Optimizing pancreas preservation for islet transplantation: mechanisms and bioenergetics of the two-layer method

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MD

I, Aditya Agrawal confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. Where others have contributed to my work, this has been acknowledged.

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Abbreviations

1H	Proton
19F	Fluorine
31P	Phosphorus
AMP	Adenosine Monophosphate
ANOVA	Analysis of Variance
APAF 1	Apoptotic protease activating factor 1
ATP	Adenosine Triphosphate
Bad	Bcl-2 associated death promoter
Bax	Bcl-2 associated X protein
Bcl2	B-cell lymphoma 2
BMI	Body Mass Index
BSA	Body Surface Area
CCL3	C-C motif chemokine 3
CI	Confidence Interval
CIT	Cold Ischaemia Time
DCCT	Diabetes Control and Complications Trial
DCD	Donation after Cardiac Death
eNOS	endothelial Nitric Oxide Synthase
FDA	Food and drug administration
FOV	Field of view
GC	Gas Chromatography
GC-FID	Gas Chromatography – Flame Ionization Detector
Hb	Haemoglobin
HBSS	Hank‟s Balanced Salt Solution
HTK	Histidine-Tryptophan-Ketoglutarate
IAP	Inhibitors of Apoptosis

ID	Internal Diameter
IEQ	Islet Equivalents
IGL-1	Institut George Lopez
IL	Interleukin
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MR	Magnetic Resonance
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NMR	Nuclear Magnetic Resonance
OLM	One layer method
OR	Odds Ratio
PaO_2	Partial pressure of oxygen in arterial blood
PCr	Phosphocreatine
Pdx1	Pancreas and duodenum homebox gene 1
PDE	Phosphodiester
PEG	Polyethylene glycol
PFC	Perfluorocarbon
PFD	Perfluorodecalin
Pi	Inorganic Phosphate
PME	Phosphomonoester
pO_2	partial pressure of oxygen
PP	Pancreatic Polypeptide
ppm	parts per million
RCT	Randomized Controlled Trial
RES	Reticuloendothelial system
RF	Radiofrequency

ROC	Receiver Operating Characteristic
RSD	Residual Standard Deviation
SD	Standard deviation
SW	Sweep width
TE	Echo time
T1DM	Type 1 Diabetes Mellitus
TLM	Two-Layer Method
TR	Repitition time
UW	University of Wisconsin
VEGF	Vascular endothelial growth factor
WI	Warm Ischaemia
WMD	Weighted Mean Difference

THESIS ABSTRACT

Background

The recent unprecedented interest in islet allotransplantation has been tempered by the observation that sustained freedom from exogenous insulin is rarely achieved and the rate of insulin independence drops to 10% at 5 years follow-up. One critical determinant of successful islet allografting is preservation injury to the pancreas. The two-layer method (TLM) of pancreas preservation was developed to mitigate the deleterious effect of cold ischaemia, but the mechanism is unclear and its clinical efficacy is controversial.

Hypothesis, Aims and Objectives

There is no clinical or experimental evidence for benefit of the two-layer method as currently employed in pancreatic islet transplantation. A potentially beneficial effect on pancreas preservation by improvement in graft ATP production is possible by modification of the two-layer method. The principal aim was to develop optimal protocols for pancreas preservation in islet transplantation by clarifying the mechanism of TLM. A second objective was to develop a dynamic model for the study of mitochondrial function during organ preservation.

Methods

1. Perfluorocarbon (PFC) content of porcine pancreases preserved in TLM and in University of Wisconsin (UW) solution for 24 hours was compared. Pancreatic samples were analysed using Varian INOVA 9.4T MR scannerspectrometer. External PFC standard was introduced for quantification. Four consecutive transverse images of 4mm thickness were obtained using a spin-echo sequence. ^{19}F MRS was performed with the same parameters except with more averages. MR data was confirmed by headspace chromatography.

2. Real-time changes in pancreas bioenergetics were studied with ^{31}P MRS for rat pancreases preserved at 4°C - 6°C in five different groups: chilled Marshall‟s, static TLM, continuous TLM with oxygen perfused at 0.5L/h, and static or continuous TLM both the latter following 30 min warm ischaemia. ^{31}P spectra were analyzed for phospho-mono-esters, inorganic phosphate (Pi) and α-, β- and γ -nucleotide triphosphate.

Results

1. PFC standard was readily detected in ^{19}F MR images. There was no signal from porcine pancreas in ^{19}F MR images following either UW or TLM storage. ^{19}F MR spectra typical of PFC were not obtained from either UW-or TLM-preserved pancreas with non-localized ^{19}F MRS. Mean concentration of PFC in TLM pancreas measured by head space chromatography was not significantly different from background concentration in UW pancreas.
2. Intergroup rates of change of [γ-ATP]/[Pi] and [β-ATP]/[Pi] throughout preservation period were significantly different. For continuous TLM there was an increase relative to baseline but decrease for both static TLM and Marshall‟s with respect to continuous TLM. Rate of decrease was similar for the Marshall‟s and static TLM groups. [γ-ATP]/[Pi] and [β-ATP]/[Pi] increased with WI continuous TLM but decreased for WI static TLM.

Conclusions

There is no evidence of penetration of perfluorocarbon into pancreas tissues investigated either by MR or chromatography in organs preserved at hypothermia. ^{31}P-MRS is an effective tool for non-invasive assessment of pancreas bioenergetics. Continuous TLM preserves cellular bioenergetics and is superior to current non-PFC based solutions for pancreas preservation.

CHAPTER 1

BACKGROUND

1.1 Diabetes – Incidence, Pathophysiology, Treatment

***Incidence*:** Diabetes is a deadly disease, accounting for 3.8 million deaths per year, similar in magnitude to AIDS (Diabetes Atlas, 2006). The International Diabetes Federation estimates that 246 million people are affected with diabetes mellitus worldwide and this figure is projected to reach a staggering 380 million by 2025 (representing 7.1% of the global adult population) (Diabetes Atlas, 2006). The incidence of type 1 diabetes is rising alarmingly, with an average annual increase of 2.5 – 3.0% per year worldwide (DIAMOND project group, 2006).

Diabetes is a crippling disease. It is the largest cause of renal failure in the developed world. An estimated 2.5 million people are affected with diabetic retinopathy and diabetes is the leading cause of adult blindness in developed countries. It is a source of major cardiovascular morbidity and mortality contributing to 50% of diabetes-associated deaths, and is responsible for over a million limb amputations every year (Diabetes Atlas, 2006).

***Pathophysiology*:** Formerly known as non insulin-dependent diabetes, Type 2 diabetes accounts for 90% of the disease burden. It is the result of abnormal insulin secretion and insulin resistance but the β cell mass remains intact. Lifestyle alterations including medical nutrition therapy and oral glucose-lowering drugs are the mainstay of treatment for this form of diabetes.

Type 1 diabetes (T1DM), on the other hand, results from selective T cell- mediated autoimmune destruction of insulin-producing β-cells and is characterized by the presence of islet cell autoantibodies. This progressive loss of β cell mass is lethal unless treated with insulin replacement.

Data available from long-term studies suggests that intensive insulin therapy has a clear benefit with regard to the development of chronic complications and retards their progression (DCCT group, 2003). However, intensive insulin treatment also results in a threefold increase in severe hypoglycaemia and coma (DCCT group, 1997), and an estimated 2-4% of the deaths of patients with T1DM have been attributed to hypoglycaemia (Cryer, 2004). Being a single cell disorder T1DM lends itself eminently to cell-based therapies and β cell replacement is, at least in theory, the ideal treatment modality for this condition.

The pancreas is a mixed exocrine-endocrine salmon-pink coloured gland situated retroperitoneally in the transpyloric plane (across the first and second lumbar vertebrae) and shaped like a flattened tongue. Both exocrine (acinar and ductal) and endocrine portions are derived from the same epithelium, developing from the dorsal and ventral endodermal anlage of the primitive foregut under the influence of the pancreas-promoting transcription factor Pdx1 (pancreas and duodenum homebox gene 1). The gland is enclosed in a thin capsule of moderately dense connective tissue and its integrity is crucial for effective distension of the gland with collagenase during the digestion phase of islet isolation.

The presence of a pancreatic capsule also has important implications in organ preservation for islet transplantation. Thus it has been shown by several investigators that ductal injection of preservation solution (as compared with simple immersion in preservation solution) improves islet isolation and reduces islet apoptosis (Sawada et al., 2003; Noguchi et al., 2008; Stiegler et al., 2009)

The endocrine pancreas is comprised of over a million islets of Langerhans, which are embedded within the exocrine portion and make up less than 2% of the entire volume of the pancreas. Each islet is comprised of several thousand cells (2500 on average) separated into a central core of insulin-producing β cells and a surrounding mantle of α (glucagon-secreting), δ (containing somatostatin) and PP (secreting pancreatic polypeptide) cells (Weir & Bonner-Weir, 1990).

Diabetes results from an absolute or relative deficiency of insulin, a heterodimeric 84 amino-acid polypeptide hormone synthesized in the B cell of the islets. The principal physiologic effects of insulin are stimulation of protein and lipid synthesis, inhibition of protein degradation, activation of

glycolytic enzymes and glycogen synthase, and inhibition of phosphorylase and gluconeogenic enzymes (Barrett et al., 2009). The overall effect is storage of carbohydrate, protein and fat. The secreted insulin is a result of protease cleavage of proinsulin into equimolar amounts of insulin and connecting peptide (C peptide). It has now become apparent that this C peptide does not merely serve as a stabilizing link between A and B peptides of insulin. It appears to be bioactive and has been shown to activate signal transduction pathways resulting in stimulation of Na^+ K^+ ATPase (Ohtomo et al. 1996 and Tsimaratos et al., 2003) and endothelial NO synthase (eNOS) activities (Kitamura et al., 2003 and Wallerath et al., 2003). In light of these observations it is now believed that C peptide has a major role in preserving neural, renal and microvascular functions in diabetic patients and potentially reversing chronic complications of diabetes (Johansson et al., 2000; Hansen et al., 2002; Marques et al., 2004; Samnegard et al., 2005; Kamiya et al., 2006; Fiorina et al., 2007), although the exact mechanisms remain elusive.

***Treatment*:** Transplantation of the pancreas is currently the most consistent method of beta-cell replacement, restoring long-term glucose homeostasis and insulin independence. These two outcomes define graft survival, which is 85%, 69% and 46% at 1, 5 and 10 years respectively for simultaneous kidney-pancreas transplants (Gruessner & Sutherland, 2005). These are the results from early eras and there have been steady improvements in outcomes with refinements in surgical and medical management. This is a remarkable achievement, considering that patients on dialysis maintained on insulin have a 5-year survival of 21% (Rayhill et al., 2000). Several studies have examined the impact of pancreas transplantation on the secondary complications of diabetes and there is evidence of significant improvements in nephropathy, retinopathy, neuropathy and micro- and macro-vascular disease (Meloche, 2007).

However, despite these impressive results, pancreas transplantation remains a formidable procedure and is associated with the highest complication rate of all routinely performed solid organ transplants. Relaparotomy rates are high (19-32%) and have a negative impact on graft and patient survival (Humar et al., 2000).

A safer, less invasive β cell replacement strategy involves transplantation of islets – a treatment option that has emerged in recent years, following the demonstration of successful reversal of diabetes by Shapiro and colleagues at the University of Alberta, Canada in 2000. Their report of insulin independence in seven consecutive patients who received portal infusions of islet allografts rejuvenated islet transplant centres worldwide. With improved peritransplant management and immunosuppression (commonly referred as the „Edmonton protocol"), dramatic clinical outcomes have been achieved. Data from the Islet Transplant Registry (Giessen, Germany) and the Collaborative Islet Transplant Centre and the results of a multicentre international trial sponsored by the National Institutes of Health suggest that overall full insulin independence is achieved in approximately 50% of patients (Shapiro et al., 2006, Bertuzzi & Ricordi, 2007). However, long-term follow-up indicates that essentially all patients lose graft function over time such that insulin therapy is again required within 5 years and the rate of insulin independence drops to less than 10% (Ryan et al., 2005). Nevertheless, C-peptide levels are maintained in more than 80% of patients. This is implicated in the effective prevention of recurrent hypoglycaemia or severe glucose lability and improved glycosylated haemoglobin profile, and is seen as a substantial benefit of islet transplantation. Moreover, although long-term data are not available there is emerging evidence that progression of secondary complications is curtailed (Fiorina et al., 2003a, 2003b, 2003c, 2005). The overall beneficial effect of islet transplantation is reflected in a recent 7-year follow-up study of 34 uraemic T1DM patients who received islet allografts. Survival among those with long-term graft function was significantly better (90% at 7 years) and comparable to those who underwent whole-organ pancreas transplantation, than in those who lost islet function (51%). The authors concluded that successful islet transplantation improved survival, atherothrombotic profile and endothelial morphology in uraemic T1DM renal transplant patients (Fiorina et al., 2003a). There is evidence from recent studies that C-peptide exerts several physiological effects and might account for the salutary metabolic effects of islet transplantation. Infusion of exogenous C peptide in T1DM patients ameliorates nerve dysfunction, increases forearm blood flow and improves myocardial vasodilatation. It has also been shown to reduce glomerular hyperfiltration and albuminuria, and to induce glomerular

vasodilatation slowing progression of nephropathy. It binds non-specifically to cell membranes and stimulates Na^+-K^+ ATPase and eNOS activities (Fiorina et al., 2007).

However, in its current state islet transplantation faces major challenges and is not yet suitable for all diabetic patients. Organ scarcity, cell loss during isolation and losses related to preservation injury, site of engraftment, apoptosis, autoimmunity, immunosuppression and allorejection are the main problems that need to be addressed in order to optimize islet transplantation.

1.2 Islet transplantation – History, Current Status, Outcomes

1.2.1 History of pancreas and islet transplantation

"By tying the pancreatic duct we have now a means of isolating the islands anatomically and of studying their chemical properties freed from the digestive ferments. This anatomical isolation of the islands will permit the testing in a rational way of an organic therapy of diabetes."

Ssobolew, 1902.

Organ transplantation is an exotic field of modern medicine that is a fascinating testament to human ingenuity, imagination and endeavour. What began as the stuff of myths and legends in ancient civilizations has become a magnificent therapeutic achievement for mankind today. The first human pancreas allograft was performed in 1966 by Dr William D Kelly and Dr Richard C Lillihei in Minnesota. But the history of islet transplantation began well over a century ago shortly after a link was established between pancreas and diabetes in 1889, when Oscar Minkowski and Joseph Freiherr von Mering serendipitously discovered that extirpation of canine pancreas resulted in hyperglycemia, polyuria and glycosuria (Luft, 1989). In actual fact, pancreatectomy-induced diabetes mellitus in dogs had been unwittingly described in exquisite detail by a Swiss physician Johann Conrad Brunner (1653-1727) in 1683 in his book Experimenta nova circa pancreas (Keck & Pfeiffer, 1989). Minkowski subsequently treated a diabetic dog with subcutaneous fragments of its own pancreas

(with intact blood supply) in 1892. His observation that no diabetes resulted until the pancreatic remnant was removed led him to surmise that diabetes resulted from lack of a pancreatic substance transported by the bloodstream. It would be more than a decade before the term „hormone" was coined to designate the body"s „chemical messengers" (Starling, 1905). Almost three decades before Banting and Best discovered insulin, the first human islet tissue transplantation was performed by Watson-Williams, a senior assistant physician in Bristol Royal Infirmary. In 1893 he transplanted a few pieces of freshly slaughtered sheep pancreas under the skin of a 13 year old end-stage diabetic boy. Following a brief response, the boy rapidly rejected the xenograft and succumbed to ketoacidosis (Williams, 1894). In 1916, Frederick Pybus described similar clinical studies, but this time sliced human pancreata were used for the subcutaneous transplants (Pybus, 1924). Unfortunately these early attempts universally failed to relieve diabetes.

Pancreaticoduodenal transplant in a rat model was first described by Lee and colleagues in 1972. Interestingly, around this time significant advances were also being made in islet transplantation – primarily in rodents.

Hellerstrom described the first technique of islet isolation by microdissection of rodent pancreas (Hellerstrom, 1964) even though the idea of separating islet tissue from the exocrine portion of the gland was broached at the turn of the nineteenth century by Ssobolew (Pratt, 1954). A year later Moskalewski described islet isolation by enzymatic digestion, using crude collagenase to disperse diced guinea pig pancreas (Moskalewski, 1965). This technique was refined and standardized by Lacy with the addition of pancreatic duct cannulation and distension with cold balanced salt solution to achieve mechanical separation before excising the gland (Lacy & Kostianovsky, 1967, Ballinger & Lacy, 1972). Further refinements led to the introduction of ductal collagenase delivery resulting in significant improvements in islet yield and purity, and the latter technique was rapidly adopted as the standard for islet isolation (Horaguchi & Merrell, 1981, Noel et al., 1982, Gray et al., 1984, Gotoh et al., 1985, Sutton et al., 1986). Subsequent developments included the introduction of a digestion/filtration chamber (Scharp et al., 1975, Gray et al., 1984, Ricordi et al., 1986) to facilitate

the process of islet digestion and of the Cobe 2991 cell separator (Lake et al., 1989) enabling large-scale purification of islet preparations from higher mammalian pancreas.

The first successful islet transplant for the treatment of a diabetic animal was performed by Ballinger and Lacy in 1972. They transplanted 400-600 islets in diabetic Lewis rats and successfully reversed their diabetes (Ballinger & Lacy, 1972). Around this time the first forays into islet xenotransplantation were also made by grafting piscine islets (Brockmann bodies) into rodents (Reemstma, 1970; Weber et al., 1975), with disappointing results. Studies of islet allotransplantation then moved on to large animal models and encouraging experiments were carried out in dogs, pigs and monkeys (Alejandro et al., 1985; Gray et al., 1986). The first fully documented report of reversal of type 1 diabetes in humans (albeit for 22 days) following islet transplantation appeared in 1990 from the St. Louis group (Scharp et al., 1990). There followed clinical trials in patients undergoing upper abdominal exenteration for advanced malignancy, conducted jointly by the groups in Pittsburg, Milan and Miami. Islets were transfused into the portal vein of the transplanted liver with promising results (Tzakis et al., 1990). The next major developments were the introduction of the automated method for isolating human islets in a Ricordi chamber (Ricordi et al., 1989) and the adaptation of the Cobe 2991 cell processor for large-scale islet purification. However attempts to reverse diabetes long-term by transplanting allogeneic islets recovered by these improved techniques remained unsuccessful. The modern era of clinical islet transplantation was ushered in with the introduction of the Edmonton protocol in 2000. This protocol was used in a trial of seven patients and resulted in 100% insulin independence (Shapiro et al., 2000).

1.2.2 Islet Transplantation and Edmonton Protocol

Islet transplantation begins with surgical removal of the pancreas followed by enzymatic digestion (with intraductal infusion of collagenase derived from Clostridium histolyticum) in a specialized GMP islet isolation facility. Islets obtained by this automated procedure are purified by centrifugation

with discontinuous gradients. Subsequently quantitative and qualitative assessment of the islet preparation is made and if standard release criteria are met, the suspension is slowly injected in the portal vein by transhepatic route under ultrasound and fluoroscopic guidance. The success of the Edmonton group was attributed to several key innovations in their protocol:

Transplanting an adequate number of islets (>10,000 islet equivalents per kilogram of recipient‟s body weight).

1. Minimizing diabetogenic effects of immunosuppressive regimen by the replacement of glucocorticoids with monoclonal antibody against interleukin-2 receptor and use of low-dose tacrolimus in combination with sirolimus.

2. Preparing islets in a xenoprotein-free medium.

3. Minimizing the duration of cold ischaemia by transplanting fresh islets immediately following harvest.

The adoption of this protocol has vastly improved islet transplantation results and the latest registry data from 31 active centres in the United States and two European centres, collected from 325 allograft recipients between 1999 and 2007 (Collaborative Islet Transplant Registry published in 2008) indicate that 70% of these patients achieve insulin independence at least once (this figure was only 12.4% in a similar number of patients transplanted before institution of the Edmonton protocol) (Alejandro et al., 2008).

1.2.3 Clinical outcomes

Clinical outcomes of islet transplantation in the current decade have changed dramatically from the dismal results of the previous two decades. Complete insulin independence is presently 70% at 1 year, although this rate drops precipitously to 15% at 5 years (Ryan et al., 2005). However, prevalence of

severe hypoglycaemic episodes is reduced from 81-85% pre-infusion to 8-35% at three years" post last infusion, and 32% of allograft recipients retain measurable C-peptide levels at three years" post last infusion (see section 1.1). The prevalence rate of HbA_{1c} at the same time point is between 36 and 64% (Collaborative Islet Transplant Registry published in 2008).

1.3 Current limitations of islet transplantation

Before islet transplantation takes its place as a conventional therapeutic option for treatment of diabetes, many major hurdles will need to be overcome:

1. The limited supply of organs and the need to use more than one (2-4 donors per recipient) pancreas for each successful outcome.
2. Huge losses of islets during the process of transport, isolation and purification.
3. Suboptimal (intra-portal) implantation site with limited engraftment potential and lack of clear understanding of inter-cellular and islet – extracellular matrix interactions.
4. Elusive mechanisms and inadequate means of preventing islet rejection.
5. Adverse reactions to immunosuppressive medications.
6. Risk of sensitization (appearance of HLA antibodies) post-transplant with a potentially negative impact on the ability of recipients to undergo further transplant procedures (Campbell et al., 2007).

While the last three are global issues in transplantation medicine, the first three hurdles are peculiar to islet transplantation and much of the effort in overcoming these obstacles has been directed towards amelioration of preservation injury to the pancreas.

1.4 Pancreas-Islet Preservation injury

Poor islet isolation efficiency continues to be a major hurdle: despite standardized isolation protocols, islet isolation outcomes have remained inconsistent and a successful islet yield is obtained from less than 50% of processed pancreases (Kin et al., 2007). Quality of the procured organ (good condition with preserved surface integrity i.e. surgically intact, presence of fatty infiltrates, heavier pancreas, and procurement team) has been identified as significant variable associated with successful islet isolations (Lakey et al., 1996; Nano et al., 2005; Hanley et al., 2008; Sakuma et al., 2008; Kaddis et al., 2010 – **table 1.1**). Goto and colleagues observed that successful clinical outcome was strongly correlated with donor and procurement related variables rather that those related to the isolation process. They identified short warm ischaemia time and low expression of tissue factor in pancreatic tissue as important variables that correlated strongly with high C-peptide levels post-transplantation (Goto et al., 2005).

Minimizing the duration of cold ischaemia is considered one of the keys to the success of the clinical islet transplantation program (Shapiro et al., 2000). The negative impact of cold storage on islet recovery has been documented in several studies both in animal models (Hesse et al., 1987; Munn et al., 1989; Tanioka et al., 1997) and in humans (Benhamou et al., 1994; Zeng et al., 1994; Lakey et al.,1995; Tsujimura et al., 2004a). Preservation of rat pancreas for 24 hours in UW solution resulted in 70% reduction in insulin secretion during perifusion compared with controls (Kneteman et al., 1994). Using UW solution, Sak‟s solution and silica gel fractionated plasma in canine and rodent models, Munn et al showed that there was a significant reduction in islet recovery and autograft success rates with increasing duration of cold preservation, irrespective of the preservation solution employed.

In their retrospective analysis of 146 adult pancreases, Lakey et al. found that cold storage for duration more than 8 hours was associated with an increased proportion of failed isolations and decreased measures of islet viability (Lakey et al., 1995). They subsequently identified prolonged cold

storage prior to islet isolation as having a significant negative correlation with respect to islet recovery from human pancreas (Lakey et al., 1996).

1.4.1 Cold ischaemia and islet injury (a. Oxidative stress b. Isolation stress)

Ischaemia and hypoxia are considered the two most detrimental factors influencing pancreatic-islet isolation outcomes (Carlsson et al., 2002; Linn et al., 2006). Current preservation techniques for whole pancreases appear inadequate for the purpose of islet isolation and transplantation: this is possibly due to additional stresses imposed on the islets during pancreatic digestion, isolation, purification and culture. Preservation inadequacy is highlighted by only 50% of isolations yielding successful outcomes (transplantable preparations) despite standardized protocols (Ponte et al., 2007). The adverse (alternating warm and cold) temperatures, shear stresses and osmotic gradients generated during the isolation process compound the ischaemia-reperfusion injury following cold preservation by the following mechanisms (Paraskevas et al., 1999, 2000, 2001; Abdelli et al., 2004; Bottino et al., 2004; Noguchi et al., 2006):

a) Withdrawal of trophic support – removal of extracellular matrix (including basement membrane) and growth factors.

b) Stress-induced activation of the pro-apoptotic NF- κB, JNK and p38 signalling pathways.

Pileggi et al. (2009) observed that cold ischaemia reduced islet recovery by 50% and this was associated with a significant reduction in islet viability and post-transplant function. Isolated islets from rat pancreas subjected to cold ischaemic storage displayed higher levels of inflammatory markers such as phosphorylated stress-activated protein kinases (c-jun N-terminal kinase and MAPK-p38) and chemokine CCL3, and correspondingly reduced levels of cytoprotective cytokines (VEGF, IL-9 and IL-10).

Moreover, gene array and western blot analysis of islets isolated from human pancreas suggests that hypoxic preservation injury also leads to reduction in mitochondrial potential with consequent release of cytochrome C and APAF 1 in the cytosol and activation of caspases. This mitochondrial-regulated apoptosis can be counteracted by reducing hypoxia using PFC for pancreas preservation (Ramachandran et al., 2006). Recently it has been suggested that hypoxia during preservation causes phosphorylation of AMP-activated protein kinase (AMPK) through ATP depletion. AMPK, in conjunction with reactive oxygen species engendered through mitochondrial oxidative stress, plays a major role in the induction of hypoxia-mediated β-cell apoptosis (Ryu et al., 2009).

Central cell death in large islets correlates with the depletion of intracellular ATP content which significantly deteriorates glucose-stimulated insulin release and morphological viability (Giuliani et al., 2005).

Much of the effort towards improving results of islet transplantation has been directed towards minimizing preservation injury to the pancreas. Subsequent chapters will focus on the existing literature and my experimental work on this aspect of islet transplantation.

Variable	Effect on islet isolation	references
DCD donor	negative	Kaddis et al., 2010
Donor age	negative	O'Gorman et al., 2005; Kaddis et al., 2010
History of diabetes	negative	Kaddis et al., 2010
High donor BMI	positive	Lakey et al., 1996; O'Gorman et al., 2005; Kaddis et al., 2010
Prolonged cold ischaemia	negative	Zeng et al., 1994; Lakey et al., 1996; O'Gorman et al., 2005; Ponte et al., 2007; Kaddis et al., 2010
pancreas weight	positive	Lakey et al., 1996; Nano et al., 2005; Kaddis et al., 2010
Intact pancreatic capsule	positive	Sakuma et al., 2008; Kaddis et al., 2010
Fatty pancreas	positive	Hanley et al., 2008; Kaddis et al., 2010
Oedema of pancreas	positive negative	Taylor et al., 2008 Mahler et al., 1999; Kaddis et al., 2010
Serum amylase / lipase	negative	O'Gorman et al., 2005; Kaddis et al., 2010
Pancreas preservation (UW vs. TLM)	no difference	Agrawal et al., 2008
Pancreas processing variables viz. enzyme, digestion period	significant not significant	Benhamou et al., 1994; Nano et al., 2005; Ponte et al., 2007; Hanley et al., 2008 Kaddis et al., 2010

Table 1.1: Variables influencing islet isolation outcome (islet yield)

CHAPTER 2

PANCREAS PRESERVATION AND THE TWO-LAYER METHOD

2.1 Solid organ preservation

Organ preservation is one of the cornerstones of successful solid organ transplantation. Utilization of organs from brain dead donors is critically dependent on maintenance of organ viability *ex vivo* to allow transportation of the organ and preparation of the recipient. The second important objective of preservation is organ optimization so that it functions immediately when transplanted.

Several phenomena have been implicated in cell injury following removal of organs from an intact circulatory system and during cold preservation. Mitochondrial damage and depletion of ATP precursors result in loss of energy-generating capabilities and cell death (D"Alessandro et al., 1994). The ischaemic environment facilitates generation of highly cytotoxic oxygen free radicals upon reperfusion (Hoshino et al., 1988; Risby et al., 1994). There is also unopposed activation of lysosomal enzymes, phospholipases and proteases and a high concentration of inflammatory mediators consequent upon the activation of the arachidonic acid cascade (McAnulty et al., 1991; Nichols et al., 1994; Pavlock et al., 1984).

Reducing ATP depletion to a minimum is critical for control of these cascades of ischaemic injury that occur during organ preservation. This is best accomplished, at least in principle, by perfusion of the organ with an oxygenated solution.

2.1.1 History of organ preservation

"Ice is civilization! " in *The Mosquito Coast* by Paul Theroux, 1981

The success of living donor kidney transplantation with the advent of azathioprine and prednisone provided the impetus for expansion of the donor pool by utilizing organs from deceased donors (Murray et al, 1967; Nolan, 1985; Murray, 2005). The vexing problem of warm ischaemia was tackled by many investigators in the 1960s after it was established by Levy in 1959 that by slowing cellular energetic metabolism, cold decreased oxygen consumption. Calne and colleagues (1963), Humphries et al.(1964), Manax et al.(1965), Ackermann, Fischer and Barnard (1966), and Ladaga et al.(1966) showed that organ preservation for 24- and 48- hours was possible with hypothermia, hyperbaric oxygen and perfusion (alone and in combination). Folkert Belzer and his colleagues (1967) at the University of California, San Francisco successfully preserved dog kidneys for three days by devising a method of continuous pulsatile perfusion at 8-12°C with cryoprecipitated human plasma. During the same period, Geoff Collins, a heart surgeon working in Paul Terasaki"s laboratory, reported effective 20-hour preservation of canine kidney by flushing with an intracellular - type phsophate-based glucose solution and storing the organ in a refrigerator at 4°C (Collins et al., 1972). Collins solution and Belzer"s method of perfusing kidneys became the standard methods of preservation and were used clinically for the ensuing 25 years with great efficacy.

The next landmark in the evolution of organ preservation was precipitated by the demonstration of the efficacy of cyclosporine and the subsequent realization of liver and pancreas transplantation. Preservation of these organs was still not possible beyond four to eight hours. In the 1980s Belzer, now at the University of Wisconsin at Madison, with his colleagues developed a cold preservation solution that contained agents to suppress hypothermic cell swelling and counteract hydrostatic perfusion pressure, a hydrogen ion buffer and a few other additives. This solution was first used effectively to extend pancreas preservation in the dog to 3 days (Wahlberg et al., 1987), and was subsequently shown to effectively preserve canine liver up to 48 hours (Jamieson et al., 1989).

Marketed as ViaSpan (DuPont Pharma, Wilmington, DE), the University of Wisconsin (UW) solution became the standard cold storage preservative for most transplant organs and retains this status today.

2.1.2 Current preservation solutions

In the current state of our knowledge, optimal organ preservation is conditional upon the following effects of preservation solutions:

- Hypothermia-induced reduction in cellular metabolism and oxygen consumption and concurrent attenuation of enzymatic activity *(the temperature effect)*
- Flushing out of blood and maintenance of vascular patency *(the solution effect)*
- Prevention of cellular swelling *(the physical effect)*
- Maintenance of physiological pH and prevention of formation of reactive oxygen species and toxic compounds *(the chemical effect)*

Thus most cold storage solutions contain impermeants (for osmotic effect), purine nucleotide precursors, antioxidants, enzyme inhibitors, and vasoactive substances. Of the different solutions in common use today (UW solution, Celsior, Histidine-Tryptophan-Ketoglutarate and Two-Layer Method), none has emerged unequivocally superior to the other.

Collin‟s solution was the first static cold preservation solution to be developed in 1969. Designed to mimic the intracellular composition of the kidney, it contained high levels of potassium with magnesium as the membrane stabilizer, phosphate as a buffer and glucose as the osmotic agent. Subsequent modifications led to the development of Euro-Collin‟s solution in 1976. Mannitol was used in place of glucose and magnesium was omitted to overcome the problem of precipitation. Although this solution has been withdrawn in the United States, it continues to be used in Europe.

Marshall solution (Hypertonic citrate)

Also developed in the 1970s in Australia, this solution contains citrate as the impermeant agent to suppress hypothermia-induced cellular swelling. This solution is still used in the UK for clinical kidney preservation.

University of Wisconsin (UW) solution

The University of Wisconsin solution is the standard preservation medium for clinical transplantation today. Developed in the late 1980s by Belzer and colleagues, it has stood the test of time and no other solution has been shown to be clearly superior to it as yet. The major ingredients in this preservative are lactobionic acid and raffinose serving as osmotic agents, hydroxyethyl starch for further oncotic support, a phosphate buffer (sodium monopotassium acid phosphate), glutathione and allopurinol as free radical scavengers, and an ATP precursor (adenosine).

Histidine-Tryptophan-Ketoglutarate (HTK) solution

Introduced as a cardioplegic solution in open-heart surgery by Bretschneider in 1980, it was successfully tested in solid organ transplantation. It contains a very potent buffer (histidine), a membrane stabilizer (tryptophan) and an energy substrate (ketoglutarate). The major differences with UW solution are:

1. Low viscosity due to lack of high molecular weight colloids resulting in improved organ perfusion.

2. Low potassium concentration obviating the need for organ flushing prior to reperfusion.

A recent meta-analysis comparing HTK with UW solution found equivalence in preservation efficacy for human livers (Feng et al., 2007). Subsequent clinical trials have confirmed this observation (Rayya et al., 2008). Similar observations for kidneys and pancreas are found on reviewing existing literature (Lynch et al., 2008, Agarwal et al., 2008). A definite conclusion cannot yet be drawn due to paucity of randomized controlled trials.

Celsior solution

Developed for cardiac grafts in 1994, this is an extracellular-type preservative that has been shown to be useful for abdominal organs as well. It combines the strengths of UW and HTK solutions, incorporating inert high molecular weight impermeants (lactobionate and mannitol) and scavengers like the former, and histidine to combat intracellular acidosis like the latter. A prospective, multicentre, non-comparative study from France suggests that it is safe and effective for use in all solid organs (Karam et al., 2005).

Perfluorocarbon-based preservatives

The philosophy that provision of oxygen is critical for organ survival during cold storage underlies the development of the perfluorocarbon- based preservation solutions. In the two-layer method (TLM) introduced by Kuroda in 1988, perfluorocarbon is usually combined with UW solution for optimal organ storage and this method has found its most useful application in pancreas preservation for islet transplantation. It has also been used experimentally for kidney, lung, heart and small bowel preservation. Large-scale use in the preservation of these organs has not been tried due to the lack of evidence of superiority over simple cold storage with the reference preservative – UW solution. Perfluorocarbon emulsions have been studied as perfusates for graft preservation. In a recent study on non-heart-beating kidney grafts, Reznik and colleagues (2008) perfused with Perftoran, a new-generation PFC-based emulsion demonstrated significant improvement in delayed graft function rates

(up to 30%). Earlier trials in liver preservation have been unrewarding largely due to the physical properties of older PFC emulsions (Klar et al., 1998).

Newer solutions

Polysol is a new preservative developed at the University of Amsterdam, with an emphasis on prevention of ischaemia/reperfusion injury. It is a complex solution with 65 components including 21 amino acids designed to confer high energy charge and potent buffering and antiproteolytic properties. It is being evaluated particularly for application to steatotic or marginal livers (Hata et al., 2007).

A new solution has been developed at the Institut George Lopez in Lyon, IGL-1 has been evaluated clinically in renal transplantation (Badet et al., 2005). In this non-randomised study of 70 renal transplants, incidence of delayed graft function was found to be significantly lower in the IGL-1 group compared to UW kidneys (5.7% against 13.9%). It is an extracellular-type fluid containing polyethylene glycol (PEG), which is believed to have anti-inflammatory and endothelial-preserving properties in addition to oncotic effects.

2.1.3 Pancreas preservation

Four methods of pancreas preservation have been described:

1. Hyperbaric
2. Simple cold storage
3. Machine perfusion
4. Two-layer method

Hyperbaric preservation

Manax et al showed in 1966, that hypothermia and hyperbaria could preserve transplant organs for 72 hours in a canine model. However pancreaticoduodenal grafts suffered irreversible damage after 20 hours with a progressive decline in insulin secretion and became hemorrhaghic on reperfusion.

Although hyperbaric preservation is an attractive option, the depth of oxygen penetration depends on the square root of the surface pO_2 and very high pressures would be required for adequate oxygenation of the pancreas. One potential solution to this problem is vascular gas perfusion (persufflation), a technique that has been tested recently in porcine pancreases with promising preliminary results (Scott et al., 2010).

Simple cold storage (hypothermic preservation)

UW solution has proven to be a very effective preservation medium for pancreas and grafts can be preserved for periods exceeding 24 hours.

Several components of the UW solution exert an inhibitory action on collagenase activity (Kneteman & Wagner, 1992; Kneteman et al., 1994; Contractor et al., 1995). There is a progressive decline in islet yield and viability with increasing storage times. The upper limit of cold storage prior to islet isolation has been determined to be 16 hours (Lakey et al., 1995). Zeng et al. (1994) have demonstrated that cold storage beyond 8 hours significantly reduces human islet yield and purity.

Machine perfusion

Machine perfusion stimulates metabolism by supplying oxygen and nutrients and removing metabolic waste products, while maintaining optimal tissue pH and allowing addition of pharmacological agents that may help preserve cell viability (D"Alessandro et al., 1994). It has been shown to be reliable and simple, and to provide better quality preservation than simple storage. For the kidney and heart, it has

been shown to minimize ischaemic and reperfusion injuries (Hassanein et al., 1998, Burdick et al., 1997).

The disadvantages of machine perfusion with respect to the pancreas include the relative complexity of the machine compared to simple ice preservation and the fact that these machines have been designed for high-flow organs (kidney, heart). Oedema and damage to the graft secondary to excessive perfusion pressures is a major obstacle and currently 24 hours is the limit of pancreas preservation by this technique. Studies comparing cold storage with machine perfusion for pancreas preservation found little difference in results after 24 hours of storage (Florack et al., 1983; Toledo-Pereyra et al., 1980)

Two-layer method (TLM)

The Two-Layer Method (TLM) of preservation for organ transplantation was developed by Dr Kuroda at Kobe University, Japan in 1988. The TLM employs oxygenated perfluorocarbon and UW solution for organ preservation – the graft being made to float at the interface of the two liquids (which differ in specific gravity almost by a factor of 2). Prior to this, perfluorocarbon, used in the form of emulsions, had been shown to be useful in preserving organs by several investigators (Berkowitz et al., 1971; Novakova et al., 1975; Dirks et al., 1980, Kamada et al., 1980, Tomera & Geyer, 1982; Menasche et al., 1983).

Although the TLM has been used almost exclusively to preserve pancreas for islet isolation, it has been shown to be superior to conventional hypothermic storage also for vascularized pancreas, kidneys (Maluf et al., 2006), heart (Kuroda et al., 1995) and small bowel (DeRoover et al., 2001, Kakinoki et al., 2004).

2.2 Pancreas (whole organ) preservation for islet transplantation

With respect to islet transplantation there are numerous studies investigating the outcomes of different preservation solutions. Hubert et al. (2007) observed a markedly reduced islet recovery from human pancreas preserved with Celsior, as compared to UW solution. They subsequently observed that Celsior induced cell swelling and pancreatic oedema within four hours of preservation in porcine pancreases and concluded that colloid-free solutions might be suboptimal for pancreas preservation. On the contrary, one retrospective study of 125 islet isolations found no significant difference between UW solution, Celsior and IGL-1 (Wojtusciszyn et al., 2005). Similarly, comparisons with HTK and UW solution have found no appreciable difference in islet isolation outcomes (Salehi et al., 2006).

Notwithstanding the equivalence of these preservation solutions, there is urgent need to improve quality of the pancreas prior to islet isolation. In 2002, several centres reported improvement in islet yield with the two-layer method (Hering et al.; Fraker et al.; Lakey et al.; Matsumoto et al; Tsujimura et al.). Subsequent studies also showed improvement in islet recovery, viability and morphology, better rates of transplantable preparations and improved graft outcomes. An improved outcome with marginal donors has been attributed to the employment of TLM preservation. The energy charge and ATP content were significantly higher in TLM-preserved pancreas (Salehi et al., 2006). Recently, investigators in Kyoto have developed a modified TLM replacing UW with Miraclid-Kyoto (M-Kyoto) solution. This preservation solution contains trehalose (cytoprotective effect) and ulinastatin (trypsin inhibitor) in addition to standard components of UW. The inhibition of trypsin activity is claimed to improve islet yield and function in comparison with UW – TLM (Noguchi et al., 2006 and 2007). Brandhorst et al. (2006) have demonstrated that simple preservation with oxygenated perfluorocarbon (one-layer method, OLM) is equally effective and less cumbersome. They observed that short-term OLM preservation improved in-vitro but not in-vivo function of pig islets damaged by warm ischaemia. More recently, other modifications have been introduced. The Miami group have

observed that addition of nicotinamide during islet processing has a synergistic effect with TLM preservation and the proportion of successful (transplantable) preparations increased up to 69% (Ichii et al., 2006). Goto et al reported that application of TLM during the digestion process, addition of perfluorodecalin to the Ricordi chamber and adenosine supplementation significantly improved islet yields and ATP levels, although there was no improvement in function when compared with controls (Goto et al., 2007).

However, the efficacy of this technique has been called into question following contradictory reports in recent literature. It seems likely that the optimal method of TLM preservation is not currently practised and the full beneficial effects of an oxygen-rich preservation medium are still to be realized. The main reason for this is that the precise mechanism of action of the two-layer method is not yet understood. The aims of this thesis are two-fold: firstly to examine the mechanism of action of the two-layer method and subsequently to develop an optimal method of pancreas preservation by TLM.

2.3 Two-Layer Method for preservation of other organs

The two-layer method of preservation has been examined for small bowel and heart, using various animal models. In a syngeneic rat model of small bowel transplantation with a Thiry-Vella loop, for preservation the lumen was filled with UW solution and the graft was then completely immersed in PFC. Whereas the simple cold storage was effective only for 24 hours, the intraluminal two-layer method with oxygen bubbling made it possible to extend the preservation time to 48 hours (Kuroda et al., 1996a). The authors also showed the efficacy of intraluminal TLM in a canine heterotopic small bowel transplant model, where the preservation time could be prolonged safely up to 24 hours with no unfavourable effects on the graft based on morphological and functional parameters (Tsujimura et al.,

2002). The cavitary TLM preservation method was also used by the authors for heart preservation with extension of preservation times by up to 48 hours (Kuroda et al., 1995).

2.4 Perfluorocarbons (PFC)

Perfluorocarbons (PFCs), or perfluorochemicals, are a class of synthetic, chemically stable compounds derived from hydrocarbons (linear, cyclic or polycyclic) in which fluorine is substituted extensively for hydrogen (**Fig 2.1**).

Following their serendipitous discovery by a 27-year old research chemist, Roy Plunkett, working at the Du Pont Research Laboratories in New Jersey in 1938, perfluorocarbons (PFC) were first produced during World War II under the auspices of the Manhattan atomic bomb project, as an outcome of the search for inert buffers against the highly reactive substrates (fluorine and uranium hexafluoride) of the fissionable isotope ^{235}U. As they were unique in their ability to resist attack by uranium derivatives, a huge secret war-time effort was mounted to produce PFCs to serve as buffers, coolants, lubricants and sealants in gas-diffusion plants for the concentration of the uranium isotope. They were code-named "Joe"s stuff" after Joe Simons, the American scientist who first described the "catalytic" method of reacting fluorine with hydrocarbon "steadily and without explosions" (direct reaction of fluorine with carbon being a highly exothermic one).

Perfluorocarbons came to the attention of medical researchers in 1966 when an article published in Science reported on the survival of mice for extended periods when immersed in an organic liquid (Clark & Gollan, 1966) (**Fig 2.2**).

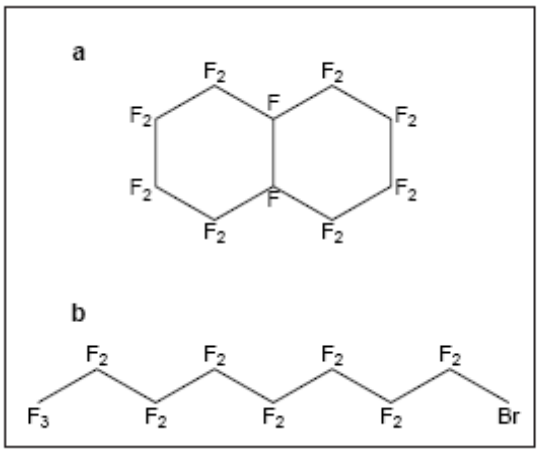


Figure 2.1: chemical structure of (a) perfluorodecalin (cyclic PFC), and (b) perfluoro-octylbromide (linear PFC)

Figure 2.2: Mouse in a breaker breathing oxygenated liquid perfluorochemical (FX-80). Adapted from Pure Appl Chem 1982;54(12):2383-3406.

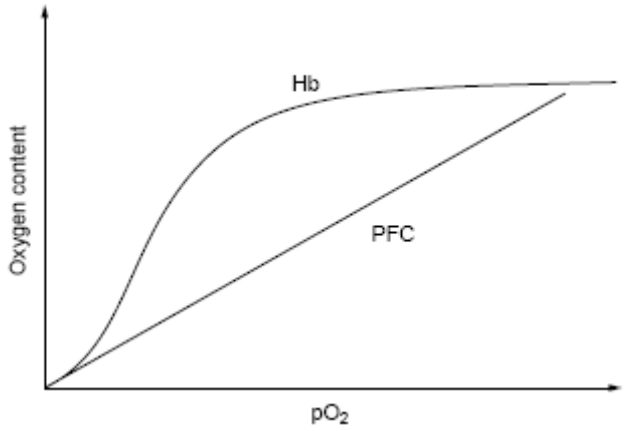


Figure 2.3: Relationship between oxygen content and partial oxygen pressures for haemoglobin and perfluorocarbon (see text).

2.4.1 Chemistry of perfluorocarbons

Perfluorocarbons are chemically inert synthetic fluorinated hydrocarbons. They owe their non-reactive properties to the exceptional strength of the C-F bond (approx 466kJ/mol) and the excellent steric and electronic protection that the fluorine sheaths confer on their underlying carbon skeletons (Lewandowski et al., 2006). Due to fluorine‟s low polarizability perfluorocarbon liquids have weak intermolecular forces – a property that explains the high solubility coefficient for respiratory gases. At 37°C the solubility of O_2 is typically 15 – 20 times higher and that of CO_2 approximately 3 times higher than that in plasma (Biro & Blais, 1987). Gas molecules dissolve physically and occupy „cavities‟ within the PFC as the latter cannot bind gases chemically (Hamza et al., 1981). Thus whereas haemoglobin exhibits the typical sigmoid oxygen dissociation curve, perfluorocarbons are characterized by a linear relationship between gas solubility and partial pressure, approximating to Henry‟s law (**Fig 2.3**). PFC-dissolved oxygen is immediately available to tissues and is characterized by high extraction ratios (90% compared with 25% for Hb). Moreover, the ease of O_2 dissociation and release to tissues is preserved at low temperatures (Riess, 2001).

Fluorine is the most electronegative and the most reactive element in the periodic table. It forms the strongest single bond to carbon encountered in organic chemistry. This is a consequence of the following properties of the fluorine atom (O'Hagan, 2008):

1. Steric effect: after Hydrogen (1.20A), F has the smallest van der Waals radius (1.47A).
2. Electronic effect: it possesses the largest electronegativity of all elements (4.0 vs. 2.5 for C). This difference in electronegativity makes the C-F bond highly polar and also contributes to its strength.
3. Bond strength: the bond energy (466 kJ/mol) is higher than other C-halogen bonds. This makes the C-F bond very stable toward metabolic transformations.

The tight binding of its valence electrons also results in low atomic polarizability, and this translates into low surface energies and weak cohesive forces. These properties explain the high compressibility of perfluorocarbon liquids and the resultant availability of interstitial spaces.

Other properties that enhance their value in medical applications are the lack of flammability and low toxicity. They are relatively resistant to thermal and radiation damage in all physical states (Geyer, 1988). They are not metabolized in the body and ultimately eliminated via the lung in expired air (Flaim, 1994). A small fraction of PFC is phagocytosed by the reticuloendothelial macrophages in liver and spleen and this leads to characteristic, predictable, and reversible biological side effects that are a consequence of normal host-defence mechanism (Flaim, 1994). Transient macrophage stimulation with systemic release of cytokines (interleukin-1, interleukin-6 and tumour necrosis factor) and arachidonic acid metabolites thromboxanes and prostaglandins) results in cutaneous flushing immediately on intravenous administration, and a delayed flu-like syndrome, all of which resolve within 12 hours of onset.

The remarkable property of PFC that underlies their efficacy for oxygen transport and delivery is their high solubility coefficients for O_2 and CO_2 and a negligible O_2 binding constant (Geyer, 1988).

Physical dissolution of gases in PFC obeys Henry's law as opposed to the Barcroft sigmoid curve for haemoglobin. PFCs can offload oxygen to the surrounding tissues far more easily in comparison with

haemoglobin, since the van der Waals interactions between gases and PFC are an order of magnitude weaker than the covalent coordination bonds that typically occur between oxygen and haemoglobin. Thus PFCs have an oxygen extraction ratio of 90%, whereas this figure is only 25% for Hb. (Faithfull, 1994; Lutz et al., 1978). However, as a consequence of this linear relationship a higher FiO_2 is required for better O_2 transport. As they are immiscible in water, in order to be used as oxygen carrier perfluorocarbons are emulsified and the droplet size is approximately 0.16μ (Kocian & Spahn, 2008). As a result, they have potential applications not only as blood substitutes but in situations of critical vascular stenoses impeding circulation of red cells viz. severe coronary or limb ischaemia (Wang & Rao, 2007). Emulsions of ozonized Perftoran have been used effectively as intra-arterial injections for treatment of critical lower limb ischaemia in Russia (Askerkhanov et al., 2007).

2.4.2 Comparison between PFC and Hb as oxygen carriers

Oxygen is carried in the blood in two forms: bound to Hb and physically dissolved in the plasma phase (much like in PFC). Oxyhemoglobin saturation depends on pO_2 and the relationship is not linear due to allosteric enzyme activity (cooperativity of Hb molecule) but sigmoidal. Oxygen affinity of the Hb molecule is reduced by increases in concentration of hydrogen, pCO_2 and temperature. Oxygen transport in the plasma phase is directly proportional to pO_2.Under normal atmospheric conditions, due to low solubility oxygen content of plasma is very small (0.3 ml/100ml blood/100mmHg pO_2). Under air-breathing conditions the contribution of plasma dissolved oxygen is small tissue oxygen delivery (DO_2) (1.6% of DO_2).

Like gases, PFCs have very low cohesive energy densities and the physical dissolution of oxygen in PFCs is characterized by loose, non-directional van der Waals interactions between like materials. In contrast, oxyhaemoglobin is the result of a strong chemical coordination bond between the dioxygen molecule and iron atom of heme (**Fig 2.4**). The relationship between partial pressure of oxygen (pO_2)

and oxygen uptake is therefore very different for these two oxygen carriers: sigmoidal for Hb and linear for PFC. Thus unlike Hb, perfluorocarbons cannot be saturated (Riess, 2006).

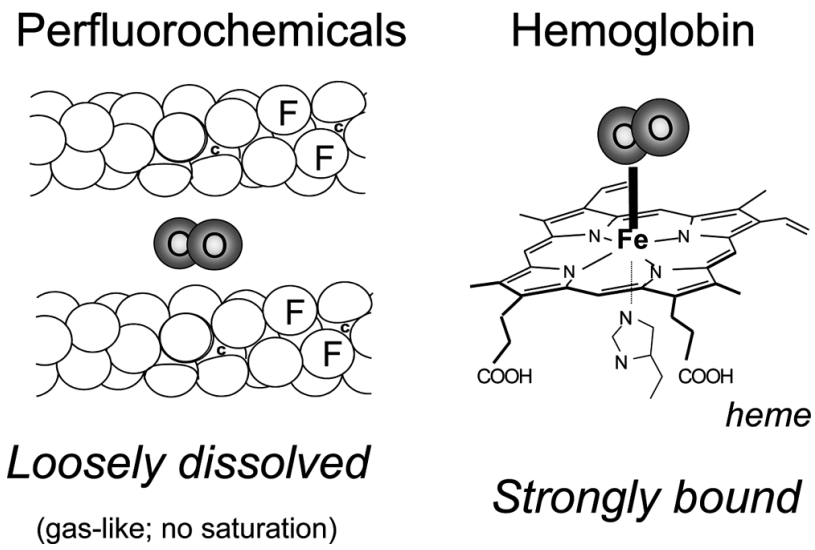


Figure 2.4: Adapted from Riess, 2006: oxygen molecule is chemically bound to iron atom of heme in Hb, unlike in PFCs where non-directional van der Waals interactions characterize the oxygen-PFC binding. Thus with the latter, there is no possibility of saturation or interaction with other reagents like NO and CO.

This lack of chemical fixation greatly facilitates oxygen extraction from PFC. Liquid gas solubility is governed by Henry‟s law: at a constant temperature, the amount of gas dissolved in a given type and volume of liquid is directly proportional to the partial pressure of that gas in equilibrium with that liquid. Oxygen solubility of PFCs is 20-25 times greater (in terms of volume percent) than in water or plasma under the same conditions (Lowe, 1999; Riess, 2005). To put things in context, solubility of oxygen is 2.5% in water and plasma, 20% in human whole blood and 40% in PFC (Ivanitsky, 2001)

It has been shown that the low oxygen solubility in plasma is the major determinant of the intracapillary resistance to oxygen transport (Federspiel, 1986, Hellums JD 1977). Because of Henry‟s

law, PFC emulsions in equilibrium with high pO_2 environments can transport more oxygen than those not exposed to high oxygen (**Fig 2.5**). Since oxygen is directly available in the soluble phase in PFC emulsions in direct proportion to the partial pressure (like in plasma), at a PaO_2 of 500 mmHg, 80-90% of the oxygen from the soluble phases (of PFC and to a much smaller extent, of plasma) will be released to the tissues prior to any oxygen being released from the haemoglobin phase. In this manner, at PaO_2 of 400 -500 mmHg, a clinical dose of 1.8g PFC/kg can supply 10-15% of the total oxygen consumption (**Fig 2.5**) – this being an equivalent to the contribution from 2-3U of packed red blood cells.

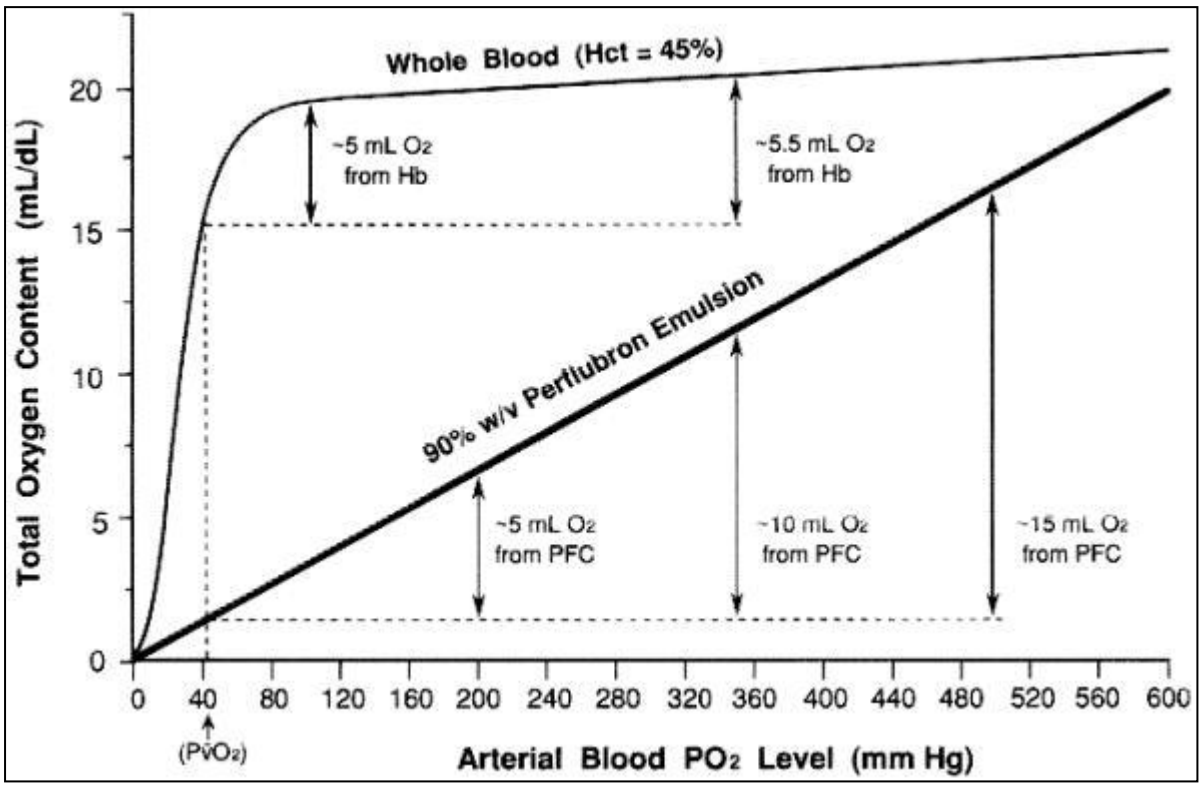


Figure 2.5: Adapted from Wahr et al., 1996. Graph depicting relationship between total oxygen content (C O_2) and partial pressure of oxygen (pO_2) for human blood and PFC emulsion. In order to offload 5 volume % O_2, higher arterial pO_2 is required for PFC compared with blood. Unloading of oxygen to peripheral tissues is more complete with PFC resulting in approximate oxygen extraction ratio (OER) of 90% compared with Hb (OER = 25%).

2.4.3 Biomedical applications of perfluorocarbons

As a result of this unique combination of properties, perfluorocarbons are of enormous biomedical interest and lend themselves well to many biological applications such as artificial blood substitution and protection of tissues against ischaemia.

Their experimental use in medicine was pioneered in 1966 by Clark and Golan at the University of Cincinnati, who demonstrated the ability of mice to survive for weeks after they had been immersed for 1 hour in perfluorochemical equilibrated with oxygen at atmospheric pressure. They also showed that an isolated beating rat heart could be supported in an oxygenated mixture of diluted blood and „FX-80" (perfluorobutyltetrahydrofuran) (Gollan & Clark, 1966). Following this dramatic introduction of the potential of perfluorocarbons, Sloviter and Kaminoto (1967) successfully employed PFC as a blood substitute for perfusion of isolated animal brain. In subsequent years, Geyer et al. showed that rats could survive for 5-6 hours after blood replacement with mixture of emulsified perfluorocarbon, electrolytes and an oncotic agent (Geyer, 1975). Extensive research by Naito and Yokoyama in Japan led to the development of Fluosol-DA in 1976, and was approved for human use by the US FDA. It was eventually discontinued because cumbersome preparation (had to be stored frozen) led to lack of interest and poor sales, and is no longer available.

A large number of studies were carried out on fluorocarbon blood substitutes in the ex-USSR under the Ministry of Defence, although very little information is available as a result of a former ban on publication of drug trials (Obraztsov, 1994). Clinical trials with Fluosol-DA, a PFC emulsion as a „complete blood substitute" were carried out in the 1980s in Japan and United States (Spence et al., 1994). These were discontinued due to concerns regarding potential toxicity and long-term tissue retention, limited amount of oxygen delivery and short duration of effect. Definite conclusions cannot be drawn from these early trials because they were poorly conceived and designed, inadequately reported and the PFC formulations were at a relatively primitive stage. These trials demonstrated

however, the feasibility of using perfluorocarbons in short-term settings, such as during haemodilution, as temporary alternatives to homologous blood transfusion.

2.4.4 Recent clinical applications of perfluorochemicals in humans

Blood substitute („redcell substitute")

Liquid PFCs have been developed for use as blood substitute (Sloviter & Mukherji, 1983). Fluosol DA has been used successfully as blood replacement during major surgical procedures and holds the distinction of being the only oxygen therapeutic approved by the FDA (Food and Drug Administration) for clinical use. Atabek (1992) reported its use in a Jehovah"s Witness undergoing Whipple"s procedure for bleeding ampullary carcinoma. Problems with stability of emulsions and requirement for patients to breathe 70 -100% oxygen during surgery were the major hurdles that needed to be overcome before PFC emulsions could become viable oxygen therapeutics.

Myocardial protection

In a multicentre trial of 245 patients undergoing percutaneous transluminal coronary angioplasty (PTCA), transcatheter perfusion of a perfluorochemical emulsion during balloon occlusion was effective in alleviating myocardial ischaemia during the procedure and global ventricular function was significantly better preserved than in controls (Kent et al., 1990). In light of this evidence, Fluosol emulsion was approved for human use as an adjunct to diminish myocardial ischaemia during coronary angioplasty in 1990 (Millard, 1994). It was withdrawn in 1994 because of difficulty of storing it frozen (a consequence of poor emulsion stability) and then bringing it to ambient temperature prior to use (Marti-Mestres & Nielloud, 2002).

Treatment of malignancies

Fluosol-DA 20% along with hyperbaric oxygen has been used as an adjuvant to conventional radiotherapy for high-grade brain tumours (Evans et al., 1993) and other head and neck malignancies.

Liquid ventilation

The first clinical trial was performed in neonates in 1989. A number of studies in infants, children and adults have concluded that liquid ventilation with perfluorochemical is safe and improves lung function (Greenspan et al., 2000).

Vitreoretinal surgery

Perfluorocarbons are widely used in vitreoretinal surgery. The high specific gravity and low viscosity of PFCs, along with a lack of significant toxicity, allow them to be employed as tamponading agents in the management of retinal tears and detachments. They also proved to be useful in the treatment of vitreous haemorrhage, partially detached posterior hyaloids membrane, and subluxed lenses from the retina. Retained perfluorochemical from previous surgery can be used for measurement of preretinal oxygen tension using 19-F MRS (Wilson et al., 1992).

Diagnostics

Their inertness, immiscibility with water, low surface tension, compressibility, make them versatile contrast agents with important applications in x-ray, ultrasound, CT and MR (Tran et al., 2007). Recent advances in the design of fluorinated nanoparticles for molecular magnetic resonance imaging enabled specific detection of ^{19}F nuclei, which provided unique and quantifiable spectral signatures. For example, integrin-targeted PFC nanoparticles were successfully used to detect early angiogenesis in sclerotic aortic valves (Waters et al., 2008).

Cell cultures

Facilitation of respiratory gas delivery by PFCs was exploited in cell biotechnology. PFC was employed in mouse hybridoma and HeLa cell culture systems, and for sperm preservation. Incorporating PFCs in culture media has been shown to have a protective effect on islets. Islet viability and functionality are preserved and so is the extracellular matrix (Maillard, 2008). In contrast, some researchers have found that PFC-cultured islets have a reduced stimulation index and show increased DNA fragmentation (Bergert, 2005).

2.4.5 Concerns regarding clinical use

No carcinogenic, mutagenic or teratogenic effects have been reported for PFCs (Tran et al., 2007). Although tissue half-life of PFCs ranges from 4 to 65 days, they are biologically inert and following reticuloendothelial uptake undergo excretion un-metabolized as vapour through the lungs (Joseph et al., 1985; Lowe, 1999).

The major disadvantages of perfluorocarbon for clinical use are:

1. Flushing, backache and flu-like reactions due to cytokine release as a result of activation of the arachidonic acid cascade secondary to particle phagocytosis.
2. Retention in reticuloendothelial system (RES) with subsequent suppression of RES. Impaired neutrophil function and increased clearance of platelets with thrombocytopaenia have also been observed. This is believed to impair defense mechanisms (Bucala et al., 1983; Flaim et al., 1994).
3. Limited shelf-life.
4. Short intravascular retention time.
5. Due to high viscosity, maximum concentration possible is only 20%. This limits their oxygen carrying capacity requiring the patient to breathe 100% oxygen.

The clinical significance of adverse effects mentioned above is doubtful as the symptoms are short-lasting and fully reversible after 4-12 hours (Schumacher & Ashenden, 2004). Due to concerns regarding an increased incidence of cerebrovascular events, phase II clinical trials of perflubron were placed on hold in 2001 (Jahr et al., 2007). However, thorough analysis of clinical safety data suggests that the adverse events may have been linked to overly aggressive autologous blood harvesting prior to cardiac surgery (Kim and Greenburg, 2004; Riess, 2006). Alliance Pharmaceuticals Corp. have resumed production of Oxygent since and new phase 2 proof of concept clinical trials have now been initiated in Europe and China (Medical News Today, 2007). A randomized clinical trial of another emulsion (Perftoran) was recently reported (Verdin-Vasquez et al., 2006). Investigators found that the PFC emulsion reduced the need for allogeneic blood and blood derivatives in patients undergoing cardiac surgery, and was safe.

2.5 PFC-based perfusates

A stable, inert, oxyphoretic liquid for perfusion of an organ during the period of preservation has obvious advantages of protecting tissues from hypoxia and preventing irreversible tissue damage. Kidneys, heart, lung, liver, pancreas, brain (Dirks et al., 1980), testis (Chubb & Draper, 1987), limbs and multiple organ blocks have been effectively preserved for transplantation using PFC-enriched perfusates (Voiglio et al., 1994). Perfluorochemical emulsions have allowed preservation of rat liver (Novakova, 1976) and kidney (Berkowitz et al., 1976) and have been used to maintain beating rat hearts at 37°C for over 10 hours (Tomera & Geyer, 1982). Similar encouraging results have been obtained with various organs from rabbits, dogs and pigs. There is experimental evidence that fluorocarbon perfusates may be superior to hypothermia in several respects. These emulsions inhibit free-radical generation and thromboxane release, thereby mitigating reperfusion injury. Perfusion of the heart results in increased synthesis of high energy phosphates and improved functional recovery. Exchange perfusion of recipient rats with Fluosol-43 to remove humoral factors leads to prolonged

survival of guinea pig heart xenografts. Canine kidneys have been successfully perfused and transplanted even under warm preservation conditions (Brasile et al., 1996) and extended preservation of intestines(DeRoover et al., 2001) and multiple organ blocks (Voigilo et al., 1996) has been demonstrated using these emulsions.

Perfluorocarbon emulsions have been also been used to improve cell cultures (Keese & Giaever, 1983) and enhance preservation of pancreatic islets (Zekorn et l., 1991; Takahashi et al., 2006; Mallaird et al., 2008).

Emulsions have been shown to not only support the metabolism and electrical activity of the isolated, perfused brain (Sloviter & Kaminoto, 1967), but also to reduce the injury associated with obstruction of a major cerebral artery (Peerless et al., 1981). It has also been observed that improvement in brain oxygenation is out of proportion to the amount of oxygen dissolved by PFC (Clark et al., 1988) suggesting that PFC emulsions facilitate capillary to tissue oxygen transfer. Evidence for this also comes from retinal oxygenation studies (Braun et al., 1992).

Fluorocarbon emulsions prevent myocardial contractility failure when used as a perfusate during coronary angioplasty procedures in humans – this led to US FDA approval of Fluosol-DA for human use (Millard, 1994). Tremper et al. (1982) demonstrated that PFC-based emulsions significantly improved tissue oxygen consumption and mixed venous oxygen saturation in severely anaemic humans.

2.6 Mechanism of the Two-layer method

In a series of experiments in a canine model of auto-transplantation, Kuroda‟s group observed that tissue oxygenation of the TLM – preserved pancreas was maintained at a high level for prolonged

periods and this correlated with a high ATP content and improved graft survival (Kawamura et al., 1989). They also observed that TLM had the ability to resuscitate pancreatic grafts that had sustained up to 90 minutes of prolonged warm ischaemia (Kuroda et al., 1993). Moreover they found that perfluorocarbons also improved the viability of vascular endothelium and microcirculation and reduced ischaemia reperfusion injury in conjunction with increased expression of heat shock proteins (Kuroda et al., 1997; Fujino et al., 2001). It has been shown in a primate model that exposure to PFC by means of the TLM lowers trypsin levels at the end of pancreatic distension and digestion. There is also evidence that zymogen granule release from exocrine tissue that normally occurs after prolonged cold storage is prevented when the pancreas is preserved by the two-layer method, suggesting that one of the functions of PFC is to stabilize endogenous enzyme activity (Iwanaga et al., 2002). Kuroda and colleagues have also observed that rat pancreas preservation by the TLM before islet isolation has a protective effect on isolated islets against apoptosis by the mitochondrial pathway (Matsuda et al., 2003). More recently, this was shown to be the case in human pancreas preserved by TLM (Ramachandran et al., 2006). There was suppression of hypoxia-induced apoptosis mediated by increase in the levels of phosphorylated bcl-2, cIAP2 and survivin. Furthermore, they observed that even after prolonged preservation of pancreas is UW solution alone, islets can be rescued from apoptotic cell death by re-oxygenating those pancreases in PFC.

2.6.1 Role of ATP

Kuroda et al from Kobe University, Japan, introduced the two-layer method of pancreas preservation for whole organ transplantation in 1988. Although his was the first description of the two-layer method, perfluorocarbons had been employed as emulsions in the past for organ preservation. The Cambridge group had reported improved results in the rat liver transplantation model using perfluorocarbon emulsion for continuous perfusion (Kamada et al., 1980).

Based on previous reports of favourable results with hyperbaric oxygenation (Idezuki et al., 1968) and with retrograde oxygen persufflation (Fischer et al., 1978), Kuroda et al. developed this less cumbersome technique of cold storage using perfluorocarbon (PFC) and Euro-Collins‟ (EC) solution in a dog auto-transplantation model (Kuroda et al., 1988). Canine pancreas grafts were placed in a jar containing PFC and EC and the bottom PFC layer was continuously oxygenated at a flow rate of 50-100 ml/min (**Fig 2.6**). Following auto-transplantation in the neck after 48 hours preservation, graft survival rates were 100% and 20% in the PFC/EC and EC groups respectively. Histological analysis of the PFC/EC grafts revealed near normal architecture while autolysis and vacuolization was observed in grafts belonging to the latter group. In subsequent studies, the same group reported survival rates of 100% for canine grafts subjected to 90 minutes of warm ischaemia, when preserved with the two-layer method (Kuroda et al., 1993).

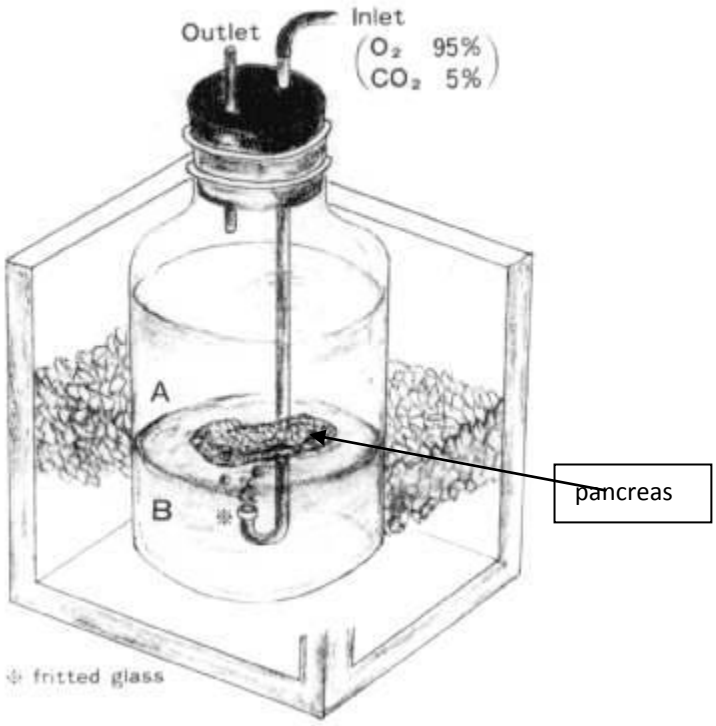


Figure 2.6: pancreas graft in Euro-Collins‟ solution (A) at the top of the perfluorocarbon layer (B) – oxygenation of graft is achieved by means of oxygen flowing continuously through the perfluorocarbon layer (Kuroda et al., 1988).

Direct diffusion of oxygen dissolved in PFC, through the under surface of pancreas, was hypothesized as the underlying mechanism (Kawamura et al., 1989). This hypothesis was substantiated by the demonstration of significantly higher tissue concentrations of ATP (measured by HPLC on tissue extracts) only in pancreases that were preserved in oxygenated perfluorocarbon (Kuroda et al., 1990). The same group also reported that graft viability was dependent on the maintenance of tissue ATP levels, oxygenation alone being insufficient. Thus, when ATP production was blocked with 2,4-dinitrophenol, pancreas viability declined sharply and 48-hour graft survival fell to 0% (Kuroda et al., 1992). In another report, they observed a statistically significant difference in tissue ATP concentrations at the end of preservation between viable and non-viable canine pancreas grafts preserved by TLM (Kuroda et al., 1991). On analysis of individual graft ATP levels, there was clear demarcation between the lowest ATP (6.3 μmol/g dry weight) in the viable group and the highest ATP (6.1 μmol/g dry weight) concentration in the non-viable pancreases. They concluded that a critical ATP value of 6.2 μmol/g dry weight of canine pancreas grafts, at the end of preservation, predicts post-transplant viability with 100% accuracy.

They also observed that TLM-preserved grafts had significantly better tolerance to rewarming injury. Vascular clamps were not released after completion of anastomosis, to induce rewarming ischaemia up to 120 minutes. All grafts preserved with the two-layer method survived whereas fresh grafts and those preserved in UW solution became nonviable after 90 minutes. These results were correlated with higher tissue ATP levels in TLM-pancreases after preservation, following rewarming ischaemia and also after reperfusion (Kuroda et al., 1994). Because ATP is an essential energy source for cell repair following damage, it was postulated that ATP synthesized during TLM preservation was utilized effectively to resuscitate ischaemically damaged pancreas during preservation. In order to further test this hypothesis, Kuroda and associates (1996) studied the effect on bioenergetics and survival of ischaemically damaged canine grafts preserved at higher temperatures (20°C) to increase the metabolic rate and accelerate ATP synthesis. After 90 minutes of warm ischaemia, auto-transplanted grafts uniformly failed to survive. However, following 1, 3 and 5 hours two-layer preservation at 20°C, corresponding graft survival rates of 0%, 60% and 100% were observed.

Resuscitation of these grafts by TLM was correlated with progressively higher tissue ATP levels for up to 5 hours of warm preservation.

2.6.2 Role of adenosine

The relationship between TLM and pancreas bioenergetics was further explored by the Kobe group in the warm ischaemia canine auto-transplant model. They reported that the Euro-Collins‟ (EC) two-layer method alone was ineffective in restoring viability after 60 minutes warm ischaemia. However, addition of adenosine to EC resulted in recovery of these ischaemically damaged grafts, suggesting that exogenous adenosine supplementation was essential for ATP synthesis (via the purine salvage pathway) in pancreas subjected to significant warm ischaemia (Kuroda et al., 1994a). They also observed that fresh pancreas grafts synthesized ATP mainly through mitochondrial oxidative phosphorylation utilizing endogenous substrates. However, following significant warm ischaemia, endogenous substrates are depleted and ATP production occurs by direct phosphorylation of exogenous adenosine as evidenced by the distribution of labelled adenosine in acid-soluble extracts (Kuroda et al., 1994b). They hypothesized that UW solution was superior to EC as a component of the two-layer method, particularly for organs from cardiac arrest donors.

2.6.3 Continuous vs. Static Two-Layer Method

The original TLM developed by Kuroda et al. (1988) required continuous oxygenation of the PFC layer. Indeed, all mechanistic studies on TLM have been performed on the original continuous TLM model.

Static two-layer (sometimes referred to as „simplified" two-layer method) was developed in 2001 by Hiraoka and colleagues (2001). They postulated that since oxygen solubility in perfluorocarbon liquids was 20 – 25 times higher than that in water or blood (Lowe, 1999), and oxygenated PFC maintained high oxygen content for a few days as observed in plant culture studies (Wardrop et al., 1997), it should be possible to employ pre-oxygenated PFC for aerobic oxygen preservation. In support of their hypothesis, they found no difference in tissue ATP levels and islet yields from rat pancreases preserved in conventional and static TLM.

Subsequently, the static TLM was tested in large animal and human pancreases by Matsumoto and colleagues in 2002. Using Clarke-type electrodes to measure oxygen tension, they found that oxygen saturation of PFC in static TLM was 95.5% and 85.5% at 18 hours, with and without pancreas respectively. The authors concluded that even with static TLM for pancreas preservation, the PFC remained oxygenated at sufficiently high levels for long periods. They also found no difference in ATP content and islet isolation outcomes among pancreases preserved in static and continuous TLM (Matsumoto et al., 2002b).

These two studies, combined with the logistic simplicity of the static method (particularly during organ transport), formed the basis of the current use of static TLM as the standard alternative to UW preservation in clinical islet transplantation. The authors" conclusion that static and continuous TLM are equivalent is based on the implicit assumption that PFC behaves as a large reservoir of oxygen and that any oxygen depletion in the TLM layer is accounted for by pancreas utilization. Direct evidence for this is lacking. Oxygen in the PFC layer is more likely to passively equilibrate with the overlying UW layer and air in the container rather than diffuse into a solid pancreas. Moreover the relatively small degree of desaturation in the presence of pancreas in the Matsumoto study suggests that pancreatic utilization is actually inefficient with static TLM. Unfortunately, the authors did not include a continuous TLM arm in their oxygen tension studies.

Another important observation from the Matsumoto study was that even after short periods of TLM preservation (5 hours), there was substantial improvement in tissue ATP levels and islet parameters (yield, viability and functionality) compared with UW-preserved pancreases.

Witkowski et al. (2005) noted that even 4 hours of PFC exposure considerably improved islet yield compared with UW storage (349,000 vs. 277,800 IEQ/g) in human pancreases. Significantly, the cold preservation time in the control (UW) group was less than 8 hours. Tsujimura and associates (2004b) found that 3 hours of additional TLM storage improved energy parameters and islet isolation outcomes in pancreases stored initially in cold UW solution for a mean duration of 11 hours.

2.6.4 Current status of the two-layer method

It is remarkable that all these studies were reported from a single institute and in the same animal model of whole pancreas auto-transplantation. They appear not to have generated much enthusiasm in the wider community of pancreas transplant surgeons and studies on the application of this preservation method to clinical pancreas transplantation are scarce. The most plausible reason for this apparent lack of interest in TLM is the contemporaneous development of UW solution (Wahlberg et al., 1987), which would become the gold standard for organ storage. The relative complexity of the continuous two-layer technique was another possible reason for its lack of appeal.

Interest in the two-layer method appears to have been rejuvenated following the perception that prolonged cold storage was detrimental for survival of islet tissue, and therefore improved pancreas preservation was vital to the success of islet transplantation. Building on the proof-of-principle experiments in canine pancreas autografts, several studies were performed in small and large animal models of pancreatic islet transplantation. A number of reports on the beneficial impact of TLM on human pancreas followed. A few of these studies attempted to explain the mechanism of action of TLM.

Ramachandran and associates (2006) have observed an up-regulation of anti-apoptotic genes (IAP, survivin), increased levels of phosphorylated Bcl2, and a concomitant down-regulation of pro-apoptotic genes (Bad, Bax, caspases) in islets derived from TLM-preserved human pancreases. This suggests that amelioration of hypoxia by PFC prevents the critical reduction in the redox potential that mediates mitochondria-dependent apoptosis.

In the clinical setting of whole organ transplantation, there is one report in literature suggesting that TLM preserved grafts are relatively protected from preservation injury (Matsumoto et al., 2000). The authors noted that none of the 10 grafts preserved in two-layer became oedematous on reperfusion, as opposed to 10 of 43 UW-preserved pancreases. They postulated that preservation of ATP-dependent Na^+/K^+ pump activity allowed maintenance of cell volume in the former group. No acute rejection episodes were observed at 3 months post-transplant in TLM grafts whereas the incidence of rejection was 15.9% in the control group.

Although preservation by TLM has been shown to influence favourably markers of graft viability in experimental animal models (Kuroda et al., 1995; Tanioka et al., 1997; Kakinoki et al., 2005; Maluf et al., 2006), there are no reports of improvement in post-transplant graft outcome in humans. Preservation by the two-layer method (TLM) using perfluorocarbon (PFC) has been advocated as a superior preservation technique for maintaining islet viability (Matsumoto et al., 2004). It is also claimed that this technique can resuscitate islets that have sustained ischaemic damage and improve islet yield and quality from marginal organs (Matsumoto et al., 2002; Ricordi et al., 2003). The success of islet transplantation from single donors has recently been attributed to the implementation of the two-layer method (Hering et al. 2005). The fact remains however, that the only report of a 100% rate of insulin independence following islet allograft transplantation was the result of transfusing fresh islets and this result has not been replicated so far even with the increasing utilisation of perfluorocarbons for pancreas preservation. Recently, there has been also debate on whether the level of tissue oxygenation at the core of pancreas is enhanced when preserved by the TLM. Using Clarke-type polarographic electrodes Matsumoto et al. (1996) measured pO_2 in canine pancreas following a short period of warm ischaemia, at a depth of 5mm from the surface and the

tissue oxygen tension was found to increase rapidly and then remains stable at about 15 mmHg during 5 hours of TLM preservation at a temperature of 20° C. In a more recent study the Minnesota group reported that pO_2 is virtually zero in the core of a 1 cm piece of porcine pancreatic tissue preserved in TLM and concluded that elevated ATP levels observed could be the result of enhanced oxygenation of only a relatively small volume fraction (about 15%) of the whole pancreas (Papas et al., 2005). In fact, in the largest study reported so far (200 islet isolations), no difference in ATP content of the human pancreas was observed between organs stored with and without PFC suggesting that contrary to popular belief, during TLM preservation oxygen might not be effectively utilized to maintain cellular bioenergetics (Caballero-Corbalan et al., 2007). These findings also bring into question the appropriateness of using the rat pancreas which is only a few millimetres thick as a model for studying the two-layer method.

We performed a meta-analysis of the evidence of the beneficial effect of perfluorocarbon in clinical islet transplantation (chapter 3).

HYPOTHESIS

There is no clinical or experimental evidence for benefit of the two-layer method as currently employed in pancreatic islet transplantation. A potentially beneficial effect on pancreas preservation by improvement in graft ATP production is possible by modification of the two-layer method.

AIMS AND OBJECTIVES

The principal aims of this project were

1. to study the mechanism of action of perfluorocarbon in the two-layer method with a view to establishing optimal protocols for its use in pancreas preservation for clinical islet transplantation.
2. to develop a clinically useful experimental model for the dynamic study of bioenergetics during pancreas preservation.

CHAPTER 3

A META-ANALYSIS OF THE IMPACT OF THE TWO-LAYER METHOD OF PRESERVATION ON HUMAN PANCREATIC ISLET TRANSPLANTATION

3.1 Background

The two-layer method of pancreas preservation using perfluorocarbon has been exhaustively studied in animal models (pigs, dogs and rats) but the mechanism of action is still poorly understood. Moreover large-scale trials in humans demonstrating significant superiority over conventional cold storage are lacking. The purpose of this review was to perform a systematic analysis of the human studies comparing the results of pancreas preserved by the TLM with those preserved in chilled University of Wisconsin (UW) solution for islet transplantation. In addition, studies looking at the mechanism of action of the two-layer method were selected for a systematic review.

3.2 Methods

Search strategy

Pubmed, CENTRAL, EMBASE, Science Citation Index and BIOSIS were searched until May 2007. The following strategy was used for Pubmed:

("Fluorocarbons"[MeSH] OR perfluorocarbon OR perfluorocarbons OR fluorocarbon OR perfluorochemical OR perfluorochemicals) AND ("Islets of Langerhans Transplantation"[MeSH] OR ((islet OR islets OR island OR islands) AND (transplantation OR transplantations)))

Equivalent strategies were used for other databases.

Inclusion and Exclusion Criteria

All studies published in the English Language which compared the outcome of preserving pancreas with UW solution vs. the two-layer method for islet transplantation were considered for meta-analysis. Exclusion criteria were as follows:

1. non-human studies
2. any preservation studies comparing the outcomes of whole-organ pancreas transplantation
3. any modification to the Edmonton protocol for islet isolation
4. any modification of the two-layer method of preservation (as defined below)
5. personal communications

These exclusion criteria were designed to ensure maximal homogeneity in the studies included for the meta-analysis. Citations that elucidated the mechanism of action of perfluorocarbons, including studies where human islets were tested in animal models, were selected for discussion in this review.

Definitions

Two-layer method – preservation of deceased donor pancreas in the interface between UW solution and oxygen-charged perfluorodecalin at 4°C on ice either immediately following organ retrieval (TLM alone) or after preliminary cold storage in UW solution (UW followed by TLM). The PFC was either continuously oxygenated by bubbling oxygen at a given flow rate or charged with oxygen before preservation (static oxygenation).

Primary end-point: Transplantable preparations – islet isolations that resulted in clinical transplantation or were judged by standard Edmonton criteria as suitable for clinical transplantation.

Secondary end-points: Islet yield – islet recovery post-purification in islet equivalents per gram of pancreas. *Islet purity* – percentage of islet to non-islet tissue. *Islet viability* – assessed by fluorescent inclusion and exclusion dyes and expressed as a percentage, all recorded in accordance with the

criteria established at the 1989 International Workshop on Islet Assessment in Minneapolis (Ricordi 1990).

Data Extracted

1. Year of publication
2. Country of study
3. Study design
4. Donor pancreas variables - age, gender, BMI and sample size
5. Methods of preservation used for comparison - UW alone, TLM alone, short or long period of UW cold storage followed by TLM
6. Duration of cold ischaemia
7. Primary outcome measure - final results in terms of the number of transplantable preparations obtained i.e. the proportion of islet isolations that were successful
8. Secondary outcome measures - islet isolation outcomes in terms of islet yield, purity and viability
9. Statistical methods used for analysis

Statistical Methods

Meta-analysis was performed using Revman Analyses 1.0 (Revman 4.2.8 provided free by the Cochrane collaboration, Copenhagen). The meta-analysis was performed by using the random-effects model if the statistical heterogeneity as measured by Higgins' I^2 was 25% or greater (DerSimonian & Laird, 1986; Higgins & Thompson, 2002). Otherwise the fixed-effect model (Demets, 1987) was used. For continuous variables, weighted mean difference (WMD) with 95% confidence interval was calculated. For dichotomous variables, odds ratio (OR) with 95% confidence interval was calculated. In the absence of suitable data available for meta-analysis from 2 or more studies, the results were tabulated and the statistical significance at p=0.05 was indicated. A subgroup analysis including only the randomized controlled trials was performed wherever possible.

3.3 Results

A total of 90 citations were identified through electronic searches of Pubmed (n=26), CENTRAL (n=0), EMBASE (n=26), Science Citation Index (n=24) and BIOSIS (n=14). Eight publications were identified that met our inclusion criteria for a meta-analysis (Matsumoto et al., 2002; Tsujimura et al. 2002; Ricordi et al., 2003; Matsumoto et al. 2004; Tsujimura et al. 2004; Kin et al., 2006; Ramachandran et al., 2006; Caballero-Corbalan et al., 2007). One of these contained a report of two different experimental sets – one studying the effect of the TLM on pancreas after a short period of storage (less than 12 hours) and the other after prolonged preservation (up to 24 hours). Another publication stratified the data into three sets based on duration of cold ischaemia (0-<6 hours, 6-<12 hours and 12-18 hours). These were considered as separate studies, giving a total of 11 studies that were finally combined for the meta-analysis.

Of the eight publications, two were randomized controlled trials (RCT) (Matsumoto et al., 2002, Tsujimura et al., 2002), three were prospective non-randomized studies (Ricordi et al., 2003, Matsumoto et al., 2004, Ramachandran et al., 2006) and the remaining three were retrospective (Tsujimura et al., 2004, Kin et al., 2006, Caballero-Corbalan et al., 2007). The sample size was by far the largest in the retrospective studies (n=142,166 and 200). The sample size in the remaining six study reports varied from 9 to 33. Three different preservation conditions were compared in these studies: cold storage in UW alone, cold storage by the two-layer method immediately after organ procurement and storage in UW solution for short or prolonged period followed by additional period of storage by TLM. Most studies compared the results of UW storage alone with either of the latter two methods (TLM alone – 6 studies, UW followed by TLM – 4 studies). There was one non-randomised trial that compared all three preservation methods (Ramachandran et al., 2006).

Results of clinical meta-analysis of TLM (Table 3.1 and appendix I)

Preservation by TLM vs. UW solution

Seven studies compared the effects of TLM alone and UW alone (Tsujimura et al., 2004, Matsumoto et al., 2004, Kin et al., 2006, Ramachandran et al., 2006, Caballero-Corbalan et al., 2007). Two of these were prospective non-randomized and the remaining five studies were retrospective. There was a statistically significant higher islet yield (WMD 711.55, 95% confidence interval [CI] 140.03 to 1283.07) in the TLM group in comparison with the UW group (Fig 3.1). There was no significant difference in purity of the islet preparations (WMD -0.32, 95% CI -3.92 to 3.27) or the islet cell viability between the groups (WMD 1.95, 95% CI -6.28 to 10.18) and the proportion of transplantable preparations obtained was not significantly different (OR 1.30, 95% CI 0.89 to 1.88) (Fig 3.1). Donor variables (age, gender, BMI and pancreas weight) did not differ significantly between the two experimental groups.

Preservation in UW followed by TLM vs. UW

Five studies compared the resuscitative effect of the two-layer method (Matsumoto et al., 2002; Tsujimura et al., 2002; Ricordi et al., 2003; Ramachandran et al., 2006). In the TLM after UW group, TLM was used after an initial period of cold storage in UW solution. There was a statistically significant higher islet yield (WMD 1325.15, 95% CI 490.86 to 2159.45) in the study (TLM) group, although islet purity (WMD -4.40, 95% CI -11.11 to 2.31) and viability (WMD 3.20, 95% CI -0.02 to 6.43) were not significantly different in the two groups. The rate of successful islet isolations (transplantable preparations) was higher in the TLM after UW group (OR 6.69, 95% CI 1.80 to 24.87 – Fig 3.2). This last outcome was reported in two of the five studies (Tsujimura et al., 2002,Ricordi et al., 2003) - one with marginal donors (age >50 yrs) and the other with marginal organs (>10 hours cold storage), both concluding that exposure to the TLM improved islet viability in marginal organs. Of particular note, in the second study the TLM was used only for an average duration of three (2.9 ± 0.7) hours. There was no significant difference in the donor characteristics between the groups.

Preservation in UW followed by TLM vs. UW (RCT only)

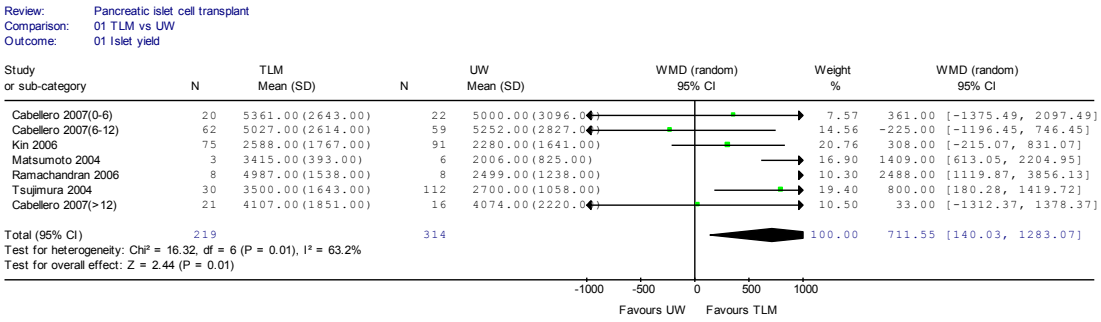
Three of the studies qualifying for the systematic review were randomized (Matsumoto et al., 2002; Tsujimura et al., 2002). Meta-analysis of these three RCTs revealed that although islet yield was significantly higher in the TLM group (WMD 1763.14, 95% CI 1102.82 to 2423.47) there was no significant difference between the two groups in terms of purity, viability and the proportion of transplantable preparations (OR 4.50, 95% CI 0.63 to 32.29) obtained. Donor age, gender and BMI were similar in the two randomized groups. However across the studies, weight of the pancreas was higher in the UW group (WMD -16.82, 95% CI -29.39 to -4.24).

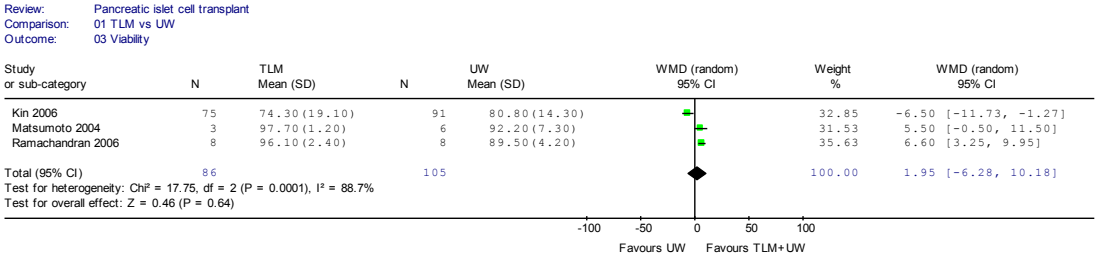
Preservation in UW followed by TLM vs. TLM

One prospective non-randomized study compared TLM after UW group (initial preservation in cold UW solution followed by the two-layer method) with TLM alone (Ramachandran et al., 2006). The islet yield, purity and viability were reduced by preliminary storage in UW solution (WMD -2793.00, 95% CI -4111.81 to -1474.19, WMD -11.00, 95% CI -29.82 to 7.82 and WMD -8.30, 95% CI -14.89 to -1.71 respectively). No data on the rate of successful isolations was available. Donor BMI and pancreas weight were not significantly different among the groups.

Comparison or outcome	Studies	Participants	Statistical method	Effect size
01 TLM vs. UW				
01 Islet yield	7	533	WMD (random), 95% CI	711.55 [140.03, 1283.07]
02 Purity	6	391	WMD (fixed), 95% CI	-0.32 [-3.92, 3.27]
03 Viability	3	191	WMD (random), 95% CI	1.95 [-6,28, 10.18]
04 Transplantable preparations	6	517	OR (fixed), 95% CI	1.30 [0.89, 1.88]
05 Donor age	4	366	WMD (fixed), 95% CI	-1.21 [-3.42, 0.99]
06 BMI	5	382	WMD (fixed), 95% CI	-0.34 [-1.49, 0.82]
07 Pancreas weight	5	382	WMD (fixed), 95% CI	2.62 [-2.69, 7.92]
08 Females	4	366	OR (fixed), 95% CI	0.74 [0.49, 1.12]
02 TLM+UW vs. UW				
01 Islet yield	5	97	WMD (random), 95% CI	1325.15 [490.86, 21 59.45]
02 Purity	5	97	WMD (fixed), 95% CI	-4.40 [-11.11, 2.31]
03 Viability	4	76	WMD (random), 95% CI	3.20 [-0.02, 6.43]
04 Transplantable preparations	2	54	OR (fixed), 95% CI	6.69 [1.80, 24.87]
05 Donor age	4	81	WMD (fixed), 95% CI	-1.02 [-3.64, 1.61]
06 BMI	5	97	WMD (fixed), 95% CI	0.58 [-1.63, 2.79]
07 Pancreas weight	5	97	WMD (random), 95% CI	-8.27 [-19.97, 3.42]
08 Females	2	27	OR (fixed), 95% CI	3.55 [0.65, 19.25]
03 TLM+UW vs. UW (RCT only)				
01 Islet yield	3	48	WMD (fixed), 95% CI	1763.14 [1102.82, 2423.47]
02 Purity	3	48	WMD (fixed), 95% CI	-3.08 [-12.50, 6.35]
03 Viability	2	27	WMD (random), 95% CI	2.75 [-1.14, 6.63]
04 Transplantable preparations	1	21	OR (fixed), 95% CI	4.50 [0.63, 32.29]
05 Donor age	3	48	WMD (fixed), 95% CI	-2.07 [-8.44, 4.30]
06 BMI	3	48	WMD (fixed), 95% CI	1.97 [-1.03, 4.96]
07 Pancreas weight	3	48	WMD (fixed), 95% CI	-16.82 [-29.39, -4.24]
08 Females	2	27	OR (fixed), 95% CI	3.55 [0.65, 19.25]
04 TLM+UW vs. TLM				
01 Islet yield	1	16	WMD (fixed), 95% CI	-2793.00 [-4111.81, -1474.19]
02 Purity	1	16	WMD (fixed), 95% CI	-11.00 [-29.82, 7.82]
03 Viability	1	16	WMD (fixed), 95% CI	-8.30 [-14.89, -1.71]
04 BMI	1	16	WMD (fixed), 95% CI	-0.90 [-7.76, 5.96]
05 Pancreas weight	1	16	WMD (fixed), 95% CI	-11.70 [-33.20, 9.80]

Table 3.1: meta-analysis results - table showing the summary of the individual meta-analyses performed for the donor variables, primary and secondary outcomes in the different sub-groups. BMI = body mass index, CI = confidence interval, OR = odds ratio, RCT = randomized controlled trial, TLM = two-layer method, UW = University of Wisconsin solution, WMD = weighted mean difference





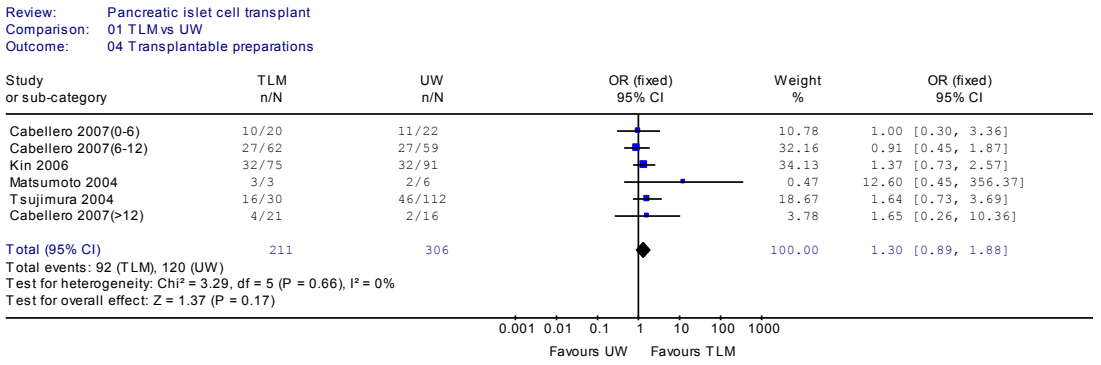


Figure 3.1: Forest plots showing the results of meta-analysis of comparison studies of islet isolation outcomes from pancreas preserved in two-layer method versus University of Wisconsin solution: weighted mean difference (random effects) plots for islet yield and islet viability and odds ratio plot (fixed effect) for proportion of transplantable preparations (successful islet isolations) obtained

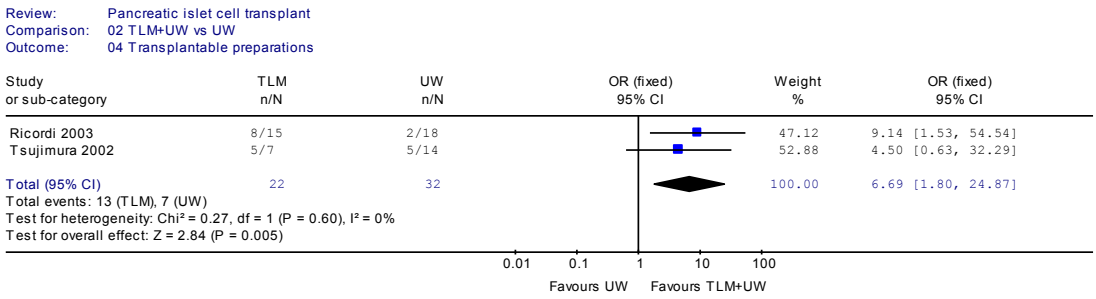


Figure 3.2: Forest plot showing the results of meta-analysis of comparison studies of islet isolation outcomes from marginal pancreases preserved in two-layer method versus University of Wisconsin solution: odds ratio plot (fixed effects) of the proportion of transplantable preparations (successful islet isolations) obtained

3.4 Discussion

The lack of a clear mechanism of action and the variable reports of efficiency from clinical studies (most of which were small) suggested a meta-analysis could help to clarify the contribution of TLM to pancreas preservation for islet transplantation. No significant clinical diversity was identified in the study reports included in the meta-analysis and overall methodological quality was good. Three of the eleven studies included in this review were randomized controlled comparisons, corresponding to 7.7% (48 of 622) of the pooled sample size. As even the pooled sample size in the sub-groups was relatively small, the possibility of a beta error for some of the outcome measures cannot be excluded (optimal information size for the meta-analysis for 80% statistical power with an alpha level of 0.05 was estimated to be 480 organs in each group) and the possibility of bias in retrospective studies cannot be excluded. The donor variables (age, gender, BMI and pancreas weight) were not significantly different between the groups with a single exception. In the randomized trial sub-group the pancreas weight was significantly higher in the UW arm. The significance of this observation is unclear and definite conclusions cannot be drawn as this difference was not significant in the individual studies and the sample size was small (n=48).

Correlation between preservation technique and human islet isolation outcomes

This meta-analysis indicated that exposure of the pancreas to the TLM significantly improves islet yield. However, the effect on islet purity and viability is not consistently superior to that obtained after conventional cold storage. Explanation for this apparent conflict is not obvious. There is evidence that some components of UW solution have an inhibitory effect on collagenase digestion of human pancreas (Contractor et al., 1995). The perfluorocarbon in TLM might counteract this effect. However, a beta error cannot be ruled out due to small data sets. The results of the final sub-group analysis (between UW + TLM and TLM) can be explained by the prolonged cold ischaemia time in the former group (26.6 ± 4.9 vs. 11.0 ± 2.2 h). This would seem to contradict the observation by other

groups that the TLM can restore islet parameters after UW-induced preservation injury (Tsujimura et al., 2002, Ricordi et al., 2003). In the third largest retrospective analysis of 142 patients, Tsujimara T et al. (2004) crucially observed that 8-10 hours of cold ischaemia in UW solution was the critical point for successful islet isolations. They also concluded that with preservation times of four hours or less, there was no difference in islet isolation outcomes between the TLM and UW groups. A more recent large-scale study analyzed islet isolation outcomes after stratification of 200 human pancreases based on the duration of cold ischaemia, and found no protective effect of TLM on organs after short or extended CIT (Caballero-Corbalan et al., 2007). They concluded that ischaemic tolerance of human pancreas could not be improved by the two-layer method.

There was no increase in the proportion of transplantable preparations using TLM. This would suggest that TLM has only limited ability to maintain islet viability. This lack of evidence of effect in the primary outcome measure between the TLM and UW groups was not consistent across studies. Only two (n=366) of the four publications analyzed concluded that there was no significant difference in the proportion of transplantable preparations obtained between the two groups. In contrast, the other two studies (n=151) found a beneficial effect in the study group. A type 2 error in the individual studies might be the reason for this discrepancy.

The only scenario where a higher rate of transplantable preparations resulted from exposure to PFC in this meta-analysis was when marginal organs were compared in two studies. The authors suggested that the TLM may have the ability to preserve and resuscitate damaged pancreas and therefore have a clinical application in this setting. However, the sample size was small (n=54) and one of the two studies was not randomized. Caballero-Corbalan et al. found that in the cohort of elderly donors (n=61/200) oxygenation with TLM failed to improve islet isolation outcomes. Definite conclusions therefore cannot be drawn and further studies are required to confirm this observation.

Correlation between preservation technique and clinical outcomes

Although not the end point for this meta-analysis, the most important outcome measure of islet isolation is the functional performance of islets following portal infusion. Eight of the eleven studies reported the success rate of islet isolation from organs in the different preservation conditions. None of these studies however, systematically recorded complete information on immediate or follow-up in vivo functional outcomes following transplantation. Tsujimara et al. (2002) observed that fasting glucose, C-peptide and glycosylated haemoglobin levels improved in all transplant recipients. Similarly Kin and colleagues found no difference in the percent reduction in insulin requirements between TLM-islet and UW-islet recipients, even after stratifying the patients by the number of islet infusions they had received. Ramachandran et al. found no significant difference between the preservation groups with respect to performance in vivo after transplantation in diabetic-SCID mice. None of the studies gave any indication of a difference between TLM preservation compared with UW solution.

Evidence of a beneficial effect of the two-layer method for routine clinical application is therefore lacking. For marginal organs, there is supportive evidence indicating that both primary and secondary outcome measures may improve with PFC-mediated oxygen exposure. Notably, none of the studies reported any specific adverse clinical outcome attributable to perfluorocarbon use.

Future studies

A large randomized controlled multi-centre study is required to further clarify the role of the two-layer method in the clinical setting of islet transplantation. The mechanism of any beneficial effect requires to be established.

CHAPTER 4

EXPERIMENTAL METHODS

Any hypothesized mechanism of TLM action that is based on enhanced gas transfer rests on the presumption that PFC in the bottom layer penetrates the capsule of the intact pancreas and infiltrates the organ. We investigated the penetration of PFC in solid pancreas during TLM preservation by ^{19}F MRI. Porcine pancreases were studied with this assay method following 24-hour preservation by TLM to examine the mode and precise macroscopic pattern of PFC infiltration. Quantification of PFC uptake was done with ^{19}F MRS. Pancreas preserved in cold UW solution served as controls. Further quantification of PFC uptake and validation of our MR data in TLM- and UW-preserved pancreases was done with headspace gas chromatography. PFC content, by GC analysis, in human pancreas preserved in UW or by TLM was also compared in a preliminary manner.

In order to validate the hypothesis that TLM preservation maintains tissue bioenergetics by allowing continued aerobic production of ATP during cold ischaemia, we measured metabolite ratios in rat pancreas in real-time by ^{31}P MRS. Since the rat pancreas is a tiny organ without a definite capsule, any beneficial effect of PFC would be independent of its ability to penetrate into the tissues. Five different preservation groups were studied: Marshall‟s (control), static TLM, continuous TLM, static TLM following warm ischaemia and continuous TLM following warm ischaemia. Signal amplitudes for phosphomonoesters, inorganic phosphate and ATP were measured continuously and analyzed to estimate changes in high-energy phosphates throughout the duration of cold preservation.

Rat islet isolations were performed after pancreas procurement and results (mean islet yield) were compared between three different groups: pancreas in fresh state (controls), pancreas after 3-hour preservation in chilled UW solution and pancreas after 3-hour preservation in TLM.

4.1 EXPERIMENTAL MODELS

4.1.1 Animal models for islet studies

Experimental islet transplantation studies in rodent models have been vital and indeed indispensable to our current understanding and application of islet transplantation in the clinical setting. Isolation and purification techniques developed in rodents have been successfully applied to mass-isolation of highly purified islets from human pancreas (Lacy & Kostianovsky, 1967; Scharp et al., 1973; Gotoh et al., 1985). Islet implantation sites (including intraportal infusion) were first studied in rodents, as were islet preservation and culture techniques (Kemp et al., 1973). These seminal studies have provided important insights and formed the basis of current clinical practice. Rats are useful experimental models because of low costs, no requirement for sterile technique, availability of inbred strains and ease of induction of experimental diabetes (Di Cataldo et al., 1989). Drawbacks of the rat model in islet transplantation studies are discussed in section 8.3.1.

Pigs are useful animal models because their physiology is comparable to that of humans (Maggee & Hong, 1965) and because the cost of purchasing and maintaining them is relatively low (compared to dogs) (Swindle, 1984). Compared to the widely used rat model, the pig pancreas is a more suitable model for pre-clinical preservation studies because of anatomical and physiological similarities with humans. Also the parenchymal tissue density is similar to that of the human gland (Swindle, 1986; Getty, 1975). Moreover, the procurement and preservation procedures in pigs can be fully adapted from the clinical situation. With regard to animal rights, emotional relations to the pig are less sensitive than with other large animals (dogs). The pig pancreas has therefore been used as a large animal model for pancreas (Troisi et al., 2000) and islet transplantation (Hubert et al., 2007, Noguchi et al., 2008) in previous studies.

4.1.2 Justification for animal models used in this research

Diffusion of oxygen from the PFC layer into the pancreas has been suggested as the most plausible mechanism of aerobic preservation with TLM (see Chapter 2: Section 2.6). This hypothesis may be applicable to rodent pancreas (which is only a few millimeters in thickness), but direct experimental evidence of increased oxygen uptake in large primate or human pancreas is not convincing. Increased oxygen uptake in a large solid pancreas preserved in TLM is only possible through direct permeation of PFC into the pancreas preserved by TLM. In order to establish whether or not PFC penetrates solid pancreases of primates and humans, we performed ^{19}F MRS in pig pancreas and analyzed PFC content of porcine and human pancreases preserved in TLM.

Given their potential as red cell substitutes, it is appropriate to explore the utility of PFC in aerobic preservation of organs for transplantation. PFC-mediated enhanced oxygen transfer is possible by direct diffusion and is not necessarily dependent on PFC infiltration into the organ. This opens up new possibilities of using PFC-based machine perfusion instead of conventional TLM for optimal pancreas preservation. In order to investigate the possible use of PFC in maintaining tissue bioenergetics during organ preservation independently of their ability to penetrate solid organs, it was necessary to use small animal pancreas. The rat pancreas is only 2mm in thickness and 30 mm long (Seki et al., 2000). For accurate estimation of changes in ATP and determination of the time-course of such changes during the preservation period we employed ^{31}P MRS as our assay technique.

4.2 PANCREAS PROCUREMENT

All animal procedures were carried out in accordance with regulations outlined in the Home Office Animals (Scientific Procedures) Act 1986, United Kingdom (Project License Number 70/5716). Deceased donor pancreases were procured for experimental purposes following appropriate consent in accordance with the Human Tissue Act (2004), United Kingdom.

4.2.1 RAT PANCREAS

Pancreases were acquired from male Sprague-Dawley rats (n=64) and were procured either immediately following anaesthesia (immediate cold preservation groups) or following 30 mins of warm ischaemia (warm ischaemia groups). As this was the first study of its kind, power calculations were not possible and and an average number of six experiments in each group was chosen. For the immediate cold preservation groups, anaesthesia induced by intraperitoneal instillation of phenobarbitone (45 mg/kg) was followed by a midline laparotomy. The right common iliac artery was exposed and cannulated with a 23G butterfly needle and flushed with 30 ml of chilled Marshall‟s solution after clamping the infra-diaphragmatic aorta and venting the right atrium by intercostals space puncture. For the warm ischaemia groups, the pancreas was procured but left in the peritoneal cavity for 30 min warm ischaemia. Following laparotomy and arterial cannulation, additional phenobarbitone (45mg/kg) was instilled into the peritoneal cavity and death confirmed by cardiac arrest. The infra-diaphragmatic aorta was clamped and inferior vena cava vented prior to perfusion with 30 ml Marshall‟s solution kept at room temperature. A different venting mechanism was chosen for the warm ischaemia group in order to ensure that cardiac arrest was spontaneous.

In order to increase the signal-to-noise ratio of the ^{31}P-MRS acquisitions, pancreases were harvested from two animals at a time and scanned together. Thus pancreases from 64 rats gave a total of 32 separate experiments.

4.2.2 PORCINE PANCREAS

Adult porcine pancreases were harvested from breeder pigs and the procurement in general followed the standard techniques for human multi-organ retrieval. Briefly, following terminal anaesthesia midline laparotomy was made and the aorta cannulated immediately. The abdominal aorta was cannulated and retrograde flushing commenced with three litres of chilled Marshall‟s solution (Soltran, Baxter Healthcare, Norfolk, UK) after clamping the supra-coeliac aorta, with effluent solution vented from the adjacent vena cava. The gastrocolic ligament was divided to expose the left pancreatic segment. The left segment was then mobilized and removed by dividing the pancreas along a line in front of the aorta downwards from the coeliac axis to the splenic – superior mesenteric vein confluence. The pancreases were immediately preserved as described in the following sections.

4.2.3 HUMAN PANCREAS

Human pancreases that did not meet the criteria for clinical pancreas or islet transplantation were used for headspace gas chromatography. All pancreases were removed from deceased donors as part of multi-organ procurement. Briefly, after the liver was removed the duodenum was divided at both ends with a linear stapler. The superior mesenteric pedicle was identified at the base of the mesentery and divided with linear stapler. The superior mesenteric artery was identified and carefully removed from the aorta with a wide patch. Spleen was mobilized by dividing the perisplenic ligaments. Finally, the

rest of the omental, mesenteric and mesocolic attachments were divided and the pancreas was lifted off the posterior abdominal wall along with spleen using the latter as a handle. Single-cannula aortic perfusion with Marshall‟s solution and UW solution was used during procurement.

4.3 PANCREAS PRESERVATION

Except for the warm ischaemia groups, all pancreases were stored in ice-filled box immediately after procurement.

4.3.1 Rat pancreas

4.3.1a Preservation for islet isolation experiments

Rats were divided into three different preservation groups as follows:

Group 1 (fresh pancreas) – islet isolation was carried out without preliminary storage as described below.

Group 2 (UW – pancreas) – following cannulation of biliopancreatic duct and harvesting, pancreas was stored in chilled UW solution for 3 hours before injection of collagenase and islet isolation.

Group 3 (TLM – pancreas) – as in group 2 but pancreas was preserved by continuous two-layer method for 3 hours. The PFC layer was continuously oxygenated at the rate of 0.5L/hr.

4.3.1b Preservation for ^{31}P MRS experiments

For the TLM, pancreases were kept at the interface between a layer of perfluorodecalin ($C_{10}F_{18}$ – F2 chemicals, Preston, UK) and an overlying layer of Marshall‟s solution (20 ml each). Marshall‟s solution, which contains citrate as the principal impermeant anion, was used for all experiments, instead of the standard UW solution, in order to avoid the confounding effect of phosphate in the latter. For the "static" TLM, the perfluorodecalin was saturated with oxygen by bubbling this gas through at 0.5 L/hr for 30 min (this was done just before preservation). For "continuous" TLM oxygen was bubbled (0.5 L/hr) through the perfluorodecalin for 20 min once every hour throughout each experiment (local laboratory safety rules did not permit continuous oxygen bubbling – due to a prior incidence of explosion in the magnet room from oxygen use).

For the immediate cold preservation groups, 38 pancreases were procured to give 19 experimental preparations (2 pancreases in each) which were then randomly assigned amongst 3 preservation groups maintained at 4°C to 6°C: Marshall‟s solution (n=6), static TLM (n=6) and continuous TLM (n=7). For the warm ischaemia group, pancreases were procured from male Sprague-Dawley rats (n=26) in 13 separate experiments. After 30 min of warm ischaemia, the procured pancreases were randomly assigned for preservation to one of two groups: static TLM (n=6) and continuous TLM (n=7). For the warm ischaemia groups, 26 pancreases were procured for 13 experiments. After 30 min of warm ischaemia (WI), each pair of pancreases was randomized to one of two preservation groups: WI static TLM (n=6) and WI continuous TLM (n=7).

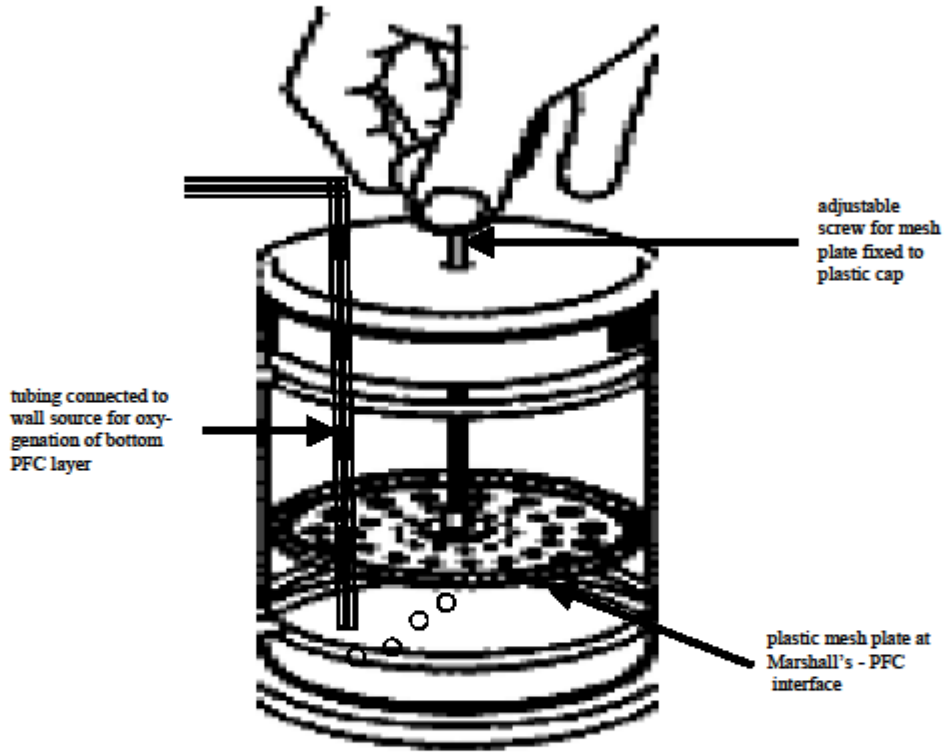


Figure 4.1: Schematic illustration of experimental set-up for rat pancreas preservation. Pancreas was kept at interface of the two preservation media by means of an adjustable mesh plate screwed to the lid as depicted. Additional tubing was fixed to plastic lid for oxygenation from external supply in the continuous TLM group.

4.3.2 Porcine pancreas

Porcine pancreas was immediately preserved in ice by one of two methods:

Group 1 - two-layer method for 24 hours in a 1.5L cylindrical jar and maintained at the interface of the bottom layer of PFC (300ml) and layer of UW solution (300ml) above by a meshed glass plate divider connected to the cap. Perfluorodecalin ($C_{10}F_{18}$ – F2 chemicals, Preston, UK), the typical compound employed in organ preservation, was the perfluorocarbon used for all the studies.

Group 2 - in the control arm pancreases were preserved in a similar fashion but using 300 ml chilled UW solution (Du Pont Pharma, Bad Homburg, Germany) for 24 hours. The jars were kept in an ice box during the storage period. Following storage, the surface of the pancreas was thoroughly irrigated with phosphate buffered saline immediately before the assays were made.

4.3.3 Human pancreas

Preservation of human pancreases was identical to that of the porcine specimens described above.

4.4 RAT ISLET ISOLATION

Rat islet isolation was based on the standard stationary digestion technique described by Gotoh et al. (1987).

Collagenase

15mg/ml collagenase P (Roche Diagnostics, Germany) was dissolved in HBSS supplemented with 5.5 mM calcium chloride solution. The collagenase was reconstituted immediately prior to use and stored on ice.

HBSS

Hank‟s balanced salt solution was reconstituted from premixed powder in 1L aliquots and stored at 4˚C for up to 2 weeks.

HBSS/BSA

5 mg/ml bovine serum albumin (Roche Diagnostics, Germany) added to HBSS.

RAT ISLET ISOLATION

Adult male Sprague Dawley rats (250-300g) were used for islet isolation. Rats were subjected to deep intraperitoneal anaesthesia with 45 mg/kg of phenobarbitone (Rhone Merieux, France). Following midline laparotomy, the small bowel was retracted to expose the liver hilum, duodenum and pancreas. The biliopancreatic duct was cannulated with PE50 tubing, which was secured with a silk ligature, at the liver hilum and the distal end of the duct was clamped with a bulldog at the entrance into the

duodenum. The animal was exsanguinated through a jugular incision. The pancreas was uniformly distended by injection of 3.3 ml of ice-cold collagenase solution into the biliopancreatic duct. The distended pancreas was excised along with adjacent duodenum and spleen. These adjacent organs were removed on the bench along with any excess mesentery and the pancreas was transferred into a 10 cm Petri dish containing 10 ml HBSS at 37˚C. Enzymatic digestion was of the pancreas was carried out by incubation in a warm bath at 37˚C for 15-17 minutes (depending on the enzyme activity of the collagenase batch). This was followed by mechanical disruption of the pancreas by aspiration through a 10G hollow needle. The resultant digest was poured into a 50 ml Falcon tube and washed three times at 400 x g for 2 minutes each at room temperature in ice-cold HBSS supplemented with 0.5% BSA (HBSS/BSA). Any remaining bits of undigested tissue were separated by sieving the tissue suspension through a mesh filter, and the final digest was repelleted in a Falcon tube and bottom loaded onto Ficoll of density 1.084 (Histopaque, Sigma Chemicals, UK). Ice-cold HBSS/BSA was layered on top of the Ficoll and the tube was centrifuged for 18 minutes at 600 x g at 4˚C. Purified islets were pipetted from the interface, re-suspended in ice-cold HBSS/BSA, washed a couple of times and transferred to a Petri dish containing HBSS/BSA for handpicking and counting under an inverted stereo microscope. These islets were stored in a 15ml test tube for further tests.

4.5 ASSAY TECHNIQUES

4.5.1 Magnetic Resonance Spectroscopy

Magnetic Resonance Spectroscopy (MRS) is a technique capable of complete structural and quantitative analysis of organic compounds. It was first described and measured in molecular beams by Isidor Rabi in 1938. Rabi‟s technique for measuring the magnetic characteristics of atomic nuclei won him the Nobel Prize in 1944, and was based on the resonance principle first described by Joseph Larmor. The technique was refined for use on solids and liquids simultaneously by two American

scientists independently in 1946, and both physicists Edward Purcell and Felix Bloch shared the 1952 Nobel Prize for their pioneering work (Bloch et al., 1946; Purcell et al., 1946). MRS employs high magnetic fields and radiofrequency pulses to manipulate the spin states of nuclei that have a nonzero-spin angular momentum. For a molecule containing such nuclei, the result is an NMR spectrum with peaks whose positions and intensities reflect the chemical environment and nucleic positions within the molecule.

Magnetic resonance or Nuclear magnetic resonance (NMR) refers to the absorption and release of non-ionizing radiofrequency (RF) energy by a nucleus in a magnetic field. It exploits the basic electromagnetic properties of small particles – charge and spin. When placed in an external magnetic field, these nuclei can be imagined to act like small bar magnets. They experience a torque which makes them precess around the direction of the field. The precession frequency of a particular nucleus (traditionally called the Larmor frequency) is proportional to the strength of the external magnetic field. When electromagnetic radiation with a matching (i.e. Larmor) frequency (RF pulse) is applied to the nuclei, the RF energy is absorbed, raising them to a higher spin state. When the RF pulse is turned off, relaxation processes restore the original equilibrium. The RF signals during relaxation induce voltage changes in a receiver coil and the resulting current is referred to as Free Induction Decay (FID) and represents the sum of resonance responses from all the excited spins. This time-domain signal is then Fourier transformed into an intensity-domain spectrum (intensity vs. frequency). The frequency of the emitted signal depends on the strength of the magnetic field and the chemical composition of the individual molecules, because the electron clouds of adjacent atoms change the local magnetic field (effect known as „shielding") that is experienced by a spin. This phenomenon is known as the chemical shift and explains why MRS is a direct probe of chemical structure. Chemists exploit this property to investigate the structure of molecules by precisely measuring the position of the resonances in the spectrum. The chemical shift values (δ) are typically of the order of 10^{-6}, and are therefore commonly referred to as the dimensionless unit, parts per million (ppm), thereby allowing direct comparison of values obtained from different instruments. The effect of the electron cloud in an atom or molecule is to slightly shield the nucleus from the applied magnetic field, thus

giving any chemical species a characteristic frequency. This gives rise to spectra where nuclei in a molecule give rise to specific signals, thus facilitating the detection of individual chemicals by means of their frequency spectra.

Although proton brain spectroscopy is the most common clinical application of MRS (for conditions such as stroke, dementia, tumours and multiple sclerosis), other MR-active nuclei have found increasing applications in vitro and in vivo studies. All nuclei that contain odd numbers of protons or neutrons have an intrinsic magnetic moment and angular momentum, and are MR-visible. The most commonly measured nuclei are 1-H, 13-C and 31-P. Other nuclei that can be observed include 19-F, 23-Na, 35-Cl, 17-O, 14-N, 15-N, 113 Cd, 195-Pt, 10-B, 11-B, 29-Si.

As a modality for molecular imaging, magnetic resonance technology has several advantages – lack of ionizing radiation, excellent resolution and soft-tissue contrast. It has the potential to precisely elucidate structural and functional alterations that occur in vivo during disease development, progress and treatment.

PFC and MRI/ MRS

As it has seven outer-shell electrons, chemical shifts for fluorine span a very wide range (approx 300 ppm). ^{19}F has a sensitivity to NMR detection that is 83% that of the sensitivity of ^{1}H (Kaneda et al., 2009). Moreover there is no endogenous background signal in vivo as fluorine has no natural occurrences in biological systems (O'Hagan, 2008). These properties make ^{19}F ideally suited for magnetic resonance studies. MRS can accurately quantify the PFC content of tissues (Pratt et al., 1992; Mason, 1994). Molecular imaging has now enabled non- invasive identification of specific tissue epitopes and observation of biological processes at cellular level. There is great interest in molecular MRI and MRS of perfluorocarbon-based nanoprobes with specific detection of ^{19}F nuclei providing quantifiable spectral signatures of targeted molecular epitopes. The perfluorocarbon core material is surrounded by a lipid monolayer which can be functionalized by incorporation of targeting

ligands, contrast agents and drugs. Ligand-targeted ^{19}F nanoparticles have been used for quantitative „magnetic resonance immunohistochemistry" and allowed accurate quantification of fibrin in atherosclerotic plaques (Morawski et al., 2004). ^{19}F MRI of cells labelled with PFC nanoparticles can be used for cell tracking (Ahrens et al., 2005, Partlow et al., 2007). An in vivo ^{19}F MRI has been described for visualization and quantification of T cell migration in a non-obese diabetic mouse model (Srinivas et al., 2007). These agents also have tremendous potential as targeted therapeutic agents. Anti-angiogenic agents for treatment of cancer can be delivered locally by cell-selective contact-facilitated mechanisms, achieving therapeutic efficacy at greatly reduced systemic doses (Winter et al., 2008). Similar studies with targeted anti-angiogenic agents have been carried out in animals for prevention of atherosclerosis (Winter et al., 2006). Fibrin-targeted nanoparticles incorporating streptokinase are being developed as thrombolytic agents (Marsh et al., 2007).

Since the biological presence of ^{19}F isotope is virtually zero and signal generated by the atoms is strong (i.e. high NMR sensitivity), perfluorocarbons can be quantified easily and precisely (Kaneda et al., 2009). Previous studies have reported the minimum detectable limit of ^{19}F at 1.5T is 1μM/ml (Schlemmer et al., 1999). In vivo magnetic resonance imaging can be used for pharmacokinetic profiling of the binding of targeted contrast agents (Neubauer et al., 2008). Integrin-targeted nanoparticles have been used for the detection and quantification of angiogenesis in experimental heart valve disease (Waters et al., 2008). PFCs have been used as positive contrast agents for detection of inflammation in heart and brain using ^{19}F MRI (Flogel et al., 2008).

^{19}F MRS has been extensively exploited in preclinical studies to measure tissue oxygenation in such diverse organs as liver, spleen, brain, heart and lung, and for various pathological conditions such as tumours and abscesses (Krohn et al., 2008). ^{19}F MR oximetry relies on the linear dependence of the spin-lattice relaxation rate R1 (= 1/T1) of perfluorocarbons on pO_2 (Parhami & Fung, 1983). The advantage of absolute pO_2 measurement with the fluorocarbon reporter molecules is that regional oxygen tension maps can be obtained simultaneously from 50-150 individual locations. Additionally, sequential pO_2 maps can be generated to reveal changes in oxygenation with respect to time and various interventions (Krohn et al., 2008).

MRS AND ATP

Unlike plants, all other organisms are heterotrophic obtaining free energy by coupling their metabolism to the breakdown of complex organic molecules in their environment. In all these organisms, ATP acts as the energy currency of the cell, transferring free energy derived from substances of higher energy potential to those of lower energy potential (exergonic-endergonic reaction). The charge repulsion of adjacent negatively charged oxygen atoms in the phosphate bonds of the ATP molecule are responsible for the high energy charge (Murray et al., 2008).

Since phosphorus is a major component of ATP and is actively transferred between different molecules during all bioenergetic processes, ^{31}P MRS is often used to study metabolism. ^{31}P MRS of tissues usually yields a spectrum approximately of 20 ppm width, with three main resonances at about -15 ppm, -8 ppm and -4 ppm from right to left labelled βATP, αATP and γATP respectively, one for each phosphorus atom in the molecule (see Figure 7.2). Phosphocreatine (PCr) peak at chemical shift 0 ppm is the standard reference point and reflects oxidative metabolism. Further along the left of the spectrum are the phosphodiester (PDE) and inorganic phosphate (Pi) peaks at 3 ppm and 5 ppm respectively. The former is related to cell membranes and the latter can be used to derive intracellular pH.

Since ^{31}P has a much lower Larmor frequency than protons, magnets with higher field strengths (4.7T) are required to provide good spatial resolution. MRS is a powerful tool for non-invasive assessment of energetic state. Phosphorus nuclei participate in energy-producing pathways necessary for all cell functions. Therefore chemical shift spectra obtained from these provide important intracellular biochemical information in a non-destructive and noninvasive way, both in vivo and ex situ. Moreover, being a non-destructive technique, MRS can be used to follow the dynamic changes of metabolites in a given tissue where the preparation can serve as its own control. ^{31}P MRS has been employed to study bioenergetics in cardiac and striated muscle. It provides a unique window on cellular energetic and has many potentially useful applications.

During cold storage of the graft, high-energy phosphates undergo degradation resulting in an increase in adenosine monophosphate (AMP) and inorganic phosphate (Pi). AMP undergoes further catabolism to adenosine and Pi (McCord, 1985).

This method can detect ischaemia in several solid organs and provides a reliable measure of organ damage/viability (Sato et al., 1996).

In an elegant in vivo experimental model of reversible rat pancreatic ischaemia, Siech and colleagues (1995) observed increase in inorganic phosphate with a concomitant decrease in ATP levels and pH in ^{31}P NMR spectra acquired during the ischaemic period when compared to controls. There was a corresponding increase in the amplitude of PME peaks and these changes were completely reversible upon restoration of blood flow.

It has been shown that pretransplant MRS parameters (PME/Pi ratio) correlate with renal viability and are predictive of delayed graft function (Bretan et al., 1989). Both reduced AMP and increased Pi concentrations have been demonstrated by biochemical analysis in ischaemically damaged kidneys, and are believed to account for this finding (Shapiro et al., 1989). ^{31}P MRS derived ratios of PME/Pi and ATP/Pi also enable noninvasive assessment of blood flow-dependent viability of in situ renal transplants (Bretan et al., 1993). Other studies have supported the use of these ratios as practical viability parameters in the clinical setting (Bretan et al., 1987, Pomer et al., 1989, Barnard et al., 1997).

It has been observed that allograft nephropathy (both acute and long-term) is accompanied by a decrease in beta-ATP/Pi ratio (Shapiro et al., 1987; Seto et al., 2001) and in a recent study a cutoff value of 1.2 was found to be an independent risk factor for graft survival (Seto et al., 2001). Another recent study found a significant correlation between high energy phosphates (^{31}P NMR-derived beta-ATP/Pi ratio) and A1C in patients who underwent combined kidney pancreas transplantation (Fiorina et al., 2007).

4.5.2 Headspace gas chromatography

Headspace chromatography measures the vapour pressure of volatile organic compounds and can be applied for accurate estimation of fluorinated hydrocarbons (Cobranchi et al., 2006). PFCs have numerous industrial applications and effluents from sewage treatment plants have been implicated as a potential pathway of PFC release into the environment. Head space gas chromatography is a well established method for detection of perfluorocarbon in biologic specimens (Backer & Pisano, 1978; Clark et al., 1992; Hoerauf et al., 2002) and traces of perfluorinated compounds have been found ubiquitously in biological tissues and in human serum (Olsen et al., 2005; Calafat et al., 2006). It is considered the most efficient assay technique for fluorocarbon compounds in biological systems (Holaday, 1970; Modell et al., 1973)

Both ^{19}F MRS and headspace gas chromatography are state-of-art standard reference methods used to investigate trace concentrations of perfluorinated compounds, both in humans and in various environmental matrices. The method detection limits of the order of 10 ng/ml and 10 ng/g can be achieved with these techniques (Jahnke & Berger, 2009).

4.6 FLUORINE (^{19}F) MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY

4.6a Proton and Fluorine Magnetic Resonance Imaging (^{1}H- and ^{19}F-MRI)

This MRS work was carried out with expert assistance from Dr Po-Wah So, medical physicist at the Hammersmith Hospital, London. Preliminary assessment of PFC penetration was made by non-invasive fluorine magnetic resonance imaging with a 9.4T Varian INOVA MR spectrometer scanner

(Varian Inc., CA, USA). For this a segment of porcine pancreas (n=4 each in groups 1 and 2), 7.5 cm in length, was divided from the tail end of each pancreas, washed and placed in a 50 ml universal tube containing UW solution. This was then centrally located in a quadrature volume coil capable of being tuned to both proton (^{1}H) and fluorine (^{19}F) resonance frequencies. A phantom (1ml syringe filled with perfluorodecalin) was taped to the universal tube containing the pancreatic segment as an external reference standard. Initially, proton imaging was performed to establish appropriate sample location using a spin-echo sequence with a repetition time (TR) of 1s, echo time (TE) of 20 ms, field of view (FOV) of 45mcm x 45mcm, 256 x 128 matrix size and 4 averages. Four consecutive transverse images 4 mm in thickness were obtained. ^{19}F MRI-Fluorine images were subsequently acquired with the same parameters except 512 averages were used.

4.6b Magnetic Resonance Spectroscopy (^{19}F-MRS)

Non-localized ^{19}F -MRS was then performed to quantify PFC uptake in the porcine pancreas using the same spectrometer scanner (9.4T). For this, 16K time data points were collected from a sweep width (SW) of 50 kHz using a 90° pulse, repetition time (TR) of 1s and 512 averages were collected. One ml syringes of Perfluorodecalin and sodium fluoride were employed as external reference for ^{19}F MRS quantifictation purposes. Four pancreas harvests were carried out in each group: in one group (TLM) the organs was preserved by the two-layer method for 24 hours, and in the other (UW alone) standard preservation in UW was employed for the same duration (n=4 in each group).

4.7 HEADSPACE GAS CHROMATOGRAPHY

Headspace gas chromatography was used as an alternative method to quantify PFC uptake in the pancreas. Six porcine pancreases preserved in TLM for 24 hours were compared with an equal number of porcine pancreases preserved for the same duration in UW solution (n=6 in each group). In addition fresh pancreatic tissue was spiked with perfluorodecalin to serve as a positive control for this experiment – 0.1µl of perfluorodecalin was added to 500mg of pancreatic tissue.

Pancreatic tissue (500mg) was transferred to 50 ml pyrex head space vials containing 2 g of anhydrous magnesium sulphate. The bottles were placed in a microwave oven and heated for 90s at 200°C to volatilize the PFC. The sample effluent from the head space was analyzed in a gas chromatograph (model 6890; Agilent Technologies, Inc. Wilmington, DE) configured with a flame ionization detector (GC-FID) and a stationary phase 20% w/w SE-30 on solid support Gas Chrom R Mesh 100-120 packed glass column (12ft x 6mm OD x 3mm ID). The carrier gas was helium flowing at a constant rate of 24ml/min. One ml of the headspace effluent was introduced into the injector port for analysis using a gas-tight syringe. The initial temperature was 100°C for 12 min followed by a ramp rate of 4°C/min for 18 min to a final temperature of 172°C held for 5 min.

Method validation for Gas Chromatography - Linearity and Precision

The method linearity and precision for head space gas chromatography were studied: Linearity data were obtained from serial diluted standards of perfluorodecalin. Linear regression analysis was performed over the range of 0.02% to 0.38% with 5 concentration levels (0.02, 0.04, 0.10, 0.19 and 0.38% w/w equivalent) and the linear equation and correlation coefficient obtained.

4.8 PHOSPHORUS (^{31}P) MAGNETIC RESONANCE SPECTROSCOPY

This MRS work was carried out with expert assistance from Dr Alan Bainbridge, medical physicist at the University College Hospital, London. In order to maintain the experimental preparation within the target temperature range, a specially designed perspex vessel contained the pancreases during MRS. Temperature was kept constant at 4° - 6° C by circulating chilled water through an outer chamber surrounding the vessel.

Preserved pancreases underwent MRS at 4.7 Tesla on a Bruker Biospec Avance (Bruker Medizintechnik, Rheinstetten, Germany). ^{31}P spectra were acquired using a 3 cm diameter surface coil operated in transmit and receive mode that could be tuned to the resonant frequencies of protons (^{1}H) and ^{31}P nuclei. Fully-relaxed ^{31}P spectra were acquired continuously during each experiment using a "single-pulse and acquire" sequence (relaxation time - 10 sec, 256 summed free induction decays). MRS was halted during oxygen bubbling in continuous TLM experiments. Spectra were analyzed by AMARES (Vanhamme et al., 1997) as implemented in the "magnetic resonance user interface" (jMRUI) analysis package (Naressi et al., 2001).

Spectrum signal amplitudes were measured for phospho-mono-esters (PME), inorganic phosphate (Pi) and α-, β- and γ -ATP. As MRS was "fully relaxed", metabolite signal-amplitude ratios were effectively concentration ratios which are independent of magnetic field strength. Without preservation, pancreatic necrosis will progress and this will be accompanied by declining ATP and increasing Pi signals: Pi/γ-ATP and Pi/ β-ATP have been previously used to investigate the endpoint ATP after TLM preservation (Yoshikawa et al., 2004).

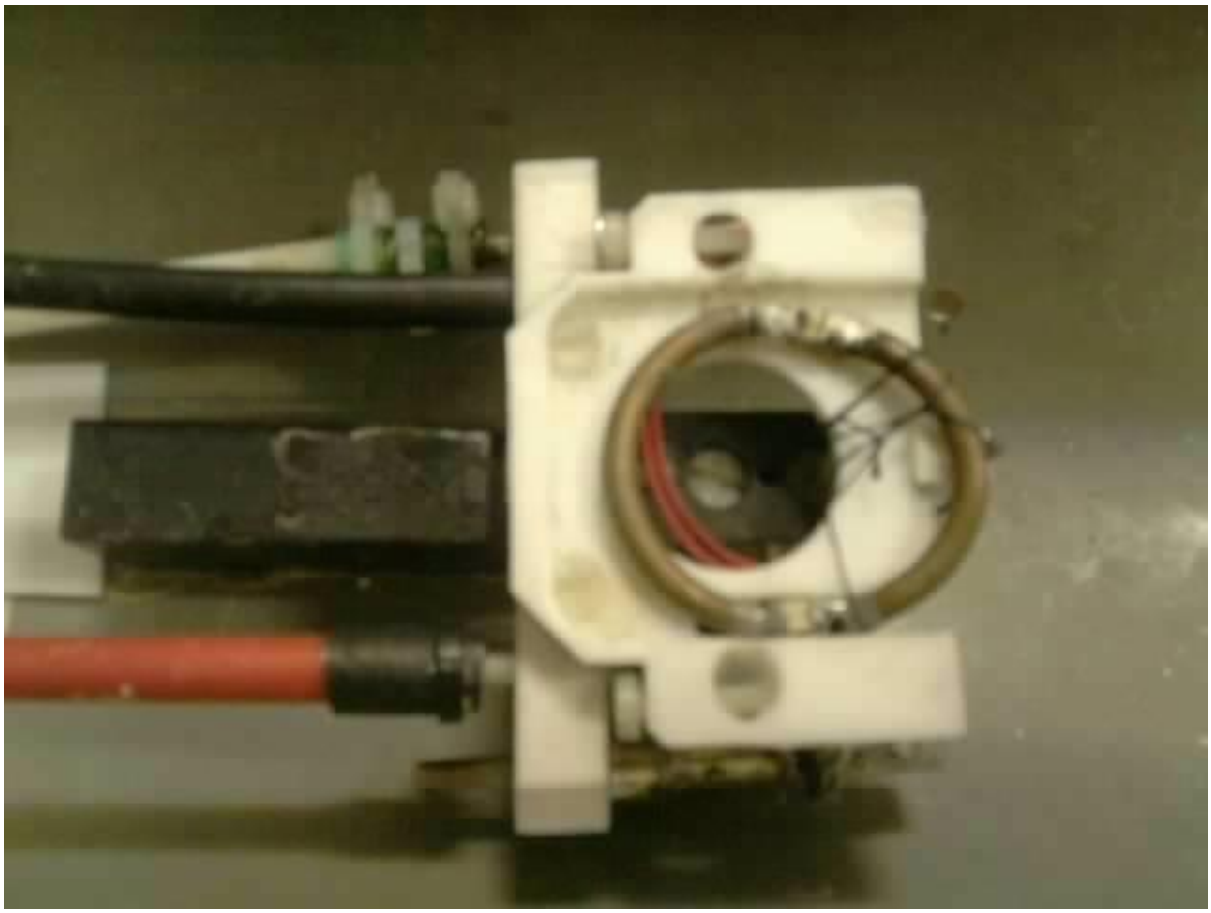
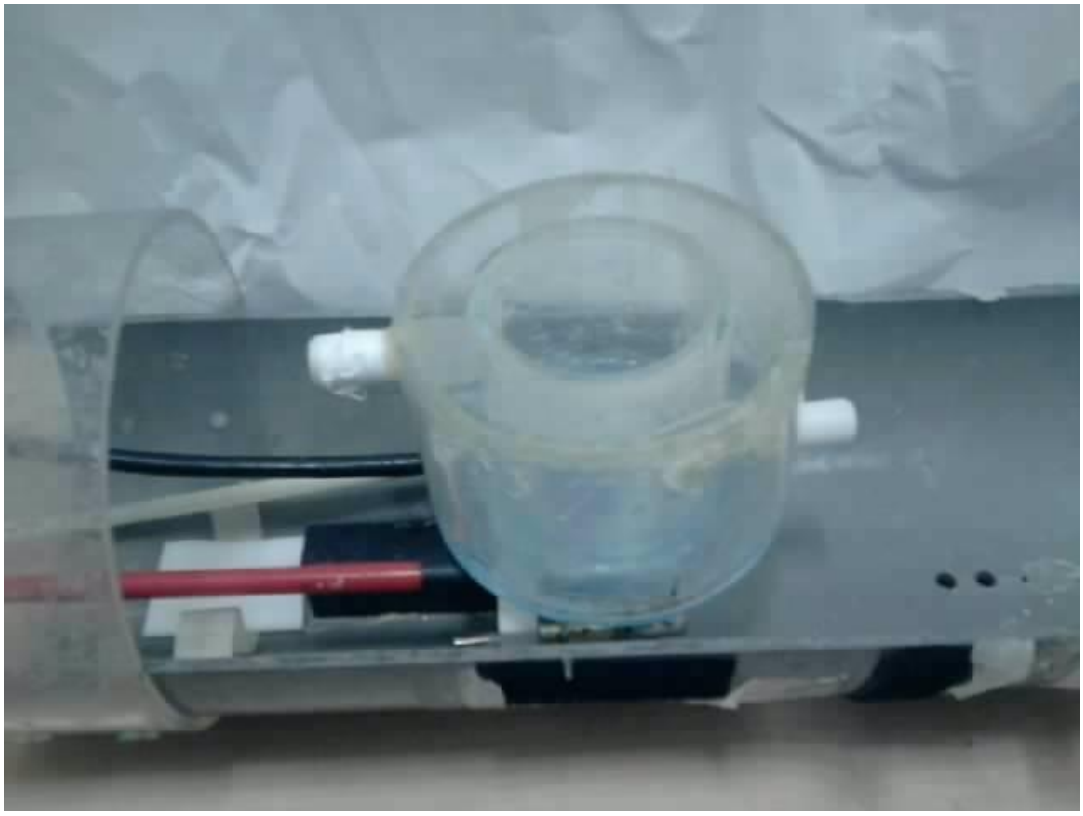


Figure 4.2a: Photograph showing surface coil designed for rat ^{31}P MRS experiments – see text for details



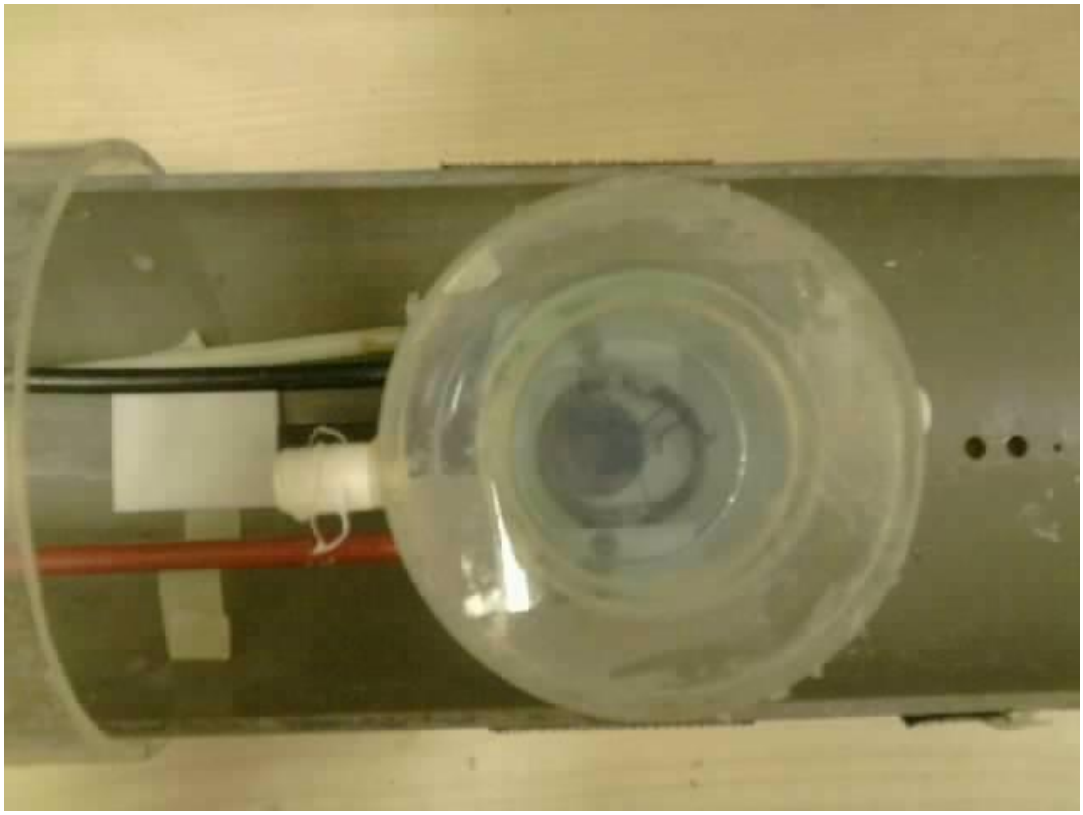


Figure 4.2b: Photographs showing the custom-made vessel for rat ^{31}P MRS experiments. Chilled fluid was circulated through the outer chamber with inlet and outlet ports for tubing connected to chiller machine. Additional tubing for oxygenation of PFC layer was attached to lid covering the vessel (not shown).

4.9 STATISTICAL ANALYSES

4.9.1 Rat islet isolation

Means between the fresh and preservation groups were compared using Student‟s t-test with significance level set at $p < 0.05$.

4.9.2 ^{19}F –MRS

Statistical analysis of spectroscopy data was not possible in view of the fact that no PFC was detected even with un-localized MRS.

4.9.3 Headspace gas chromatography

Statistical significance for headspace gas chromatography was assessed using Student‟s t test to compare the means between the two groups. A p value of less than 0.05 was considered as significant.

4.9.4 ^{31}P-MRS

We nominated [γ-ATP]/ [Pi] and [β-ATP]/ [Pi] as our principal outcome measures for the purpose of statistical analyses. [β-ATP]/ [Pi] and [γ-ATP]/ [Pi] were plotted against time from pancreas procurement for each experiment: linear regressions were performed and rates of change derived which were grouped according to preservation method. If rates of change were normally distributed, intergroup tests for difference used one-way ANOVA with pair-wise comparisons using a 2-tailed t-test without equal variance within the groups. If rates of change were not normally distributed,

ANOVA on ranks was used with Dunn‟s method for pair-wise comparisons. The threshold for statistical significance was $p = 0.05$: because of the strong correlation between [β-ATP]/ [Pi] and [γ-ATP]/ [Pi] a Bonferroni correction for multiple comparisons was not employed despite testing for differences in both these ratios.

CHAPTER 5

PFC UPTAKE AND DISTRIBUTION IN PORCINE PANCREAS - ^{19}F MRS

5.1 Purpose of study

Little is known about the precise mechanism of action of the two-layer method of pancreas preservation for islet transplantation. It is currently held that penetration of perfluorocarbon results in aerobic preservation of cellular bioenergetics and organ viability. If indeed this is the case, it will be instructive to examine the mode and time-course of such penetration so that optimal protocols for clinical use of perfluorocarbon can be devised. To my knowledge ^{19}F MRI/MRS has not been employed to study PFC-based organ preservation so far. Because of the unique properties of PFC and its exceptional visibility on ^{19}F NMR imaging, we used this technique to investigate the precise macroscopic pattern of tissue distribution of PFC when pancreas was preserved by the two-layer method. In addition to PFC detection, it was our aim to quantify the PFC uptake at the end of the preservation period using ^{19}F MRS.

5.2 **Methods** (see Sections 4.2.2, 4.3.2 and 4.6 for details)

Whole porcine pancreases were preserved in chilled UW solution or static two-layer method for 24 hours. The organs were thoroughly washed with phosphate buffered saline immediately prior to ^{19}F MRS experiments. Preliminary assessment of PFC penetration was made by non-invasive fluorine magnetic resonance imaging with a 9.4T Varian INOVA MR spectrometer scanner (Varian Inc., CA, USA). For this a segment of porcine pancreas (n=4 each in groups 1 and 2), 7.5 cm in length, was divided from the tail end of each pancreas, washed and placed in a 50 ml universal tube containing UW solution. This was then centrally located in a quadrature volume coil capable of being tuned to both proton (^{1}H) and fluorine (^{19}F) resonance frequencies. A phantom (1ml syringe filled with perfluorodecalin) was taped to the universal tube containing the pancreatic segment as an external reference standard. ^{1}H and ^{19}F MRI was performed on these samples. This was followed by non-

localized ^{19}F -MRS to quantify PFC uptake in the pancreas using the same spectrometer scanner (9.4T).

5.3 Results

5.3.1 Results of Proton and Fluorine Magnetic Resonance Imaging

The results of MR imaging are shown for a typical pancreas preserved by TLM in Figures 5.1 and 5.2. Proton and Fluorine MR Imaging was carried out in all pancreatic segments (n=4 pancreases in each group) as a preliminary to spectroscopic evaluation. With proton imaging, the pancreatic segment was clearly imaged but the PFC syringe was not visualized. The PFC syringe was readily detected with fluorine imaging but the pancreatic segment was not visible. The observations in both UW- and TLM –preserved pancreas were the same.

5.3.2 Results of ^{19}F Magnetic Resonance Spectroscopy

Due to the lack of fluorine signal from imaging of the pancreas, non-localized ^{19}F-MRS was performed to estimate the PFC level in the whole pancreatic sample. Syringes containing 1ml of sodium fluoride and Perfluorodecalin were used as positive controls. Figure 5.3 illustrates the typical ^{19}F -MRS spectra obtained from these experiments. Qualitatively there was no difference between the spectra obtained from TLM-preserved pancreases (n=4) and those in UW-solution (n=4), and the characteristic ^{19}F MRS spectrum of PFC was not seen in either (Figure 5.3).

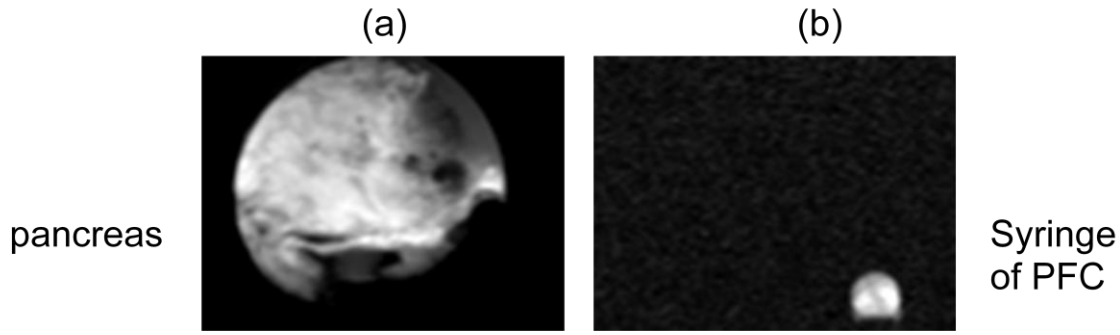


Figure 5.1a and b

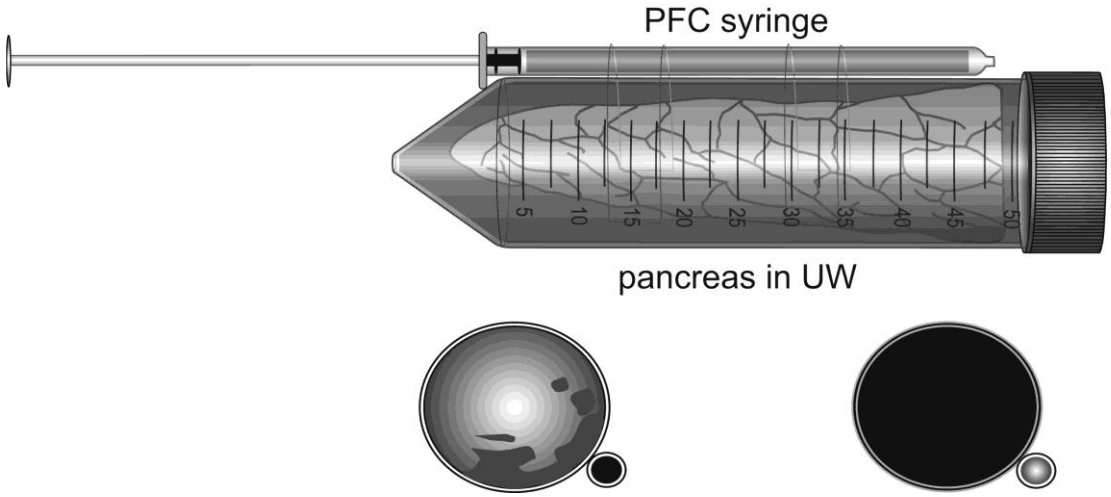


Figure 5.2

Corresponding ^{1}H (Figure 5.1a) and ^{19}F (Figure5.1b) transverse MR images of a typical porcine pancreas stored in TLM for 24 hours. Although the experimental phantom is clearly visualized, there is no fluorine signal from the adjacent pancreas indicating that PFC does not infiltrate into the parenchyma (Figure5.1b). This was consistent in all pancreases from both UW- and TLM- groups. Figure 2 illustrates these images diagrammatically (scale - the universal tube has a diameter of 30 mm).

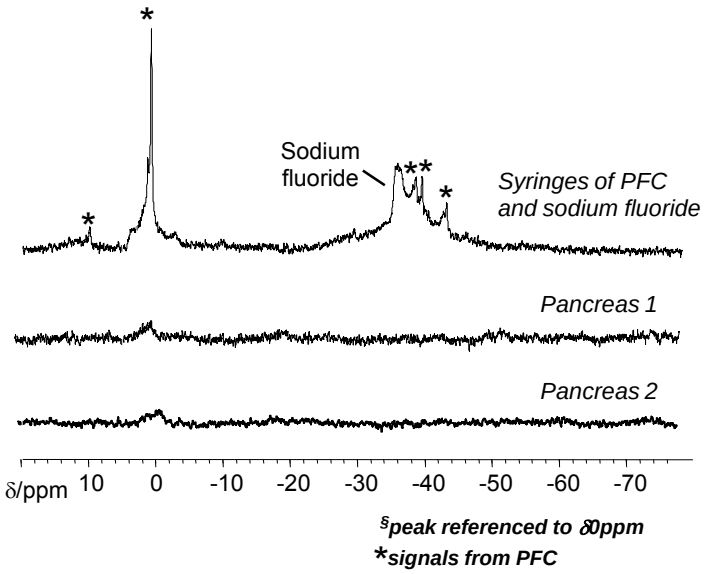


Figure 5.3

^{19}F NMR spectra obtained from pancreas preserved by simple cold storage (pancreas 2) and by two-layer method (pancreas 1) compared with representative spectra of PFC and sodium fluoride. Both TLM- and UW-preserved pancreases have similar NMR spectrum and no PFC signal is evident.

5.4 Discussion

In this study, with proton signal MR imaging the pancreas was clearly seen but the reference syringe containing PFC was not visualized – this was expected as there are no hydrogen atoms in the perfluorodecalin molecule. However the PFC syringe was readily seen with the fluorine imaging MR sequence used, whereas no signal was detected from the pancreatic segment. This indicates that any PFC present in the tissue was below the detection limit of the MR experiment as no fluorine signal could be obtained from the pancreas from a series of volumes of 0.25mm^3. In addition, the characteristic spectrum of PFC was not obtained from the TLM-preserved pancreas by ^{19}F MRS. Further confirmation of this was obtained when ^{19}F NMR spectra of UW- and TLM- preserved pancreas were compared with typical spectra obtained from the experimental phantoms (syringes filled with perfluorodecalin and sodium fluoride). There was no difference between the ^{19}F NMR spectra between UW- and TLM- preserved pancreas: no ^{19}F resonance was observed in either spectra and they both lacked "PFC" regions.

It is unlikely that leakage of PFC from the pancreas tissue from sample could have contributed to the absence of signal. Since the scans were performed immediately after the end of the preservation period and since the samples interrogated were whole blocks of pancreas with an intact capsule, there was very little possibility of PFC leakage prior to the measurements. In order to avoid PFC signal from the preservation solution, these measurements were not carried out in situ and the blocks were suspended in UW solution instead. Initially, un-localized spectroscopy was performed on the whole sample volume because un-localized spectroscopy has greater sensitivity than ^{19}F MRI to measure PFC since the former allows the detection of any PFC throughout whole sample. Here again, PFC was not detected. Conversely ^{19}F-MRI will only detect PFC if above the limit of detection in a defined volume, which in this instance is 25mm^3.

Our current study was designed to focus on the potential for PFC penetration into pancreases under conditions close to those found in the clinic. For this reason, the two-layer method was set up with

pre-oxygenation of the PFC, although changes in oxygen tension are unlikely to make any difference to the penetration of the chemical itself. Oxygen tension in the PFC would become important if metabolic effects of the two-layer method were to be investigated, but ^{19}F MR spectroscopy cannot provide information about bioenergetics.

Although ^{19}F MRS has been used to quantify fluorinated compounds (Martino et al., 2005; Mandal & Pettegrew, 2008) and to identify tracer nanoparticles for diagnostic and therapeutic purposes (Waters et al., 2008; Kaneda et al., 2009), its current application to investigate retention of perfluorocarbon in organs stored in two-layer method is new. Since no quantifiable fluorine signal was obtained in the pancreatic samples interrogated, we proceeded to validate the ^{19}F MR findings with gas chromatography.

CHAPTER 6

PFC UPTAKE IN PORCINE PANCREAS - HEADSPACE GAS CHROMATOGRAPHY

6.1 Purpose of study

With ^{19}F magnetic resonance spectroscopy, no evidence of PFC uptake was found in porcine pancreases preserved in TLM for 24 hours. This would suggest that any PFC present in the stored pancreases was below the level of detection by MRS. Gas chromatography is a standard reference method for quantification of trace amounts of perfluorocarbons (see Section 4.1.2b).

6.2 **Methods** (see Section 4.2.2, 4.2.3, 4.3.2, 4.3.3 and 4.7 for details)

Porcine pancreases were preserved for 24 hours in one of two groups immediately after procurement: chilled UW or chilled static TLM (n = 6 in each group). Gas chromatography was performed on 500 mg tissue samples after thoroughly washing the preserved organs. In addition fresh pancreatic tissue was spiked with perfluorodecalin to serve as a positive control (n = 3) for this experiment – 0.1µl of perfluorodecalin was added to 500mg of pancreatic tissue.

Identical analysis was done for human pancreases. Due to paucity of organs available for research there was only one pancreas in each of the preservation groups (results in **Appendix III**).

6.3 Results

Table 6.1 shows the results of gas chromatography for each of the three experimental groups and Figure 6.1 compares the mean PFC content between TLM-preserved, UW-preserved and PFC-spiked pancreas. Figure 6.2 shows the typical GC-FID chromatograms of pancreas preserved for 24 hours in the two-layer method (first panel), pancreas preserved in UW solution for 24 hours (second panel) and pancreas spiked with Perfluorodecalin (third panel). The mean concentration of perfluorodecalin, as identified by the retention times of the PFD isomers, in the pancreatic tissue samples preserved in TLM was 0.011nl/g (SD ±0.006). This was not significantly different from the concentration of PFC in the control samples preserved in UW solution (0.012nl/g, SD ± 0.006, p = 0.42). The mean concentration in the spiked standards (0.1µl perfluorodecalin added to 500mg of fresh pancreas) was 166.07nl/g.

Method validation for Gas Chromatography - Linearity and Precision

The method linearity and precision for head space gas chromatography were studied: Linearity data were obtained from serial diluted standards of perfluorodecalin. Linear regression analysis was performed over the range of 0.02% to 0.38% with 5 concentration levels (0.02, 0.04, 0.10, 0.19 and 0.38% w/w equivalent) and the linear equation and correlation coefficient obtained. A correlation coefficient (R) of more than 0.99 indicates that the proposed method has a good linearity. The correlation coefficient of the curve for our method was 0.9981and the factor of curvature 0.9846 (Figure 6.3). In addition to this, six separate analyses of the 0.10% w/w standard produced a residual standard deviation (RSD) of 4.5% indicating good precision.

	UW pancreas ppm	UW pancreas nl/g	TLM pancreas ppm	TLM pancreas nl/g	spiked control ppm	spiked control nl/g
1	0.028	0.015	0.024	0.012	313.79	165.15
2	0.031	0.016	0	0	321.72	169.22
3	0.019	0.001	0.033	0.017	311.28	163.83
4	0.029	0.015	0.025	0.013		
5	0.022	0.011	0.021	0.011		
6	0.023	0.012	0.025	0.013		
mean (±SD)	0.025333 (±0.004676)	0.011667 (±0.005574)	0.021333 (±0.011183)	0.011 (±0.005762)	315.5967 (±5.449443)	166.0667 (±2.80949)

Table 6.1

Results of headspace gas chromatography to determine perfluorodecalin content of porcine pancreases preserved in UW solution and two-layer method (expressed in both ppm and nl/g of pancreatic tissue). The last two columns give the concentration of PFC in pancreatic tissue spiked with perfluorodecalin.

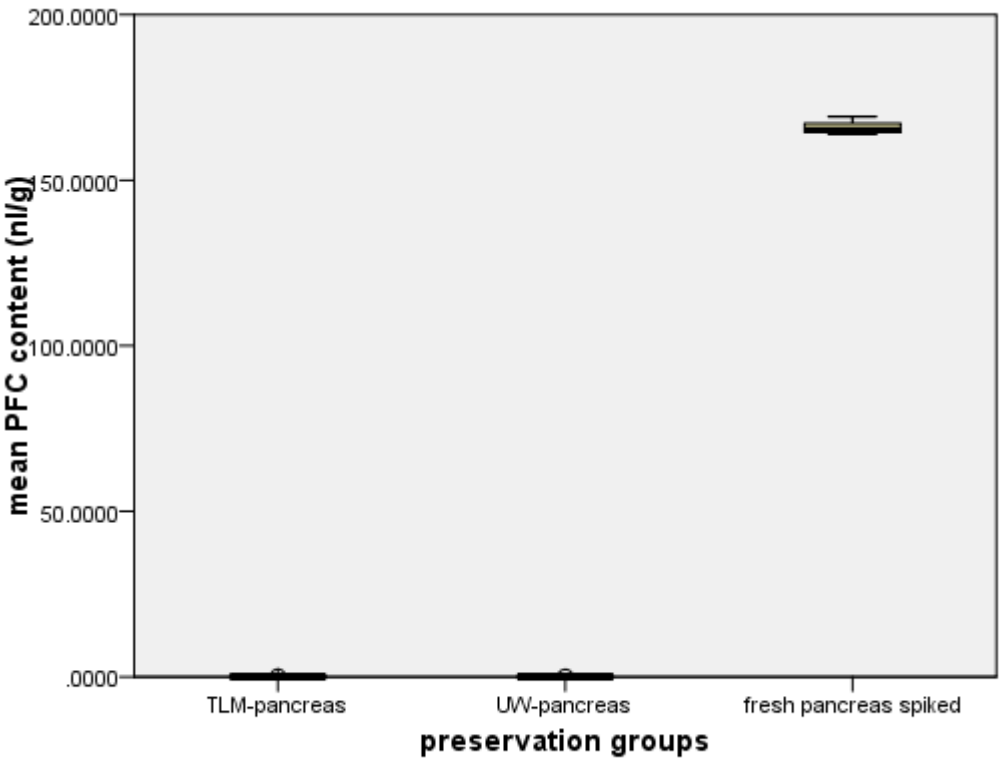


Figure 6.1

Boxplot comparing the mean PFC content (as determined by headspace gas chromatography) of TLM- and UW-preserved pancreas (n=6 each) with that of fresh porcine pancreas spiked with PFC (n=3).

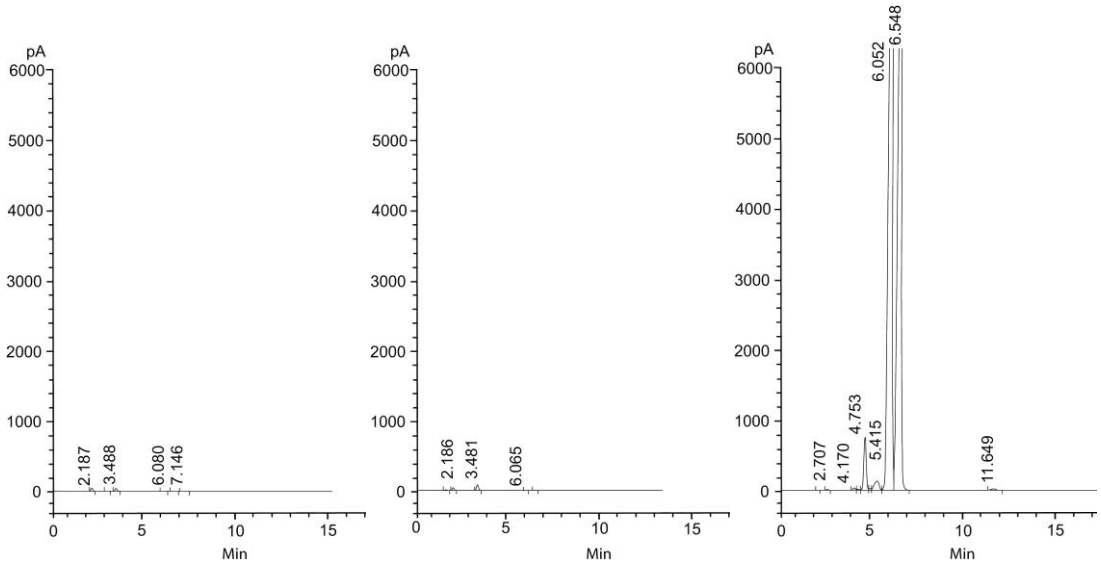


Figure 6.2

Headspace chromatograms of PFC from pancreas preserved by TLM for 24 hours and washed with phosphate buffered saline i.e. test sample (first panel), from pancreas preserved in UW solution alone i.e. control (second panel) and from fresh pancreas spiked with perfluorodecalin i.e. positive control (third panel). PFC levels are minimal in both TLM- and UW- preserved pancreas suggesting lack of PFC penetration in pancreatic tissue.

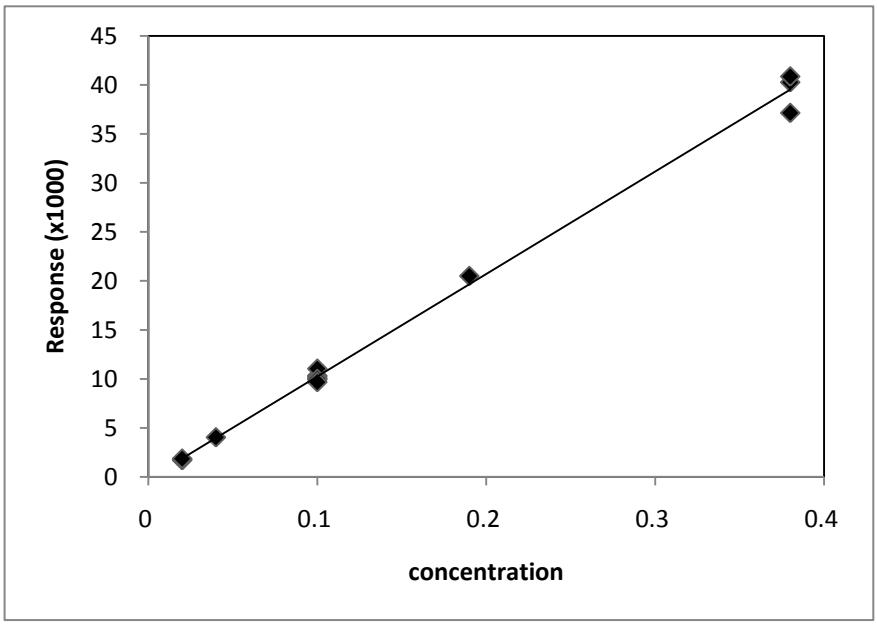


Figure 6.3

Standard Curve (linear regression graph) of the response obtained from analysis of 5 different concentration levels of diluted standard for method validation indicating good method linearity and precision (see text).

6.4 Discussion

In chapter 5 pancreatic tissue concentration of PFC was measured by magnetic resonance spectroscopy and in this chapter headspace gas chromatography was used to corroborate the findings. No evidence for the uptake of PFC into pancreas during cold preservation by TLM was seen.

Compared to the widely used rat model, the pig pancreas is a more suitable model for pre-clinical preservation studies because of anatomical and physiological similarities with humans. Also the parenchymal tissue density is similar to that of the human gland (Swindle et al., 1986; Troisi et al., 2000). Moreover, the procurement and preservation procedures in pigs can be fully adapted from the clinical situation. The pig pancreas has therefore been used as a large animal model for pancreas (Troisi et al., 2000) and islet transplantation (Hubert et al., 2007; Noguchi et al., 2008) in previous studies.

^{19}F NMR (nuclear magnetic resonance) is a specific and accurate method for measurement of tissue PFC concentration. It has been used to quantify the biodistribution of PFC blood substitutes in porcine tissues following intraperitoneal administration of a perfluorocarbon emulsion (Pratt et al, 1992). Recently, perfluorocarbon content of targeted nanoparticles has been exploited for the purposes of ^{19}F imaging and spectroscopy to accurately quantify fibrin content in atherosclerotic plaques (Morawski et al., 2004; Kaneda et al., 2009). Headspace gas chromatography has been employed for detection of trace amounts (of the order of nl/g of tissue) of perfluorocarbon in blood and tissues of premature lambs during liquid ventilation experiments (Shaffer et al., 1996). Headspace chromatography has been recently employed to study the elimination and exhalation kinetics of a perfluorocarbon ultrasound contrast agent administered intravenously to healthy volunteers (Landmark et al., 2008).

In this study headspace gas chromatography confirmed our prior MR data. There was no significant difference in GC measurements between samples of tissues from pancreases stored either with UW alone or with TLM.

No significant difference was found in the PFC content of pancreases stored by TLM and the background concentration of PFC (which would result from environmental contamination) in pancreas stored in University of Wisconsin solution. Both ^{19}F MR and headspace gas chromatography studies demonstrate that perfluorocarbon does not permeate into a large animal pancreas when preserved by TLM even for 24 hours. Preliminary studies with human pancreas using headspace gas chromatography in our laboratory have shown similar results (see **Appendix III**). These findings may explain the observation by Papas et al. that pO_2 level in the core of solid pancreas preserved in TLM is zero and that there is no difference in tissue ATP levels between TLM- and UW-preserved human pancreases (Papas et al., 2005). These results may also explain the findings in chapter 3 of limited benefit with the two-layer method of preservation in human islet transplantation, as observed in a recent meta-analysis (chapter 3). This would suggest that any beneficial effect of PFC in the two-layer method clearly does not depend on PFC penetration. Alternative mechanisms that require further investigation include the possible ability of PFC to act as a sump for CO_2 or other organic molecules.

Perfluorocarbons, in other circumstances, have been shown not to cross biological membranes. When used for liquid ventilation, there is evidence that PFC is partially absorbed across the alveolus. However, even though the blood-gas barrier is only 0.2 – 0.3μm thick (Gehr et al., 1978), only trace amounts of perfluorocarbon (0.001mg/g tissue) were detected in peripheral tissues by gas chromatography (Modell et al., 1973; Calderwood et al., 1975). There is no histologic evidence of presence of residual perfluorocarbon liquid in tissues except for local accumulation of vacuolated macrophages in the lungs and associated lymph nodes. It has been estimated that only approximately 0.5ml of perfluorocarbon escapes into the circulation during standard experimental liquid ventilation in dogs (the endotracheal tube being connected to a reservoir containing 1-1.2 litres of liquid fluorocarbon for one hour), either directly or via macrophages (Holaday et al., 1972; Calderwood et al., 1975).

A number of investigators have observed that when used for liquid ventilation, perfluorochemicals have a beneficial anti-inflammatory action in the alveolar space. In vitro studies have demonstrated that PFC decreases cytokine and reactive oxygen species production from, and attenuates

chemiluminescence of lipopolysaccharide-stimulated rabbit and human alveolar macrophages (Smith et al., 1995; Thomassen et al., 1997). Although, it was speculated in the past that this immunosuppressive property of PFCs might translate into reduced rejection episodes and improved graft survival following islet transplantation (Matsumoto et al., 2002a) on the presumed basis that PFC might permeate the pancreas, none of the trials comparing TLM with conventional preservation has demonstrated such an advantage and recent studies have revealed that there is no significant difference in immunogenicity of islets isolated from fresh pancreas and those preserved in TLM (Toyama et al., 2003).

In summary, we have evaluated by two sensitive methods of PFC detection that there is no evidence of penetration of perfluorocarbon into large animal pancreases preserved by the two-layer method. The hypothesis of a physiological effect mediated by improved gas transfer in a solid pancreas is not plausible. The mechanism of action of the two-layer method remains unclear and its precise role in pancreas preservation for islet transplantation needs to be revisited.

CHAPTER 7

DYNAMIC BIO-ENERGETIC ASSESSMENT OF RAT PANCREAS WITH ^{31}P MRS

7.1 Purpose of study

Animal models have shown that TLM maintains a high level of ATP within the pancreas graft in comparison with UW preservation (Goto et al., 2007; Yoshikawa et al., 2004). However this observation is not universal and recent porcine studies have suggested no benefit of TLM as regards graft ATP levels (Scott et al., 2009), bringing into question the validity of the hypothesized TLM mechanism or the relevance of certain animal models.

In the clinical setting, the benefit of TLM is also controversial. A recent meta-analysis by our group concluded that routine use of TLM engendered no improvement in islet isolation outcomes and post-transplant function (Agrawal et al., 2008). Although small clinical trials have reported a beneficial impact (Tsujimura et al., 2004; Witkowski et al., 2005) most recent large retrospective series comparing human islet isolation and transplantation outcomes between TLM- and UW- preserved pancreases have found no difference in ATP content, islet yield and successful isolations between the two preservation methods (Kin et al., 2006; Caballero-Corbalan et al., 2007).

Several reports have identified donor organ variables that influence islet isolation outcomes (Lakey et al., 1996; Sakuma et al., 2008; Kaddis et al., 2010) but there is currently no means of rapidly and reliably assessing the metabolic health and measuring the extent of preservation injury of stored pancreas in order to predict islet survival and graft success. ATP content is a sensitive marker of cell viability (Sandker et al., 1993; Yoshikawa et al., 2004). Since repair and defense mechanisms are energy consuming (Sandler et al., 1983), ATP level is a good indicator of the graft‟s ability to recuperate following implantation. Although correlation between graft survival and ATP levels has been demonstrated for pancreas (Sandker et al., 1993; Tanioka et al., 1996) and other solid organs in several reports (Kamiike et al., 1988; Pegg et al., 1989; Inci et al., 2001), there has been no attempt to

examine the dynamic real-time changes in ATP metabolism of TLM-preserved pancreas for islet transplantation.

One aim of this study was to develop a clinically relevant experimental model for dynamic assessment of pancreatic energy during preservation using 31-Phosphorus magnetic resonance spectroscopy (^{31}P-MRS). ^{31}P-spectra from viable tissue display 3 prominent ATP peaks originating from the γ-, α-, and β-phosphate groups. ^{31}P-MRS is non-invasive and has been previously applied clinically (Weiner, 1987; Barbiroli et al., 1999; Rico-Sanz et al., 1999) and in animal models (both *in vitro* and non-invasively *in vivo*) to assess ATP levels and, hence, graft viability (Bretan et al., 1989; Fuller et al., 1990; Kanno et al., 1993; van Dobbenburgh et al., 1996; Davidson et al., 1997; Changani et al., 1997 & 1999; Niekisch et al., 2004; Caus et al., 2006) but only to a limited extent to study pancreas preservation (Yoshikawa et al., 2004) and islet tissue viability (Hesse et al., 1994). Real-time assessment of ischaemic damage would identify non-viable pancreases, thus avoiding the expense of processing such tissue for islets. It would also enable appropriate selection of marginal organs for clinical use. A validated model would also allow preservation conditions to be optimized. Therefore a second objective was to use this model to investigate the relative efficacy of different pancreas preservation methods, including the TLM, to maintain viable pancreatic bioenergetics, and to establish optimal protocols for their use in clinical islet transplantation. We investigated both immediate cold preservation and the experimental equivalent of the non-heart beating donor situation in which pancreases were subjected to a prior period of warm ischaemia. The latter model was studied.

7.2 METHODS (See Sections 4.2.1, 4.3.1, 4.4, 4.8 and 4.9.4 for details)

In the "static" TLM arm, perfluorodecalin was saturated with oxygen by bubbling this gas through at 0.5 L/hr for 30 min (this was done just before preservation). For "continuous" TLM oxygen was

bubbled (0.5 L/hr) through the perfluorodecalin for 20 min once every hour throughout each experiment.

Five different preservation groups were compared. ^{31}P MRS spectra were acquired from rat pancreases preserved immediately in Marshall"s solution, static TLM or continuous TLM. Spectra were also acquired from pancreases preserved in static or continuous TLM after 30 min warm ischaemia. For the three immediate cold preservation groups, 38 pancreases were procured from male Sprague-Dawley rats to give 19 experimental preparations (2 pancreases in each) which were then randomly assigned amongst 3 preservation groups maintained at 4°C to 6°C. For the two warm ischaemia group, pancreases were procured from male Sprague-Dawley rats (n=26) in 13 separate experiments. Spectrum signal amplitudes were measured for phospho-mono-esters (PME), inorganic phosphate (Pi) and α-, β- and γ -ATP.

In a separate series of experiments rat islet isolations were performed after pancreas procurement from Sprague-Dawley rats - in the fresh state (serving as controls), and after 3-hour preservation in chilled UW solution or in the TLM (n=5 in each group). Mean islet yield from each group was compared.

7.3 RESULTS

For clarity results have been divided into two sections: immediate cold preservation groups (Marshall"s or control, static TLM and continuous TLM) and warm ischaemia groups (static TLM post-warm ischaemia and continuous TLM post-warm ischaemia)

Immediate cold preservation groups

- There was no significant difference in the time (mean (SD)) between pancreas procurement and first MRS for these 3 preservation groups: Marshall‟s 0.69 (0.06) hr, continuous TLM 0.72 (0.05) hr, static TLM group 0.74 (0.11) hr, (p=0.62, ANOVA).
- Preservation durations based on the time of last MRS were similar in each group: Marshall's 3.08 (0.35) hr, continuous TLM 3.08 (0.16) hr, and static TLM 3.00 (0.27) hr.
- At first MRS after pancreas procurement there were no intergroup differences in [γ-ATP]/[Pi]: Marshall‟s 0.21 (0.07), continuous TLM 0.28 (0.05), static TLM 0.24 (0.09) (p=0.26, ANOVA) or [β-ATP]/ [Pi] (Marshall‟s 0.10 (0.04), continuous 2-layer 0.18 (0.07), static 2-layer 0.15 (0.06)) (p=0.12, ANOVA).
- Plots of [γ-ATP]/[Pi] and of [β-ATP]/[Pi] against time from pancreas procurement for the three immediate cold preservation groups are shown in Figures 7.1a and 7.1b respectively. Linear regression residuals were normally distributed with constant variance confirming that linearity was an appropriate fitting model. Table 7.1 gives rates of change of [γ-ATP]/[Pi] and of [β-ATP]/[Pi]. There were significantly different intergroup rates of change for both [γ-ATP]/[Pi] ($p < 0.001$, ANOVA) and [β-ATP]/[Pi] ($p<0.001$, ANOVA). [γ-ATP]/[Pi] and [β-ATP]/[Pi] decreased in pancreases preserved in Marshall‟s solution compared to continuous TLM (both $p < 0.001$, t test) but not compared to static TLM ($p =0.21$ and $p = 0.22$ respectively, t test). For static TLM [γ-ATP]/[Pi] and [β-ATP]/[Pi] decreased compared to these ratios with continuous TLM ($p < 0.001$ and $p < 0.05$ respectively, t test). Representative spectra from pancreases preserved in Marshall‟s solution and in continuous TLM are shown in Figure 7.2.

Warm ischaemia groups

- There was no significant intergroup difference in the time between pancreas procurement and first MRS: continuous TLM 0.79 (0.25) hr and static TLM 0.79 (0.15) hr, (p=0.97, t test).
- Preservation durations based on the time of last MRS were similar: WI continuous TLM 6.03 (0.41) hr and WI static TLM 6.18 (0.45) hr.
- At first MRS after pancreas procurement there were no significant intergroup differences in [γ-ATP]/[Pi] (WI continuous TLM 0.17 (0.03); WI static TLM 0.21 (0.04), p=0.11, t test) or [β-ATP]/[Pi] (WI continuous TLM 0.10 (0.04); WI static TLM 0.13 (0.03), p=0.07, t test).
- Plots of [γ-ATP]/[Pi] and [β-ATP]/[Pi] against time from procurement are shown in Figures 7.3a and 7.3b respectively. Table 7.2 gives the rates of change of [γ-ATP]/[Pi] and of [β-ATP]/[Pi] derived by linear regression: both ratios decreased during static TLM (both $p < 0.001$, t test vs. continuous TLM) but baseline levels were maintained during continuous TLM.

	Rate of change hr^{-1}	
	[γ-ATP]/[Pi]	[β-ATP]/[Pi]
Marshall's	-0.049 (0.025)	-0.036 (0.019)
Continuous 2-layer	0.043 (0.033) * #	0.029 (0.029) ##
Static 2-layer	-0.023 (0.016)	-0.015 (0.026)

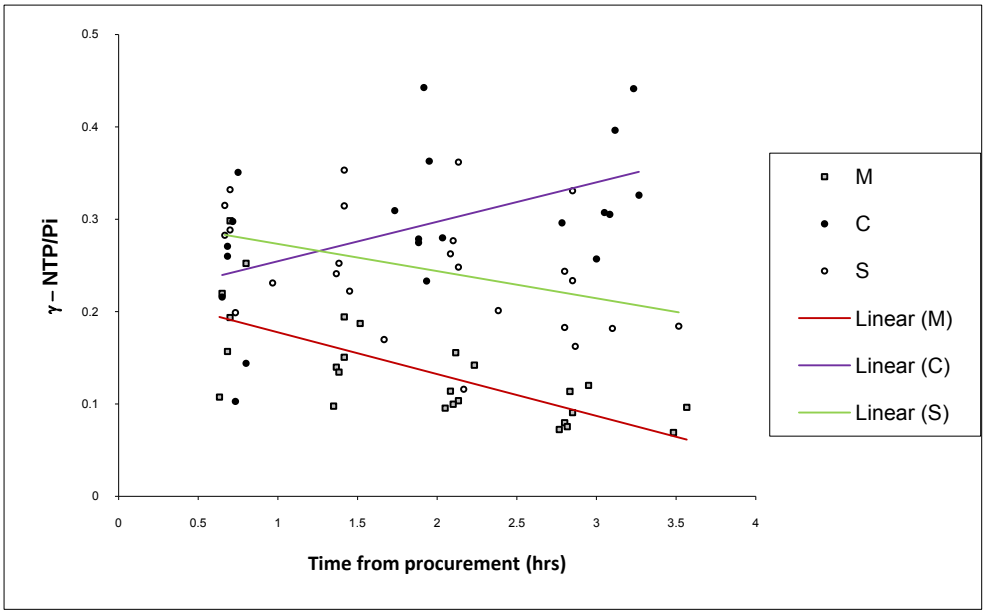
* $p < 0.001$ compared to Marshall"s (t-test)

# $p < 0.001$ compared to static 2-layer (t-test)

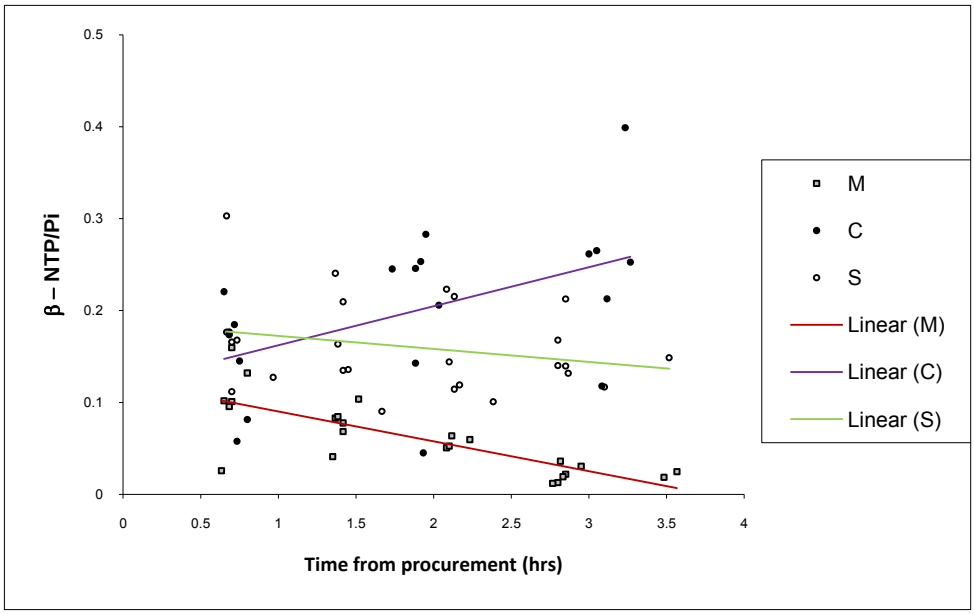
$p < 0.05$ compared to static 2-layer (t-test)

Table 7.1

Rates of change of [γ-ATP]/[Pi] and [β-ATP]/[Pi] during the immediate cold preservation study. Using standard preservation (Marshall"s) the [ATP/Pi] ratios progressively reduced during the preservation period. This reduction was less with static TLM whereas there was a slight increase (significant) in the ratios during continuous TLM preservation.



(a)



(b)

Figure 7.1

Immediate cold preservation groups: **a)** [γ-ATP]/[Pi] and **b)** [β-ATP]/[Pi] against time (in hours) from pancreas procurement. Both [γ-ATP]/[Pi] and [β-ATP]/[Pi] increased during continuous TLM preservation (C) whereas for static TLM (S) there was no change and for Marshall's preservation (M) these ratios declined.

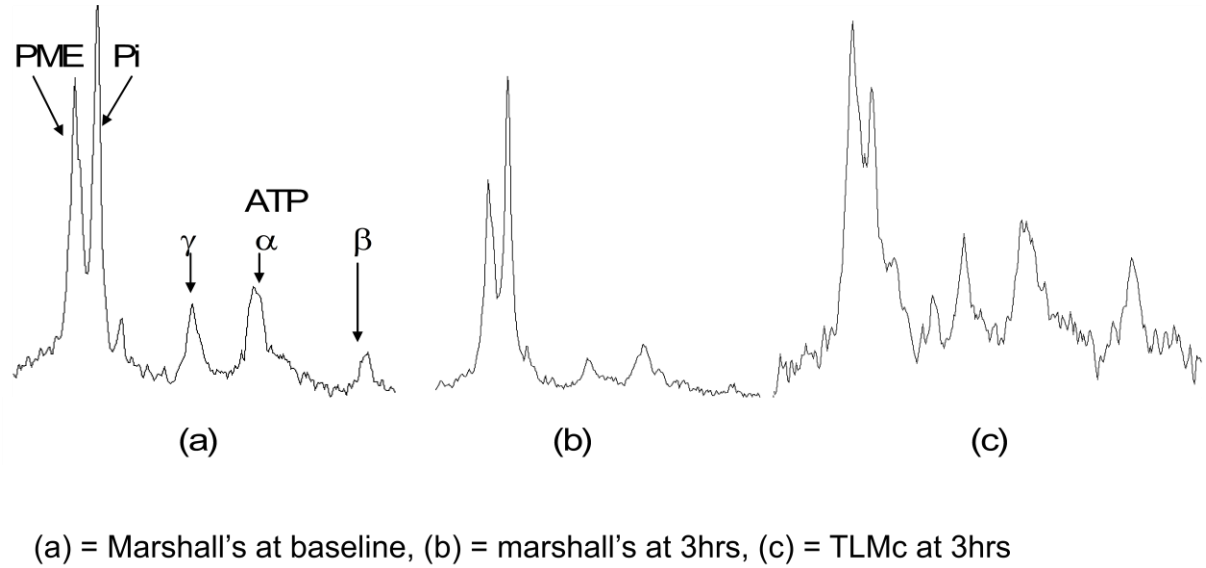


Figure 7.2

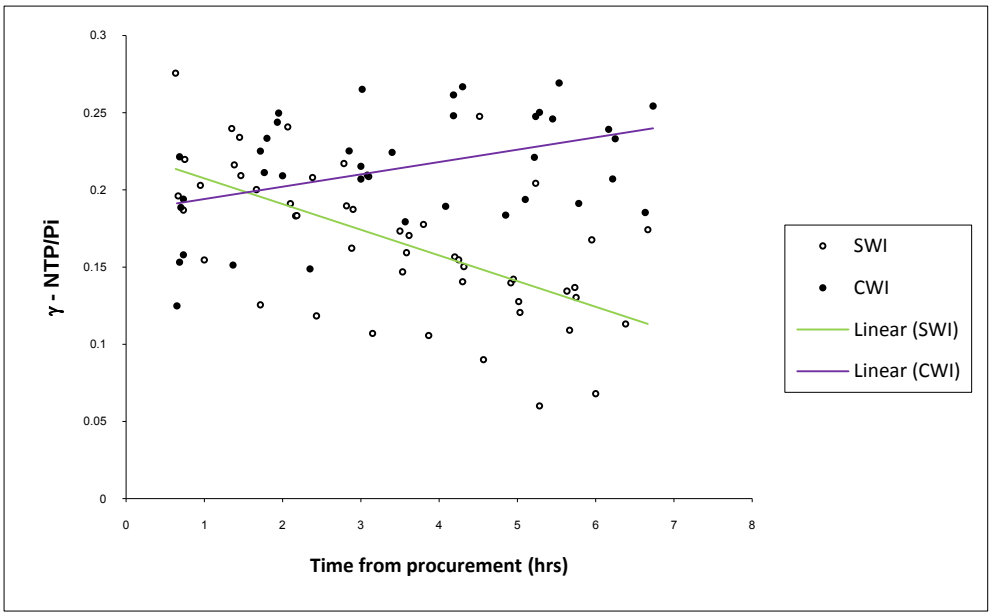
Example ^{31}P spectra from pancreases preserved in Marshall"s taken at **a)** baseline and **b)** 3 hr post-procurement and **c)** preserved with continuous TLM 3 hr post-procurement. The signal to noise ratio is reduced in c) because the pancreases were at the interface of the two layers thus further from the surface coil. [ATP]/[Pi] decreased Marshall"s preservation but increased during continuous TLM.

	Rate of change hr^{-1}	
	[γ-ATP]/[Pi]	[β-ATP]/[Pi]
Continuous 2-layer	0.008 (0.009) #	0.007 (0.008) #
Static 2-layer	-0.018 (0.008)	-0.014 (0.004)

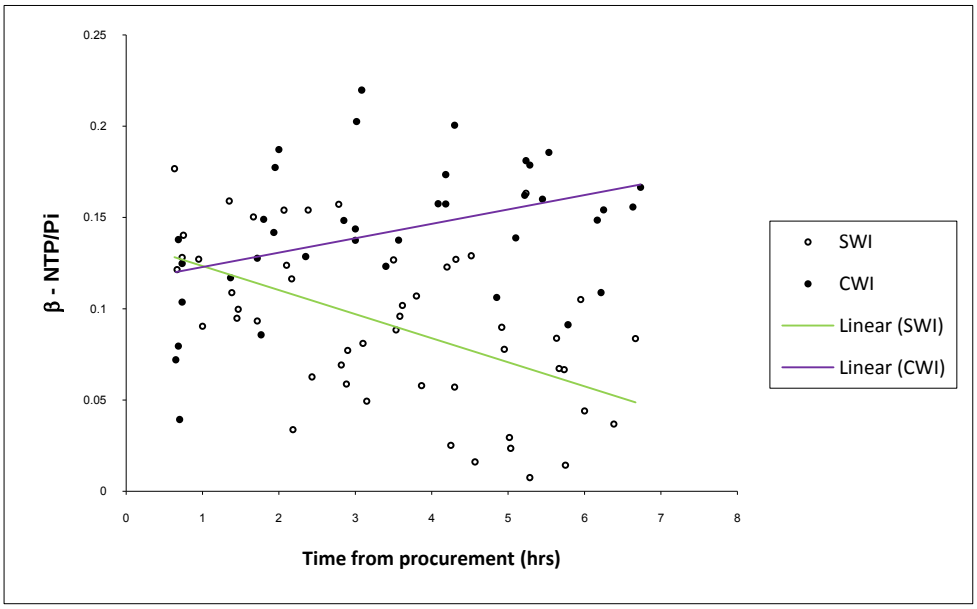
# $p < 0.001$ compared to static 2-layer (t-test)

Table 7.2

Rates of change of [γ-ATP]/[Pi] and [β-ATP]/[Pi] during the warm ischaemia study. Following warm ischaemia preservation with static TLM results in reduction of [ATP/Pi] ratios progressively, whereas with continuous TLM preservation there is slight (significant) increase in the ratios.



(a)



(b)

Figure 7.3

Warm ischaemia groups: **a)** [γ-ATP]/[Pi] against time (in hours) from procurement, **b)** [β-ATP]/[Pi]against time from procurement. Both [γ-ATP]/[Pi] and [β-ATP]/[Pi] increased with continuous TLM preservation throughout the period of observation. Key: SWI = static TLM warm ischaemia, CWI = continuous TLM warm ischaemia, linear = linear regression

Rat islet isolation

Results of islet isolation from the three pancreas preservation groups are shown in **appendix V**. Mean islet yield was 423, 57 and 63 from fresh, UW- and TLM-preserved pancreases respectively. Single factor ANOVA showed a highly significant difference between the experimental and control groups but no significant difference between the two experimental arms.

7.4 Discussion

These results suggest that continuous TLM at 4^{o}C to 6^{o}C achieves the goal of hypothermic preservation of pancreas while maintaining the organ‟s capacity to generate ATP – an essential pre-condition for graft viability in the rat model.

Ischaemia and hypoxia are considered the two most detrimental factors influencing pancreatic islet isolation outcomes (Carlsson et al., 2002; Linn et al., 2006). Current preservation techniques for whole pancreases appear inadequate for the purpose of islet isolation and transplantation: this is possibly due to additional stresses imposed on the islets during pancreatic digestion, isolation, purification and culture. Preservation inadequacy is highlighted by only 50% of isolations yielding successful outcomes despite standardized protocols (Ponte et al., 2007).

The viability of transplanted grafts is fundamentally dependent on the capability of the cell to restore active energy metabolism and to regenerate ATP swiftly (Calman, 1974). There is currently no rapid yet accurate method of assessing donor pancreas viability applicable between pancreas procurement and processing for islets. Several islet isolation studies have demonstrated a correlation between pancreatic ATP levels islet recovery and functional viability (Tsujimura et al., 2004; Salehi et al., 2006). A number of studies on other organs have established strong correlations between viability and

MRS measures of bioenergetics. A recent report concluded that intra-islet ATP content predicted engraftment outcome with a sensitivity and specificity of 83.3% and 73.3% respectively (Kim et al., 2009). This suggests that measurement of pancreatic ATP levels prior to islet isolation may similarly correlate with islet isolation outcome. In the present study we used ^{31}P-MRS to measure changes in pancreatic ATP under different preservation conditions.

When the pancreas is made ischaemic, Pi signal increases whereas ATP signals decrease; ATP reaches a measurable minimum 15-20 min after ischaemia onset (Siech et al., 1995).

Previously, Pi/γ-ATP and Pi/β-ATP have been used as measures of graft viability in TLM cold storage (Yoshikawa et al., 2004): successfully transplanted canine whole-organ grafts had lower Pi/ATP than non-viable pancreases suggesting that this ratio reflected the extent of graft damage. Analyzing the "receiver operating characteristic" (ROC) curves for these ratios between viable and non-viable groups, Yoshikawa et al observed that with optimum Pi/γ-ATP and Pi/β-ATP cut-off levels, the predictive accuracy was 83% for each ratio (respectively, sensitivity 75% and 75%, and specificity 100% and 90%). In the present study γ-ATP/Pi and β-ATP/Pi were measured and their temporal changes considered linear during cold preservation; linear regression residuals were tested for normality and constant variance confirming that linearity was an appropriate model. The linear-regression slopes were used as measures of the decline in pancreas viability. [γ-ATP]/[Pi] and [β-ATP]/[Pi] are not wholly independent variables – an increase in [Pi] might lead to declines in both ratios. Therefore Bonferoni corrections were not applied to the statistical p-values resulting from testing for intergroup differences [γ-ATP]/[Pi] and [β-ATP]/[Pi].

In the present study, temporal changes in pancreatic [γ-ATP]/[Pi] and [β-ATP]/[Pi] depended on the cold preservation method. With the continuous TLM, these ratios increased during preservation (Figure 1 and Table 1) suggesting that this method not only prevents pancreatic ATP depletion but actually increases energy levels. The ATP/Pi increases imply that (most likely by providing sufficient oxygen) continuous TLM stimulates oxidative phosphorylation, restores ATP concentration, and limits organ damage without the complexity, expense, and potential deleterious effects of continuous

perfusion. Contrastingly, in both cold Marshall‟s solution and the static TLM [γ-ATP]/[Pi] and [β-ATP]/[Pi] decreased monotonically suggesting progressive energy depletion during preservation.

With increasing experience, expanded acceptability criteria pancreases are being used more and more for clinical islet transplantation, including those from non-heart-beating donors. In our meta-analysis on the effect of TLM on human pancreatic islet isolation we observed a beneficial effect of the TLM in marginal pancreases (chapter 3). In addition to cold preservation we also investigated the ability of TLM to maintain viability of rat pancreas subjected to a prior period of warm ischaemia in the present study: this would be analogous to the situation of pancreas procurement from a deceased cardiac donor.

After 30 min warm ischaemia, pancreatic ATP/Pi ratios decreased in the static TLM but maintained their baseline levels with the continuous TLM over the entire preservation period.

The prolonged oxygenation in the continuous TLM group effectively prevented mitochondrial dysfunction, and was more efficient compared to the pre-oxygenated static TLM. Our observations suggest that pancreases from non-heart-beating donors and other marginal situations could derive significant benefit from continuous TLM preservation. As tissue metabolism is temperature-dependent, organs lose high energy phosphates (e.g. ATP) rapidly during warm ischaemia. ATP depletion continues during subsequent static-TLM preservation as oxygen deprivation engenders reliance on energy generation via anaerobic glycolysis (an inefficient ATP generator). However, provided mitochondria remain viable, such ATP depletion can be reversed by continuous-TLM aerobic preservation thereby restoring energy status.

A correlation between PME/Pi decay during cold ischaemia and graft function has been observed in kidney transplantation (Niekisch et al., 2004). However, ^{31}P MRS studies on pancreas are few and no such correlation with graft performance has been documented. It has been observed that hypoxia in perfused rat pancreas increases [Pi] accompanied by a smaller [PME] increase (Matsumoto et al., 1988). In our study [PME]/[Pi] was independent of preservation method and this was probably due to

the levels of both these metabolites increasing during preservation (whereas [ATP] and [Pi] change reciprocally).

Reversal of ischaemic mitochondrial dysfunction and resumption of the cellular capacity for ATP synthesis is critical for successful graft outcome. Our results suggest that the TLM with continuous PFC oxygenation restores mitochondrial function best and should be the preferred pancreatic preservation method for islet transplantation. However, limitations of using animal models must be recognized including differences in organ size and morphology, duration of preservation, and stress levels prior to procurement (unlike human donors, animals in this study were not subjected to any physiological stress before donation). This study should therefore be validated in large animal (pig) model and/or discarded human pancreases.

So far, human studies on the TLM mechanism have compared ATP content of pancreatic biopsies taken at the end of the preservation period with conflicting outcomes. Also conventional biochemical determination of [ATP] is not considered a clinically suitable test of transplant organ viability because it requires biopsy and is time-consuming. Our experimental method could measure real-time ATP changes in human pancreas, under different preservation conditions, in order to establish whether perfluorocarbons improve clinical organ viability and optimal preservation methodology. Our ^{31}P-MRS method is a practical way to assess mitochondrial dysfunction in pancreas non-invasively and expeditiously.

To our knowledge, ours is the first ^{31}P-MRS study examining dynamic changes in pancreatic bioenergetic profile during cold preservation with both static and continuous TLM. The present study has shown that continuous oxygenation of the preservation medium effectively maintains pancreatic oxidative phosphorylation capacity and this is superior to static PFC oxygenation in the TLM. One recent ^{31}P-MRS study has shown a similar benefit in rat pancreas from intermittent re-oxygenation of PFC in rat pancreas (Scott et al, 2009). Interestingly, the authors did not detect any discernible ATP peaks in TLM-preserved porcine pancreas. However in the absence of sample numbers and numerical data it is not possible to draw any definite conclusions from this study.

In clinical TLM application, pre-oxygenated PFC is commonly used for logistic reasons. This practice is also based on the prevailing notion from experimentation (Matsumoto, et al., 2002b) that there is no significant difference in pancreas preservation efficiency between pre-oxygenated PFC and continuous oxygenation. Our findings suggest that this is not the case and lend further support to recent concerns regarding the effectiveness of the static TLM in clinical pancreas preservation for islet isolation (Agrawal et al., 2008). It seems likely that appropriate TLM application should involve continuous oxygenation of the PFC layer thus preventing ATP depletion and ensuring aerobic preservation of the pancreas. Based on our results, this would be a valid hypothesis for a subsequent study in humans or large animal models.

One limitation of this study is the lack of correlation with islet isolation outcomes. In a separate series of experiments we observed that islet yield from both UW- and TLM-preserved rat pancreas (after 3 hours preservation) drops to approximately 15% of that from freshly procured pancreas (**appendix V**). A number of investigators have documented the difficulty of islet recovery from rat pancreas subjected to cold storage, especially without preliminary distension of the pancreatic duct with enzyme or other additives (Kneteman et al., 1990; Ohzato et al., 1990). Such an intervention would introduce extrinsic variables in our experimental protocol designed primarily to compare the efficacy of different cold storage solutions in restoring pancreatic bioenergetics. In order to correlate pancreatic bioenergetic changes after preservation with islet isolation outcomes, this study must be repeated in large animal pancreases using our model to measure real-time changes in ATP metabolism. The value of ^{31}P-MRS for islet viability assessment can also be confirmed by repeating this study on procured research human pancreases using reversal of diabetes following islet transplantation in nude mice as endpoint.

An interesting observation in our study relates to the use of Marshall‟s solution instead of UW preservation medium in the TLM in order to avoid contamination of ^{31}P spectra by signal from phosphate in the latter. ATP regeneration was observed with the TLM despite the lack of exogenous adenosine in Marshall's solution. Adenosine has previously been considered an essential component of UW solution, particularly with respect to preservation of ischaemically damaged pancreas for islet

isolation. This observation is important because adenosine (along with several other components of UW solution) has shown inhibitory action on collagenase activity during pancreas digestion for islet isolation (Contractor et al., 1995). Removing adenosine from UW solution may improve islet preparations without compromising energy metabolism during preservation although further studies are required before definite conclusions can be made.

CHAPTER 8

DISCUSSION

A renewed impetus was provided to clinical islet transplantation following the success of the Edmonton protocol in achieving 100% insulin independence in 2000 (Shapiro, 2000). This success however, has been tempered by the observation that sustained freedom from exogenous insulin is rarely achieved (rate of insulin independence drops to 10% at 5 years follow-up [Ryan, 2005]). One critical determinant of successful islet allografting is preservation injury to the pancreas (Agrawal 2008).

The two-layer method (TLM) of pancreas preservation was developed to mitigate the deleterious effect of cold ischaemia. A number of studies in small and large animal models have shown improved islet isolation results in pancreas preserved by this technique. Investigators have observed a correlation between increased islet yield and tissue ATP content in TLM-preserved pancreas and hypothesized that the perfluorocarbon (PFC) in TLM acts as an oxygen reservoir allowing aerobic preservation during cold storage. Large-scale retrospective analyses of islet transplantation however have failed to confirm this beneficial impact in humans suggesting that the two-layer method as currently applied to clinical islet transplantation is of no value (Agrawal, 2008).

One crucial observation that is relevant to the clinical application of TLM was the lack of PFC penetration into solid primate pancreas. Using ^{19}F magnetic resonance imaging and spectroscopy, and headspace gas chromatography, we found no evidence of PFC infiltration into porcine and human pancreas preserved by TLM.

In order to further clarify the mechanism of the two-layer method, we employed ^{31}P magnetic resonance imaging to measure real-time bioenergetic changes in rat pancreas during cold preservation. We found that ATP/Pi ratios in rat pancreas fell progressively during conventional cold storage in Marshall‟s solution. The ratios were however, maintained in TLM-preserved pancreases throughout the duration of preservation (mean 3 hours). Similar changes in bioenergetics were observed when the

pancreases were subjected to preliminary warm ischaemia to simulate the deceased cardiac donor situation. However this beneficial effect of TLM was only observed when the perfluorocarbon was continuously oxygenated from an external supply. TLM with static pre-oxygenation of PFC, as currently practised, did not maintain the pancreatic bioenergetics and was equivalent to preservation in Marshall‟s solution. This is the first study to demonstrate superiority of continuous TLM and may explain the lack of benefit observed with conventional TLM for human pancreas. This is also the first study to demonstrate that adenosine supplementation in preservation fluids is not necessary.

Taken together, these results suggest that current application of TLM in humans is suboptimal and improved methods of preservation with perfluorocarbons, such as continuous TLM and perfusion with perfluorocarbon emulsion, must be explored.

8.1 TLM META-ANALYSIS

This meta-analysis of the clinical impact of the two-layer method found no clear evidence that the TLM is beneficial in human islet transplantation.

Study limitations:

Only three of the eleven studies included in this review were randomized controlled comparisons, corresponding to 7.7% (48 of 622) of the pooled sample size. As even the pooled sample size in the sub-groups was relatively small, the possibility of a beta error for some of the outcome measures cannot be excluded (optimal information size for the primary outcome measure of the proportion of successful islet preparations for 80% statistical power with an alpha level of 0.05 was estimated to be 480 organs in each group) and the possibility of bias in retrospective studies cannot be excluded. The

donor variables (age, gender, BMI and pancreas weight) were not significantly different between the groups with a single exception. In the randomized trial sub-group the pancreas weight was significantly higher in the UW arm. The significance of this observation is unclear and definite conclusions cannot be drawn as this difference was not significant in the individual studies and the sample size was small (n=48).

Future studies:

A large randomized controlled multi-centre study is required to further clarify the role of the two-layer method in the clinical setting of islet transplantation. The mechanism of any beneficial effect requires to be established.

The conclusion from this meta-analysis leads to a dilemma: given their great clinical potential as a „blood substitute" and as „oxygen therapeutic", it remains to be explained why perfluorocarbons should have little effect on organ preservation. Moreover how can we explain the discordant observation by a few investigators that TLM supports pancreatic bioenergetics and maintains tissue ATP content?

8.2 PFC UPTAKE IN TLM-PRESERVED PANCREAS

Direct diffusion of oxygen in a large pancreas is an extremely slow process. Improved oxygen transfer with perfluorocarbon would suggest that the perfluorocarbon itself penetrates the organ (Matsumoto et al., 1996). The hypothesis that PFC penetrates the pancreas in order to facilitate oxygenation is based on previous observations in paediatric patients with adult respiratory distress syndrome. Ventilation with perfluorocarbon is associated with absorption across the alveolus and subsequent elimination by evaporation (Matsumoto et al., 1996, Gauger et al., 1996). To date measurement of

perfluorocarbon uptake in pancreas preserved by the two-layer method has not been attempted. The purpose of this study was to quantify and examine the pattern of distribution of perfluorocarbon within adult porcine pancreas preserved by the two-layer method.

In the present study no evidence of PFC uptake was found with two different analytic methods (^{19}F MRS and headspace gas chromatography).This finding is not surprising, given that PFCs are biologically inert and immiscible with blood and biological fluids. Indeed, the original hypothesis of PFC penetration into the pancreas is based on little scientific evidence. From clinical experience with PFCs as blood substitutes, we know that there is no chemical interaction in PFC gas transport. Following reticuloendothelial uptake, PFCs are excreted un-metabolized by exhalation. Any anaphylactoid reaction from their clinical use has been attributed to the surfactants used in emulsifying PFCs. In fact production of stable perfluorocarbon emulsions with an acceptable shelf-life has been a considerable challenge.

Given that PFC does not infiltrate primate pancreas preserved in TLM, it follows that aerobic solid organ preservation with TLM, as currently practiced, will be ineffectual. A recent oxygen tension study in porcine pancreas corroborates this conclusion (Papas et al., 2005).

To my knowledge, there are only two reports of direct measurements of oxygen partial pressure in solid pancreases preserved by TLM. Matsumoto et al. (1996) used Clark oxygen electrodes to measure tissue oxygenation in the core of canine pancreas preserved in TLM. They observed that even under warm ischaemic conditions, when preserved by TLM at 20°C oxygenation at the core of the pancreas improved rapidly, reached a level of 15 – 17 mmHg within 30 minutes and was maintained at this level. In the control arm where pancreas was preserved in oxygenated UW solution at 20°C, tissue pO_2 and ATP levels continued to decrease progressively with time. They concluded that TLM could restore the viability of pancreas damaged by warm ischaemia. The authors speculate that PFC might permeate the organ to facilitate core oxygenation. This observation was challenged in a more recent study by Papas and colleagues (2005). They used a homogeneous diffusion reaction model (in the absence of pO_2 gradients in preservation media equilibrated with pure oxygen i.e. pO_2 =

760 mmHg at medium / organ interface) for theoretical calculations of pO_2 profiles of a cylindrical pancreas and found that the predicted depth of oxygen penetration was just 1mm under cold storage conditions and that the oxygenated volume fraction dropped further as the temperature was raised. They confirmed these results by experimental measurements of pO_2 with fiber optic sensors placed in the core of 1cm thick piece of porcine pancreas. Within 12 minutes of insertion of the probe, the pO_2 in the pancreatic core dropped to zero. The authors concluded that primate pancreas remains largely anoxic during TLM preservation. Our findings validate this conclusion.

Our results also substantiate recent reports of lack of benefit of TLM preservation in clinical islet transplantation. In human islet isolations, Kin et al. (2006) reported no significant increase in ATP content of the pancreases that were preserved by the two-layer method, calling into question the validity of the hypothesis that the two-layer method efficiently delivers oxygen to the pancreas. More recently another retrospective analysis of 200 human islet isolations arrived at the same conclusion (Caballero-Corbalan et al., 2007).

Study Limitations:

The major limitation of this study is the lack of correlation with tissue oxygenation or utilization. Tissue oxygen content is difficult to measure reliably. Current methods of direct pO_2 measurements appear to be highly dependent on the type of probe used, the experimental conditions (temperature) and the possibility of contamination of results due to seepage of PFC around the probes and are therefore unreliable. Moreover as oxygen is being continuously metabolized in viable tissue, actual tissue pO_2 level may not correlate with oxygen availability and utilization. Relative levels of energy substrates and metabolites are more reliable measures of oxygen utilization and indirectly of tissue oxygenation. Another major limitation of oxygen electrodes is the extremely limited area of measurement, with penetration depths of approximately 15μm (Koch et al., 2001). Since tissue oxygenation is heterogeneous, pO_2 levels in the immediate vicinity of the electrode will not reflect true oxygen tension of the organ.

Future studies:

In order to ensure maximum benefit from oxygenated PFC, it should be used as an emulsion to perfuse pig or human pancreas. The pattern of PFC uptake can then be studied by performing ^{1}H and ^{19}F magnetic resonance imaging synchronously on perfused pancreas. ^{19}F MR oximetry can be simultaneously performed to obtain accurate measurement of tissue pO_2 (as a novel application of this technique). These studies should be performed in real-time with varying temperatures (i.e. both cold and warm preservation) to identify the optimal preservation conditions for PFC.

8.3 BIOENERGETICS OF TLM PRESERVATION

Existing reports on the bioenergetic benefits of TLM organ preservation are contradictory. There are two possible explanations for this: first, animal and human studies on the TLM mechanism have compared enzymatic ATP content of frozen pancreatic biopsies taken at the end of the preservation with conflicting outcomes. Inference from these reports of the dynamic effects of perfluorocarbons on energy metabolism is therefore not scientifically sound. Secondly, the current clinical application of TLM organ preservation is unscientific and may not be optimal for exploitation of the unique oxygen delivering properties of perfluorocarbons.

In order to ascertain the true nature of interaction between perfluorocarbon and mitochondrial activity during hypothermic preservation, we developed an experimental model for dynamic assessment of pancreatic energy during preservation using 31-Phosphorus magnetic resonance spectroscopy (^{31}P-MRS). ^{31}P MRS can be used to study high-energy phosphate metabolites to provide in vitro and in vivo assessment of the bio-energetic status of tissues.

In the rat model we found that TLM supports bioenergetics only when PFC is continuously oxygenated. Pre-oxygenation of PFC was insufficient to maintain tissue ATP levels during hypothermic preservation. This observation is crucial: rat pancreas is only a few millimeters in thickness and simple diffusion can, at least in theory, oxygenate 60 – 100% of the pancreatic volume at 4°C (Agvoustiniatos et al., 2006). The observation that static TLM fails to maintain adequate ATP levels implies that preoxygenated PFC is inefficient in oxygen delivery (assuming that the continuing depletion of ATP is the result of lack of oxygenation – see section 8.3.1). Continuous oxygenation of PFC, on the other hand, preserves mitochondrial function effectively – even after a warm ischaemic insult. Thus a hyperbaric oxygen environment appears to be essential for adequate PFC-mediated gas transfer during TLM.

8.3.1 Why is continuous TLM more effective than static TLM?

Static TLM is currently accepted as the standard method of aerobic preservation with perfluorocarbon and this is based on the belief that continuous and static TLM are equally effective. The rationale underlying this concept is discussed in chapter 2 (section 2.6.3).

However, the dramatic results obtained with research human pancreases have never been replicated in the clinical situation (Agrawal et al., 2008). All large retrospective clinical series have found no beneficial effect of static TLM. More recently, a multicentre database (Islet Cell Resource Centre Consortium) analysis of 806 human islet isolation outcomes found no difference between UW and TLM preservation (Kaddis et al., 2009).

Our results from ^{31}P MRS study suggest that this discrepancy is the result of incorrect application of the two-layer method and that the beneficial effect of static TLM in previous experimental reports is likely to be a type 2 error. A careful scrutiny of scientific literature on perfluorochemicals suggests that the assumptions on which the static TLM is based are fundamentally flawed, and a result of an

incomplete understanding of the oxygen transport characteristics of perfluorocarbons and of the crucial mechanistic differences between these liquids and haemoglobin.

In blood, oxygen is chemically coordinated to the four iron atoms in hemoglobin resulting in the characteristic sigmoid oxygen uptake curve which rapidly levels off to a straight line. In contrast, the volume of oxygen dissolved in PFC increases linearly according to Henry‟s law.

Since PFCs dissolve oxygen physically and there is no binding function (in contrast with haemoglobin), the amount of oxygen that can be carried at any given temperature is directly proportional to the oxygen partial pressure and oxygen transport is governed by diffusion only (rather than being dependent on changes in oxygen affinity of hemoglobin, as in blood). A corollary to this is that a high partial pressure gradient is required to dissolve a large quantity of oxygen in the PFCs. In addition, similar gradients are necessary between PFC and peripheral tissues in order to achieve a biologically useful degree of oxygen unloading. Thus supplementary oxygen is a prerequisite for any beneficial PFC effect to be realized. In other words, an elevated partial pressure is required to maximize the oxygen transport capacity of perfluorocarbon emulsions. Additionally, another crucial difference is that the release of oxygen from PFC is considerably simpler compared to haemoglobin, where the gas must cross the red cell membrane, pass through plasma and endothelial cells (Spiess, 2009).

The fundamental difference in oxygen transport characteristics between PFC and haemoglobin can be illustrated by the following example:

Under physiological conditions when breathing air, the pO_2 gradient between the alveoli and peripheral tissues is approximately 60 mm Hg. Normal blood (Hb 15 g/L, packed cell volume 45%) releases ca. 5 ml O_2 per 100 ml, representing an extraction ratio of 25%. In contrast, the corresponding value for a 60% (w/v) perflubron emulsion under ambient pO_2 would be only approx. 2 ml O_2 per 100 ml. However, if pure oxygen (pO_2 100%) were breathed, arterial pO_2 would be raised 500 mm Hg and approx. 10 ml O_2 per 100 ml would be released giving an extraction ratio of 90% (Faithfull, 1992 and 1994). Thus, breathing oxygen-enriched air can increase oxygen saturation of

PFC in a linear fashion and enhance tissue oxygenation when perfluorocarbon emulsions are employed. This is in sharp contrast to the physiological situation where further increases in inspired pO_2 can have little effect on oxygen saturation of haemoglobin (or on the maximal amount of oxygen carried by blood).

Stated differently, when equilibrated with 100% O_2 (pO_2 = 760 mmHg) PFCs carry about the same amount of oxygen as equal volume of packed erythrocytes (the major difference being that in the former most of the O_2 is physically dissolved and thus easily available). However when equilibrated with air or alveolar gas (pO_2 = 100 – 150 mmHg), oxygen content of PFCs is only 15-20% of that of an equal volume of RBCs (Sloviter, 1985). It is thus necessary for subjects to inspire gas containing at least 60% oxygen to enable PFC to substitute adequately for erythrocytes. In an elegant experiment Sloviter and Kamimoto (1967) were able to successfully preserve isolated rat brains by perfusing them with perfluorocarbon emulsions – but only when the perfusates were continuously oxygenated with 95% oxygen: 5% carbon dioxide.

Experimental and clinical evidence indicates that PFCs work optimally only in the presence of high O_2 partial pressures. A recent study found that efficacy of perfluorocarbons in oxygen delivery was reduced at high altitude where partial pressure of oxygen is lower (Gardeazabal et al., 2008). Clinical studies with intravenous perfluorocarbon emulsions have also observed that these chemicals cannot dissolve sufficient oxygen under ambient inspired air conditions. Efficacy of the emulsion is noted only when patient breathes an oxygen-enriched atmosphere (Lane, 1995; Keipert, 1995 and 1998; Goodnough et al., 1998; Spahn et al., 1999; Spahn et al., 2002; Kocian & Spahn, 2008) and these studies have proven that higher FiO_2 is a prerequisite for better oxygen transport. Phase 3 clinical trials with Oxygent in major elective non-cardiac (Spahn et al., 2002) and cardiac surgery (Verdin-Vasquez et al., 2006) have shown significant benefit with reduction in allogeneic blood requirements. FiO_2 in all these trials was increased to 100%.

From these observations, it follows that an appropriate protocol for aerobic preservation must ensure continuous oxygenation of the PFC layer. Our ^{31}P MRS study in rat pancreas provides empirical evidence for this fundamental principle.

Study Limitations:

There are several limitations in the study.

1. Given that MRS signal was obtained from whole pancreas, applicability of this data to islets may not be entirely appropriate.
2. ^{31}P MRS study on rat pancreatic bioenergetics is not correlated with islet viability or post-transplant outcome.
3. Lack of validation of ^{31}P MRS rat pancreas model with mammalian or human pancreas.

The phosphorus signal obtained from a whole pancreas is not strictly representative of the bioenergetics of the endocrine portion of the gland. There were two main reasons why whole pancreas rather than islets were used in this study:

a) Due to the very nature of the procedure, islets are subjected to various physical and chemical damaging processes during islet isolation and purification (see section 1.4.1). MRS study of isolated islets would represent the cumulative effect of preservation and isolation / purification injuries. The only way in which preservation injury can be studied in isolation is by interrogating the whole pancreas prior to the isolation process.
b) There is clinical and experimental evidence that in comparison with its endocrine counterpart, exocrine pancreas is significantly more susceptible to perfusion injury. In the setting of clinical whole pancreas transplantation, while endocrine dysfunction is rarely observed the incidence of post-transplant graft pancreatitis (17 – 87.5%) is substantial (Vollmar et al., 1999). Experimentally, in the rat it has been demonstrated that with cold storage there is significant microvascular injury in the exocrine pancreas while the endocrine tissue is

relatively resistant with well-preserved capillary perfusion following re-implantation (Vollmar et al., 1999). Morphological studies of human pancreatic allografts with clinical and biochemical evidence of post-transplant pancreatitis have shown acinar disruption with massive granulocyte infiltration typical of ischaemia-reperfusion injury (Busing et al., 1993). Endocrine function was well-preserved in these patients. This implies that if a preservation method is shown to maintain pancreatic bioenergetics during organ storage, endocrine portion of the gland will be excluded from the damaging effects of cold ischaemia. On the contrary, when bioenergetic status deteriorates islets may or may not have been irreversibly damaged during preservation.

One major limitation of using surrogates is the transferability and validity of extrapolating data. Human trials are always predicated on data obtained from small and large animal studies. Animal models are invaluable subjects for proof-of-concept studies and to determine adverse effects and toxic doses of new treatments. However results obtained from such studies are only partially predictive for humans and cannot be translated directly into clinical medicine. Successful tolerance strategies developed in rodents and even non-human primates provide a case in point. Although there are fundamental similarities, humans have infinitely more complex physiology than specific pathogen-free laboratory animals.

The role of the rodent model as translational proving ground has been indispensable in the field of pancreas and islet transplantation, as in other areas of transplantation and medicine in general. However direct extrapolation into the clinical setting of experimental results in rodents has been problematic in a few areas of islet transplantation:

1. „Chopped" tissue digestion of the rat pancreas with collagenase was a landmark development in the history of islet transplantation (Lacy and Kostianovsky, 1967). Because rat pancreas is considerably softer, thinner and less fibrous than human organs it was possible to obtain good results with simple dispersion of the gland with collagenase after chopping the gland into several fragments. That „stationary phase" digestion involving ductal distension with the

enzyme was necessary for effective digestion of the human pancreas, was a realization that came years later (Gotoh et al. 1987 and 1990).

2. The two-layer method has been shown to work remarkably better than UW storage in the rodent islet transplant model but no clear-cut superiority is evident on analysis of the human trials (Agrawal et al., 2008). Once again, anatomical differences help to explain the observed discrepancies (Papas et al., 2005, Avgoustiniatos et al., 2006). We have shown that the PFC in two-layer preservation method does not penetrate a solid porcine pancreas after 24-hour storage.

The rat pancreas is not a distinct and well-defined gland as in humans. Rather it is diffuse organ enclosed in a dorsal mesentery. Whereas in humans the interlobular ducts converge to empty into a main pancreatic duct and the latter enters the duodenum adjacent to the common bile duct, interlobular ducts in rat pancreas directly join the biliopancreatic duct (Mann et al., 1920; Takahashi et al., 1977). Thus the TLM as currently employed, may not be optimal for human pancreas preservation. With mechanistic and proof-of-principle data, the rat islet transplant model has enabled us to move on to (anatomically and physiologically more appropriate) large animals for further tests, with appropriate modifications. For instance, the ^{31}P-MRS experiments can be conducted in porcine model comparing UW and TLM preservation with perfusion of perfluorocarbon emulsion following organ harvest.

Another problem with the rodent model that is peculiar to islet transplantation research is the difficulty in isolating islets following a period of cold preservation. Unlike freshly harvested rat pancreas, organs stored in preservation solution undergo degenerative changes with loss of islet isolation efficiency. Several studies have shown that preliminary ductal distension with enzyme and/or preservation solution is necessary for any measurable islet recovery from cold-preserved rat pancreas (Munn et al., 1989; Kneteman et al., 1990; Shapiro et al., 1996). This introduces another

variable in preservation studies making correlations of bioenergetics with islet isolation outcomes between different preservation groups impossible. Ohzato and colleagues (1989 and 1990) found that irrespective of the preservation solution used for simple storage of rat pancreas, islet recovery fell rapidly even with preservation times of 2 hours. The islet yield decreased to one fifth to one tenth of that of a fresh pancreas after 6-hour preservation. Islet recovery from cold-preserved rat pancreas improved to some extent only when the pancreas was distended with collagenase at the time of harvesting. This failure to isolate islets following preservation has been attributed to loss of ductal integrity (Munn et al., 1989; Ohzato et al. 1989 and 1990). However, islet isolation studies in rats have several limitations. As opposed to the compact and fibrous nature of pancreas in higher animals, the rat pancreas is a soft, diffuse organ embedded in the dorsal leaf of the greater omentum, mesoduodenum and mesojejunum and weighs about 1g in a 300g rat.

The fragility of rat pancreas has been borne out in elegant experiments by Ohzato and colleagues (Ohzato 1991). They observed a significant reduction in islet yield when the rat pancreas was preserved in cold storage solution prior to commencing islet isolation. Islet yield decreased uniformly in proportion to cold ischaemic time with as little as 2 hours preservation, irrespective of the solution used. By six hours the islet yield dropped to less that 25% of that from fresh pancreas. Ohzato reported that the pancreatic duct integrity was rapidly lost with preservation resulting in a significant drop in the plateau pressure seen with intraductal injection of Hank‟s balanced salt solution (HBSS). Pancreatic ductography with gelatin-barium sulphate solution revealed poor ductal distension and tissue infiltration of contrast in spite of massive leakage outside the parenchyma. In addition, there was infiltration of contrast within the islet capillaries resulting in loss of selective digestion of non-islet tissue. These findings suggest that the loss of ductal integrity during preservation was responsible for the poor isolation result in rat pancreas. He concluded that islets should ideally be harvested from fresh rat pancreas and that ductal collagenase injection should be done at the time of harvesting for maximum yield and viability.

In order to correlate pancreatic bioenergetic profile after preservation with islet isolation outcomes, this study must be repeated in a large animal model. Our model has been designed to measure real-time changes in ATP metabolism and is applicable to large animal and human pancreas as well.

Future studies:

.A major limitation of this study is the lack of correlation with islet isolation outcomes. A number of investigators have documented the difficulty of islet recovery from rat pancreas subjected to cold storage, especially without preliminary distension of the pancreatic duct with enzyme or other additives (Kneteman et al., 1990; Ohzato et al., 1990 and 1991). Such an intervention would introduce extrinsic variables in our experimental protocol designed primarily to compare the efficacy of different cold storage solutions in restoring pancreatic bioenergetics. In order to correlate pancreatic bioenergetic changes after preservation with islet isolation outcomes, this study must be repeated in large animal pancreases using our model to measure real-time changes in ATP metabolism.

The value of ^{31}P-MRS for islet viability assessment can also be confirmed by repeating this study on procured human pancreases using reversal of diabetes following islet transplantation in nude mice as endpoint. Alternatively, a clinical study could use our ^{31}P-MRS pancreas assessment method and randomize into TLM or UW preservation groups: the proportion of transplantable islet preparations in each group and clinical outcome would be the primary endpoints.

Our experimental model has several potential applications in islet transplantation and organ preservation research in general. Bioenergetic status of organs can be directly and non-invasively determined by measuring the relative abundances of phosphorylated metabolites during preservation. Rapid assessment of the viability of marginal donor pancreases can be particularly useful in the clinical setting. This evaluation method can also be potentially utilized to increase the precision of prognosis in graft performance post-transplantation.

The technique can also be applied experimentally to test the efficacy of pharmacological interventions to ameliorate preservation injury. Recent studies with a newer generation of PFC emulsions for

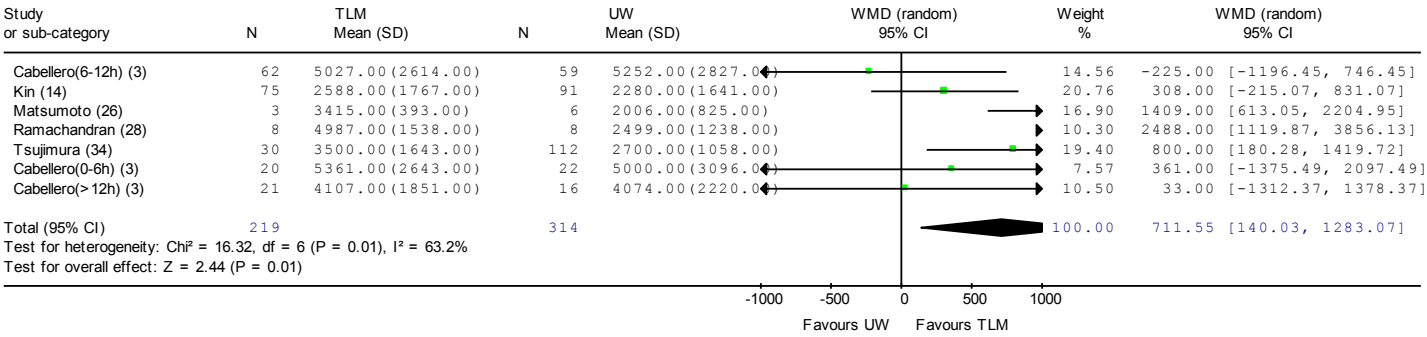
human kidney perfusion are encouraging (Reznik et al., 2008). PFC emulsions can potentially be employed for machine perfusion of pancreases procured for islet isolation and ^{31}P-MRS would be invaluable in assessing the efficacy of this method.

Another technique that can have potential application in the study of pancreas bio-energetics during preservation by different techniques is microdialysis. Metabolomics using this technique has been experimentally tested in kidney, liver and intestinal grafts (Keller et al., 2009; Hrydziusko et al., 2010; Birke-Sorensen & Andersen, 2010). This technique can be similarly applied to pancreas preservation alongside MRS and the efficacy of metabolic profiling of the two technologies compared.

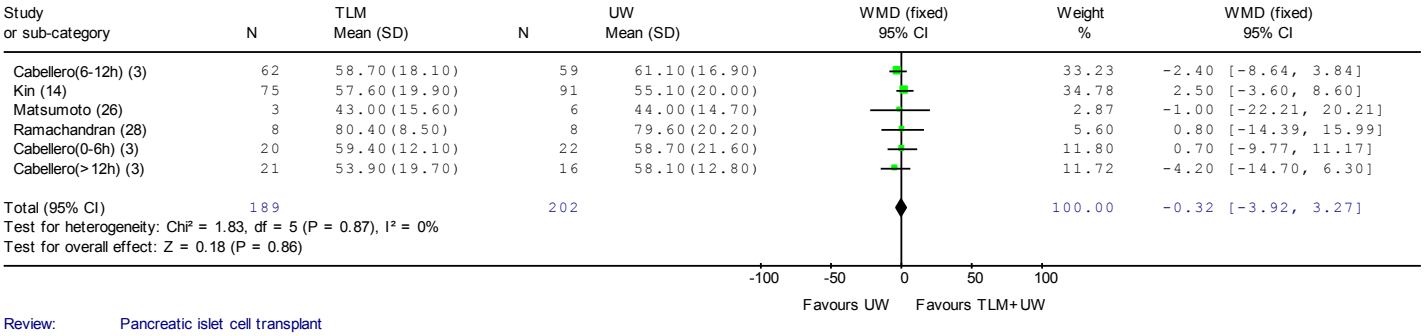
APPENDIX I

FOREST PLOTS OF TLM META-ANALYSIS

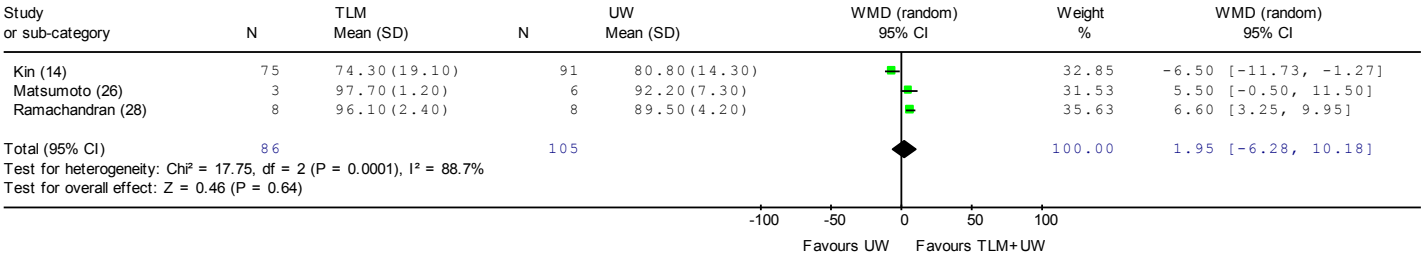
Review: Pancreatic islet cell transplant
Comparison: 01 TLM vs UW
Outcome: 01 Islet yield



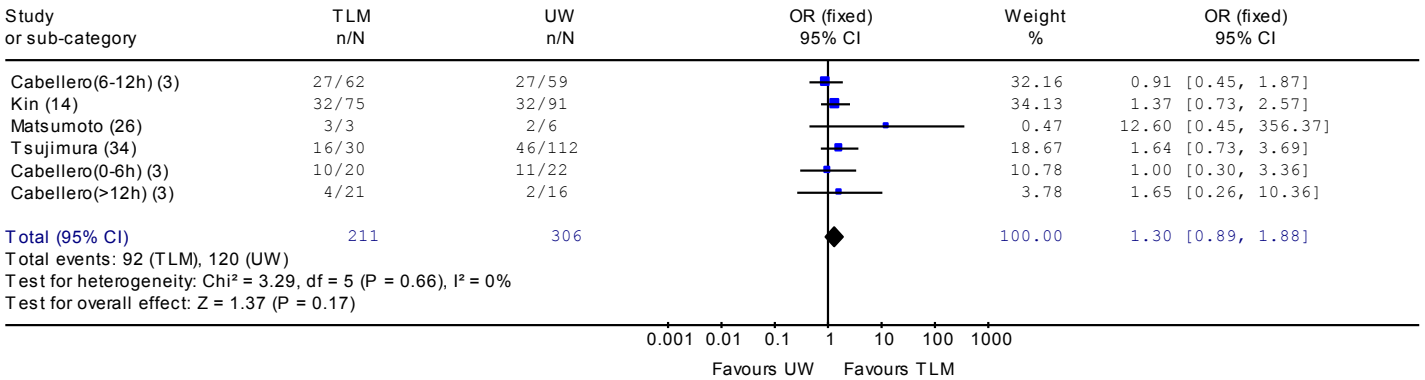
Review: Pancreatic islet cell transplant
Comparison: 01 TLM vs UW
Outcome: 02 Purity



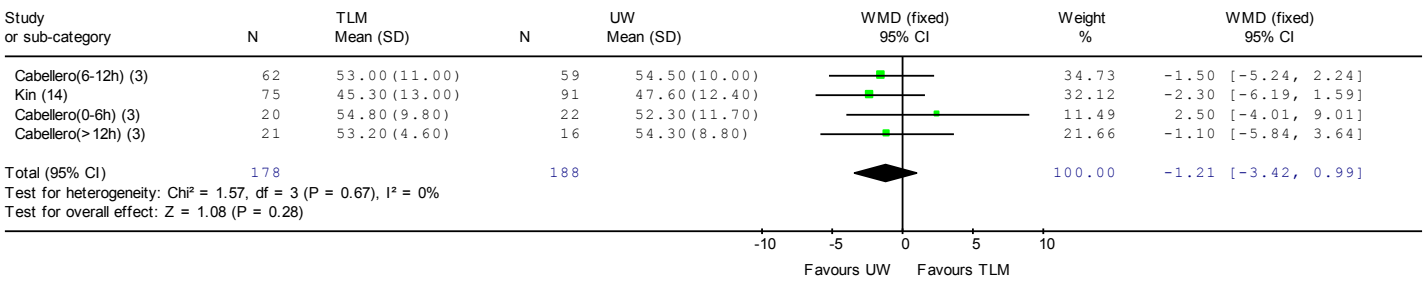
Review: Pancreatic islet cell transplant
Comparison: 01 TLM vs UW
Outcome: 03 Viability



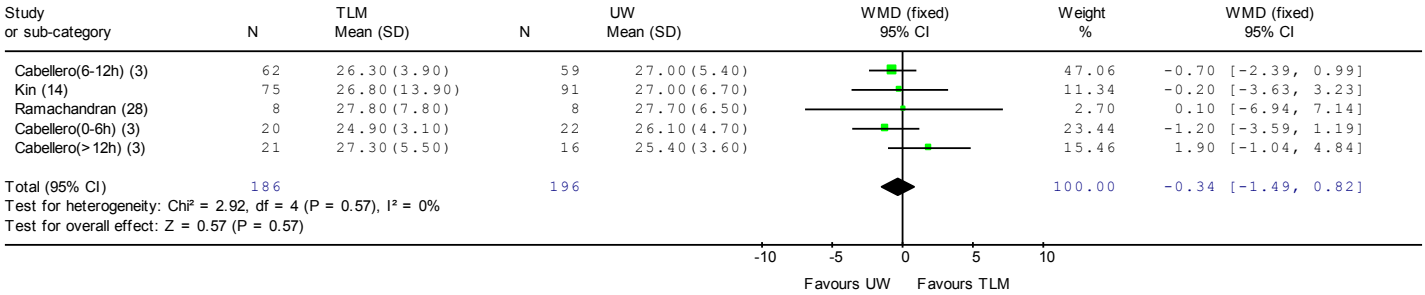
Review: Pancreatic islet cell transplant
Comparison: 01 TLM vs UW
Outcome: 04 Transplantable preparations



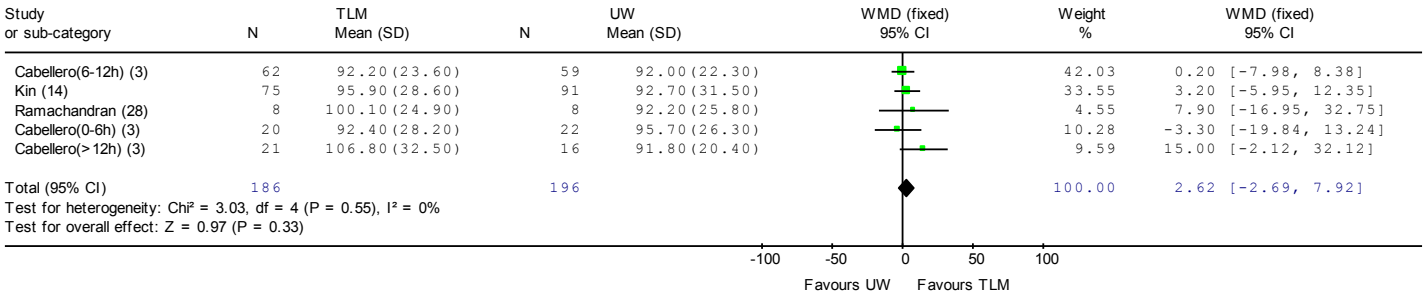
Review: Pancreatic islet cell transplant
Comparison: 01 TLM vs UW
Outcome: 05 Donor age



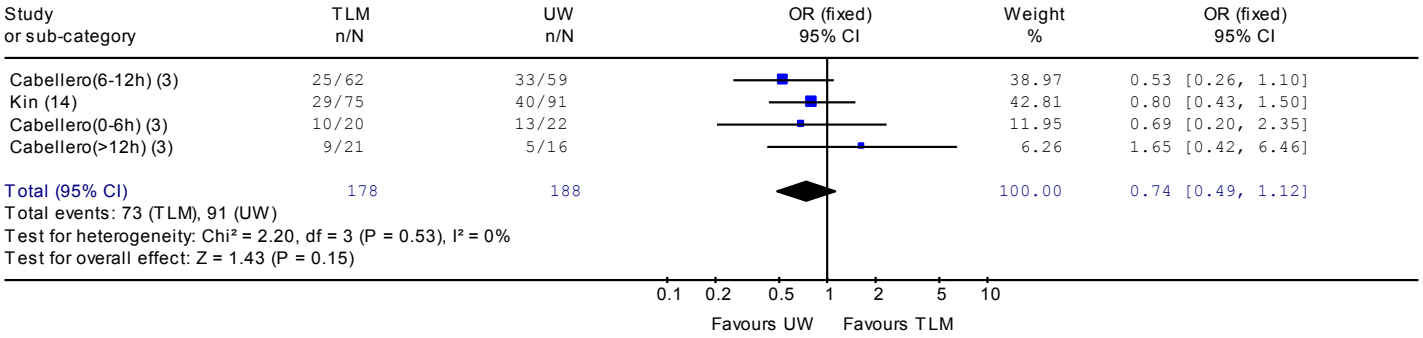
Review: Pancreatic islet cell transplant
Comparison: 01 TLM vs UW
Outcome: 06 BMI



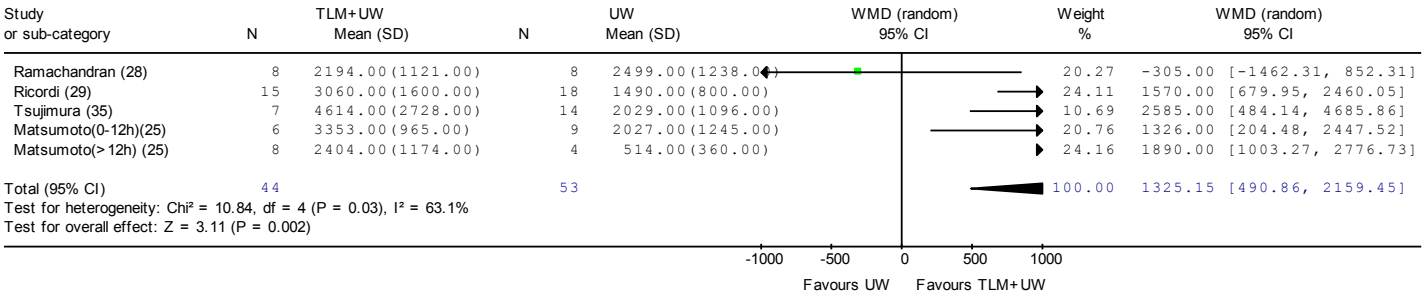
Review: Pancreatic islet cell transplant
Comparison: 01 TLM vs UW
Outcome: 07 Pancreas weight



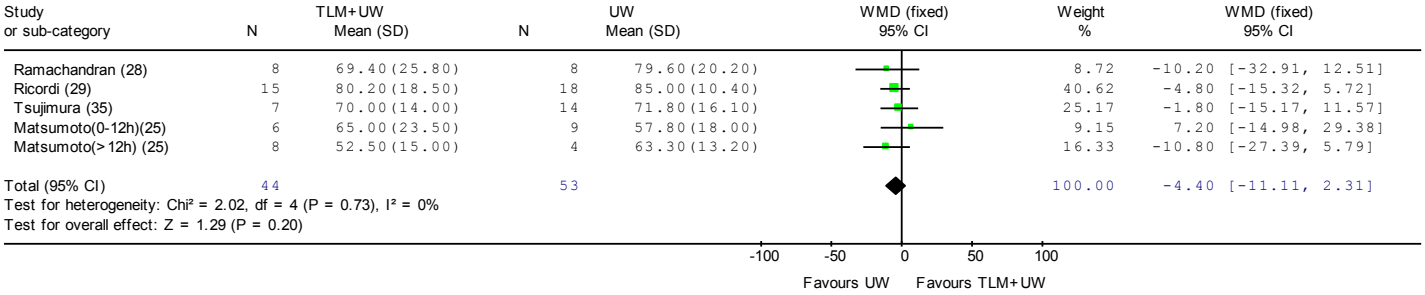
Review: Pancreatic islet cell transplant
Comparison: 01 TLM vs UW
Outcome: 08 Females



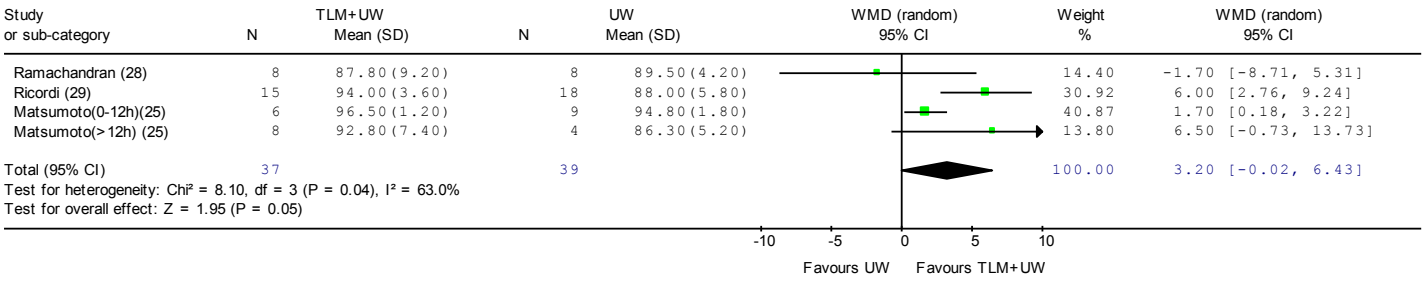
Review: Pancreatic islet cell transplant
Comparison: 02 TLM+UW vs UW
Outcome: 01 Islet yield



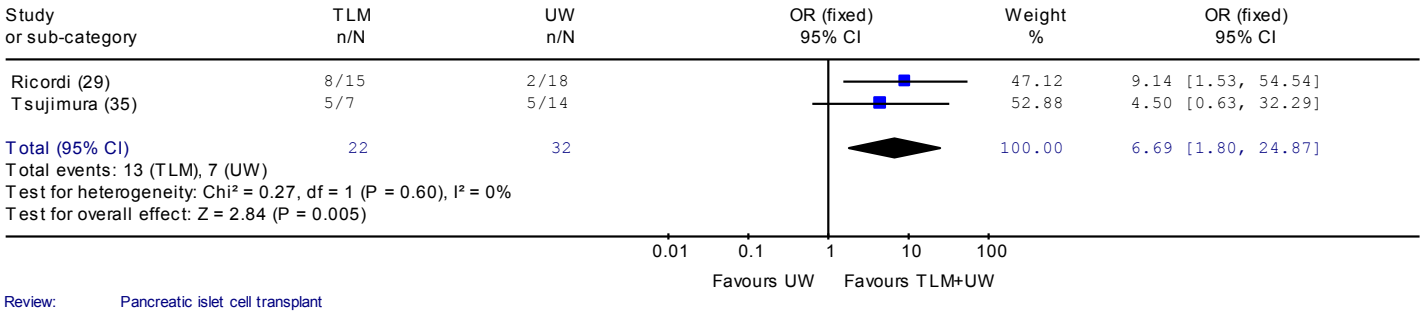
Review: Pancreatic islet cell transplant
Comparison: 02 TLM+UW vs UW
Outcome: 02 Purity



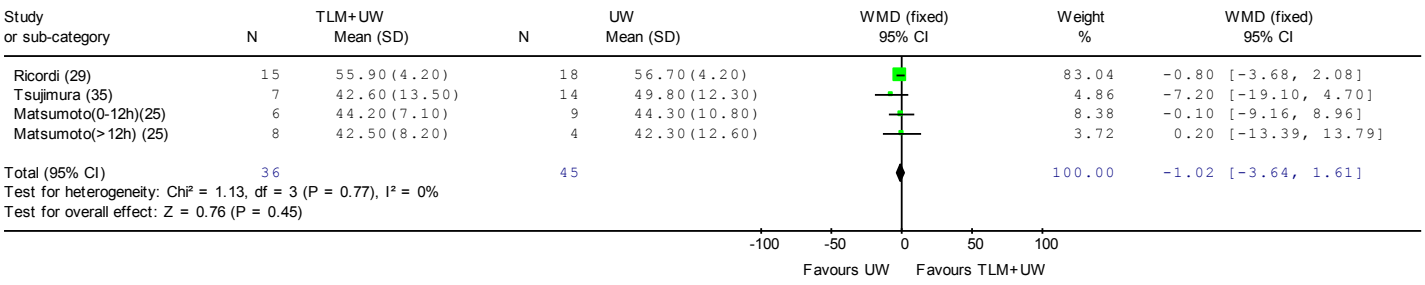
Review: Pancreatic islet cell transplant
Comparison: 02 TLM+UW vs UW
Outcome: 03 Viability



Review: Pancreatic islet cell transplant
Comparison: 02 TLM+UW vs UW
Outcome: 04 Transplantable preparations



Review: Pancreatic islet cell transplant
Comparison: 02 TLM+UW vs UW
Outcome: 05 Donor age



Review: Pancreatic islet cell transplant
Comparison: 02 TLM+UW vs UW
Outcome: 06 BMI

Study or sub-category	TLM+UW N	TLM+UW Mean (SD)	UW N	UW Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI
Ramachandran (28)	8	26.90(6.10)	8	27.70(6.50)		12.82	-0.80 [-6.98, 5.38]
Ricordi (29)	15	26.00(3.90)	18	27.20(7.20)		32.69	-1.20 [-5.07, 2.67]
Tsujimura (35)	7	25.40(3.70)	14	23.40(3.40)		45.77	2.00 [-1.27, 5.27]
Matsumoto(0-12h)(25)	6	32.60(15.90)	9	29.80(4.50)		2.87	2.80 [-10.26, 15.86]
Matsumoto(>12h) (25)	8	29.10(11.30)	4	27.80(4.80)		5.86	1.30 [-7.83, 10.43]
Total (95% CI)	44		53			100.00	0.58 [-1.63, 2.79]

Test for heterogeneity: Chi² = 1.87, df = 4 (P = 0.76), I² = 0%
Test for overall effect: Z = 0.51 (P = 0.61)

-100 -50 0 50 100
Favours UW Favours TLM+UW

Review: Pancreatic islet cell transplant
Comparison: 02 TLM+UW vs UW
Outcome: 07 Pancreas weight

Study or sub-category	TLM+UW N	TLM+UW Mean (SD)	UW N	UW Mean (SD)	WMD (random) 95% CI	Weight %	WMD (random) 95% CI
Ramachandran (28)	8	88.40(18.50)	8	92.20(25.80)		19.72	-3.80 [-25.80, 18.20]
Ricordi (29)	15	119.20(36.20)	18	107.50(20.80)		21.46	11.70 [-8.99, 32.39]
Tsujimura (35)	7	86.60(28.80)	14	109.30(28.10)		15.52	-22.70 [-48.62, 3.22]
Matsumoto(0-12h)(25)	6	87.20(22.50)	9	102.70(22.80)		18.10	-15.50 [-38.87, 7.87]
Matsumoto(>12h) (25)	8	89.60(13.00)	4	104.30(16.20)		25.20	-14.70 [-32.95, 3.55]
Total (95% CI)	44		53			100.00	-8.27 [-19.97, 3.42]

Test for heterogeneity: Chi² = 5.77, df = 4 (P = 0.22), I² = 30.7%
Test for overall effect: Z = 1.39 (P = 0.17)

-100 -50 0 50 100
Favours UW Favours TLM+UW

Review: Pancreatic islet cell transplant
Comparison: 02 TLM+UW vs UW
Outcome: 08 Females

Study or sub-category	TLM n/N	UW n/N	OR (fixed) 95% CI	Weight %	OR (fixed) 95% CI
Matsumoto(0-12h)(25)	4/6	3/9		54.55	4.00 [0.45, 35.79]
Matsumoto(>12h) (25)	4/8	1/4		45.45	3.00 [0.21, 42.62]
Total (95% CI)	14	13		100.00	3.55 [0.65, 19.25]

Total events: 8 (TLM), 4 (UW)
Test for heterogeneity: Chi² = 0.03, df = 1 (P = 0.87), I² = 0%
Test for overall effect: Z = 1.47 (P = 0.14)

0.01 0.1 1 10 100
Favours UW Favours TLM+UW

Review: Pancreatic islet cell transplant
Comparison: 03 TLM+UW vs UW (RCT only)
Outcome: 01 Islet yield

Study or sub-category	TLM+UW N	TLM+UW Mean (SD)	UW N	UW Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI
Tsujimura (35)	7	4614.00(2728.00)	14	2029.00(1096.00)		9.88	2585.00 [484.14, 4685.86]
Matsumoto(0-12h)(25)	6	3353.00(965.00)	9	2027.00(1245.00)		34.67	1326.00 [204.48, 2447.52]
Matsumoto(>12h) (25)	8	2404.00(1174.00)	4	514.00(360.00)		55.45	1890.00 [1003.27, 2776.73]
Total (95% CI)	21		27			100.00	1763.14 [1102.82, 2423.47]

Test for heterogeneity: Chi² = 1.25, df = 2 (P = 0.54), I² = 0%
Test for overall effect: Z = 5.23 (P < 0.00001)

-1000 -500 0 500 1000
Favours UW Favours TLM+UW

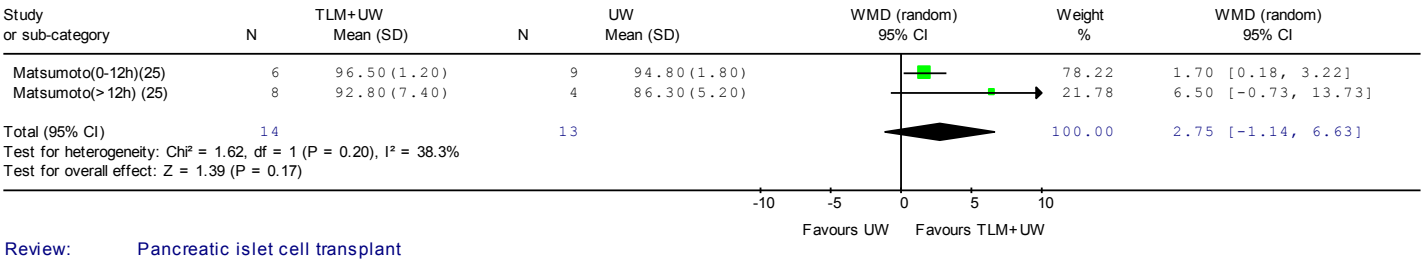
Review: Pancreatic islet cell transplant
Comparison: 03 TLM+UW vs UW (RCT only)
Outcome: 02 Purity

Study or sub-category	TLM+UW N	TLM+UW Mean (SD)	UW N	UW Mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI
Tsujimura (35)	7	70.00(14.00)	14	71.80(16.10)		49.70	-1.80 [-15.17, 11.57]
Matsumoto(0-12h)(25)	6	65.00(23.50)	9	57.80(18.00)		18.05	7.20 [-14.98, 29.38]
Matsumoto(>12h) (25)	8	52.50(15.00)	4	63.30(13.20)		32.25	-10.80 [-27.39, 5.79]
Total (95% CI)	21		27			100.00	-3.08 [-12.50, 6.35]

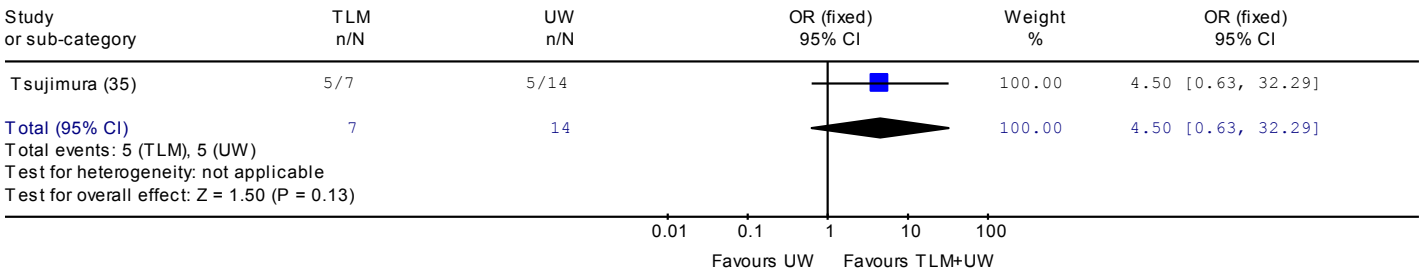
Test for heterogeneity: Chi² = 1.69, df = 2 (P = 0.43), I² = 0%
Test for overall effect: Z = 0.64 (P = 0.52)

-100 -50 0 50 100
Favours UW Favours TLM+UW

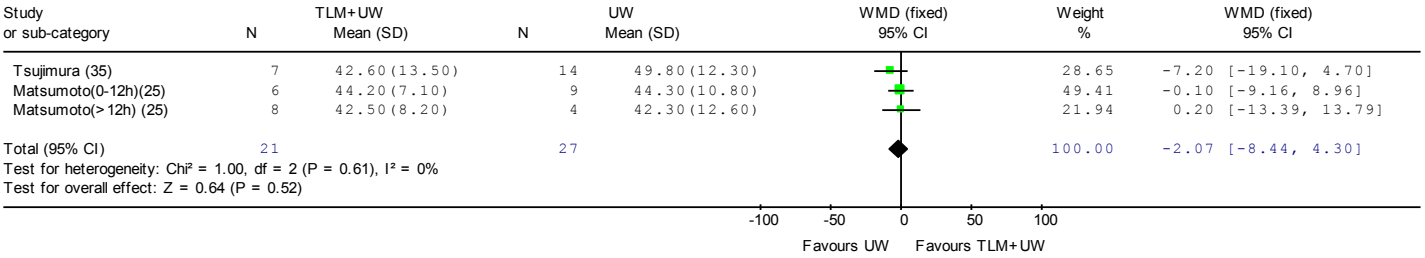
Review: Pancreatic islet cell transplant
Comparison: 03 TLM+UW vs UW (RCT only)
Outcome: 03 Viability



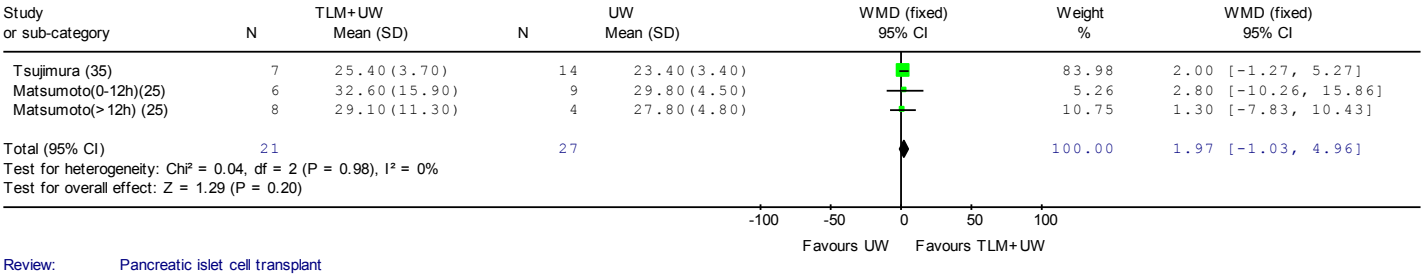
Review: Pancreatic islet cell transplant
Comparison: 03 TLM+UW vs UW (RCT only)
Outcome: 04 Transplantable preparations



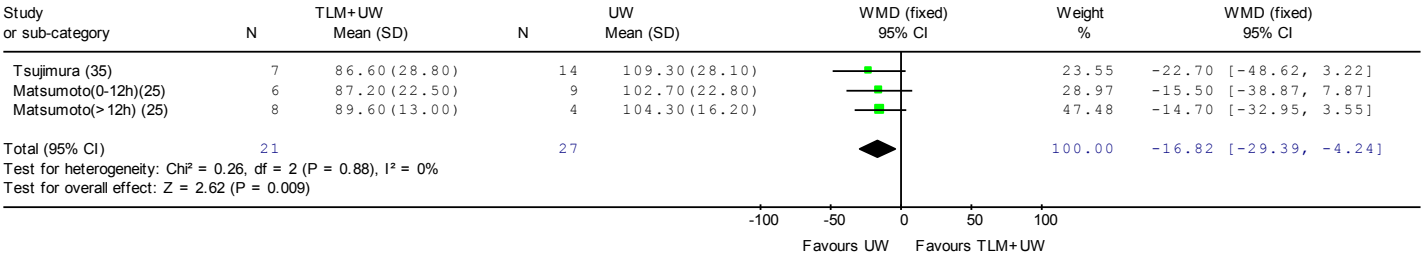
Review: Pancreatic islet cell transplant
Comparison: 03 TLM+UW vs UW (RCT only)
Outcome: 05 Donor age



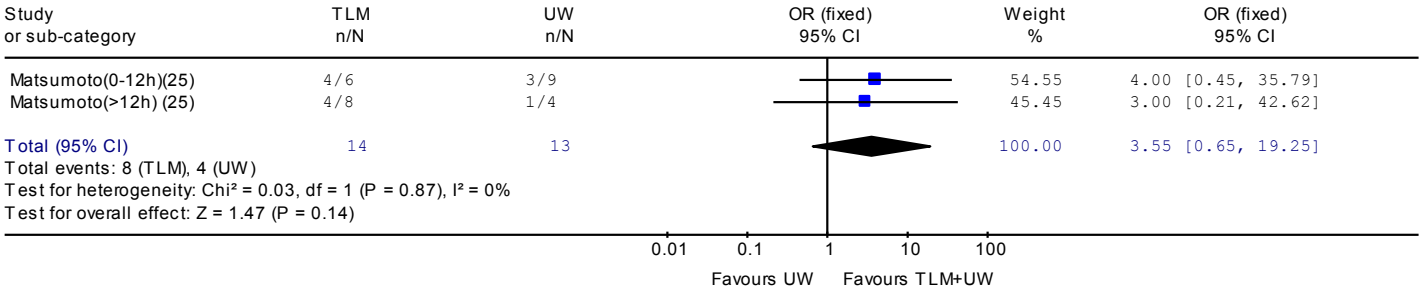
Review: Pancreatic islet cell transplant
Comparison: 03 TLM+UW vs UW (RCT only)
Outcome: 06 BMI



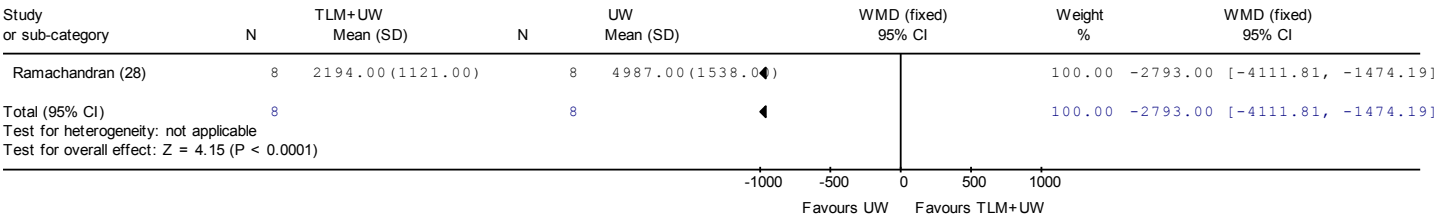
Review: Pancreatic islet cell transplant
Comparison: 03 TLM+UW vs UW (RCT only)
Outcome: 07 Pancreas weight



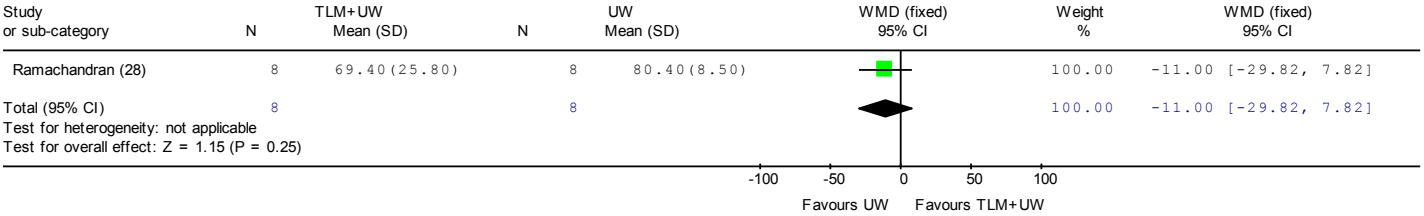
Review: Pancreatic islet cell transplant
Comparison: 03 TLM+UW vs UW (RCT only)
Outcome: 08 Females



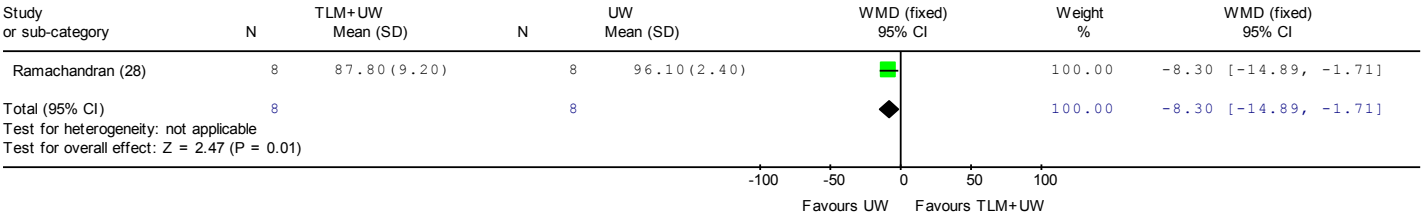
Review: Pancreatic islet cell transplant
Comparison: 04 TLM+UW vs TLM
Outcome: 01 Islet yield



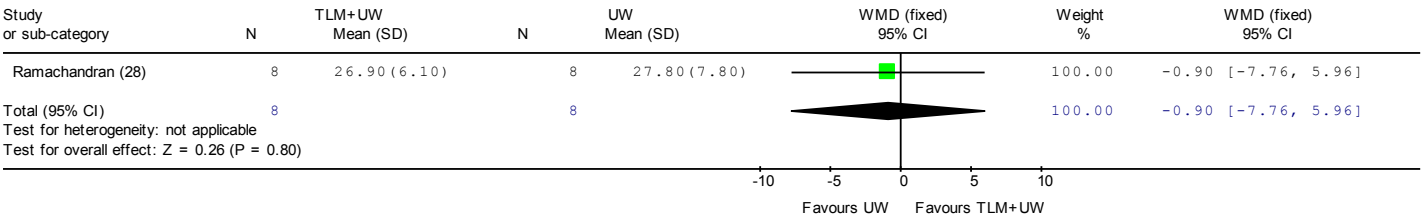
Review: Pancreatic islet cell transplant
Comparison: 04 TLM+UW vs TLM
Outcome: 02 Purity



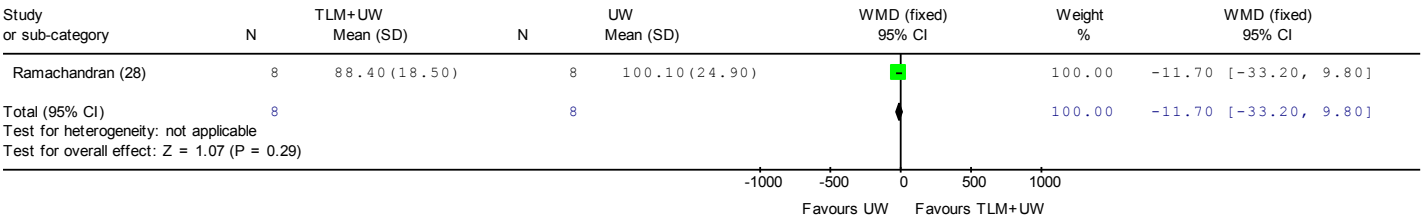
Review: Pancreatic islet cell transplant
Comparison: 04 TLM+UW vs TLM
Outcome: 03 Viability



Review: Pancreatic islet cell transplant
Comparison: 04 TLM+UW vs TLM
Outcome: 04 BMI



Review: Pancreatic islet cell transplant
Comparison: 04 TLM+UW vs TLM
Outcome: 05 Pancreas weight



APPENDIX II

^{19}F MRS SPECTRA

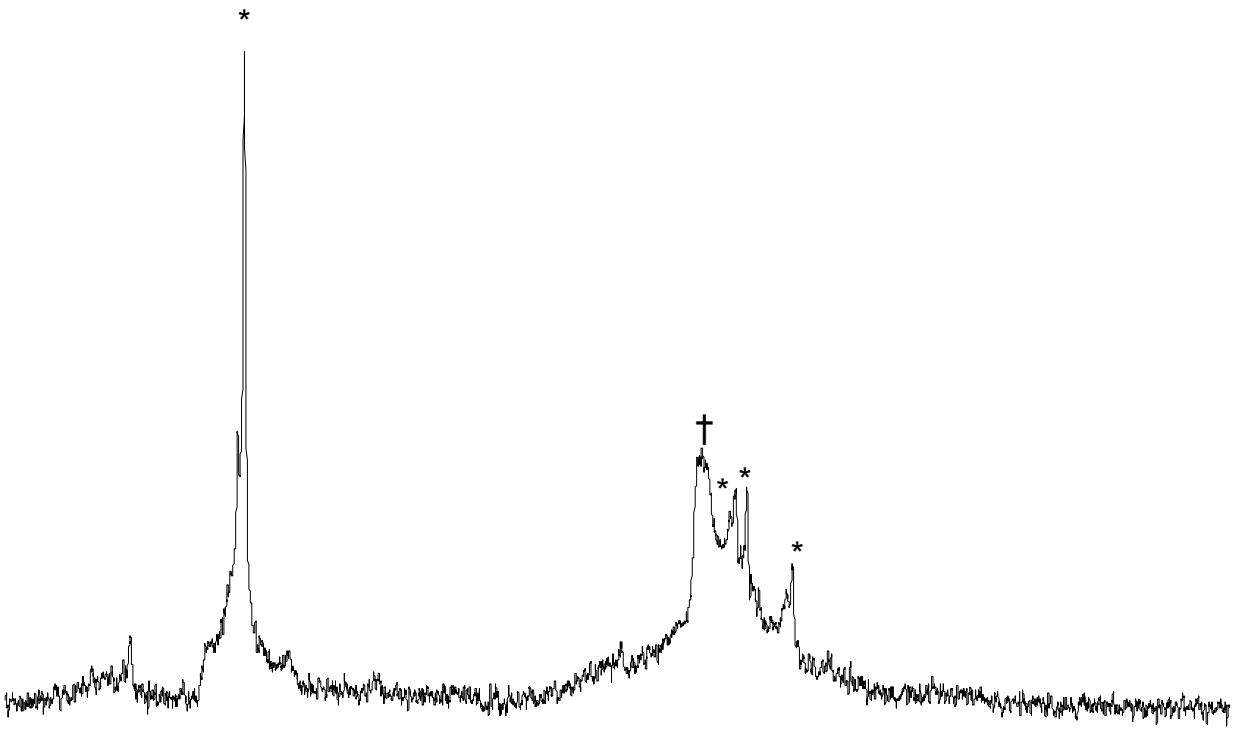


Figure II.1: ^{19}F spectra showing peaks obtained from phantom syringes filled with * perfluorodecalin and †sodium fluoride.

Figure II.2: ^{19}F MRS SPECTRA (Porcine pancreas preserved in TLM for 24 h: any PFC in these samples is below the level of detection).

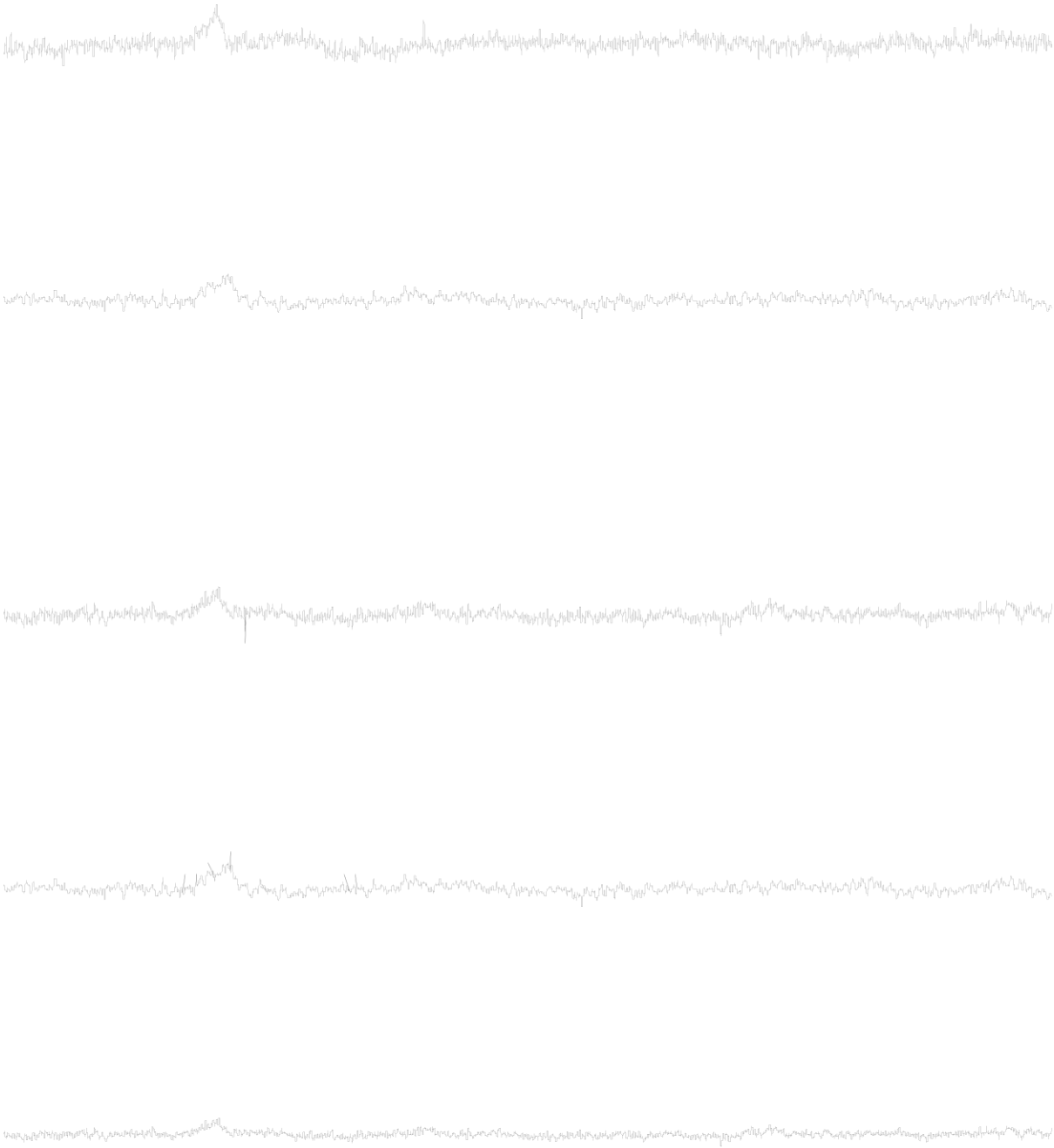
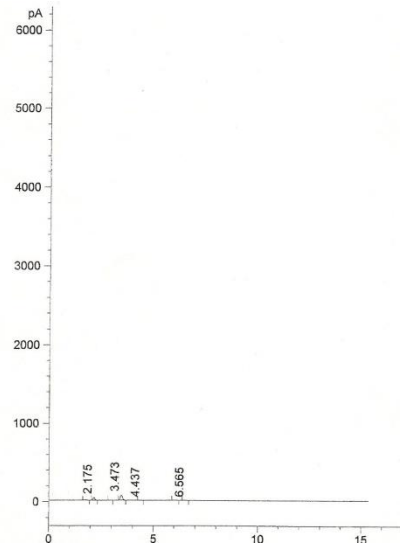


Figure II.3: ^{19}F MRS SPECTRA (Porcine pancreas preserved in UW solution for 24 hours: no PFC is detectable)

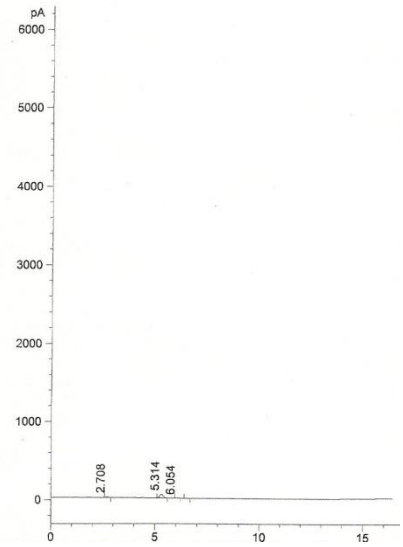
APPENDIX III

HEADSPACE GAS CHROMATOGRAPHY OF HUMAN PANCREAS



Human pancreas – TLM

Figure III.1: Headspace chromatogram depicting concentration of perfluorodecalin in 500 g of human pancreas preserved in TLM for 24 hours – it is negligible.



Human pancreas in UW

Figure III.2: Headspace chromatogram depicting concentration of perfluorodecalin in 500 g of human pancreas preserved in UW solution for 24 hours – negligible traces detectable represent environmental contamination.

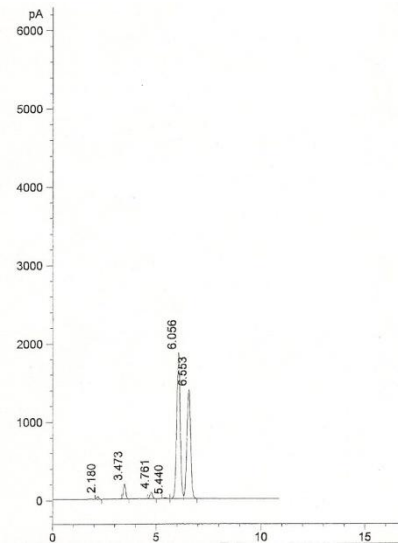


Figure III.3: Headspace chromatogram depicting concentration of perfluorodecalin in 500 g of human pancreas spiked with PFC (0.1µl perfluorodecalin). Large concentration peaks are observed corresponding with PFC retention times.

PFC concentration	UW-pancreas (n=1)	TLM-pancreas (n=1)	spiked control (n=1)
ppm	0.0185	0.027	361.57
nl/g	0.009	0.014	190.3

Table III.1: showing concentration (in ppm and nl/g) of PFC in human pancreas preserved in UW, TLM and positive control.

APPENDIX IV

^{31}P MRS DATA

RP	Type	int 1 - 2.75hrs Pi/gammaATP	Pi/BetaATP	RP	Type	int 1 - 2.75hrs Pi/gammaATP	Pi/BetaATP	RP	Type	int 1 - 2.75hrs Pi/gammaATP	Pi/BetaATP
27				29				37			
28	M	14.74	37.05	30	C	4.79	7.77	38	S	6.49	14.03
31				33				43			
32	M	15.1	46.75	34	C	7.39	14.62	44	S	9.2	17.38
35				39				49			
36	M	10.82	25.68	40	C	9.12	36.14	50	S	6.69	11.54
41				45				51			
42	M	19.23	82.17	46	C	6.2	8.67	52	S	11.23	13.46
47				53				57			
48	M	10.72	34.69	54	C	6.01	7.13	58	S	7.33	7.98
59				55				61			
60	M	16.5	30.35	56	C	6.52	7.55	62	S	4.98	8.4
				63							
				64	C	4.34	7.04				
mean		14.5183 3333	42.781 6667			6.33857 1429	12.702 8571			7.65333 3333	12.131 6667
SD		3.30512 5817	20.554 9453			1.60178 4719	10.671 2132			2.22339 0804	3.5895 6497

RP	Type	int 1 - 2.75hrs Pi/gamaATP	Pi/Beta ATP	int 1 - 5.5hrs Pi/gamaATP	Pi/Beta ATP	RP	Type	int 1 - 2.75hrs Pi/gamaATP	Pi/Beta ATP	int 1 - 5.5hrs Pi/gamaATP	Pi/Beta ATP
73	S					65	C				
74	WIS	8.93	31.48	26.86	87.07	66	WIC	7.4	19.79	17.54	30.45
77	S					75	C				
78	WIS	7.38	11.12	24.23	35.05	76	WIC	7.55	12.48	21.31	31.19
79	S				161.4	83	C				
80	WIS	13.79	22.72	44.06	8	84	WIC	8.98	10.2	20.49	24.63
81	S					85	C				
82	WIS	8.77	16.74	26.82	70.36	86	WIC	8.63	14.65	20.06	31.12
67	S					87	C				
68	WIS	8.92	18.24	27.56	70.66	88	WIC	8.32	18.79	21.66	43.18
71	S					89	C				
72	WIS	8.58	12.45	21.67	36.4	90	WIC	8.28	13.52	19.44	31.11
			18.79	28.533	76.83			8.1933	14.90	20.083	31.946
		9.395	16667	33333	66667			33333	5	33333	66667
		2.2303	7.483	7.9168	46.32			0.6125	3.713	1.4858	6.0649
		78892	45486	72278	61545			90129	68685	62264	37483

Pi/Gamma ATP 1 to 2.75 hours

M	S WI	C WI	S	C
14.74	8.93	7.4	6.49	4.79
15.1	7.38	7.55	9.2	7.39
10.82	13.79	8.98	6.69	9.12
19.23	8.77	8.63	11.23	6.2
10.72	8.92	8.32	7.33	6.01
16.5	8.58	8.28	4.98	6.52
				4.34

One Way Analysis of Variance Monday, October 29, 2007, 15:59:56

Data source: Data 1 in Notebook

Normality Test: Passed (P = 0.145)

Equal Variance Test: Passed (P = 0.275)

Group Name	N	Missing	Mean	Std Dev	SEM
M	6	0	14.518	3.305	1.349
S WI	6	0	9.395	2.23	0.911
C WI	6	0	8.193	0.613	0.25
S	6	0	7.653	2.223	0.908
C	7	0	6.339	1.602	0.605

Source of Variation	DF	SS	MS	F	P
Between Groups	4	247.518	61.879	13.244	<0.001
Residual	26	121.48	4.672		
Total	30	368.998			

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
M vs. C	8.18	5	9.619	<0.001	Yes
M vs. S	6.865	5	7.779	<0.001	Yes
M vs. C WI	6.325	5	7.168	<0.001	Yes
M vs. S WI	5.123	5	5.806	0.003	Yes
S WI vs. C	3.056	5	3.594	0.112	No
S WI vs. S	1.742	5	1.974	0.636	Do Not Test
S WI vs. C WI	1.202	5	1.362	0.869	Do Not Test
C WI vs. C	1.855	5	2.181	0.546	Do Not Test
C WI vs. S	0.54	5	0.612	0.992	Do Not Test
S vs. C	1.315	5	1.546	0.808	Do Not Test

Pi/Beta ATP 1 to 2.75 hours

M	S WI	C WI	S	C
37.05	31.48	19.79	14.03	7.77
46.75	11.12	12.48	17.38	14.62
25.68	22.72	10.2	11.54	36.14
82.17	16.74	14.65	13.46	8.67
34.69	18.24	18.79	7.98	7.13
30.35	12.45	13.52	8.4	7.55
				7.04

One Way Analysis of Variance Monday, October 29, 2007, 16:03:08

Data source: Data 1 in Notebook

Normality Test: Failed (P = <0.001)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Monday, October 29, 2007, 16:03:08

Data source: Data 1 in Notebook

Group	N	Missing	Median	25%	75%
M	6	0	35.87	30.35	46.75
S WI	6	0	17.49	12.45	22.72
C WI	6	0	14.085	12.48	18.79
S	6	0	12.5	8.4	14.03
C	7	0	7.77	7.235	13.133

H = 16.207 with 4 degrees of freedom. (P = 0.003)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
M vs C	18.952	3.747	Yes
M vs S	16.333	3.112	Yes
M vs C WI	12	2.286	No
M vs S WI	9.833	1.873	Do Not Test
S WI vs C	9.119	1.803	No
S WI vs S	6.5	1.238	Do Not Test
S WI vs C WI	2.167	0.413	Do Not Test
C WI vs C	6.952	1.374	Do Not Test
C WI vs S	4.333	0.826	Do Not Test
S vs C	2.619	0.518	Do Not Test

Pi/Gamma ATP 1 to 5.5 hours

S WI	C WI
26.86	17.54
24.23	21.31
44.06	20.49
26.82	20.06
27.56	21.66
21.67	19.44

t-test Monday, October 29, 2007, 16:06:24

Data source: Data 1 in Notebook

Normality Test: Failed (P = 0.003)

Test execution ended by user request, Rank Sum Test begun

Mann-Whitney Rank Sum Test Monday, October 29, 2007, 16:06:24

Data source: Data 1 in Notebook

Group	N	Missing	Median	25%	75%
S WI	6	0	26.84	24.23	27.56
C WI	6	0	20.275	19.44	21.31

T = 57.000 n(small)= 6 n(big)= 6 P(est.)= 0.005 P(exact)= 0.002

Pi/Beta ATP 1 to 5.5 hours

S WI	C WI
87.07	30.45
35.05	31.19
161.48	24.63
70.36	31.12
70.66	43.18
36.4	31.11

t-test Monday, October 29, 2007, 16:07:20

Data source: Data 1 in Notebook

Normality Test: Failed (P = 0.008)

Test execution ended by user request, Rank Sum Test begun

Mann-Whitney Rank Sum Test Monday, October 29, 2007, 16:07:20

Data source: Data 1 in Notebook

Group	N	Missing	Median	25%	75%
S WI	6	0	70.51	36.4	87.07
C WI	6	0	31.115	30.45	31.19

T = 55.000 n(small)= 6 n(big)= 6 P(est.)= 0.013 P(exact)= 0.009

The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = 0.009)

APPENDIX V

ISLET ISOLATION OUTCOMES FOLLOWING PANCREAS PRESERVATION IN RATS

pancreas	group 1	group 2	group 3
1	384	89	41
2	470	62	112
3	359	68	28
4	388	34	65
5	512	32	71
Mean (±SD) islet yield	423 ± 65	57 ± 24	63 ± 32

Table V.1: Islet yield from rat pancreas: fresh (group 1), preserved in cold UW solution for 3 hrs (group 2) and preserved in TLM for 3 hrs (group 3).

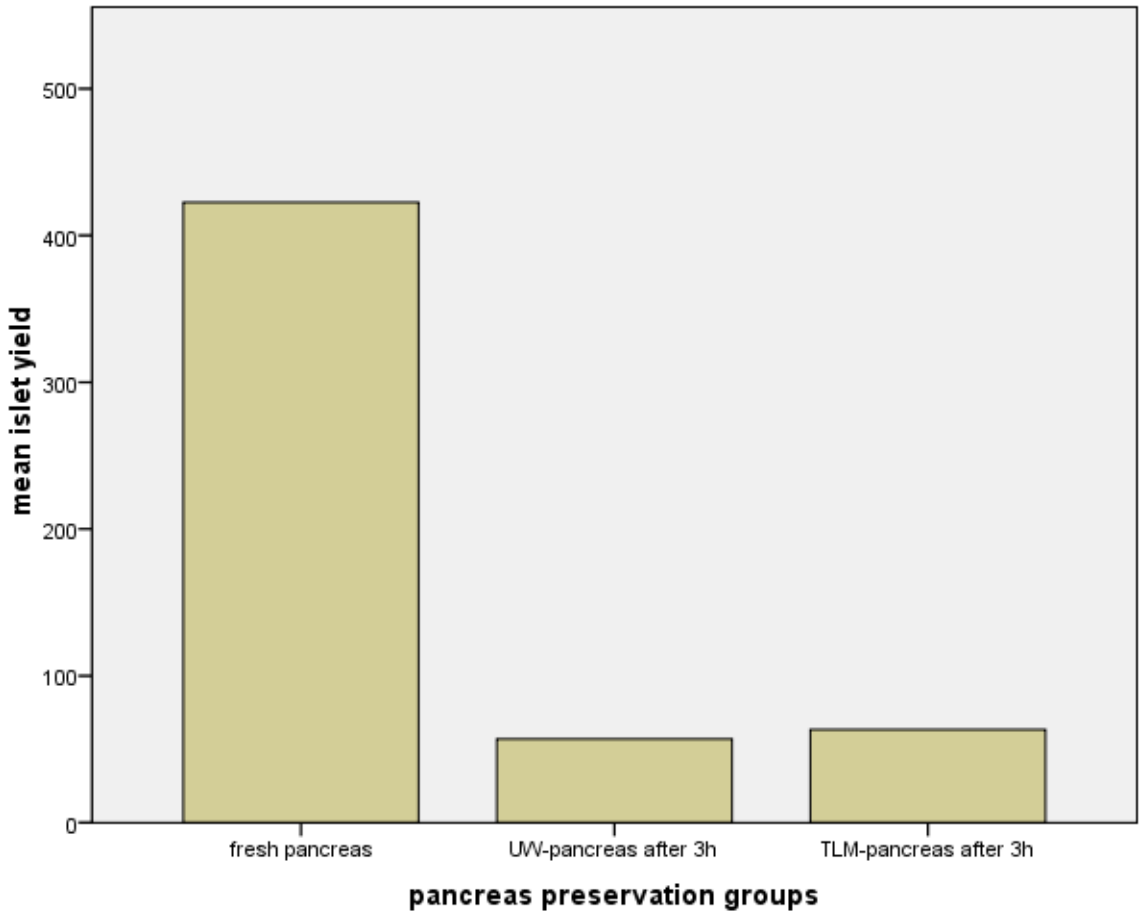


Figure V.1: Graph comparing mean islet yield from rat pancreas preserved in UW solution and in TLM with that from fresh pancreas (n=5). There was no significant difference in islet yield between the two experimental groups but the yield was significantly lower compared to that obtained from fresh pancreas.

Multiple Comparisons

mean islet yield

Tukey HSD

(I) pancreas preservation groups	(J) pancreas preservation groups	Mean Difference (I-J)	Std. Error
fresh pancreas	UW-pancreas after 3h	365.60*	27.972
	TLM-pancreas after 3h	359.20*	27.972
UW-pancreas after 3h	fresh pancreas	-365.60*	27.972
	TLM-pancreas after 3h	-6.40	27.972
TLM-pancreas after 3h	fresh pancreas	-359.20*	27.972
	UW-pancreas after 3h	6.40	27.972

Based on observed means.

The error term is Mean Square (Error) = 1956.033.

*. The mean difference is significant at the 0.05 level.

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
		211		
group 1	5	3	422.6	4242.8
group 2	5	285	57	581
group 3	5	317	63.4	1044.3

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	437881.6	2	218940.8	111.931	1.73E-08	3.885294
Within Groups	23472.4	12	1956.033			
Total	461354	14				

Table V.2: Results of ANOVA showing that the difference between control and experimental groups is significant and that there is no significant difference in islet yield between the experimental groups.

PUBLICATIONS AND PRESENTATIONS

Publications

Agrawal A**,** Gurusamy K, Powis S, Gray D, Fuller B, Davidson B. A meta-analysis of the impact of the two-layer method of preservation on human pancreatic islet transplantation. *Cell Transplantation* 2008;17(12):1315-22.

Agrawal A, Po-wah So, Penman A, Powis S, Davidson B, Press M, Fuller B. Limited Penetration of Perfluorocarbon in Porcine Pancreas Preserved by Two-Layer Method with ^{19}Fluorine Magnetic Resonance Spectroscopy and Headspace Gas Chromatography. *Cell Transplantation* 2010; in press

Presentations

31-phosphorus magnetic resonance spectroscopy for dynamic assessment of ATP levels in pancreas preserved by the two-layer method.

British Transplant Congress. Liverpool April 21-24, 2009 and

International Society for Magnetic Resonance in Medicine, Honolulu 18-24 April 2009

^{19}F MRI and Head space Chromatography to determine penetration of perfluorocarbon in pancreas preserved by two-layer method for islet transplantation.

British Transplant Congress. Manchester April 28-30, 2007 and

44th Annual meeting of the Society for Cryobiology Jul 28 – Aug 1, 2007

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Abdelli S, Ansite J, Roduit R, Borsello T, Matsumoto I, Sawada T, Allaman-Pillet N, Henry H, Beckmann JS, Hering BJ, Bonny C. Intracellular stress signalling pathways activated during human islet preparation and following acute cytokine exposure. Diabetes 2004;53(11):2815-23.

Ackermann JR, Fisher AJ, Barnard CN. Live storage of kidneys: a preliminary communication. Surgery 1966;60(3):720-4.

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