turn activate the specific MAP2Ks namely MAP2K3 and MAP2K6. The MAPK for this pathway is the p38 protein. p38 activates a variety of transcription factors and inflammatory mediators such as the p53 pathway,ELK-1, sap1a, gadd153, transcription factors such as MAX,MEF2c,ATF2, ATF4,heat shock proteins and ATF4. The net result is the progression of the inflammatory process

and induction of apoptosis. The activation of this pathway is kept under control by the dual specificity phosphatases (DUSP) and protein tyrosine phosphatases (Ptpn) which inhibit the activation of p38.

Jun amino-terminal kinases (JNK/MAPK) pathway is activated by a variety of environmental stresses and inflammatory cytokines. Stress signals are delivered to this pathway by GTPases such as Rac and Rho. These activate a member of the mixed lineage kinases (MLK) that phosphorylate and activate MAP2K4 or MAP2K7. This activates JNK proteins which translocate to the nucleus and regulate the activity of multiple transcription factors involved in inflammation and apoptosis.

Differential expression of pathways in two preservation groups

There was significant over-representation of the ERK/MAPK pathway in the static cold storage group but not in the portal venous oxygen persufflation group (Figure 56). Under-expression of growth factor receptors was seen predominantly in the portal venous oxygen persufflation group. Decreased expression of the RAS guanine nucleotide exchange factor and protein kinase C was seen in the static cold storage group. These proteins are involved in the activation of the MAP3K (Raf) through calcium signalling and diacyl glycerol pathway respectively. There was no differential expression of Raf and MEK1/2 noted in either preservation groups. However increased expression of MAPK1/ERK was noted in the static cold storage group. Dual specificity phosphatases were over-expressed in both preservation groups. Fifteen

downstream targets of the ERK/MAPK were differentially expressed in the two preservation groups (Figure 56) . Ten were over-expressed in both groups. These included proteins involved in transcription (Myc, Fos,MKNK2, STAT3) and translation (EIF4E). Four were uniquely expressed in the static cold storage group. Phospholipase A2, ELF1 and Talin 1 were over-expressed while ETS1, a transcription factor, was under-expressed. One target gene p90 Ribosomal S6 kinase 1 was uniquely over-expressed in the portal venous oxygen persufflation group.

There was significant over-representation of the p38 MAPK pathway in the static cold storage group but not in the portal venous oxygen persufflation group (Figure 57). In both preservation groups the inducers of p38 pathway such as TNFα, IL1β were over-expressed. Components of TNFα receptor, TRAF 2 and 6 were differentially expressed in portal venous oxygen persufflation (over expressed) and static cold storage (under-expressed) respectively. The MAP3K for this pathway were not differentially expressed in either group. Over-expression of Map2k3 and Map2k6 was seen in the static cold storage group while portal venous oxygen persufflation had up regulation of Map2k3 alone. None of the p38 isoforms were differentially expressed in either group. Amongst the downstream products for this pathway, eight downstream targets of the p38/MAPK were differentially expressed in the two preservation groups. Five were over-expressed in both groups. These included proteins involved in transcription such as Ddit3, My, SRF, MKNK2 and the histone protein H3F3C. One gene was under-expressed in both groups (Cell division cycle homolog B).

Two were uniquely expressed in the static cold storage group (Phospholipase A2 and MYC associated factor X).
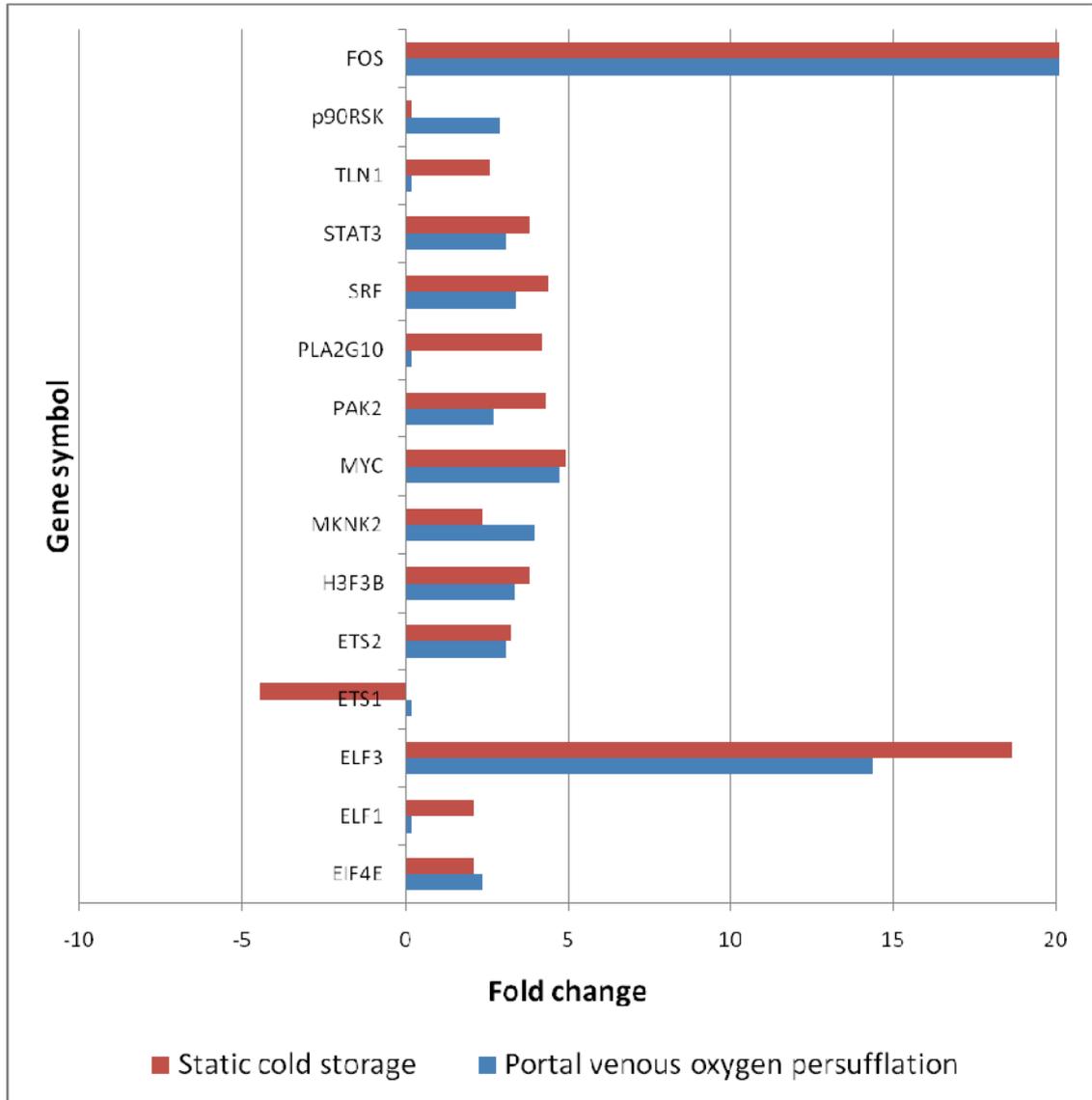
Figure 56 Bar diagram comparing the differential expression of fifteen target genes of the ERK/MAPK pathway in both preservation groups

Fifteen downstream targets of the ERK/MAPK were differentially expressed in the two preservation groups. Eleven were over-expressed in both groups. These included proteins involved in transcription (Myc, Fos,MKNK2, STAT3) and translation (EIF4E). Three were uniquely expressed in the static cold storage group. Phospholipase A2, ELF1 and Talin 1 were over-expressed while ETS1 was under-expressed. p90 Ribosomal S6 kinase 1 was uniquely over-expressed in the portal venous oxygen persufflation group.

Figure 57 MAPP showing the MAPK pathways and the differential expression of genes in the two preservation groups

Figure in opposite page.

This MAPP was modified from the version available at GenMAPP version 2.1. Genes are denoted by rectangles with arrows showing the relation between individual genes. The rectangle for each gene is split into two parts to show the nature of differential expression of that particular gene for each of the two preservation groups. The colour codes for over-expression and under-expression for each preservation group are noted in the diagram legend.

MAPK signaling pathway

Heteromeric G-protein

Phosphatidylinositol signaling system

IP3

cAMP

Pr1

Ca2+

DAG

MKP

Dusp1
Dusp6

STAT3
Tau
STMN1

PTP

Classical MAP kinase Pathway

Ngfb
Bdnf
Ntf5

Ntrk1

Rasgrf2
Rasgrp4
Rasgrp1

Prkcd
Prkcc
Prkcb1
Prkcz
Prkch

Rap1b
RAP-1A

RGD:708516
Ptpn5

PLA2G10
p90-RSK2
MKNK2

ATF4

HBEGF
Egf
Fgf4
Pdgfb
PDGFA

Egfr
fgfr
Pdgfrb
PDGFRA

Grb2

SOS1

Kras2
Nras
Mras

Nf1
Rasa1

Braf
Raf1
Mos

Map2k1
Map2k2

Mapk1
Mapk3

elk-1
ELK-4
Myc
ELF1
EIF4E

SRF → DNA → Fos → DNA → Proliferation, differentiation

Ppp3cc
Ppp3ca
Ppp3cb
Ppp3r1
Ppp3r2

Gna12

Gap1m

flna   JSAP1
Arrb1   Crk
Arrb2   Crkl

JNK

Hspa8
Hspa1a
Hspa5

nfat2/4

JNK & p38 MAP kinase pathway

Integrin-mediated cell adhesion

Map4k3   Map3k8

Rac2
Rac1
Cdc42

hgk
hpk1
Pak1
Pak2

Map3k1
mlk3
Map3k2

mkk4
mkk7

Mapk8
Mapk10
Mapk9

Jun
Jund

DNA

Serum, cytotoxic drugs, irradiation, heatshock, reactive oxy species, LPS and other stress

Casp6
Casp2
Casp8
Casp7
Casp9
Casp3
Casp1

Stk3
Stk4

Izk
Map3k12
mltk

Akt1
Akt2
Akt3

Mapk8ip

PTP

Ppm1a RGD:708516
Ptpn5
Ptprr

Atf2
Elk-1
Tp53 → p53 signaling

Tnf
Il1a
Il1b
Tnfsf6

Prkcd
Il1r2
Il1r1
Tnfrsf6

Traf2 → gck

ask2

Map3k5

Map2k3
Map2k6

Mapk13
Mapk12
Mapk14

p38

PLA2G10
H3F3C

Proliferation, differentiation, inflammation

Apoptosis

SRF
MYC
MKNK2
Ddit3
Max
mef2c

DNA

Tgfb3
Tgfb1
Tgfb2

Acvr1c
Acvr1b
Tgfbr1
Tgfbr2

Daxx

Map3k7

Ppp5c

Ppm1b

LPS

Cd14

tab1
tab2
ecsit

Traf6

prak
Mapkapk2
msk1/2

Hspb2
Hspb1
Atf4

DNA damage

Gadd45a

mekk4

Taok1
Taok2

Cdc25b

nlk → Wnt signaling pathway

ERK5 pathway

Serum, EGF, reactive oxygen species, or Srk tyrosinkinase downstream

Map2k5 → Mapk7 → Nr4a1 → DNA → Proliferation, differentiation

MAPKKKK   **MAPKKK**   **MAPKK**   **MAPK**   **Transcription factors**

Ikbkb → Nfkb1 → Proliferation, inflammation, anti-apoptosis

Detailed legend in the previous page

6.3.8 Expression of genes related to cell repair and metabolic activity

Transporter activity

Genes coding for the α and β subunits of haemoglobin were under-expressed in both groups. The suppression was greater in the oxygen persufflation group. When expression of genes related to transporter activity was compared, 42 genes were differentially expressed. Of these, 32 genes in the oxygen persufflation group were over-expressed when compared to ten genes in the static cold storage group. Over-expressed genes in the portal venous oxygen persufflation included transporters of glucose, amino-acids and phospholipids.

Enzymes involved in the glycolytic pathway

Hexokinase which catalyses the first rate-limiting step of glycolysis was over-expressed in both static cold storage and Portal venous oxygen persufflation (2.1 fold vs. 4.9 fold). Phospho-fructokinase (PFK) is the enzyme catalysing the most important and irreversible step in glycolysis. Over-expression of the platelet isoform of PFK (PFKP) was noted in the portal venous oxygen persufflation group (6.1 fold) while the liver isoform was under-expressed in the static cold storage group. Downstream to glycolysis, static cold storage group was characterised by increased activity of the pyruvate dehydrogenase kinase enzyme which inactivates pyruvate dehydrogenase. Pyruvate dehydrogenase catalyses the rate limiting step in the entry to Krebs cycle. Expression of pyruvate dehydrogenase phosphatase which dephosphorylates and activates PDH were over expressed in both groups (Figure 58).

Sterol biosynthesis

Over-expression of genes related to sterol biosynthesis was noted in the portal venous oxygen persufflation group (Figure 59).

Glycolysis and Gluconeogenesis

Glucose

Hk1
Hk2
Hk3
Gck

G6pc

↓ Glycolysis
↑ Gluconeogenesis

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Gene Database
Rn-Std_20070817.gdb
Expression Dataset

Name: three merge set
Color Sets:
Merged PVOP
Merged SCS
Gene

Legend: Merged PVOP
PVOP>=2.0
PVOP<=-2.0
No criteria met
Not found

Legend: Merged SCS
SCS>=2.0
SCS <= -2.0
No criteria met
Not found

Glucose-6-phosphate

Gpi

Fructose-6-phosphate

Pfkm
Pfkl
Pfkp

Fbp1

Fructose-1,6-bisphosphate

Aldoa
Aldob
Aldoc

Glyceraldehyde-3-phosphate ← Tpi1 → Dihydroxyacetone Phosphate

Gapd

1,3-Bisphosphoglycerate

Pgk1
Pgk1

3-Phosphoglycerate

Pgam2
Pgam1

2-Phosphoglycerate

Eno1
Eno3
Eno2

Phosphoenolpyruvate

Pck1

Got1

Aspartate

Aspartate

Got2

Oxaloacetate

Oxaloacetate

Mdh1

Mor1

Cytosol
Mitochondrion

Pkm2
Pklr
Pkm2

Malate

Malate

Pyruvate

Ldha
Ldhb
Ldhc

Pyruvate

Pc

TCA Cycle

Lactate

PDK2
PDK3
PDK4

Dlat
Pdha1
Pdha2
Pdhb

PDP1
PDP2

Acetyl-CoA

Figure 58 MAPP of the glycolytic pathway comparing the differential expression of genes in the two preservation groups.

Both the rate-limiting enzymes of glycolysis, hexokinase and phospho-fructokinase were over-expressed in the portal venous oxygen persufflation group. The liver isoform of phosphofructokinase was under-expressed in the static cold storage group. Lactate dehydrogenase converts pyruvate to lactate, clears the end-product of glycolysis and drives glycolysis. This was under-expressed in the static cold storage group.

Cholesterol Biosynthesis
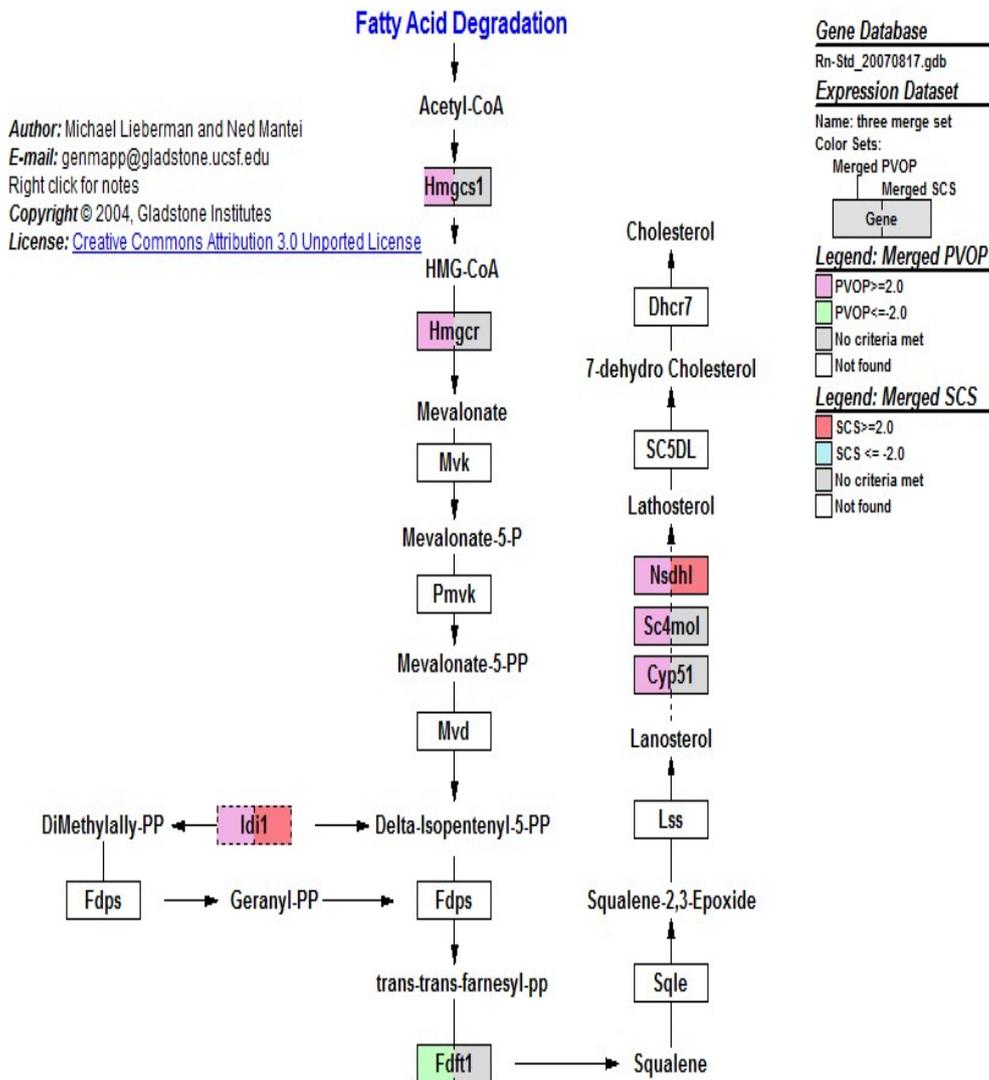
Figure 59 MAPP of the cholesterol biosynthetic pathway showing the differential expression of genes in the two preservation groups.

The cholesterol biosynthetic pathway includes the melavonate pathway. The products of this pathway include dimethyl-allyl-pyrophosphate, isopentenyl pyrophosphate and cholesterol. These are required for the synthesis of molecules involved in cell membrane maintenance, protein folding, protein attachment to cell membranes and steroid biosynthesis. The rate-limiting enzyme for this pathway is Hydroxy-Methylglutaryl CoA reductase (Hmgcr). Portal venous oxygen persufflation was associated with the increased expression of several genes coding for enzymes involved in sterol biosynthesis including the rate-limiting enzyme Hmgcr.

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6.4 Discussion

Gene microarray data give information regarding the relative difference in the amount of mRNA related to the genes of interest. Best practice suggests that microarray data should be confirmed with quantitative PCR. We have not done this for the rat pancreas dataset. However, the validity of the microarray technique has been confirmed by our group in parallel microarray experiments using RNA from rat kidneys. Good concordance of microarray data and quantitative PCR of selected genes in this dataset has been confirmed (Ms Kibondo, PhD student, University of Sunderland).

The amount of gene specific mRNA present in the cell depends both on gene transcription and the half-life of the specific mRNA. Half-life of mRNAs can vary from a few minutes to several hours and is related to their function rather than size[262-264]. Cellular mechanisms have been described which modulate this in response to the cell state [265, 266]. Cheadle *et al.*[267] reported that 50% of the differential expression data obtained using microarrays can be directly attributed to differences in half-lives of various mRNAs. Finally, gene expression is only the beginning of the overall cellular response. Ultimate response depends on the levels of proteins, their phosphorylation status and integration of multiple cellular pathways. We have not attempted to investigate these in our study. In view of these factors, the results from these experiments should be considered preliminary data only and to be further investigated by suitable experiments.

We have identified a large number of differentially expressed genes in both study groups compared to the control group. It is not possible to decide on a statistically sound cut-off fold change due to the absence of replicates in the study. The 2 fold change cut-off is an accepted compromise when there are no replicate samples to apply stricter tests to assess the validity of the data. This cut-off also gives gene lists of reasonable size for downstream analysis (Dr H.Peters, Center for Life, University of Newcastle, personal communication).

Genes related to transcription, heat shock proteins and cytokine response were over-expressed in both preservation groups as previously reported by other studies[253, 268]. Nearly three times more genes were uniquely under-expressed in the static cold storage than in the portal venous oxygen persufflation group. This could be suggestive of a partial shut-down of transcriptional activity or more rapid turnover of mRNAs in the static cold storage group.

Analysis using the gene ontology terms database showed marked similarities in the terms over-represented in both two preservation groups. Biological processes such as cytokine production, antigen processing and presentation, chemotaxis, apoptosis were identified in both preservation groups and have been previously reported. The overrepresented cellular component terms in both groups were related to the membrane structures and components involved in intercellular communications. The molecular processes over-represented in both groups were related to transcription and the immune response. Activation of NF-

κB pathway, was significantly over-represented in the portal venous oxygen persufflation group.

Our results suggest that the genes related to the death receptor pathway of apoptosis were over-expressed in the portal venous oxygen persufflation group. This included the caspases 3 and 8 and Bid. This suggests an increased propensity for the portal venous oxygen persufflation group pancreas to undergo apoptosis. Though this might suggest a worse outcome for this preservation group, it is not necessarily so. Ischaemia and reperfusion will induce severe cellular and mitochondrial injury in both preservation groups. Mitochondrial injury with mitochondrial permeability transition has been reported to be an important step during both apoptosis and necrosis[244]. Cells undergo apoptosis only when sufficient ATP reserves are available. Progression of apoptosis cannot occur in ATP depleted cells which undergo death by necrosis. Necrotic death has the distinct disadvantage of exacerbating the inflammatory process leading to continued recruitment of pro-cell death pathways. In an interesting study, Zhao *et al.* investigated the effect of artemisinin, a known inducer of cellular apoptosis in experimental acute pancreatitis[245]. They found that administration of this drug increased apoptosis, decreased necrosis and improved the markers of inflammation such as serum amylase, histological grading and serum cytokine levels. Similar conclusions have been reached by other studies [246, 247]. In a study by Lemasters and co-workers in 2003 [248] the nature of hepatocyte death after ischaemia reperfusion was investigated. They found that 4 hours of culture in anoxic medium followed by two hours of

oxygenated reperfusion caused necrosis in 60% of cells. Provision of fructose for 20 minutes prior to start of reperfusion and during the reperfusion period lead to increased glycolysis, increased ATP, increased apoptosis and decreased necrosis. The authors concluded that both apoptosis and necrosis occur through mitochondrial permeability transition. Induction of glycolysis increases the energy status of tissues and redirects cellular outcome from necrosis to apoptosis.

Increased expression of anti-apoptotic proteins was seen in both study groups. This was more prominent in the portal venous oxygen persufflation group indicating a more robust cellular response to ischaemia reperfusion injury. The increased expression of genes related to the glycolytic pathway, decreased suppression of transporter gene expression and increased sterol synthesis are again markers of increased metabolic activity and initiation of cellular repair processes in the portal venous oxygen persufflation group.

PI3K/Akt and the ERK/MAPK are important survival pathways involved in the recovery of cells after ischaemia reperfusion. Studies examining the biology of post-conditioning during myocardial reperfusion have identified these pathways to be responsible for the beneficial effect [269]. Activation of both these pathways occurs classically through growth factor receptors. Activation of the ERK/MAPK pathway has also been reported through the protein kinase C pathway[103]. Similarly, activation of Akt by cytokines through PI3K and heat shock proteins has been described[110]. All these pathways are relevant in our model. Protein kinase C expression was decreased in the static cold storage

group. Similarly decreased expression of the catalytic subunit of PI3K and increased expression of the regulatory unit indicate an overall decreased activity of the enzyme. This suggests a decreased induction of these survival pathways in the static cold storage group.

Increased activation of the NF-κB pathway was also identified in the portal venous oxygen persufflation group. NF-κB is a transcription factor with numerous target genes. Its role in ischaemia reperfusion injury has been previously studied. The conclusions have varied in different studies. Chang and co-workers in 2003 reported that NF-κB activity decreased apoptosis induced by TNFα[125]. Sarkar and co-workers in 2009 reported that activation of NF-κB by cytokines in beta cells increased production of the anti-apoptotic proteins[270]. NF-κB activation has also been reported to inhibit the pro-apoptotic JNK/MAPK pathway through multiple mechanisms[126].

Activation of NF-κB can occur through the TNFα/FAS receptor, Toll like receptor[117] and PI3K/Akt pathways. We have identified that all three pathways were relevant in our study. While increased expression of a variety of genes which are known downstream targets of NF-κB were identified in both groups this was more prominent in the portal venous oxygen persufflation group. Expression of IκBε which acts as marker of NF-κB activity was over-expressed in the portal venous oxygen persufflation group suggesting its preponderance in this group.

Activation of NF-κB through reactive oxygen species has been studied with interest in the context of portal venous oxygen persufflation. It has been

suggested that portal venous oxygen persufflation when carried out in the presence of superoxide dismutase leads to the conversion of superoxide ions to hydrogen peroxide[198]. Hydrogen peroxide is well known to be an activator of NF-κB [271, 272]. Superoxide dismutase was used as a final flush in pancreases of both preservation groups in our study. This could also have contributed to the activation of NF-κB.

NF-κB has a central role in the cellular inflammatory response. Target genes of NF-κB are involved in every aspect of the inflammatory response. Controversy persists as to whether NF-κB activation is primarily pro-survival or anti-survival. This is probably academic as the ultimate effect depends on the integration of NF-κB activation with other inflammatory pathways.

To summarise, reperfusion in both groups resulted in a significant up regulation of the processes of transcription and inflammatory cytokine response. We postulate that the increased expression of apoptotic and anti-apoptotic genes in the portal venous oxygen persufflation group reflects an improved energy status in the graft which helps it to circumvent cell death by necrosis. Increased activation of the pro-survival pathways and evidence of increased metabolic activity and cell repair noted in the portal venous oxygen persufflation group suggest improved preservation of the pancreas.

Chapter 7 Overall discussion

7.1 Discussion

The present work was aimed at identifying improved methods of preservation of the non-heart-beating-donor pancreas. Hypothermic machine perfusion and portal venous oxygen persufflation were studied in comparison with static cold storage which is the current standard for pancreas preservation. This is the first known report of the use of venous oxygen persufflation in the preservation of the pancreas. The data obtained from this study has been extremely encouraging. Portal venous oxygen persufflation caused a significant improvement in the yield of purified islet equivalents as compared to static cold storage or hypothermic machine perfusion. The increased islet yield with this preservation was also associated with better islet morphology and marginally improved viability and *in vitro* function compared to the static cold storage group. The severity of reperfusion injury in the pancreas after preservation for 16 hours with portal venous oxygen persufflation or static cold storage. There was no definite evidence of decreased reperfusion injury in the portal venous oxygen persufflation group. Nevertheless, several indicators of increased metabolic recovery were noted in the oxygen persufflation group. These included active duodenal peristalsis during reperfusion which was seen in some persufflated pancreases but in none of the static cold storage pancreases.

This is also the first known report of the effect of oxygen persufflation on the global gene expression in any organ. Ischaemia reperfusion injury is well known

to stimulate an intense transcriptional response. Genes related to cytokine and chemokine activity, chaperone proteins and innate immunity are well known to be over-expressed in this setting. A similar transcriptional response was identified in our study. Interestingly, pancreas preservation with portal venous oxygen persufflation was noted to cause over-expression of genes related to apoptosis, survival pathways such as PI3K/Akt and NF-κB. These are markers of improved recovery of the pancreas graft.

Non-heart-beating-donor pancreases are not commonly used for islet transplantation because of the concern of warm ischaemia induced islet injury affecting post transplant graft function. Early studies have shown poor islet yield from pancreases that have endured warm ischaemia time greater than 30 minutes[46]. These experiments have shown that the islet yield decreases with increasing warm ischaemia times. The yield of islets in pancreases with warm ischaemia duration of 30 minutes was less than 50% of the yield in control animals.

The success of clinical islet transplantation depends almost entirely on the total mass of viable islets transplanted. The use of pancreases for islet transplantation is limited to isolations that can provide at least 5000 IEQ/kg of recipient weight[32]. Studies have also shown that on average approximately 12000 IEQ/kg body weight is required in humans to make them independent of insulin therapy[30]. This is the reason why patients need 2-3 islet transplants each to become insulin independent. Decreased islet yield from non-heart-beating-donor pancreases means that a significant proportion of these

pancreases will not be suitable for islet transplantation as the islet yield does not make the above cut-off. It also means that a greater number of islet transplants will be necessary to make each of these patients insulin independent. Loss of islets early after transplantation due to ischaemia reperfusion injury is an important cause of graft failure. Pancreas preservation techniques that can increase the islet yield and decrease the severity of ischaemia reperfusion injury can therefore increase the chance of success following transplantation.

The present work has demonstrated that novel preservation methods can improve the yield of islets from pancreases which have sustained significant warm ischaemia injury. A non-heart-beating-donor pancreas model with a warm ischaemia time of 35 minutes was developed. This ensured a significant decrease in the islet yield with traditional methods of pancreas preservation i.e. static cold storage. These experiments have demonstrated that preservation techniques such as hypothermic machine perfusion and portal venous oxygen persufflation improve both the crude and purified islet yield as compared to static cold storage.

Hypothermic machine perfusion of the pancreas has been attempted in previous studies[169, 173, 175]. This method of preservation presented logistic difficulties for everyday clinical use due to the size of the equipment. This has changed with the current availability of portable kidney perfusion equipment. Lifeport™ (Organ Recovery Systems Inc, Chicago, IL) a portable kidney perfusion machine has revolutionised the use of hypothermic machine perfusion in kidney transplantation. The Lifeport™ pancreas preservation equipment used in our

porcine experiments was the kidney perfusion machine modified for human/large animal pancreases preservation (Appendix 1). The feasibility of machine perfusion of porcine pancreases has been confirmed by the study. After an initial learning curve, the back-table preparation of the pancreas and hypothermic perfusion of the graft could be carried out without major problems. Machine perfused pancreases become oedematous even with low pressure perfusion (perfusion pressure of 10mm Hg). However histological assessment showed that the oedema is primarily restricted to the interlobular septae and the exocrine compartment of the pancreas with relative sparing of the islets. This has the potential to improve islet isolation as pancreatic digestion and dispersion of the digested tissue could be easier when the pancreas is oedematous. Taylor and co-workers in 2008 using the same machine have shown that the islet yield from pancreases preserved for 24 hours with machine perfusion was similar to fresh controls. In addition they reported that dithizone staining of the islets had revealed better quality digestion in the machine perfused pancreases [170]. Our work with preservation of rat non-heart-beating-donor pancreas using machine perfusion has also shown improved islet yield as compared to static cold storage. Hypothermic machine perfusion for 4 hours lead to a 30% improvement in the purified islet equivalent yield as compared to static cold storage. However this improvement was not as impressive as preservation with portal venous oxygen persufflation. The concept of viability prediction using parameters that can be calculated from machine perfusion is well established in non-heart-beating-donor kidney transplantation. Perfusion parameters such as the flow

resistance and perfusate biochemical markers such as glutathione-s-transferase have been reported to predict the risk of primary non-function and delayed graft function in non-heart-beating-donor grafts[167, 168]. Its use during pancreas preservation with machine perfusion will provide information that can help decide whether a pancreas is suitable for islet isolation. This is particularly important as islet isolation of a human pancreas is a costly and labour intensive process. Decreasing the frequency of unsatisfactory isolates by screening non-heart-beating-donor pancreases prior to islet isolation makes financial sense as 30% of the total cost of islet transplantation is for islet isolation [273]. The role of machine perfusion in assessing pancreas viability was first reported by Kenmochi and co-workers in 1992 [175]. They used non-heart-beating-donor canine pancreas grafts which were perfused for one hour. They found a correlation between the tissue flow rate (perfusate flow per sec per gram of pancreas), super-oxide dismutase levels and post-transplant pancreas graft function. The present porcine pancreas perfusion experiments have shown a significant positive correlation between the initial flow resistance to perfusion and activated caspase 3 levels in the pancreatic tissue at 18 hours. Similarly the rat pancreas preservation experiments have shown that higher perfusion pressures are associated with lower islet viability.

Portal venous oxygen persufflation was found to be the most promising technique of preservation of the non-heart-beating-donor rat pancreas in our study. Portal venous oxygen persufflation improved the purified islet yield (islet equivalents) significantly. Islet viability and isolation index were also better with

portal venous oxygen persufflation as compared to static cold storage and hypothermic machine perfusion. Portal venous oxygen persufflation islets were also more likely to be functional (stimulation index>1) than the islets from static cold storage group. Portal venous oxygen persufflation was associated with improved preservation of islet morphology. Islets isolated from portal venous oxygen persufflation pancreases were more globular with less fragmentation and less peripheral irregularity. Islet morphology has been reported to be an important prognostic marker for post transplant graft function in experimental and clinical settings[217, 218].

The mechanism of action of portal venous oxygen persufflation in the preservation of the non-heart-beating-donor rat pancreas is unclear. Previous reports of the use of venous oxygen persufflation in liver preservation have reported increased tissue ATP levels[186]. Venous oxygen persufflation has been reported to decrease the amount of apoptosis in the grafts[185]. Venous oxygen persufflation has also been reported to maintain cellular and mitochondrial integrity [184]. It is possible that the oxygen provides for the production of ATP to initiate the repair of injury sustained during warm ischaemia. Portal venous oxygen persufflation may also improve duct epithelial viability and maintain the pancreatic ductal architecture. This would ensure uniform distension of the entire pancreas graft during intra-ductal injection of collagenase solution. Uniform distension implies homogenous digestion and thus better islet yield. This hypothesis is supported by our experiments where pancreases preserved by portal venous oxygen persufflation had a more

uniform distension of the entire gland as compare to the static cold storage group. One effect of portal venous oxygen persufflation may simply be to recruit a larger proportion of the pancreas for collagenase digestion. This could play a significant role in digestion of larger pancreases (porcine/human) as disrupted peripheral ductal architecture may prevent exposure of the entire pancreas to the collagenase injected into the main pancreatic duct.

The reperfusion experiments were set up to investigate the severity of reperfusion injury in pancreases preserved by static cold storage and portal venous oxygen persufflation. It was expected that the pancreas will experience a more severe ischaemia reperfusion injury as compared to livers or kidneys because of the high concentration of autolytic enzymes present in the exocrine component[140]. This is a clinical reality as ischaemia reperfusion injury after pancreas transplantation causes significant morbidity in the form of pancreatitis, graft thrombosis and bleeding. Ischaemia reperfusion injury is also responsible for the early loss of nearly 60-80% of the transplanted islet mass described as instant blood mediated inflammatory reaction (IBMIR)[14].

Our experiments failed to identify significant differences in the markers of ischaemia reperfusion injury. There was a trend towards increased lipid peroxidation in the oxygen persufflation group. There was also a suggestion of increased metabolic activity with higher lactate:pyruvate ratio at the beginning of reperfusion in the oxygen persufflation group. The lack of significant difference between the two groups could be due to the severity of the ischaemic insult sustained by the pancreas and the prolonged cold ischaemia period. Any small

differences between the two groups may also not have been evident due to the small numbers in each group.

Duodenal peristalsis was identified in three of portal venous oxygen persufflation pancreases but in none of the static cold storage pancreases. Though the incidence of peristalsis was not statistically different in the two preservation groups, its occurrence only in the portal venous oxygen persufflation group is an important indicator of tissue viability.

RNA microarray analysis was carried out to clarify the cellular mechanisms and pathways that were over-expressed in the portal venous oxygen persufflation group as compared to the static cold storage group. This is the first known report of the pattern of gene expression in an organ preserved by portal venous oxygen persufflation. Despite the sub-optimal quality of RNA extracted from the pancreatic tissue we have been able to identify important similarities and differences in gene expression between the two preservation groups. We found increased expression of genes related to cellular stress, transcription and inflammatory cytokines in both groups. Portal venous oxygen persufflation was associated with an over-representation of genes related to apoptosis and anti-apoptosis. The portal venous oxygen persufflation group was characterised by up-regulation of the pro-survival PI3K/Akt and the NF-κB pathways. The pro-inflammatory p38 MAPK pathway was preferentially over-represented in the static cold storage group. Increased expression of genes encoding for carrier proteins, glycolytic enzymes and steroid biosynthesis also suggest increased metabolic activity and cellular repair in the portal venous oxygen persufflation.

Provision of oxygen to the non-heart-beating-donor graft during the cold ischaemia phase is increasingly accepted as an important means of ameliorating post-reperfusion injury. Availability of oxygen enables the production of ATP. ATP helps in the maintenance of the function of the sodium potassium ATP pump thus preventing cellular swelling. The ideal mode of delivering this is however contentious. Two layer method appears to work because of the oxygenation of the outer rim of pancreatic tissue. Its effectiveness in oxygenating the entire human pancreas is controversial as the diffusion of oxygen from the surface is limited[156]. In a recent review of the effectiveness of two layer method, Noguchi and co-workers in 2009 suggested that the reported lack of benefit of TLM in large studies was due to problems with the technique used by inexperienced teams[150]. A recent report reported the possibility of using ductal injection of oxygenated perfluorocarbon solution as a means to improve pancreas preservation. They reported improved ATP levels in rat pancreases preserved by this technique[274]. Despite the attractiveness of this technique, it is not clear if ductal injection can ensure adequate oxygenation of large animal pancreases.

Alternate methods of oxygenation of organs using the vascular system of the pancreas are being investigated. These have the advantage of ensuring effective oxygenation of all parts of the organ. Warm oxygenated perfusion of the grafts has been reported to improve graft outcomes[275]. However, this is complicated and can involve the use of blood products. Oxygenated hypothermic machine perfusion is another attempt in this direction[276]. This

has the advantage of using standard preservation solutions saturated with oxygen. Studies have reported that even a short period of oxygenated machine perfusion prior to warm reperfusion can improve the tissue viability and reperfusion injury[225, 277, 278]. They have suggested a post-conditioning like effect of oxygenated perfusion prior to warm reperfusion[278]. In other studies oxygenated machine perfusion especially when prolonged was found to increase endoplasmic reticulum stress leading to increased apoptosis and cell death [279].Minor and co-workers in 2009 compared the effectiveness of long-term oxygenated machine perfusion and venous oxygen persufflation in preserving ischaemic rat livers. They found improved preservation and greater functional recovery with venous oxygen persufflation [280].

Scott and co-workers in 2010 reported initial data of pancreas preservation using oxygen persufflation through the arterial system. They reported improved ATP levels, better preserved histology in the pancreases as compared to preservation by TLM[281, 282].

7.2 Limitations of the study

Several limitations in the methodology used in the study research have been identified both during and since the completion of the laboratory work. Some of these were unavoidable due to restrictions of animal usage, equipment availability and funds. With the benefit of hindsight some problems could have been avoided. A few of them are detailed below. This should help place the findings of this research in the correct perspective.

7.2.1 Experimental model

The choice of the animal model plays an important role in extrapolating the results to clinical application. The rat model of pancreas preservation was chosen because of the simplicity of rat islet isolation and the lower expenses involved. However, this model is not best suited for studying pancreas preservation techniques. Rat pancreas is a delicate and flimsy organ that is only a few millimetres thick. It cannot be ascertained whether the beneficial effect of portal venous oxygen persufflation seen in our study can be replicated by direct diffusion of oxygen across the pancreas capsule. This could have been clarified by including an additional arm in the study protocol where the pancreas was stored in UW solution with oxygen bubbled into the preservation fluid directly.

Male Wistar rats were selected for the experiment once they had reached the required weight. Though this usually meant that the animals were of similar age, this was not specifically confirmed. In addition the weight range used (250-350g) meant that some of the pancreases were fattier than others. Age and adiposity of the pancreas has been reported to affect actual islet numbers in the pancreas and the quality of islet isolation and purification.

In view of the small size of the pancreas it was expected that leakages on the arterial or the portal venous side could not be avoided. Each pancreas was checked on the back-table for leaks with gentle flushing or persufflation. Any obvious leaks were sutured. Pancreases with significant leaks that could not be

quickly controlled were discarded. However minor leaks during hypothermic machine perfusion or portal venous oxygen persufflation might have remained. This could have compromised the effectiveness of the preservation techniques. Some studies have reported the use of dye injection to identify and repair small leaks. This was not considered feasible with the rat pancreas. Previous work on portal venous oxygen persufflation has been carried out in livers and kidneys. Here small punctures made on the organ surface acted as outlets for the oxygen persufflated into the organ. In the case of the rat pancreas this was complicated as punctures into the already flimsy pancreas would have made effective ductal injection of collagenase impossible. Punctures were instead made in the surface of the spleen and duodenum for efflux of oxygen. This had the disadvantage of not providing oxygen to the pancreatic capillaries directly and the recorded persufflation pressure may not be the actual pressure in the pancreatic capillary bed. However visible distension of the venules on the surface of the pancreas during persufflation was used as marker for satisfactory pancreatic persufflation. Some researchers have stressed the importance of confirming the effectiveness of oxygen persufflation by demonstrating increased tissue ATP levels. This could not be done.

7.2.2 Islet isolation and assessment experiments

Shared use of perfusion and persufflation equipment in the labs meant that all preservation techniques were not continuously available. Random allocation of treatments, though initially planned was not always feasible. This problem was

handled by ensuring that continuous series of animals were not included in one preservation arm. Similarly change of the planned method of preservation was avoided once the pancreas retrieval procedure was started to minimise sampling error.

Rat pancreas islet isolation was carried out using collagenase ductal injection and digestion and discontinuous gradient separation. While this is the most commonly used technique in published literature there are several drawbacks to the technique as used in this study. The collagenase used had an incubation period of 50 minutes in accordance with the manufacturer's recommendations and our own lab trials. This was carried out in a static water bath. Ongoing ischaemia to the pancreas during the digestion period would have had an effect on the viability of the isolated islets. There are recent reports of the benefit of oxygen treatment during the digestion phase either by direct oxygenation or by using oxygenated perfluorocarbon solution[193].

Whilst all attempts were made to carry out the entire digestion and isolation process in a clean environment this was not always feasible due to the exposure of the pancreas to enteric contents during retrieval. The affect was minimised by rinsing the pancreas after retrieval in fresh wash medium prior to start of preservation. Sterile environment and equipment was used for the final stages of islet purification and the islets were cultured overnight in sterile culture medium. It is possible that some bacterial growth would have occurred during the preservation-digestion-culture period.

The viability testing of islets with exclusion dyes are not sensitive techniques to assess islet viability[217]. They identify dead cells but do not identify dying cells with still intact cell membrane barrier function. Hence these would tend to underestimate the extent of cell death. It is also well known that after islet separation the core of the islet is dependent on diffusion for the supply of oxygen and nutrients. As the size of the islet increases there is an increasing risk of the core of the islet becoming ischaemia. The islets that were produced from the portal venous oxygen persufflation group tended to be larger than the static cold storage and hypothermic machine perfusion groups. We have attempted to account for the core effect by standardised assessment in which islets with more than the central 25% staining blue are counted as being non-viable. However this is still a subjective method of assessing islet viability. Improved methods of viability assessment such as oxygen consumption rate[283] are more accurate methods of viability assessment.

Static glucose stimulated insulin secretion is a rudimentary assay to explore *in vitro* function. Results are variable and depend of the exact protocol used. Standardisation of the technique with modifications to the incubation medium was helpful in decreasing the variability of the results. The variability can be further decreased and accuracy of islet function improved using real time methods of *in vitro* islet assessment such as the micro-perfusion technique.

Islets with good viability on staining and good functional ability on *in vitro* testing may still not succeed in providing insulin independence after clinical transplantation. The only true predictor of islet viability and post-islet transplant

success is its ability to reverse hyperglycaemia when transplanted into diabetic immuno-deficient mice. We have not done this in our study. Further evaluation of the use of portal venous oxygen persufflation should include *in vivo* assessment of the islets.

Two layer method was not part of the initial study plan. It was included as an additional study midway through phase 1 of the study when we were able to acquire a small quantity of perfluorocarbon solution. This group hence did not have sufficient numbers to compare the effectiveness of two layer method with the other preservation methods.

7.2.3 Warm oxygenated reperfusion

The warm reperfusion study was an exploratory study to evaluate the benefit of portal venous oxygen persufflation in ischaemia reperfusion. Sample size calculations were not done. A sample size of at least six animals per preservation group was considered based on literature review of studies involving animal models of organ preservation and ischaemia reperfusion injury. The number of animals in each group ultimately depended on the constraints of time.

The cold ischaemia time in the islet isolation phase of the study was 5 hours. For the warm reperfusion study, the duration of cold ischaemia was extended to 16 hours. It is hence not possible to compare the data from the two studies directly. The usual cold ischaemia times in clinical pancreas and islet transplantations range from 12-14 hours[12, 32]. The cold ischaemia time for the

study was extended as 4-5 hours was too short to be relevant in clinical transplantation.

Duodenal peristalsis identified during oxygenated reperfusion is an important finding in this study. Though the phenomenon was video-recorded for two pancreases, objective documentation of the duration and intensity of peristalsis was not done. We had attempted continuous monitoring of the intra-duodenal pressure using a luminal pressure probe during the reperfusion part of the experiment. Problems with the technique such as leakage of duodenal contents, blockage of the transducer cannula and slippage of the cannula were encountered during the model development stage. Hence attempts of intra-duodenal pressure monitoring were abandoned.

Caspase 3 assay was used to measure the extent of apoptotic activation in the tissues. The protocol for the assay had to be modified to use it for estimation of caspase 3 levels in the tissue extracts. The modification included increasing the volume of the extracts in each well and by using appropriate negative control for each sample to account for the background. This is unlikely to be as accurate as the standard assay. However it is useful in giving some idea of the extent of caspase 3 activity in the extracts.

Using a single assay to identify the extent of apoptosis is inadequate. Most studies report the use of a battery of tests such as caspase activation, Tunel staining of tissue sections and characteristic DNA fragmentation to quantify apoptosis.

The major form of cell death in these experiments is cell necrosis. Confirmation of necrotic cell death is difficult as it does not have any characteristic features. Staining of tissue sections to quantify necrotic, apoptotic and viable cells could have helped in further clarifying the predominant form of cell death in the two preservation groups.

7.2.4 Microarray experiments

The quality of RNA extracted in the initial part of the study was of poor quality and could not be used for microarray experiments. The RNA from pancreases in the later part of the study was of better quality due to modifications in the extraction technique. However, there were not enough good quality RNA samples in each study group to run replicates. Hence the data available for each study group was from a single RNA sample pooled from three RNA samples of that group. This is expected to increase the risk of sampling errors. Confirmation of microarray findings using quantitative rtPCR is recommended by most researchers. This has not been done. In a parallel study of microarray analysis of RNA extracted from rat kidney, the microarray data was validated by PCR and showed good correlation between the two.

7.3 Future work

Further work in this area should concentrate on validating the benefit of portal venous oxygen persufflation on a large animal model of non-heart-beating-donor. Following our experiments on porcine pancreas machine perfusion we have gained experience in porcine pancreas retrieval, back table preparation for machine perfusion. Using this model to study portal venous oxygen persufflation should be feasible. Porcine islet isolation is complicated and needs specialised equipment and expertise which is currently not available for our group. Use of normo-thermic oxygenated reperfusion in the *in vitro* setting can be used to investigate the effects of this preservation technique.

The feasibility of short term oxygen persufflation lasting 1-2 hours after a period of warm ischaemia and static cold storage to improve the outcomes of these grafts should be investigated. This technique of post-conditioning has been reported by researchers using venous oxygen persufflation in liver grafts and two layer method in pancreas grafts. The advantage of this technique in the clinical setting would be to avoid the use of complex preservation techniques at the donor hospital. Instead the pancreas can be transported in cold storage and then put through venous oxygen persufflation in the islet isolation facility for 1-2 hours before the start of islet isolation or pancreas transplantation.

The results of microarray analysis are a guideline for further work in this area. We have identified increased expression of apoptotic and anti-apoptotic genes in the study. The net effect of this gene expression should be quantified by

techniques such as Tunel staining of pancreas tissue sections. Tissue sections can also be used to compare the relative extent of cell necrosis and apoptosis in each preservation method. This would validate our conclusion that increased apoptotic activation in the venous persufflation group is associated with decreased cell necrosis. The microarray study has suggested upregulation of survival pathways in the portal venous oxygen persufflation. Confirmation of this through cellular protein measurements and pathway specific reporter gene studies will further strengthen these conclusions.

The ultimate aim of this research area is to develop a suitable form of large animal pancreas preservation that can improve the viability of the marginal grafts and identify pancreases that have sustained irreversible damage. A possible system could include a short period of hypothermic machine perfusion of non-heart-beating-donor pancreases to evaluate viability using perfusion parameters. Grafts with good viability can undergo immediate islet isolation. Short period of venous oxygen persufflation for grafts with marginal viability can be followed by islet isolation. Grafts with obviously poor viability on machine perfusion can be discarded. This system can ensure full utilisation of the donor resource without the unnecessary expense of poor quality islet isolations. The technology for these kind of interventions is available today. If validated with large animal pancreases, it can be immediately applied to clinical pancreas preservation.

APPENDICES

Appendix 1 List of reagents and equipment used in the study

Reagents and chemicals

Carbogen (95% oxygen, 5% carbon-di-oxide)	BOC
Caspase 3 colorimetric assay kit	Sigma-Aldrich
Collagenase P	Roche
Dulbecco's Modified Eagle Medium (1X) without glucose	Invitrogen
Griess reagent kit	Invitrogen
Hanks' Buffered Salt Solution (10X) with phenol red	Invitrogen
High range rat insulin Elisa kit	Mercodia AB,Sweden
KPS-1 preservation solution	Organ Recovery Systems
Lithium chloride (3.5N)	Ambion
Microdialysis multiassay kit	CMA Microdialysis AB
Nycoprep	Axis-shield
Oxygen (100%)	BOC
Quant-iT Protein Assay Kit	Invitrogen
RNAsecure	Ambion
RNAzap	Ambion
Streptokinase (Streptase™)	CSL Behring
Trizol reagent	Ambion
Viaspan (UW solution)	Du Pont pharma

Equipment

1.5ml microcentrifuge tubes	Eppendorf
50ml Centrifuge tubes	Falcon
Centrifuge (lab)	Harrier 15/80
Centrifuge (refrigerated)	Mistral 3000
Class II safety cabinet	Biomat-2
CMA 600 Microdialysis analyser	Microdialysis AB
Elisa plate reader	Dynatech Laboratories
Eyepiece Graticule	Pyse-SGI Ltd
Fluorescence microscope	Nikon
Freezer (-20°C)	Beko
Freezer (-80°C)	Lab Impex Research
Gilson Pipettes	Anachem
Incubator	Sanyo
Inverting Microscope	Olympus CK2
Microcentrifuge	Hettich mikro 12-24
Microvials for Microdialysis analyser	CMA Microdialysis AB
ML110 Bridge Amp	AD Instruments
Multi-well plates (24,96 wells)	Beckton Dickinson
Multiwell plates (6,24,96 wells)	Iwaki
Peristaltic perfusion pump	Watson Marlow 323
pH meter	AII Orion
Powerlab 4/25 Data Acquisition system	AD Instruments

Refrigerator	Electrolux
Sonicator	Langford Ultrasonics
Spectrophotometer	Uvikon
Syringe filter (0.2µm)	Sartorius
Syringe pump driver	Graseby 3150
Syringes (5ml,10ml,20ml,50ml)	Beckton Dickinson
Water bath	Grant Instruments

Appendix 2 Methodology of assessment of lactate, pyruvate and glycerol in portal venous effluents

These assays were carried out using the CMA 600 Microdialysis analyser. Batch analysis of portal effluent samples stored at -20°C was carried out. Glycerol, lactate and pyruvate assays were used in the study.

Glycerol assay

Glycerol is phosphorylated by adenosine triphosphate (ATP) and glycerol kinase (GK) to glycerol-3-phosphate. This is subsequently oxidized in the presence of glycerol-3-phosphate oxidase (GPO). The hydrogen peroxide formed reacts with 3,5-dichloro-2-hydroxy-benzene sulphonic acid (DCHBS) and 4-amino-antipyrine. This reaction is catalyzed by peroxidase (POD) and yields quinoneimine. Presented as mmoles/l.

Pyruvate assay

Pyruvate is enzymatically oxidized by pyruvate oxidase (PyrOx). The hydrogen peroxide formed reacts with N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS) and 4-amino-antipyrine. This reaction is catalyzed by peroxidase (POD) and yields quinonediimine. Presented as mmoles/l.

Lactate assay

Lactate is enzymatically oxidized by lactate oxidase. The hydrogen peroxide formed reacts with 4-chlorophenol and 4-amino-antipyrine. This reaction is catalyzed by peroxidase (POD) and yields quinoneimine. Presented as mmoles/l.

Appendix 3 Low pressure hypothermic machine perfusion of non-heart-beating-donor porcine pancreas

Background

Hypothermic machine perfusion has been successfully used in the preservation of non-heart-beating-donor kidneys. It has been shown to improve preservation, decrease the duration of DGF and help in the assessment of the viability of graft kidneys. There is now increasing interest in the use of this technique to preserve non-heart-beating-donor pancreases. Organ Recovery Systems Inc (Chicago, IL) has been providing portable systems for kidney machine perfusion. They have developed a protocol of a pancreas perfusion system on the same platform. One sample machine was provided to our unit to evaluate the feasibility of machine perfusion of non-heart-beating-donor porcine pancreases.

Aims

- To determine the perfusion mechanics of non-heart-beating-donor porcine pancreas on low pressure hypothermic machine perfusion.

- To determine if perfusion parameters can predict warm ischaemic tissue injury.

- Compare the preservation of non-heart-beating-donor porcine pancreas by low-pressure hypothermic machine perfusion and two layer method of preservation.

Methods

The Pancreas perfusion system was based on the current kidney perfusion system and shares the same platform. The organ chamber is modified to hold the pancreas.

The perfusion circuit has a split arterial inflow to simultaneously perfuse the splenic and the superior mesenteric artery so the entire pancreas and the adjoining duodenum can be perfused. Kidney Preservation Solution-1 (KPS) which is similar to the UW solution in composition was used as the perfusate (Figure 60).

278
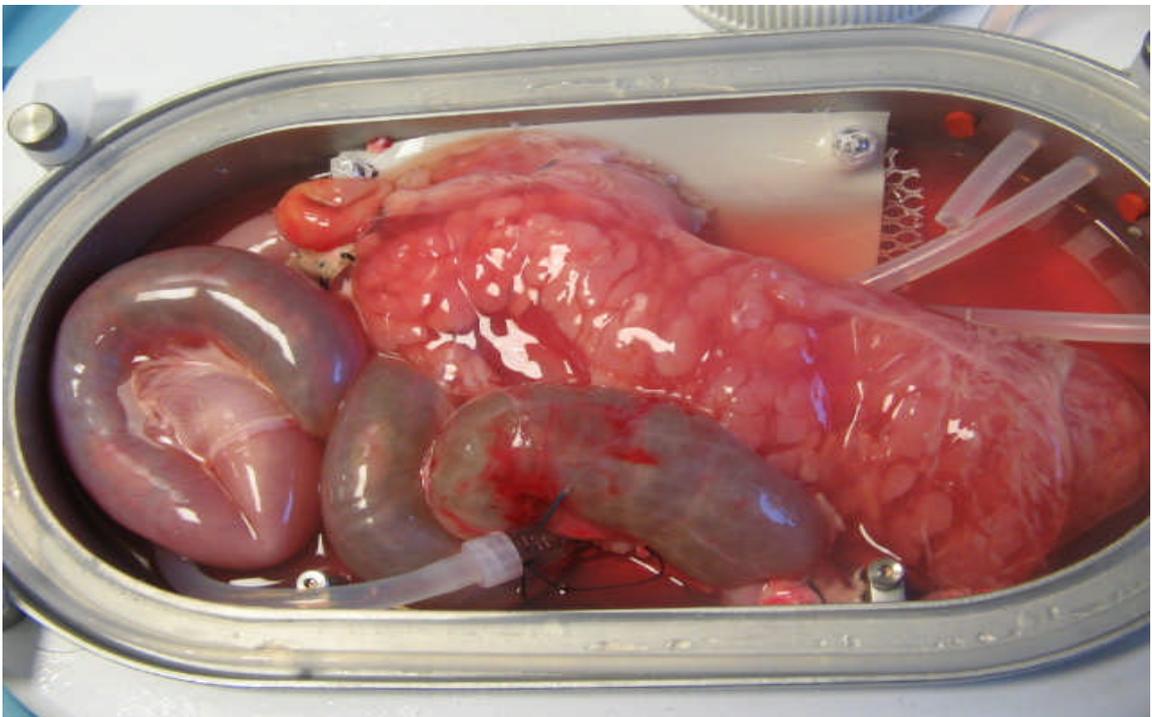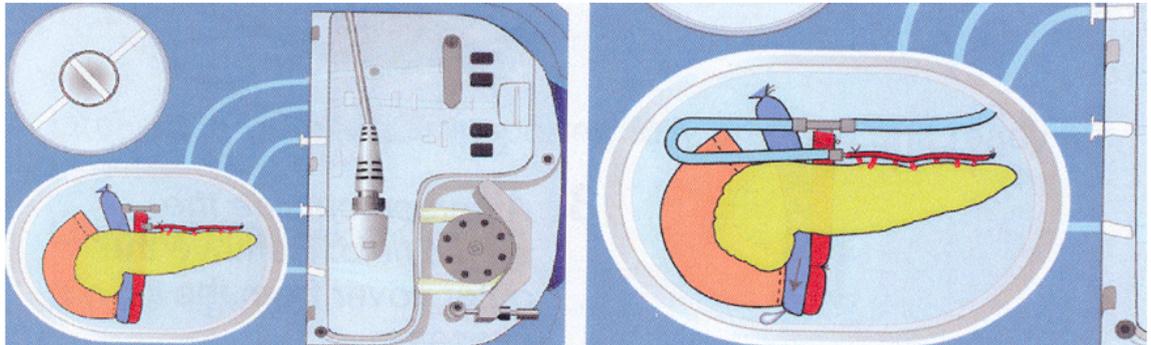
Figure 60 Hypothermic machine perfusion of porcine pancreas
Top figure shows the schematic representation of the machine perfusion circuit. It is based on the same platform as the LifePort™ kidney perfusion machine. The inflow for the pancreas is split to ensure perfusion through both the splenic artery and the superior mesenteric artery. This ensures perfusion of the entire pancreas. Outflow from the pancreas is through the cut portal vein. Bottom image shows the porcine pancreas after 24 hours of machine perfusion. The duodenal distension and the pancreatic oedema is clearly seen.

Retrieval of NHB porcine pancreases

Juvenile landrace pigs used for another project investigating a laparoscopic renal cooling device were used as the source of the non-heart-beating-donor pancreases. Briefly the pigs underwent a laparoscopic renal procedure under general anaesthesia. On completion of the experiment, the animal was sacrificed by a barbiturate overdose. After a period of warm ischaemia (30 minutes), laparotomy was done. The infra-renal aorta was cannulated after ligating the distal aorta. The supra-coeliac aorta was ligated below the diaphragm. 1litre of cold Marshall's solution containing 10000 units of unfractionated heparin was perfused through the aortic cannula. The IVC was incised to vent the blood. The lesser sac was packed with ice slush. The pancreas along with the spleen and adjoining duodenum along with a segment of aorta containing the celiac and superior mesenteric arteries was removed and placed in a bowl of ice-cold Marshall's solution. Back-table dissection involved splenectomy, shortening of the duodenal segment, and cannulation of the coeliac and superior mesenteric arteries. Leakages from the arterial side were identified by gentle flushing of the arterial cannulas and ligated. The pancreas was weighed.

Techniques of pancreas preservation

Two layer method

150 ml of perfluorocarbon (PFC) solution was transferred to the preservation chamber and connected to a source of 100% oxygen. Oxygen was bubbled through the solution for at least 30 minutes prior to start of preservation. The pancreas was placed in the oxygenated PFC and 500ml of cold KPS was transferred to the preservation chamber to completely submerge the pancreas. The pancreas was stored for 24 hours.

Hypothermic machine perfusion

The perfusion circuit was primed with 750ml of KPS. The pancreas was transferred to the preservation chamber. The two arterial cannulas were connected to the perfusion circuit according to suppliers recommendations. Perfusion was started at a mean perfusion pressure of 10mmHg and continued for 24 hours.

Data collection

The perfusion parameters were recorded at 2, 4 & 6 hours of perfusion. Pancreas biopsies were collected before the start of preservation and after 6 and 24 hours of preservation. Biopsies were stored immediately in liquid nitrogen or fixed in formalin.

Caspase 3 assay

Pancreas biopsies were snap frozen in liquid nitrogen and stored at -80°C. Biopsies were homogenized in bead beating tubes using a Precellys 24 Tissue Homogeniser. The supernatant was transferred to labelled tubes and stored at -80°C until analysis.

Substrate based colorimetric Caspase 3 assay was used. The assay was carried out according to manufacturer's instructions. 5µl of the test sample in duplicate was used. The plate was read after overnight incubation. The protein content in the tissue extracts was assayed using the Bradford assay after diluting the extract 1:200 and comparing it with prepared protein standards. The Caspase 3 activity in terms of µmoles of pNA released per min per mg of tissue protein extract was calculated.

Histology

Formalin fixed biopsies were used to make paraffin blocks. 6µm sections were cut manually. The sections were stained with eosin and haematoxylin by standard technique. The stained sections were examined for a qualitative estimation of the extent of tissue oedema.

Results

Eight pancreases were used for the study. Five underwent machine perfusion while three were stored by two layer method (Table 14).

The median warm ischaemia time was 35 minutes. One pancreas had a warm ischaemia time of 60 minutes as in-situ cold perfusion could not be done and the pancreas was retrieved and flushed on the back-table (PP8).

Median weight of the pancreas was 175gm. The median weight gain after 6 hours of machine perfusion was 17.6%. The weight gain was 40% for PP3 which could not be cooled below 14°C due to problems with the perfusion circuit. There was no weight gain in the pancreases stored by TLM.

Perfusion parameters

The perfusion pressure was 10mmHg. Good initial flow rates were achieved by all pancreases except PP8 which had a high initial resistance and low flow rate. During the preservation period a gradual increase in flow rate with a decrease in resistance was noted in all but one pancreas (PP6) (Figure 61).

Table 14 Details of the eight non-heart-beating-donor porcine pancreases preserved by two layer method or hypothermic machine perfusion.

TLM: Two layer method, HMP: Hypothermic machine perfusion

Code	Preservation type	Warm ischaemia (minutes)	Initial weight (grams)	Weight gain at 6 hours (%)	Initial resistance	Flow rate (ml/min)	Problems
PP1	HMP	35	170	17.60%	0.95	12	
PP3	HMP	25	180	44%	1.03	10	Perfusion fluid temperature did not decrease below 12°C
PP6	HMP	35	200	10%	0.45	24	Good initial flow rate but did not improve during the preservation period
PP7	HMP	35	220	18%	0.45	20	
PP8	HMP	60	220	13.60%	1.99	5	In-situ aortic flush not possible. Flushed on the back-table. Low initial flow rate. Patchy perfusion of the pancreas.
PP2	TLM	30	130	0			
PP4	TLM	40	140	0			
PP5	TLM (could not perfuse)	30	160	0			
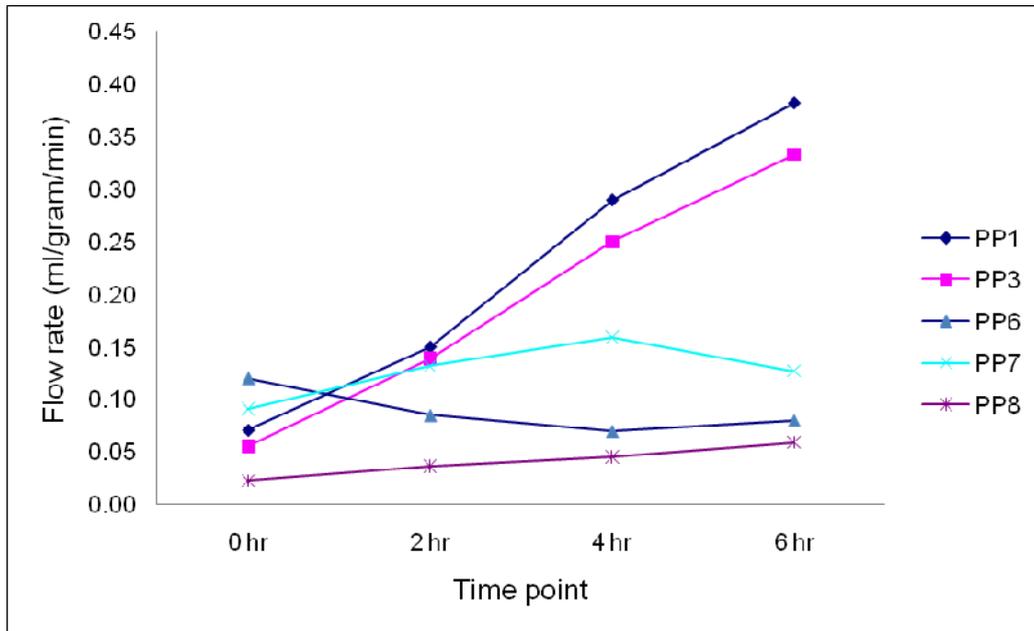
Figure 61 Trend in perfusion flow rate in the five machine perfused porcine pancreases
Perfusion flow rates for the initial 6 hours of machine perfusion are shown in the graph. The general trend was of gradual increase in the flow rates over time. This was associated with a fall in the flow resistance (data not shown). PP8 was associated with a much longer warm ischaemia period and had the lowest initial flow rate with some improvement over the perfusion period. PP6 had a good initial flow rate which failed to increase.

Caspase 3 assay

Caspase 3 levels were highest at the start of preservation and then decreased over the preservation period in the machine perfused pancreases. The only exception was PP8 which registered the highest levels at 6 hours of preservation. This persisted after 24 hours of preservation. No particular trend in serial caspase 3 estimations was noted in the TLM group. There was no significant difference in caspase 3 levels between the two preservation groups at all time points (Figure 62)

Significant positive correlation was noted between the initial flow resistance and the caspase 3 levels after 24 hours in the machine perfused pancreases (Pearson's product correlation=0.948, p=0.014).
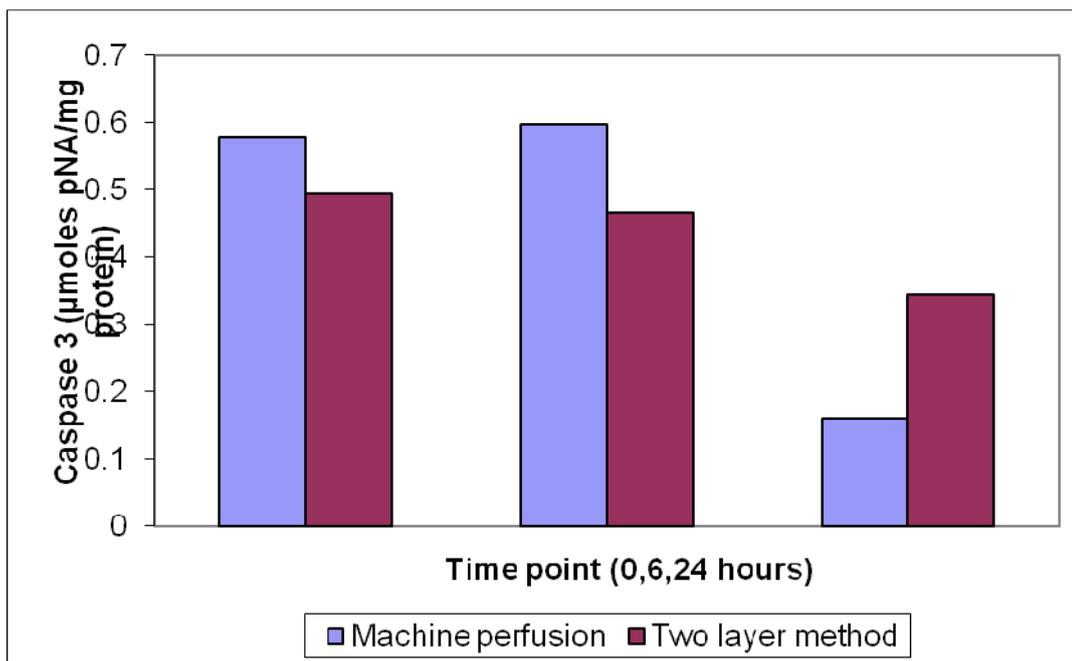
Figure 62 Comparison of tissue caspase 3 levels in pancreases preserved by two layer method or hypothermic machine perfusion at different time points.
No difference in caspase 3 levels between the two preservation groups was noted at all three time points.

Histology

Sections showed increased oedema in the interlobular septa in the machine perfused pancreases. The oedema appeared to spare the islets. There was no significant oedema

in the two layer method preservation pancreas (Figure 63).

| Hypothermic machine perfusion | Two layer method |
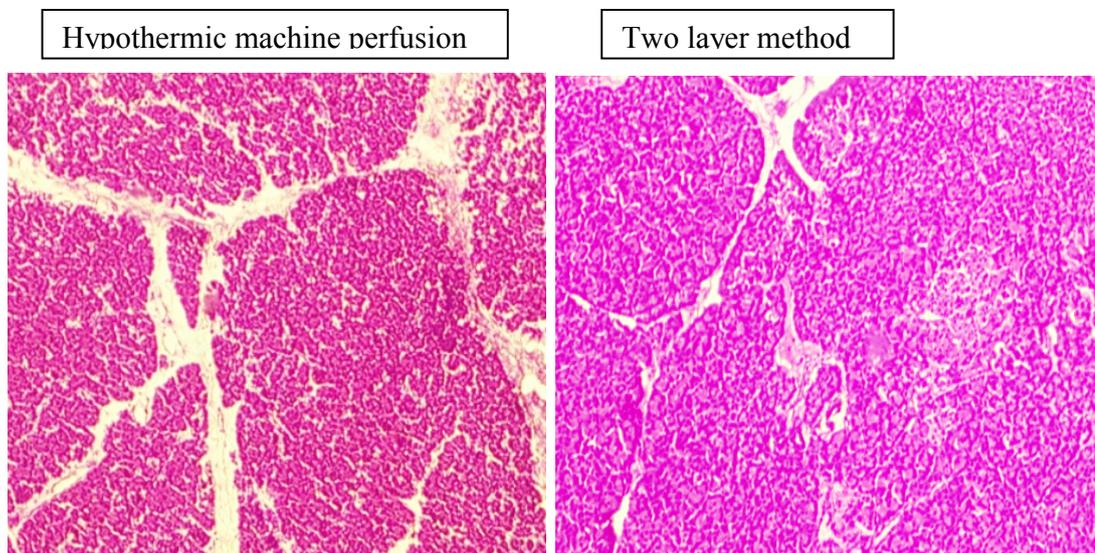
Figure 63 Comparison of the extent of interstitial oedema in pancreas biopsies after 6 hours of preservation
Photomicrographs of 6μm sections stained with H&E staining and examined under a microscope at 40X magnification. Interstitial edema is prominently seen in the hypothermic machine perfusion group pancreas.

Discussion

This was a feasibility study to evaluate the use of the modified LifePort™ machine for large animal pancreas perfusion. We have been able to delineate the perfusion dynamics during low pressure hypothermic machine perfusion.

The pancreases were retrieved after the death of the animal to simulate the warm ischaemia in a non-heart-beating-donor donor. The retrieval procedure was similar to the pancreas retrieval in a clinical setting. In one case, retrieval was complicated by the inability to properly carry out the aortic flush. This lead to prolonged warm ischaemia time. This pancreas flushed on the back-table with difficulty and had high resistance and low flow rates at start of perfusion. This improved only partially after six hours of perfusion. Tissue from this pancreas showed elevated caspase 3 levels.

There was increased weight gain in the machine perfusion group as compared to the TLM group. Initial weight in the perfusion group was higher as compared to TLM group (198 *vs.* 143 g). The flushing with preservation solution during back-table preparation in machine perfused pancreases to identify and repair leaks could be responsible for this.

The caspase 3 levels were not different in the TLM and HMP groups. The size of the study was however not designed to show a significant difference in the two groups. Caspase 3 is an effector enzyme of apoptosis and its levels increase significantly only in the reperfusion phase[284]. The fall in caspase 3 levels during the preservation period could be due to the gradual breakdown of caspase 3 formed during warm ischaemia.

The significant correlation between the initial flow resistance and the caspase 3 levels at 24 hours of machine perfusion was interesting. It is possible that both are markers of significant warm ischaemia injury in the pancreas. Use of perfusion parameters as viability tests have been reported previously in pancreas preservation[175]. They are routinely used to assess non-heart-beating-donor kidneys. Further studies to evaluate perfusion parameters as markers of viability during pancreas preservation are needed.

Pancreatic oedema was seen exclusively in machine perfused organs. Histology suggested that the oedema was mainly located in the interlobar and interlobular septae.

Pancreatic oedema increases the risk of capsular tears and bleeding in the setting of whole organ pancreas transplantation. However it could be beneficial in islet isolation as it may improve the disruption of the pancreatic parenchyma and improve separation of islets from the exocrine tissue. Similar conclusions were reached by a recent study of pancreas perfusion using the same perfusion apparatus[170].

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