AN *EX VIVO* NORMOTHERMIC PORCINE PANCREAS PHYSIOLOGICAL MODEL: IMPLICATIONS FOR PANCREAS & ISLET TRANSPLANTATION

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ABSTRACT

An Ex Vivo Normothermic Porcine Pancreas Physiological Model: Implications for Pancreas and Islet Transplantation

Introduction: The aim of the work performed for this thesis was to establish an *ex vivo* normothermic porcine pancreas perfusion model which is physiological and has the potential to facilitate detailed study of both its exocrine and endocrine function. The *ex vivo* porcine pancreas model was used to investigate the effects of machine perfusion on parameters that may be important in graft preservation and the success of whole organ pancreas and islet transplantation.

A porcine islet isolation and digestion protocol was also established to enable the assessment and quantification of islet yield and viability.

Methodology: During the preliminary phase of the thesis eight porcine pancreata were recovered and perfused in order to establish a reproducible experimental protocol for an *ex vivo* physiological pancreas model. Subsequently nine porcine pancreata were successfully recovered and normothermically perfused with autologous blood at a mean blood pressure of 50 mmHg (normotensive). Graft viability was then compared against a further four *ex vivo* porcine pancreata normothermically perfused at 20 mmHg (low) pressure. Serological and haematological parameters measured were: arterial and venous oxygen gas differential, routine biochemistry, glucose concentration and graft insulin responses to glucose stimulation. Exocrine function was assessed by measuring the rate of production of pancreatic juice (volume/time) and the level of serum amylase. Immunohistochemistry for cellular viability was assessed by haematoxylin and eosin staining, M30 cytoDEATH, anti-Caspase 3 antibody, and anti-ATP synthetase complex V antibody.

Results: All normotensive pancreata were perfused for a median of 3 hours (range 2–4 hours) with a mean perfusion pressure of 50 mmHg and graft flow rate of 141 $mL.min^{-1}$ (95% confidence intervals 122.4 to 160 $mL.min^{-1}$). In comparison, all of the 'low' pressure models were successfully perfused for a minimum of 4 hours, with mean perfusion pressure of 20 mmHg and graft flow rate of 40 $mL.min^{-1}$ (95% confidence interval 31 to 48 $mL.min^{-1}$), $p < 0.05$. All pancreata in both the normotensive and low pressure groups demonstrated cellular viability with evidence of oxygen consumption and preserved endocrine and exocrine function. Following statistical analysis, the 'low' pressure perfused porcine pancreata compared favourably in important biochemical and immunohistochemistry cellular profiles, suggesting the potential for an improved method of graft preservation with improved viability.

Conclusion: The physiological behaviour of this *ex vivo* perfused pancreas model allows the changes that occur in recovered pancreata and the potential effect they have on islet isolation, yield and whole organ viability to be studied in detail. The model also avoids use of live animals, is reproducible and mimics a "donation after circulatory death" pancreas transplant scenario. The work in this thesis demonstrated the successful application of the model to investigate the effect of machine perfusion on variables that may influence and potentially optimise both a whole organ pancreas and islets prior to transplantation.

STATEMENT OF ORIGINALITY

I certify that the intellectual and written content of this thesis is a collection of my original, independent work excepted where it has been acknowledged. This thesis has not been submitted elsewhere for any other degree.

Rohan Kumar

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ABBREVIATIONS

∑	total; the sum of
µ	micro
<	less than
=	equals
>	greater than
/	divide
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
ATP Synthetase	Adenosine Triphosphate Synthetase Complex V Enzyme
CI	Cold ischaemia
CP	Chronic pancreatitis
CS	Cold (static) storage
DCD	Donation after Circulatory Death
ECD	Extended criteria donor(s)
ELISA	Enzyme Linked Immuno-Sorbent Assay
ELT	Experiential Learning Theory
ENPP	*Ex vivo* Normothermic Porcine Pancreas
FBS	Foetal Bovine Serum
FDA	Fluorescein Diacetate
g	grams
H&E	Haematoxylin and Eosin
HBSS	Hank's Balanced Salt Solution
HMP	Hypothermic Machine Perfusion
HTK	Histidine-Tryptophan-Ketoglutarate Solution
IAT	Islet autologous transplantation
IMS	Industrial Methylated Spirits
IPMN	Intra-ductal papillary mucinous neoplasm
kPa	kilopascal (i.e. unit of pressure)
MEM	Minimum Essential Medium
mg	milligrams
$mL.hr^{-1}$	Millilitres per hour
$mL.min^{-1}$	Millilitres per minute
mmHg	millimetres of Mercury (standard unit of blood pressure measurement)

IPTR	International Pancreas Transplant Registry
mM	millimoles per 1 Litre (i.e. millimolar concentration)
MP	Machine Perfusion
NMP	Normothermic Machine Perfusion
p	Probability value
pH	$-\log_{10}[H^+]$
p=NS	Probability value is non-significant
P_aO_2	Partial Pressure of Oxygen in the artery (aorta inflow segment)
P_vO_2	Partial Pressure of Oxygen in the vein (Portal venous outflow)
SCD	Standard criteria donor(s)
SD	Standard Deviation
SOP	Standard Operating Protocol / Procedure
TBS	Tris buffered Solution
TP	Total pancreatectomy
TPIAT	Total pancreatectomy with islet auto-transplantation
$U.L^{-1}$	International Units per Litre
UNOS	United Network for Organ Sharing
UW	University of Wisconsin Solution
WI	Warm ischaemia

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CHAPTER 1: INTRODUCTION

1.1 AN OVERVIEW OF PANCREAS AND ISLET TRANSPLANTION

1.1.1 BACKGROUND

In December 1966 the world's first pancreas transplant was performed in the USA at the University of Minnesota Hospital, Minneapolis [1]. Over the next five decades there have been 40 000 pancreas transplants worldwide with an annual rate of approximately 2000 [2] and pancreas transplantation has moved from an experimental to a routine clinical procedure. Despite these advances however, pancreas transplantation lacks the clinical impact of either kidney or liver transplantation principally because the absolute numbers transplanted are much smaller.

Pancreas transplantation is employed for the treatment of diabetes mellitus. Traditionally, patients suffering with immune mediated loss of their insulin-producing islets of Langerhans were considered candidates for a pancreas transplant. In these patients, a transplanted pancreas may restore the physiological hormone profile and ameliorate the long term end organ complications of diabetes mellitus. In addition to the traditional indications approximately 7% of pancreas transplants are also carried out in a subset of patients with obesity and peripheral insulin resistance (Type II diabetes mellitus) [2-4].

Pancreata have been transplanted alone, simultaneously or following a previous kidney transplant. Simultaneous pancreas and kidney transplantation is from a single deceased organ donor, usually in the setting of the recipient suffering from diabetes mellitus and end stage chronic renal failure. Simultaneous pancreas and kidney transplantation accounts for 80% of total pancreas transplant procedures. A pancreas may also be transplanted following a previously established kidney transplant from a living organ donor and this sequential pancreas

transplantation accounts for 10% of all cases. Those patients with normal kidney function but who suffer with severe diabetes related life-threatening complications or rapidly progressive diabetes may undergo a pancreas transplant alone which makes up the remainder of cases [2, 3].

In islet allo and auto transplantation, the insulin producing endocrine component of the pancreas is 'infused or transfused' following digestion of the gland and isolation of the islets of Langerhans. This strategy of transplantation of only the endocrine component of the pancreas has advantages over whole organ transplantation because it avoids the morbidity consequent upon complications from the exocrine component of the organ. Islet transplantation from donor pancreata is called allogenic islet transplantation and two or more donors are generally required together or sequentially to achieve glucose homeostasis.

When islets are re-transplanted into a patient immediately following a total pancreatectomy (TP) for conditions such as chronic pancreatitis (CP) this is referred to as autologous islet transplantation or islet auto-transplantation (IAT). CP is a progressive inflammatory condition which frequently leads to intractable abdominal pain. In selected patients a total or near TP may be the only means of achieving a satisfactory quality of life, reducing or abolishing the need for opiates. TP is a major procedure with the potential for significant morbidity but it does nevertheless remove the root cause of the pain. Without an IAT, the procedure leaves the patient devoid of beta cell mass and consequently diabetes which can be very difficult to control, often described as "brittle". Following TP there is in addition the total loss of pancreatic exocrine function, although in the vast majority of patients considered candidates for surgery for CP, exocrine function is already minimal or absent [5]. TP with islet autologous transplantation (TPIAT) is now an accepted treatment modality for CP in patients with normal or near-normal oral glucose tolerance tests. Patients must be rigorously assessed and a multidisciplinary approach is essential prior to consideration of surgery [6].

For islet isolation, following resection of the gland, the pancreas is digested and islets are isolated with or without purification. The islets are then returned into the patient (autologous) or infused into the recipient (allogenic), usually via the portal vein directly into the liver, accessed radiologically via a trans-hepatic approach. Portal pressures are monitored during the infusion to avoid portal hypertension and/or acute portal vein thrombosis [7].

TPIAT was previously undertaken almost exclusively for CP but recently has been considered and performed for an increasing number of conditions including benign cystic lesions [8], pancreatic trauma [9], premalignant conditions such as intra-ductal papillary mucinous neoplasms (IPMN). Usually TPIAT is indicated for hereditary conditions if gene mutations result in CP or recurrent acute pancreatitis and include; cystic fibrosis, protease trypsin 1 and serine protease inhibitor Kazal type 1 gene mutations [10]. Even overtly malignant conditions have been treated by TP (or segmental pancreatectomy) arguing that the presence of malignancy should not, in itself, be an exclusion criterion for IAT [11].

IAT requires the combination of a high volume pancreatic surgical unit and expertise in islet isolation. In 2001, the International Islet Transplant Registry reported that a total of 240 IAT operations had been performed by 15 centres across the world although only four centres had successfully carried out more than 5 cases; Minneapolis 54, Leicester 34, Geneva, 14 and Indianapolis 11 cases [12]. By the end of the following decade seven centres had contributed to the literature to report on their clinical experiences with six or more IAT cases [13].

Historically, islet transplantation (irrespective of whether it is autologous or allogenic) has been viewed as 'inferior' to whole organ transplantation in respect of the ability to achieve an adequate islet mass to reverse diabetes and restore glucose homeostasis. Generally with

allogenic islet transplantation more than one donor is required to achieve insulin independence and in IAT, the presence of impaired glucose tolerance prior to pancreatectomy is considered a contraindication to islet transplantation [14]. In contrast whole organ pancreas transplant confers a greater chance of achieving insulin independence from a single donor although there have been significant improvements which have questioned this paradigm. Islet isolation and immunosuppression in allogenic islet transplantation and in combination with improved technical aspects in IAT have led to reproducible, high insulin independence rates following islet engraftment [15].

1.1.2 WORLDWIDE PANCREAS TRANSPLANTATION ACTIVITY

By the beginning of 2011 almost two thirds of the 40,000 worldwide pancreas transplants had been performed in the United States of America. The data from the International Pancreas Transplantation Registry (IPTR) with respect to the pancreas transplants that have taken place in the USA are accurate because it is shared data with the United Network for Organ Sharing (UNOS). Data reporting to UNOS is a compulsory obligation as opposed to the results following pancreas transplants outside of the United States which are voluntarily reported to the IPTR. It is estimated that this represents about 90% of the non-USA reported pancreas transplants. Nevertheless despite this under reporting there is a still a discrepancy between utilisation of pancreas transplantation internationally and in the USA [2, 4, 16].

Analysis of the UNOS and the IPTR data demonstrated that pancreas transplantation appeared to have peaked in the late 1990's and early 2000's for pancreas alone and pancreas with kidney transplantation, respectively. Since the early 2000's however the annual numbers have steadily declined [2].

The current limiting factor for whole organ pancreas and islet transplant programmes across the world is the shortage of suitable donors. This shortage of suitable pancreata for transplantation makes it imperative to develop and establish reproducible and effective methods of pancreas preservation to ensure that all suitable organs are available for implantation [4].

1.1.3 THE OPERATIVE STEPS FOR PANCREAS RECOVERY AND TRANSPLANTATION

In the pancreas retrieval and transplantation process there are a number of important operative steps that warrant description and these are discussed in this section. The description refers to the retrieval process in the context of retrieval from the multi-organ abdominal donor. The pancreas is particularly susceptible to ischaemic damage and cold ischaemic tolerance in pancreas allografts, preserved with University of Wisconsin solution (UW) during retrieval, is limited to 20 hours for successful preservation. The median cold ischaemic (CI) time in the USA has been under 12 hours since 2006.

During retrieval it is routine practice to flush the donor duodenal segment with antiseptic or antibiotic solution, instilled via the donor's nasogastric tube. Duodenal contents must be sent for bacterial and fungal culture prior to flushing, as these cultures may be helpful in guiding antimicrobial therapy in the case of post-transplant sepsis. Povidone-Iodine solution may be toxic to the duodenal mucosa and therefore the duodenum is generally only flushed with this solution on the preparation bench prior to transplantation.

The removal of the donor organs *en bloc* is the quickest and safest retrieval method and the pancreas allograft is removed with the duodenal segment, spleen and liver. Following removal

and preservation the pancreaticoduodenal allograft can be prepared on the bench in the recipient centre. It is good practice to mark the gastroduodenal and splenic arteries and check the patency and collateral circulation by flushing them with preservative solution. The graft arterial inflow is from the gastroduodenal artery and splenic artery which are anastomosed to the donor iliac artery, usually fashioned as a 'Y' graft. Alternatively, the arterial supply of the pancreas is isolated on an aortic segmental patch, dissected with the coeliac axis. During the preparatory bench work, meticulous attention is given to the lymphatic tissue and small vessels around the pancreas and this reduces the risk of primary haemorrhage and lymphatic leakage post-operatively. The truncated duodenal segment staple suture lines may be inverted to reduce the risk of 'stump blow out' [17].

The pancreas is usually implanted in the patient's right hand side because the access facilitates the vascular reconstruction and subsequent implantation of the organ. This leaves the left iliac vessels free for the kidney to be implanted if a simultaneous pancreas and kidney transplantation operation is planned. The pancreatic vascular anastomosis is fashioned first as it has a lower ischaemic tolerance than the kidney.

Alternatively, the kidney may be placed extra-peritoneally on the left adjacent to the iliac vessels or even in the right iliac vessel intra-abdominally, distal to the pancreas anastomosis. If a kidney transplant has previously been sited on the right iliac vessels it is still preferable for the pancreas to be transplant on the right-hand side because of the natural lie of the pancreas to the left lateral side and it is in this case, placed cranially on the right iliac vessels, above the previously transplanted kidney. The donor iliac 'Y' graft, often offers flexibility when it comes to dealing with a recipient's calcified, atherosclerotic vessels which are commonly encountered in the recipient due to longstanding diabetes [17].

Following the arterial anastomosis consideration is given to the management of the venous and exocrine secretion drainage. The most common approach to the venous drainage from the

pancreas allograft is to anastomose directly to the systemic venous system at the level of the inferior vena cava. It is also possible to drain the allograft into the portal venous system, via the superior mesenteric vein by placing the allograft more cranially. Irrespective of technical issues the argument in favour of the portal system is based upon physiological and metabolic considerations. A portal anastomosis is intuitively more appropriate as it allows insulin drainage which reproduces the natural situation and avoids the hyperinsulinaemia associated with direct systemic venous drainage. From a technical perspective a portal venous anastomosis requires a longer donor iliac 'Y' graft in order to reach from a suitable arterial anastomotic site on the recipient iliac artery. Of note, in the obese recipient or those with a shortened small bowel mesentery, it may not be feasible to anastomose the venous drainage to the recipient superior mesenteric vein and therefore the option of a portal venous drainage is not available [17].

The early approach to the management of the pancreatic exocrine secretions was via drainage into the recipient's urinary bladder. It was believed that this was safer when compared to an enteric anastomosis because of the often catastrophic consequences of an anastomotic leak. Additionally, the ability to monitor urinary amylase levels was considered an advantage, allowing the earlier detection of allograft pancreatitis. In the USA enteric drainage of the pancreatic exocrine secretions has replaced urinary drainage because of the urinary complications which are often chronic and can lead to disabling symptoms. This differs from Europe where the enteric anastomosis for control of exocrine secretions has always been favoured. A pancreatico-enteric anastomosis between the duodenal segment of the pancreatic duct and small bowel can utilise any part of the small bowel using either a "Roux-en-Y" anastomosis or a side to side enteroenterostomy with the latter rapidly becoming more common [17].

1.1.4 COMMON COMPLICATIONS WITH PANCREAS TRANSPLANTATION

In stark contrast to renal transplantation, the results of pancreas transplantation demonstrate a greater incidence and variety of complications and twenty-five to thirty percent of patients require a re-look laparotomy. This may be in part due to patient factors, such as the high rates of co-morbidity associated with a patient population predominantly suffering from long standing poorly controlled diabetes which results in poor wound healing and impaired resistance to infection. The pancreas graft is also unique because it contains and produces proteolytic enzymes and pro-enzymes which predisposes to complications including secondary haemorrhage, fistulation, anastomotic dehiscence, pancreatitis and vascular thromboses [17].

Pancreas graft venous thrombosis is twice as common as arterial complications and the usual cause of early graft loss occurring in approximately 5.1% to 7.4% of recipients. There are a number of factors that may be of importance in graft thrombosis and a randomised controlled trail suggests that tacrolimus may be associated with less thrombosis when compared to ciclosporin [18, 19]. Intravenous immunoglobulins [20] and the use of venous graft extensions for anastomosis to the portal vein are also associated with an increased risk of graft thrombosis [17].

Once graft thrombosis is confirmed, the treatment is re-laparotomy and not infrequently graft pancreatectomy. Occasionally, if the thrombosis is truly segmental, then the graft may be rescued, following a thrombectomy or segmentectomy, but this is rare. Anticoagulation with heparin as prophylaxis against vascular thrombosis of the allograft is not standard practice, because of the risk of bleeding and equivocal evidence in respect of the reduction in rates of thrombus [17].

Primary haemorrhage intra-operatively is 'revealed' following the release of the vascular clamps and reperfusion of the allograft. There may be multiple bleeding points and meticulous bench work prior to implantation is of paramount importance to minimise this issue. Attention must also be given to the lymphatics and tiny surface vessels as well as the major in and out flow vessels. The other type of primary 'reactionary' haemorrhage, occurs within the first few hours following implantation and is usually a result of the pancreatic exocrine secretions leaking from the surface of the allograft which then comes into contact with the small vessels or major vascular anastomoses. Primary haemorrhage is the commonest reason for a 'return to theatre' although in comparison with thrombosis, haemorrhage rarely results in graft loss. True secondary haemorrhage is uncommon, but can be catastrophic when it does occur. it is often due to the formation of a pseudoaneurysm which subsequently ruptures or as a result of anastomotic dehiscence following direct exposure to leaking exocrine secretions [17].

Immunosuppression following transplantation is associated with opportunistic infections in the recipient. *Cytomegalovirus* occurs more commonly following pancreas transplantation than liver or kidney transplantation and antiviral prophylaxis is mandatory when the donor and recipient are mismatched for *Cytomegalovirus*. Other infections from fungi or bacteria can occur following transmission from the donor or precipitated by an anastomotic dehiscence. It remains unclear whether duodenal segment washout and decontamination during graft retrieval are of any benefit but as discussed above a sample of the duodenal commensal flora should be taken and sent routinely for analysis to aid in guiding antimicrobial therapy in the event of an episode of sepsis post-operative. Some centres undertake peritoneal lavage with antibiotic or antifungal agents following implantation, although again there remains no objective evidence to support this practice in terms of reduced infection rates post-operatively [17].

Acute graft rejection remains difficult to diagnose and from data reported to the IPTR is estimated to occur in up to 25% of cases [4, 21]. Most clinical signs and symptoms of acute rejection of a pancreas transplant are non-specific and thus make it difficult to diagnose and in addition changes in glycaemic control in the early post-transplant period may be due to allograft pancreatitis rather than early graft rejection. In patients that undergo a simultaneous pancreas and kidney transplant there is the potential for acute rejection to be diagnosed at an earlier stage due to the relative ease of diagnosing kidney rejection. This may help to explain in part the improved survival rates of allografts when the pancreas is transplanted simultaneously with a kidney compared with pancreas transplant alone. A late sign of allograft dysfunction is hyperglycaemia as a result of pancreas dysfunction that occurs from rejection.

The mainstay of the diagnosis of rejection is a 'core biopsy' of the allograft obtained by radiologically controlled image guidance. The *Banff Schema* is a standardised nomenclature for grading graft biopsies and confirming rejection [22, 23]. Treatment of presumed or proven acute graft rejection is with a steroid bolus (prednisolone 500 mg to 1000 mg) over a period of three days, or if the rejection process is steroid resistant, then treatment is with an infusion of antibodies (monoclonal or polyclonal). Acute graft rejection is an important risk factor for long term graft loss resulting from chronic rejection. Micophenalate mofetil with tacrolimus or in combination with ciclosporin is a better regime at preventing rejection than ciclosporin and azathioprine [15, 24].

Cold static storage (CS) for pancreas preservation is followed by reperfusion injury which results in allograft pancreatitis with oedema and inflammation. There is no accepted definition of rejection in terms of elevated serum amylase levels, however when re-laparotomy is required some degree of allograft oedema is always encountered although allograft pancreatitis is rarely life or graft threatening. Pancreatitis and acute rejection may both result in an oedematous

and swollen graft initially and often have an association with abdominal pain and a slight elevation in serum amylase, making them difficult to distinguish from one and another [17]. If the pancreas graft has been drained into the urinary bladder in the presence of bladder autonomic neuropathy, then a high intra-vesicle pressure may cause 'reflux' pancreatitis. This is managed by urinary catheterisation in the acute setting. In the long term, the exocrine anastomosis may need revision to an enteric diversion.

As previously discussed in the technical aspects of the operation, exocrine drainage is sometimes performed via the bladder, which can result in a number of complications including a chemical cystitis, an increased risk of urinary bladder infections, chronic transfusion dependent haematuria and long term dysuria. In men, the longstanding urethritis can progress to urethral disruption and the removal of irreversible loss of exocrine secretion via connection to the urinary bladder results in chronic dehydration and acidosis [17].

1.2 WHY IS THERE A NECESSITY FOR ORGAN PRESERVATION?

When clinical organ transplant programmes were being developed, both the donor and recipient were in the same hospital which greatly facilitated the direct transfer of the graft without the need for advanced preservation beyond simply the 'flushing' out of blood from the organ. With the advent of national and international transplant programmes organ preservation became necessary and with time the required preservation period became progressively longer. The development of prolonged, effective preservation periods allowed organs to be transported over greater distances and stored at the recipient site if the implantation was delayed. The ability to maintain organs in a suitable condition for implantation also allows for a more detailed approach and hence improved tissue matching. Organ preservation may only be deemed 'adequate' retrospectively when the organ has been implanted into the recipient and demonstrated to function adequately.

Initially, organs were preserved by rapid cooling and storage 'on ice' but this method of hypothermic and hypoxic organ preservation has been shown to cause significant tissue damage. This prompted an active programme of development, investigating a number of different techniques and preservation solutions. Over recent decades this research has resulted in improvements in both the short and long term outcomes for organ transplantation with the result that the greatest remaining challenge is not rejection or failure of the transplant process but the supply of suitable donor organs. As a consequence, there is an ever-increasing mismatch between the supply and demand of suitable organs. The response of transplant centres to this mismatch is research exploring the use of older, 'higher risk' organs (which may be prone to early graft failure) and in addition attempts to optimise the condition of these organs and even potentially reverse some of the consequences of warm and cold ischaemia [25].

1.2.1 THE VARIOUS TYPES OF ORGAN DAMAGE IN TRANSPLANTATION

If an organ remains at body temperature (normothermic) following circulatory arrest, then cellular metabolism will continue for a period of time. In the absence of venous drainage respiratory metabolites accumulate and without any arterial inflow nutrients and oxygen for metabolism cannot be delivered. As a consequence, anaerobic metabolism occurs and a stepwise mechanism of cellular injury takes place to differing degrees during all organ recovery. 'Higher risk' organs are particularly prone to this injury process and may be rendered unusable relatively easily. The injury begins with the death of the donor and continues during organ recovery and preservation until successful transplantation has occurred. This organ injury is due either to a total absence or an inadequate supply of oxygen and nutrients. A reduction in the supply of nutrients and oxygen leads to the rapid depletion of intracellular energy stores, a loss of cell membrane integrity due to osmotic swelling, cellular acidosis and ultimately cell death. This type of cellular damage is defined as warm ischaemia (WI).

The simple act of lowering the temperature of an organ following retrieval will reduce the cellular metabolic rate and in turn improve the WI damage although even at cold temperatures cellular metabolism continues, albeit at a much lower level. The hypothermia and hypoxia leads to alterations in cellular metabolism with the synthesis of adenosine triphosphate (ATP) but causes the sodium-potassium cell membrane pumps to slow down and allows passive, unopposed entry of sodium into the cell. The increased intra-cellular sodium causes the entry of water into the cell by osmosis and the subsequent swelling causes cellular breakdown and lyse. This type of cellular damage during cold preservation of organs is called cold ischaemia (CI).

When the donor organ is implanted into recipient and 're-connected' with blood at body temperature and physiological pressures, another type of injury results known as reperfusion injury. The mechanisms of reperfusion injury have been studied within the context of liver and kidney transplantation and the pathophysiology is multifaceted, involves similar processes and is observed in both organs. Upon organ reperfusion following implantation, there is an up-regulation of pro-inflammatory cytokines and endothelial adhesion molecules, which include P-selectin and intracellular adhesion molecule-1 [26]. The microcirculatory blood inflow is impaired because the inflammation and upregulation of adhesion molecules cause leucocytes and platelets to become activated and adherent to the vessel wall. These adherent white cells and platelets cause a reduction in perfusion of the graft resulting in on-going ischaemic injury and cellular ATP depletion. Additional damage occurs with the production of reactive oxygen species and proteolysis from enzymes released from activated neutrophils [27].

1.2.2 THE VARIOUS CLASSIFICATION OF ORGAN DONORS

In standard criteria donors (SCD), following brain death, both the WI and CI damage components are acceptable for transplantation. In extended criteria donors (ECD) and donation after circulatory death (DCD), the cellular injury may be so great that the organ is irrecoverable [25].

DCD donors are usually categorised using the Maastricht Criteria [28]. The DCD donors have a higher risk of delayed graft function and primary graft non-function in renal transplantation [29] and increased biliary complications and risk of requiring re-do surgery following liver transplantation [30]. Category I, II and IV are designated 'uncontrolled' DCD because of the long WI times in comparison with category III, DCD from withdrawal of supportive treatment (Table 1).

Table 1 Maastricht categories for DCD organ donation

Maastricht category of deceased organ donors after cardiac death (DCD)	Description
I	Dead on arrival into hospital
II	Unsuccessful resuscitation within the hospital
III	Withdrawal of supportive treatment
IV	Cardiac arrest following established brain death

The tissue injuries associated with a prolonged WI time from these donors led to the development of new organ preservation techniques to reduce WI damage and resuscitate the donated organ. In SCD, the donor organ comes from a deceased, heart beating donor, following a diagnosis of brain death.

Conventional methods to preserve organs (following recovery until transplantation into the recipient) focus around 'flushing' the donor organ with specialist organ preservation solutions and cooling the organ in 'cold (static) storage' (CS) at ice temperature (0˚C to 4˚C). Although CS is the ubiquitous preservation technique across the transplant specialties; marginal grafts (ECD and DCD organs) are highly sensitive to preservation injury and the use of CS alone is increasingly seen as the limiting factor in graft success following transplantation [25, 31].

1.3 A REVIEW OF THE CURRENT METHODS IN PANCREAS PRESERVATION

1.3.1 COLD STATIC STORAGE

CS remains the 'gold' standard method for preservation of the pancreas following SCD and the technique is utilised in the majority of cases [32]. The principle of CS is the reduction of cellular metabolism and during cooling the cellular consumption of ATP is reduced and intracellular enzymes are inhibited which leads to a reduction in cellular degradation. Although cellular metabolism is reduced it does not cease completely and with increased 'cold storage' times some cellular injury does occur via the on-going, albeit diminished cellular metabolism. Preservation solutions are so-called because they aim to counteract these processes and reduce as far as possible this CI damage.

Belzer and Southard examined this problem almost 50 years ago and worked to develop a hypothermic preservation solution for the preservation of canine kidneys which contained a high concentration of sodium, gluconate, adenine, ribose and glucose [33]. This solution was modified during the 1980s to the preparation referred to as University of Wisconsin solution (UW). UW has been used for the preservation of the canine pancreas with a high potassium concentration in order to maintain cellular ion balance. UW was designed to contain lactobionate to maintain osmotic concentrations due to being metabolically inert, phosphate buffers to assist in hydrogen ion stability, allopurinol and glutathione as antioxidants, hydroxyethyl starch as a colloid and adenosine as a precursor for continued ATP production during the CI phase of preservation [34, 35].

Preservation solutions may be classified according to their composition, particularly their sodium and potassium concentrations, type of cellular impermeants, hydrogen ion buffer

system, antioxidants and type of energy precursors they contain. The principle aim of all preservation solutions is to prevent cellular oedema, delay cellular destruction and to minimise ischaemic organ damage until reperfusion is established.

In pancreas preservation UW is the most commonly used preservation solution worldwide and despite being used for over a quarter of a century still remains the 'gold' standard preservation solution [32, 36]. However, a variety of preservation solutions are now available commercially and some solutions offer an alternative to the lactobionate UW system and contain citrate to chelate magnesium ions (e.g. Ross Marshall citrate, Soltran®). All these compound solutions aim to stabilise the extracellular environment to form an impermeable compound and they replace the glucose of UW with mannitol. Histidine-Tryptophan-Ketoglutarate solution (HTK) was developed as a cardioplegic solution by Bretschneider *et al*., in the 1970s [37] and in comparison with UW and Ross–Marshall citrate solutions, it has a much lower potassium concentration. The lower potassium content allows it to be safely introduced into the peripheral circulation and it has been adopted for kidney, liver and pancreas preservation and HTK also has a low viscosity which makes it ideal for organ 'flushing'. The composition of HTK is similar to that of extracellular fluid and all of the components occur naturally in the body. The histidine is an inert buffer in the extracellular space (histidine/histidine hydrochloride) and in clinical practice ketoglutarate supports anaerobic metabolism as a precursor and tryptophan for cellular membrane stability [38, 39].

There is one other preservation solution also used in pancreas allograft preservation which was initially developed for the preservation of cardiac allografts. Celsior has a high sodium content, a low potassium concentration, the main impermeant is lactobionate, histidine is the buffer and reduced glutathione is the antioxidant.

Currently in SCD pancreas allograft preservation, the most common and considered 'gold standard' preservation solution remains UW. There is an increasing body of data supporting the

use of HTK and Celsior as suitable alternatives and indeed they are preferentially used in some transplantation programmes. CS remains the dominant form of hypothermic pancreas preservation used in current clinical practice but there is certainly scope to further reduce the CI damage sustain during CS by the additional delivery of oxygen either by machine perfusion or directly in CS via the 'two layer method' and 'oxygen presufflation' [32, 39].

1.3.2 THE 'TWO LAYER METHOD' OF PANCREAS PRESERVATION

Oxygen delivery during organ preservation has the potential to allow for continued aerobic cellular metabolism and therefore reduces ischaemic damage. The 'two-layer method' of pancreas preservation describes the addition of oxygen carrier perfluorocarbons to the preservative solution although even in this context the preservative solution is usually UW. The perfluorocarbons have a high capacity for dissolving oxygen and they are denser than the preservation solution so two layers are formed spontaneously with the pancreas allograft suspended in between these layers with the oxygen being delivered to the perfluorocarbon layer continuously at a rate of 500 to 1000 $mL.min^{-1}$ [14]. There is some evidence that the two-layer method in pancreas preservation, both for whole organs [40, 41] and islets, may improve the ATP to precursor ratio [42, 43] although others have questioned the ability of oxygen to penetrate into deeper tissue layers [44]. Nevertheless, the two-layer method appears to have the potential to improve the CI damage sustained during pancreas preservation and warrants further investigation.

1.3.3 OXYGEN PERSUFFLATION AS A METHOD FOR PANCREAS PRESERVATION

Persufflation of an allograft organ with oxygen is another method of oxygen delivery during CS where filtered and humidified oxygen is bubbled through either the arterial inflow or venous

outflow. Scott 3rd *et al.,* have demonstrated that antegrade oxygen persufflation via the splenic artery in human pancreas and the coeliac ostium in the porcine pancreas has demonstrated even oxygen distribution, with restoration of ATP levels and a reduction of cellular necrosis [45, 46]. There remains however a paucity of evidence for the use of persufflation in the clinical setting and the safety profile of the technique has not been established and requires detailed further study (and comparisons with established methods of CS) before it can be introduced into the clinical setting.

1.3.4 MACHINE PERFUSION FOR ORGAN PRESERVATION

Machine perfusion (MP) requires a device to pump preservation fluid or blood through the organ following retrieval and can be performed either normothermically (NMP) or hypothermically (HMP). With MP, the organ is kept metabolically active during storage which potentially prevents the injury associated with CS. MP also has the potential to repair and even wash out the toxic metabolites that accumulate during ECD and DCD organ recovery. In *ex vivo* MP, the organ is connected and perfused immediately following retrieval until the time of transplantation. Alternatively, the retrieved organ is preserved in CS solution initially and then connected to the MP system for a short period immediately prior to transplantation [25, 31, 47].

MP as a means of organ preservation has been successful in the context of human kidney and lung transplantation. In both these fields, organs deemed unsuitable to meet the criteria for SCD, have been successfully transplanted following a period of MP preservation. The Toronto Lung Transplant Program, Canada [48] and The Heart and Lung Centre, Sweden [49] reported that rejected donor lungs, following *ex vivo* perfusion, performed similarly to lungs that were initially selected for transplant. Twenty one discarded human lungs were connected to MP for

four hours of *ex vivo* NMP and then successfully transplanted into human recipients [48] and these results suggested a potentially important role for *ex vivo* perfusion in lung salvage.

Eighteen patients have each undergone a successful kidney transplant from an ECD organ which was preserved using NMP immediately prior to transplantation [50]. Furthermore, the Leicester Renal Transplant unit has developed an *ex vivo* porcine kidney perfusion model. This porcine model has facilitated their understanding of MP within the context of a renal transplant programme and Leicester has pioneered the use of MP in a bench to bed-side fashion from a porcine MP model to use in clinical practice. A similar MP circuit to the one used for porcine perfusion (an oxygenated red cell–based perfusion) has been employed with kidneys from marginal human donors to restore the graft circulation *ex vivo* prior to clinical transplantation [50].

The pancreas is a 'low flow' organ with a delicate endothelium which is intolerant of sheer flow stress and the use of MP within the context of pancreas preservation for whole organ or islet isolation has therefore not been extensively studied. As a consequence there remains considerable scope for optimising both the perfusate and perfusion parameters in experimental MP of the pancreas to determine whether the results from clinical renal and lung transplantation can be reproduced [14]. Furthermore, experimental models of MP of the pancreas may provide the foundations for future *ex vivo* pancreas preservation prior to transplantation [32, 51]. The particular attraction with MP use in pancreas preservation is the potential to improve the preservation in marginal grafts. This potential benefit will result from the continued circulation of oxygen and nutrient with maintenance of vascular inflow and endothelial protection. MP will also allow a detailed assessment of allograft viability, therapeutic manipulation and gene therapy with substrates such as those that may reduce

ischaemic reperfusion injury or anti-apoptosis (e.g. p52 inhibitor pifithrin-alpha) and facilitate long distance organ sharing [52].

MP in pancreas preservation is currently being investigated in the pre-clinical experimental phase and more detailed studies are required to establish the optimal perfusion parameters. Key variables that need to be specifically addressed are pressure, flow, perfusate temperature, duration of perfusion and composition of the perfusate solution. Eventually MP may revolutionise pancreas graft preservation especially within the ECD and marginal graft setting, but considerable research into all the potential variables is still required [52].

1.4 A REVIEW OF EX VIVO PORCINE PERFUSION MODELS AND THEIR RELEVANCE TO TRANSPLANTATION

1.4.1 INTRODUCTION AND ADVANTAGES OF EX VIVO PORCINE PERFUSION IN TRANSLATIONAL RESEARCH

The porcine organ system is similar (in terms of size and density) to human organs and porcine organs are readily available and inexpensive. They may even be recovered from a commercial abattoir as a by-product of meat production negating the ethical implications of using live animals for experimentation. The use of porcine organs dates back at least 2500 years to the time of Aristotle and Erasistratus, who both performed studies on live animals [53]. In 2nd century Rome, Galen dissected live goats and pigs to become the father of vivisection [54].

Ex vivo perfusion systems describe models which are disconnected from extrinsic regulatory control mechanisms (e.g. neuro-hormonal, chemical) and this allows for detailed evaluation of the organ and its response to different situations without the confounding systemic responses that are present with *in vivo* or clinical studies. *Ex vivo* porcine models are of particular relevance in transplant surgery, since the process of organ recovery causes both WI and then CI damage from storage in ice cold preservative solutions (CS) followed by a period of MP in a laboratory. This mimics the entire transplantation retrieval process.

Ex vivo porcine perfusion models allow for the reproduction of physiological and metabolic conditions and the manipulation of haemodynamic parameters in a similar fashion to *in vivo* models but with greatly reduced costs and ethical considerations. *Post mortem* animal studies also avoid the lengthy application process for ethical approval which is a requirement of live animal studies [55].

The porcine model is often appropriate as a model for human studies as organ size is comparable and facilitates comparison and application to clinical trials something which is not generally possible with data from other smaller animal models, such as the murine model. In addition, and of relevance to allogenic transplant surgery the porcine model often has a greater similarity in its genome sequence to humans [56]. Twenty-two papers have been published on this topic and are summarised (Table 2). The studies all discussed *ex vivo* porcine organ perfusion within the context of transplant preservation surgery: eight liver, three kidney, three lung, two pancreas/islet, four combined liver–kidney multi-organ models, one small bowel and one cardiac (Table 2).

1.4.2 PORCINE LIVER PERFUSION

Six of the eight liver perfusion studies examined NMP, while two used HMP. Markers of ischaemic reperfusion injury were investigated in two studies the first of which argued that hyperglycaemia in the perfusion fluid was due to mobilisation of liver glycogen and a greater ischaemic insult to the liver led to an increased hyperglycaemia [57]. The second study investigated incremental WI damage in a DCD HMP model. Liu *et al.,* developed a 'damage index' score following multifactorial analysis of biochemical and haemodynamic parameters in the perfusion fluid [58]. Four studies compared MP against CS as a method of liver preservation. One of these investigated MP against CS in a 'split liver' DCD model [59]. Three studies compared CS against MP in a DCD liver model as a preservation technique prior to transplantation. Two of these studies concluded that function was maintained [60, 61] and that NMP may even be superior to CS preservation [62].

One study investigated the feasibility of NMP in porcine liver and presented data on technique and hazards in 40 successful models [31]. One study developed a steatotic liver perfusion model by feeding pigs with a high fat diet and streptozotocin prior to DCD liver recovery [63].

1.4.3 PORCINE KIDNEY PERFUSION

The renal transplant group in Leicester investigated the role of *ex vivo* porcine kidney perfusion and the role of a short period of NMP to resuscitate the kidney by replenishing adenosine triphosphate levels prior to transplantation. They compared CS against a short period of NMP [64] and further investigated the effects of different arterial perfusion pressures [65] and role of a nitric oxide synthetase inhibitor [66].

1.4.4 PORCINE LUNG PERFUSION

Three papers discussed porcine lung perfusion within the context of transplantation with one paper described a 'biodome' to house the retrieved DCD lung such that it could be perfused and simultaneously connected to a ventilator. Nelson *et al.,* argued that their model was suitable to investigate acute lung injury and investigate effects of trans-tracheal aerosolised agent delivery [56]. Meers *et al*., focussed on developing an 'aspiration' model of lung perfusion by artificially filling lungs with gastric contents and comparing them with control porcine lungs [67] while Mulloy *et al*., compared CS against NMP as a modality of lung DCD preservation [68].

1.4.5 PORCINE CARDIAC PERFUSION

One paper discussed the resuscitation of five porcine hearts following cardiac arrest, induced by the cessation of ventilation. The hearts were subjected to fifteen minutes of WI to mimic a

cardiac DCD model of transplantation. The return of satisfactory contractility and improvement in perfusate lactate concentrations was demonstrated following NMP [69].

1.4.6 PORCINE PANCREAS PERFUSION & ISLET ISOLATION

Two papers investigated the effects of HMP of the pancreas prior to digestion and isolation of islets. In the first of these papers following HMP the pancreas became oedematous prior to islet isolation and they argued that the oedema may actually be beneficial in the digestion and islet isolation process [70]. The second paper assessed islet viability following a 24 hour period of pancreas HMP and although islet viability was increased following HMP when compared with controls but this did not reach statistical significance [71].

1.4.7 PORCINE SMALL BOWEL PERFUSION

A model for the HMP of eight metres of small bowel was reported by one group. The perfusion machine had two circuits, one circulating hypothermic preservation solution through the bowel mesentery whilst the other circulated it through the lumen of the bowel in a pulsatile fashion, mimicking peristalsis. HMP compared favourably against CS of small bowel in terms of inflammation within the graft [72].

1.4.8 PORCINE MULTI-ORGAN LIVER & KIDNEY COMBINED

Chung *et al.,* developed a multi-organ *ex vivo* perfusion model by combining their established NMP of porcine liver model with a kidney, recovered from the same animal. They argued that the combination of a kidney led to a more stable biochemical milieu and more favourable perfusion when compared with NMP of liver alone. The cytokine profile was shown to be

improved, presumably by filtering the metabolites produced from liver. Furthermore, they went on to develop a functional 24 hour porcine liver perfusion model [73-76].

Table 2 Summary of the Porcine *Ex Vivo* Transplantation Models

Reference	Organ	Experiment focus	Experimental design	Conclusions
[77]	LIVER NMP	Investigated the effects of hypoxic MP by using an isovolumic but haemodiluted perfusate.	Hypoxia created by isovolumic haemodilution of autologous blood collected at time of liver retrieval in 12 porcine models. Biochemistry collected at regular intervals and compared with undiluted perfusion models	Ischaemic reperfusion injury leads to mobilisation of glycogen and an increase in liver transaminases and lactate levels.
[61]	LIVER NMP	The NMP of livers as an alternate preservation method was compared with established CS.	4 hours of NMP immediately prior to transplantation of liver using German Landrace pigs.	NMP was found to be a viable method to preserve donor liver grafts. It was comparable to the CS method of preservation.
[63]	LIVER Steatotic liver model	Development of a steatotic liver model using high fat diet in combination with streptozotocin caused mild steatosis to develop in pig livers.	The MP of steatotic livers was compared against normal pig livers. Outcome measures were bile production, albumin, transaminases, triglycerides and percentage steatosis on histology.	NMP successfully preserved the steatotic liver and even facilitated the reduction of steatosis.
[60]	LIVER DCD model	A comparison of NMP against CS as method of liver preservation prior to transplantation	8 hours of NMP was compared against 4 hours of CS preservation. The outcome measures were: hepatocyte injury, hepatic artery perfusion and bile duct function.	A two-fold decrease in hepatic artery perfusion and an increase in bile duct necrosis rates in the CS organs compared with NMP group. Additionally, following transplant, the CS group had greater evidence of hepatocyte injury as marked by a rise in transaminase levels.
[31]	LIVER DCD model	Investigated the feasibility of NMP in a porcine liver model.	40 Yorkshire female pig livers were recovered following DCD and then underwent NMP. The study investigated feasibility of the technique as a suitable model.	There were minimal technical failures and minimal safety hazards in the 40 experiments. The use of porcine livers was concluded to be safe, simple and reliable.
[62]	LIVER DCD model	A comparison of NMP against CS as method of liver preservation prior to transplantation	One hour of warm ischaemia followed by 10 hours of CS compared against 10 hours of NMP with autologous blood. All livers were then re-perfused for 24 hours with blood to mimic transplantation. Outcome measures were histology of liver and biliary epithelium.	CS group had an increase rate of necrosis and ischaemic injury. The biliary epithelium in the NMP group actually showed evidence of regeneration with an increase in Ki-67 staining.

[58]	LIVER DCD model	The stepwise increase in warm ischaemic injury to livers was quantified.	Livers were subjected to warm ischaemic damage for 0 to 120 minutes and then underwent preservation using HMP for four hours. During HMP biochemical and haemodynamic markers of liver damage were measured in the perfusate.	Longer warm ischaemia caused the perfusate to become more acidotic and led to increased vascular resistance. Additionally, transaminases, redox-active iron, liver fatty acid binding protein were all increased in the perfusate. A damage index based on multi-factorial analysis was generated to quantify warm ischaemic and reperfusion injury.
[59]	LIVER Split liver transplant model	HMP preservation technique was compared against CS in a split liver transplant model	12 livers were recovered and six placed in CS at 4˚C for 100minutes and six placed on HMP at 20˚C to 22˚C. The left lobe was removed towards the end of the preservation time. The split liver was then transplanted into anaesthetised animal for 12 hours.	No significant difference in biochemical parameters between CS and HMP groups. However sinusoidal spaces were widened in the CS group as compared with the HMP group.
[64]	KIDNEY NMP	Warm ischaemic damage followed by CS was compared against NMP.	Following retrieval, porcine kidneys were subject to CS at 4˚C for 24 hours (control group) and compared with 23 hours of CS at 4˚C and 1 hour of NMP at 38˚C. Following the period of preservation, 3 hours of reperfusion was used to assess the difference in preservation technique.	The NMP group of kidneys had better metabolic function and reduced tubular injury. Heat shock protein 70 and Interleukin 6 were also upregulated in the NMP group arguing NMP may upregulate mechanisms that condition the kidney.
[65]	KIDNEY NMP	Arterial perfusion pressures during NMP of kidney were investigated. They aimed to optimise perfusion pressure in renal NMP.	Porcine kidneys were subject to CS at 4˚C for 24 hours (control group) and compared with 23 hours of CS at 4˚C and 1 hour of NMP at either 55 mmHg or 75 mmHg mean perfusion pressures. Following the period of preservation, 3 hours of reperfusion was used to assess the difference in preservation technique.	Less tubular damage and less endothelial injury occurred in the 75 mmHg mean arterial perfusion pressure when compared to the 55 mmHg mean pressure group.
[66]	KIDNEY DCD model	Effects of inducible nitric oxide synthase inhibitor, 1400W, were investigated in ischaemia reperfusion.	Kidneys were recovered after 25 minutes of in situ warm ischaemic injury, then CS for 18 hours at 4˚C and then NMP for 3 hours with or without 1400W.	Improved renal function and reduced oxidative stress in the 1400W group
[56]	LUNG DCD Model	Ex vivo lung perfusion within the context of transplantation were investigated.	Porcine lung placed in a 'biodome' and connection to a centrifugal pump, a membrane oxygenator and leucocyte filter. Additionally the 'biodome' was developed so that the attached porcine lungs could be ventilated via attachment to endotracheal tube.	Porcine ex vivo lung perfusion is feasible and may be used to evaluate organ function and useful to model an acute lung injury and investigate the effects of trans-tracheal and aerosolised agent delivery.
[67]	LUNG MP	The effects of gastric juice contents to mimic aspiration injury perfusion of lungs.	Lung graft function was assessed during two hour period of ex vivo perfusion when porcine lungs were subject to gastric content damage compared against no such damage.	Gastric content lung damage led to increased vascular resistance, reduction in pulmonary blood flow and decreased compliance.
[68]	LUNG NMP DCD model	Investigated the optimal timing for NMP in a DCD porcine lung model.	60 minutes of warm ischaemic damage in lungs and then stratified into three groups.1) CS preservative solution at 4˚C. 2) 4 hours of NMP. 3) 4 hours of CS at 4˚C and then 4 hours of NMP. After preservation lungs went on to be allotransplanted for fours to assess ischaemia-reperfusion.	Oxygenation, mean airway pressure and pulmonary artery pressure were all better in the donor lungs that were preserved by method 3) i.e. CS for 4 hours and then followed by 4 hours of NMP group. Additionally pro-inflammatory cytokines were reduced.

[69]	CARDIAC NMP DCD model	Ex vivo resuscitation and assessment of DCD porcine hearts. Induction of cardiac death occurred following cessation of ventilation. The porcine hearts were retrieved following 15 minutes of warm ischaemia.	Five porcine hearts were connected to MP to be perfused and oxygenated at normal physiological temperature for four hours. Lactate levels were used to monitor the graft.	Four grafts were successfully resuscitated following DCD cardiac arrest. Of these, three grafts had satisfactory contractility and lactate levels improved with NMP.
[70]	PANCREAS / ISLET HMP or CS of pancreas (prior to Islet recovery)	Juvenile Yorkshire porcine pancreata were retrieved with a warm ischaemic time of 30 minutes. Then subjected to various preservation methods.	24 hour preservation following warm ischaemic damage with different preservation solutions in HMP compared with CS. No preservation solution was set as a control. Following the 24 hour period of preservation the pancreata were digested and islets isolated. The preservation solutions were varied in turn and compared via CS against HMP.	24 hours of HMP was found to contribute to oedema of the pancreas. This oedema may improve the islet digestion yield. HMP was discovered to be a viable pancreas preservation method prior to islet digestion.
[71]	PANCREAS / ISLET HMP (Comparison of islet yield)	24 hours of HMP of pancreata was followed by islet isolation. The temperature of MP was 4°C to 8°C. The islet viability was then assessed.	Each pancreas acted as its own control as the model was a ‘split lobe’ model. One lobe was perfused, one lobe was not perfused and one lobe was stored at at 4°C for less than 3 hours. Oxygen consumption rate was compared to DNA as an assessment of islet viability following the preservation method.	Islet viability was deemed to be higher following HMP compared to controls, but did not reach statistical significance.
[72]	SMALL BOWEL HMP (8m of small bowel)	To develop a method of simultaneous perfusion of the small bowel mesenteric vessels and the lumen.	Developed a MP with a double circuit of preservation fluid. One circuit perfused the mesenteric vessels whilst the second circuit perfused the small lumen bowel simultaneously in a 'peristaltic' fashion. Histology was used to confirm viability of the graft for comparison with the control.	The intestinal preservation system with dual circuit was successful in maintaining viability of an 8 metre length of graft. Cold preservation solution was circulated with HMP (4˚C to 8˚C) for 8 hours and histology showed that the HMP preservation reduced inflammation in the graft.
[73, 75]	MULTI-ORGAN NMP Liver-Kidney	A combined, simultaneous liver and kidney NMP system was compared against an established liver model NMP system by Chung et al.	8 livers underwent NMP and were compared against 5 liver - kidney (combined simultaneous) NMP system. The group also went on to develop a 24 hour liver perfusion NMP system.	An additional kidney allowed for a more stable biochemical milieu and argued for more consistent reproducibility. Adding a kidney to the liver porcine perfusion circuit at normal physiological temperature provided a better biochemical environment, without worsening the cytokine profile because the kidney was found to filter the liver metabolites.

1.4.9 SUMMARY AND LIMITATIONS OF EX VIVO PORCINE PERFUSION

There are a number of experimental studies examining different organs which argue that *ex vivo* animal models are a suitable tool for the investigation of ischaemic reperfusion injury. *Ex vivo* porcine perfusion models lend themselves naturally as a suitable model for DCD transplantation with the organs usually recovered following electromechanical cerebral stunning and exsanguination which causes hypovolaemia-induced pulseless electric activity of the porcine heart. This leads to circulatory death and readily mimics the DCD transplantation process and it is at this point that the WI injury begins and the extent of injury has been the focus of a number of studies (as the independent variable) [31, 57, 58, 64, 69, 76, 77].

Several studies have endeavoured to make comparisons of preservation methods and the use of *ex vivo* porcine perfusion models has facilitated the comparison of established CS preservation techniques with HMP or NMP. In other studies, the optimisation of MP parameters, such as perfusion pressure or hypothermic versus normothermic perfusion have been examined. Finally, studies have focused on the optimal timing of a fixed period of MP with respect to CS, arguing that a short period is the optimal approach to 'wash out' toxic metabolites. All of these findings have led to an iterative approach to, and adjustment of translational research all facilitated by the use of *ex vivo* porcine models and the feasibility and reproducible nature of the porcine perfusion model was the focus of one publication [31].

Ex vivo porcine perfusion has also allowed the biochemical, haemodynamic and histological markers of retrieval injury to be investigated simultaneously potentially also paving the way to establish transplant organ injury scores or indices relevant to human clinical transplantation.

An ECD model using marginal grafts has been replicated in liver and lung experiments. Jameison *et al*., fed their pigs a high fat diet in conjunction with streptozotocin to induce steatosis prior to liver retrieval and NMP [63]. Since the incidence of liver steatosis is on the rise, the ability to re-create a suitable porcine model to investigate primary graft non-function in steatotic liver transplantation has implications in ECD transplantation. The aspiration of gastric contents into lungs is a common reason to discard human lungs for organ donation. A porcine perfusion model has been developed to further investigate how to mitigate this damage [67]. This model may be useful in the investigation of the effects of MP in order to precondition the graft prior to donation.

The effects of a variable WI times have been examined by delaying the recovery incrementally following circulatory death. Additionally, once organs are recovered and transported to the laboratory, there is the potential to examine the effects of ischaemic reperfusion injury at a cellular and histological level. There is additional scope for altering, observing and comparing the effects of a blood based perfusate against acellular perfusate in either normothermic or hypothermic models.

The use of an *ex vivo* perfusion model is limited to the lifespan of the organ which is usually several hours rather than days although the limited lifespan of porcine *ex vivo* organs may be improved by the use of commercially available perfusion kits. The use of these MP pumps has allowed for accurate and consistent organ perfusion parameters to be strictly maintained in either a continuous or pulsatile fashion, with autologous blood or preservation fluid. These sophisticated perfusion pumps and circuits have played a fundamental role in allowing researchers to maintain porcine *ex vivo* organs in a viable state for up to 24 hours *post mortem* [71, 76].

The interaction of autologous blood with the inert, non-biological surface of a commercially available MP pump creates a systemic response and the data must be interpreted in this context. With progressive iterations of the same experiment, the effects of any systemic response should be negated because they are the same between experiments. Repeated *ex vivo* experimental runs allow relative comparisons between preservation methodology, solutions or temperatures to be meaningful and amenable to statistical analysis. Several runs of the same experiment are inexpensive and easy to perform in an *ex vivo* porcine model when compared with an *in vivo* model.

In conclusion, porcine *ex vivo* perfusion models have allowed a significant number of organ preservation experiments to be investigated in an ethical, safe and inexpensive setting before being incorporated into clinical kidney and lung transplantation. From the lessons learnt in these two fields of translational transplant research, there may be scope for extrapolation to liver, pancreas, islet, small bowel and cardiac organ preservation and transplant research.

1.5 THE ANATOMY OF THE PORCINE PANCREAS

1.5.1 GROSS LOBULAR ANATOMY

The porcine pancreas is a retroperitoneal organ and is comparable anatomically to the human pancreas. The main gross anatomical variation when compared to the human pancreas is that the porcine pancreas has three lobes with irregular margins and a nodular surface. One study has described pancreatic anatomy from dissection of 65 porcine pancreata and found the mean weight of the whole trimmed pancreas was 347 +/- 103 grams with a range of 190 to 698 grams [78]. The lobes are designated as a 'splenic lobe', a 'duodenal lobe' and a 'connecting lobe'. The splenic lobe corresponds to the tail and body of the human pancreas. The duodenal lobe is analogous to the human head of the pancreas. The connecting lobe is analogous to the human uncinated process of the pancreas and is attached and associated with the portal vein anteriorly [78]. Often, a 'bridge' of pancreatic tissue connects the the splenic and connecting lobes (Figure 1A – image taken from Ferrer *et al*., 2008 [78]).

1.5.2 ARTERIAL SUPPLY

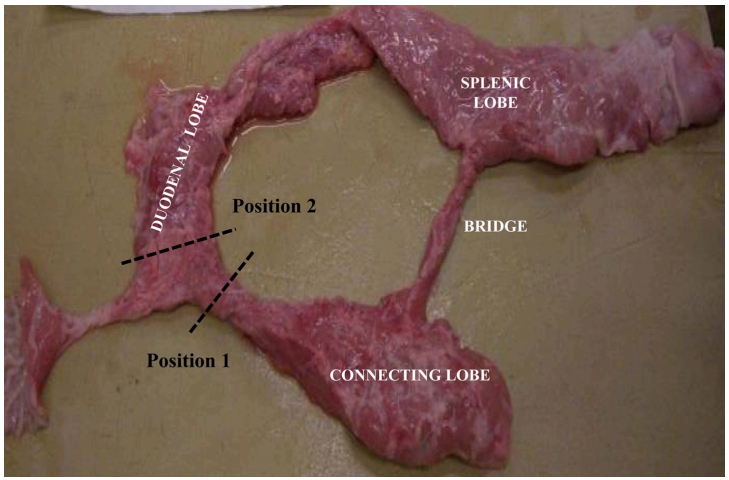
The arterial supply of the porcine pancreas arises from the abdominal aorta, which is retroperitoneal. The coeliac trunk is the first infra-diaphragmatic abdominal branch from the midline and similarly to humans gives rise to the common hepatic, left gastric and splenic arteries. The posterior pancreatic artery arises from the splenic or the hepatic artery [78]. The gastroduodenal artery arises from the common hepatic artery and supplies the region of the pylorus and duodenum. The gastroduodenal artery usually has two branches after bifurcation and these supply the duodenal lobe of the porcine pancreas via the superior pancreaticoduodenal and the right gastro-omental arteries. The inferior pancreaticoduodenal

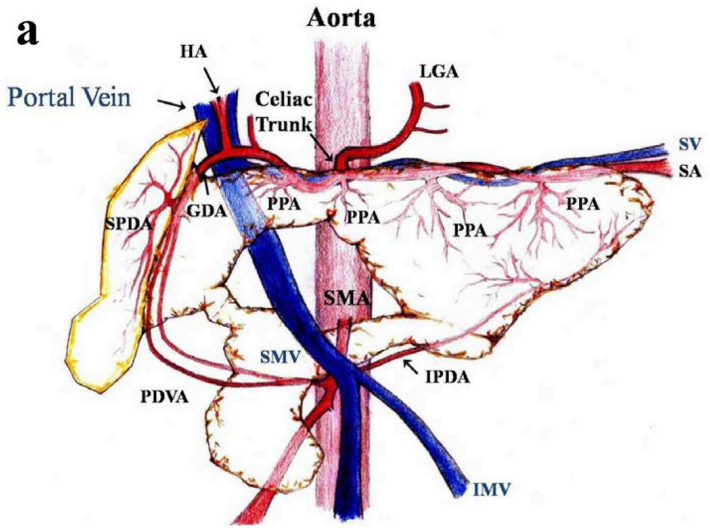
artery, arising from the superior mesenteric axis, forms an anastomosis with the superior pancreaticoduodenal artery. These two arteries form a 'pancreatico-duodenal vascular arcade' [78]. The superior mesenteric artery gives other branches: jejunal, right colic, and middle and ileocolic arteries. The connecting lobe of the pancreas and the 'bridge' of parenchyma are supplied by the inferior pancreatic artery, arising from the inferior pancreaticoduodenal arterial arcade, which in turn is from the superior mesenteric artery (Figure 1B – image taken from Ferrer *et al*., 2008 [78]).

1.5.3 VENOUS DRAINAGE

The splenic vein drains the body and tail of the pancreas and is similar to man in being partially encased in the pancreatic parenchyma. The splenic vein receives the left gastric, left gastroepiploic, inferior pancreaticoduodenal veins and small tributaries directly from the connecting lobe. The inferior mesenteric vein usually flows into the superior mesenteric vein which is a deviation from the usual anatomy of the venous drainage of the human gut. The gastroduodenal vein drains the duodenal lobe of the pancreas and it receives the superior pancreaticoduodenal vein following anastomosis with the inferior pancreaticoduodenal vein. The gastroduodenal vein then drains directly into the superior mesenteric vein immediately before its confluence with the splenic vein. The splenic vein and the superior mesenteric vein confluence marks the origin of the portal vein (Figure 1B – image taken from Ferrer *et al*., 2008 [78]).

Figure – 1A (Left) and Figure – 1B (Right). Figure 1A demonstrates the lobular anatomy of the porcine pancreas. There are three lobes: splenic, duodenal and a connecting lobe. Figure 1B demonstrates the vascular supply to and from the porcine pancreas. Positions 1 & 2 represent the two common routes that the pancreas duct takes in the porcine pancreatic parenchyma [78]. Abbreviations: hepatic artery (HA), left gastric artery (LGA), gastroduodenal artery (GDA), posterior pancreatic artery (PPA), splenic vein (SV), splenic artery (SA), superior mesenteric artery (SMA), superior mesenteric vein (SMV), superior pancreaticoduodenal artery (SPDA) inferior pancreaticoduodenal artery (IPDA), inferior mesenteric vein (IMV). Images have been taken from Ferrer *et al*., 2008. Referenced: [78]





Pancreatic Duct Anatomy

Ferrer *et al.,* used coloured saline infusion to delineate the pancreatic ductal anatomy and demonstrated that in 39% of specimens the duct in the connecting lobe was 'connected' to the other two lobes, splenic and duodenal. In 31% of cases the ductal communication only extended from the connecting lobe to the splenic lobe [78] and therefore taken together the connecting lobe was connected to the splenic lobe in 70% of their cases. When Ferrer *et al*., did not find a ductal communication between the connecting and splenic lobes then all communications were solely from the duodenal lobe to the splenic lobe [78]. In their study the connecting lobe duct was variably connected to the splenic and duodenal lobes. But in our experiments the splenic lobe was connected to the duodenal lobe in every case (unpublished observations).

1.5.4 PORCINE PANCREAS PHYSIOLOGY

The physiological role of the porcine pancreas is analogous to that of the human pancreas with essential functions as both an endocrine and exocrine gland. The endocrine portion of the pancreas is organised as discrete islets (of Langerhans) and contain four cell types. The most numerous are the beta islets and produce insulin. The alpha islets produce glucagon, delta cells produce somatostatin, F or PP cells produce pancreatic polypeptide. The endocrine component is predominantly concerned with glucose homeostasis [79].

The synthesis of insulin has two phases. In the acute phase, preformed insulin is released in response to an increase in blood glucose concentration. Alternatively, during a chronic phase, insulin pro-protein is synthesised and stored. The metabolism of insulin involves the proteolysis

of two chains, designated 'A' and 'B' which are the active peptides. The role of insulin in the pig is as an anabolic hormone [79].

Glucagon has three main important functions, decreasing glycogen synthesis and increasing glycogenolysis and gluconeogenesis. Glucagon synthesis is stimulated by a reduction in blood glucose concentrations [79]. The main function of somatostatin is to inhibit the hormonal secretion of the pancreas and inhibits the production of insulin, glucagon and pancreatic polypeptide [79].

Pancreatic exocrine secretions are stimulated by secretin and are independent of the release of insulin or glucagon, the basal concentrations of which are stable and non-fluctuating. Data from porcine physiological study have indicated that a sudden increase in glucose concentration of 20mM is sufficient to raise the insulin concentration and significantly suppress the glucagon output [80].

Jensen *et al.,* also carried out physiology experiments on the isolated porcine pancreas to investigate gastrin production and gastric secretion. Secretion was shown to be stimulated by all isomers of gastrin (gastrin-34, gastrin-17, gastrin-14 and component I) in a dose-dependent manner. Gastrin increased the exocrine secretion rates at *in vivo* physiological concentrations and the endocrine system was stimulated at abnormally high gastrin concentrations that mimicked levels found in gastrinomata [81].

Experiments investigating the effect of cholecystokinin on an isolated porcine pancreas revealed that it had an important role in pancreatic peptide production without significantly affecting the endocrine component [82]. These studies also demonstrated that the composition of pancreatic juice was similar to that of humans in terms of bicarbonate and protein concentrations [83]. The similarity of porcine and human anatomy, vascular supply,

drainage and physiological studies allow an *ex vivo* model to be utilised for studies of pancreas isolation and preservation.

1.6 THE CLINICAL PROBLEM: A NECESSITY FOR AN *EX VIVO* NORMOTHERMIC PANCREAS PERFUSION MODEL

1.6.1 INTRODUCTION

In human pancreas transplantation the major barrier at the present time is a shortage of suitable organs to meet the demand. This has led to the utilisation of 'high risk' marginal donor organs in order to make up the shortfall, although these have an increased susceptibility to early graft failure and post-operative complications. Pancreas preservation techniques assume even greater importance when using 'high risk' (DCD and ECD) marginal donors compared with SCD pancreata. Unfortunately, pancreas preservation techniques *per se*, are complex and associated with a number of problems largely due to the organ having such a propensity for ischaemic damage when compared with other transplanted organs. CS using UW is the 'gold standard' pancreas preservation technique and this has been the case for the last three decades. In order to use marginal grafts successfully in pancreas transplantation, it is likely that preservation techniques may need to evolve from simple CS and adopt MP preservation, which has produced significant progress in clinical renal and lung marginal donor transplantation.

With MP, the organ is kept metabolically active during storage, which may prevent the injury associated with CS and it also has the potential to repair and even wash out toxic metabolites that accumulate during ECD and DCD organ recovery. In *ex vivo* MP, the organ is connected to a circuit and perfused immediately following retrieval until the time of transplantation. Alternatively, the retrieved organ is preserved in CS solution initially and subsequently connected to the MP system for a short period immediately prior to transplantation [25, 31, 47]. MP as a means of organ preservation has been successful in the context of human kidney and lung transplantation and in both of these fields, organs deemed unsuitable to meet the criteria for SCD have been successfully transplanted following a period of preservation [48, 50].

Another issue when considering MP in the context of pancreas preservation is the technical parameters that are optimal for a 'low flow' organ. This is one of the reasons that MP for pancreas preservation is at such a rudimentary stage in comparison with kidney and lung transplantation, where it has evolved into a procedure that is being increasingly used routinely in clinical practice. The success of MP in renal and liver transplantation however is encouraging and it is hoped that similar progress in the field of pancreas transplantation may be able to increase the donor pool by facilitating the safe use of organs from the marginal 'high risk' donor.

A recent meta-analysis demonstrated a correlation between the absolute number of islet cells infused, islet equivalents per unit of body mass and islet size with increased rates of insulin independence following IAT [84]. The current immediate success of TPIAT relies on the recovered yield from the islet cell isolation process and the proportion of insulin producing islet cells which successfully engraft and survive following infusion [6, 32]. Pre-transplantation MP preservation techniques may help to address and optimise the islet yield and size in IAT and allogenic islet transplantation.

Unfortunately, there remains a paucity of reproducible and reliable experimentation models in pancreas whole organ and islet preservation and transplantation. Advances in pancreas preservation techniques do not compare to those made for other solid organs which are recovered for human transplantation and CS remains the only technique in widespread clinical use despite it being developed three decades ago [32, 51]. Furthermore, much of our understanding in the field of transplantation surgery comes from our knowledge of organ physiology, gleaned from studies in *ex vivo* porcine perfusion systems. To this author's knowledge, there is no such porcine pancreas MP model that has been established to perform and investigate techniques, both in whole organ pancreas preservation or for MP pancreata prior to islet isolation techniques.

1.6.2 RESEARCH METHODOLOGY, RESEARCH QUESTION AND NULL HYPOTHESIS

The overall aim of the thesis was to investigate whether machine perfusion in a porcine pancreas ex vivo model could be developed and if it was possible then to establish whether its use could ameliorate the ischaemic damage sustained during retrieval with particular emphasis on pancreas allograft and/or islet yield and viability?

The initial focus of this thesis was to establish an *ex vivo* normothermic porcine pancreas (ENPP) perfusion model. It was hoped that when established it would behave physiologically in the short term while accepting the limitations and advantages of an *ex vivo* perfusion system as discussed above. It was felt important that both endocrine and exocrine pancreatic function were preserved in the model during perfusion. Additionally, it was hoped that the model would be viable histologically for at least several hours *post mortem*. Chapters 2, 4 and 5 describe these aspects of the *ex vivo* porcine pancreas perfusion model in detail through the preliminary failed models and invaluable lessons learnt (Chapter 2), to the established physiological model in Chapter 4. In Chapter 5 the model is used to compare a single perfusion parameter, attesting to and confirming the reliable and reproducible nature of our *ex vivo* porcine pancreas physiological perfusion model to test a specific hypothesis. Chapter 6, describes how the methodology for islet isolation was established and the quantification of viability and yield, setting the scene for future planned work to investigate the effects of MP in the context of islet isolation and subsequent transplantation.

The linking of theoretical models with empirical data produces two types of research strategy; *deductive* and *inductive*. Deductive research is defined as theory first followed by research whereas inductive is research followed by theory [85]. Deductive research methods endeavour

to identify a relationship (*or the extent of*) a relationship that may exist between theory and the research findings [86]. The 'relationship' that may or may not exist is assessed by hypothesis-testing, which is subject to statistical rigour. Furthermore once a relationship is identified, it may then form the foundations for, or lead to the generation of a novel, alternative theory [85, 86]. Inductive theory in contrast focuses upon an empirical research entity in order to generate a novel theorem or model [86].

The inductive research phase of the thesis was concerned with establishing a reproducible porcine *ex vivo* perfusion model, to answer the following research question:

Is it feasible to establish a physiological ex vivo porcine pancreas model that is both reproducible and reliable?

Following on from this phase of inductive research, the model was used to address the following null hypothesis using a deductive research methodology, subjected to statistical analysis:

During ex vivo normothermic porcine pancreas machine perfusion, organ viability is no different when the porcine pancreas undergoes perfusion with normotensive pressure compared with a low pressure system.

CHAPTER 2: THE PRELIMINARY PORCINE MODELS AND LESSONS LEARNT

"By three methods we may learn wisdom: First, by reflection, which is noblest; Second, by imitation, which is easiest; and third by experience, which is the bitterest." Confucius (551BC – 471BC)

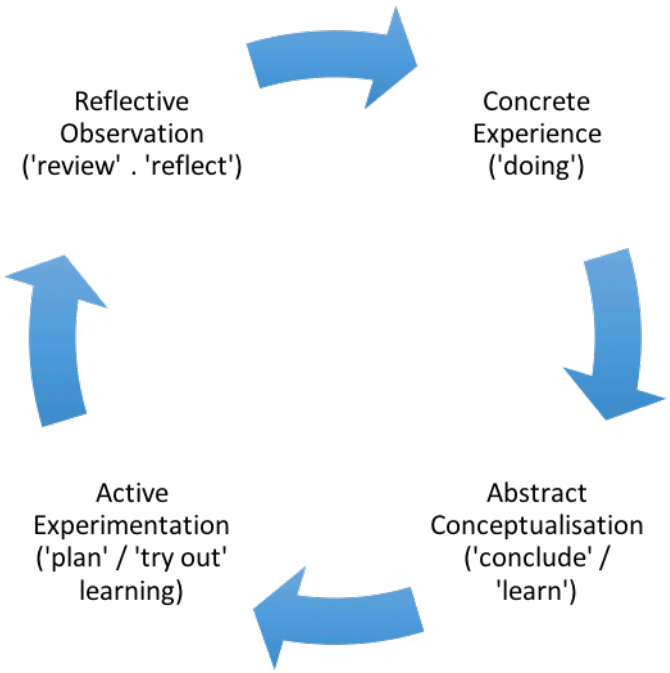
2.1 INTRODUCTION

In 1984, Kolb defined an Experiential Learning Theory (ELT) which combined three domains; *experience* with *cognition* and *behaviour [87]*. The ELT model has its roots in Confucius's learning by reflection theory; to learn without reflection is a waste and experiences must be transformed into learning via appropriate reflection [88]. Without reflection, learners may make the same mistakes repeatedly. Furthermore, reflection makes sense of the situation and confers a fuller comprehension, leading to richer learning and an enhanced learning effectiveness [88].

ELT consists of a four stage framework: *concrete experience* (active involvement in the activity), *reflective observation* (consciously reflecting back on the event), *abstract conceptualisation* (consider a theory or model of what is to be observed) and *active experimentation* (think about how to test this theory at the next experience) [89]. The process of using and application of Kolb's experiential learning cycle in the preliminary phase of the research in this thesis, has led to the establishment of the final perfusion protocol. The final protocol is defined as the standard operating protocol (SOP), described in the next chapter.

This chapter demonstrates the ELT cycle as applied to each iteration of the porcine perfusion

experiment and discusses the learning in turn. Eight porcine pancreata were recovered in the preliminary experiments with the aim of learning the anatomy and to develop the operative steps in the retrieval and perfusion protocol. The ELT with appropriate reflection was applied such that invaluable lessons were learnt and not repeated in the next experiment. This resulted in the minimisation of WI, the optimisation of the perfusion circuit, the reduction in graft thrombosis and important operative steps were understood. The detail of each iteration and the link to the stages in ELT has been tabulated at the end of this chapter (Table 3).



Schematic of the Experiential Learning Cycle, which was applied to each iteration of the eight preliminary porcine models [87].

2.2 MINIMISATION OF THE WARM ISCHAEMIC TIME

It has been argued that the pancreas is the most difficult organ to preserve. It suffers significantly from ischaemic damage and is associated with highest rates of primary non-function graft complication. Additionally, within the field of transplantation surgery, ischaemic reperfusion injury rates are highest for pancreas [17]. The kidney and liver, in contrast, have a better preservation, complication and ischaemic damage profiles.

Our laboratory has previously established expertise in *ex vivo* organ perfusion and preservation. From this knowledge, experience and understanding, Dr Chung *et al.*, have further highlighted the paramount importance of a minimal WI time [73, 74, 90]. In their liver perfusion and liver-kidney (simultaneous) perfusion models, WI was minimised by the early cannulation of the inflow artery for immediate delivery of ice cold preservation solution. This important principle of a 'controlled' and short WI time was observed. Improvements in this area were made but not without difficulty.

The arterial inflow to the pancreas was intially accessed via cannulation of the coeliac axis ostium. Although the delivery of ice cold preservation solution was feasible using this method, it was difficult to perform sufficient dissection quickly enough. The coeliac axis was invariably retroperitoneal and a safe dissection was lengthy even after the coeliac axis was identified and skeletonised. Subsequent cannulation of a vasoconstricted and non-compliant ostium, usually with a calibre of 1.5mm, was technically difficult and these two problems caused inefficient delivery of large volumes of preservation solution and resulted in a prolonged WI time.

Poiseuille's law of fluid flow dynamics for the efficient delivery of larger volumes per unit time (i.e. increased flow rate), is dictated via a larger cross-sectional area, a shorter length external

cannula and one with a greater pressure gradient. Applying this principle it was decided to avoid any further attempts to cannulate the coeliac ostium. Consequently an alternative approach was considered, to deliver preservation solution via a wide bore blood vessel such as directly via the abdominal aorta. Unfortunately rapid access to the abdominal aorta proper was fraught with difficulty, particularly adhesiolysis of crural lumbar muscles and the pre-requisite clearance of the diaphragmatic attachments within the retroperitoneum. Unfortunately these technical challenges again resulted in an prolonged WI time approximately equal to the original technique of coeliac axis cannulation.

After consideration of these issues and increased familiarity with the anatomy of the area it was decided to adopt a 'top down' approach. Access into the thorax was achieved via a median sternotomy, and subsequent division of diaphramatic and parietal pleural attachments conferred improved mediastinal access. The supra-hepatic inferior vena cava and oesophagus were clamped, divided and ligated. This allowed the thoracic (descending) aorta to be accessed for cannulation. This was followed by cannulation of the thoracic (descending) aorta, which was rapidly achievable by division between haemostats and then followed by cannulation. Access via the thoracic aorta allowed for the delivery of large volumes of ice cold preservation fluid and using this method of aortic cannulation, the delivery of ice cold preservation solution could be achieved within seven minutes from circulatory death.

Even using the 'top down' approach, further lessons were learnt. On one occasion the bespoke aortic cannula was inadvertently advanced beyond the coeliac and superior mesenteric axes. This was only later realised when there was a total absence of preservation solution within the portal outflow and the resultant thrombosed, non-functional graft served as an important lesson for future experiments. The tip of the cannula was advanced only as far as the beginning

of the abdominal aorta in subsequent experiments, taking care never to advance beyond the coeliac ostium.

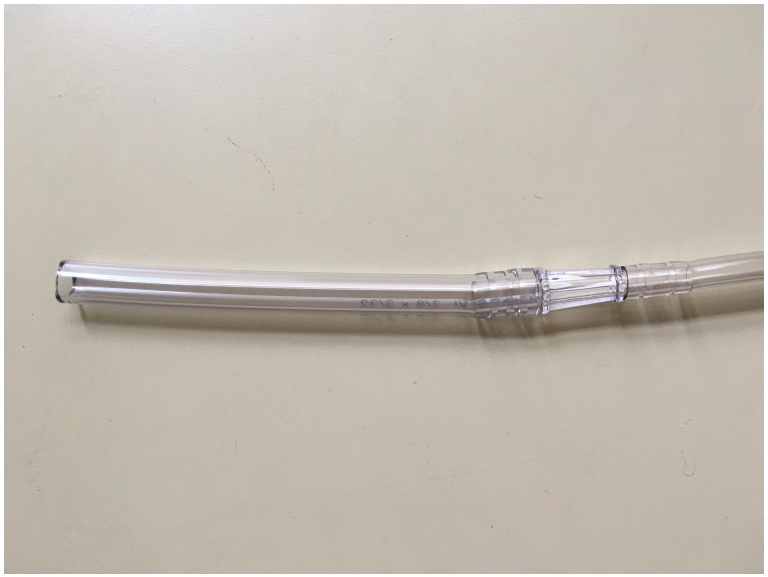
2.3 THE DESIGN OF A BESPOKE AORTA CANNULA

There were two porcine retrievals which precipitated the design of the present, bespoke, aortic cannula. The previously used aortic cannula was a simple flexible, plastic tubing with a calibre of 10mm (Figure 2A) taken from discarded tubing supplied as a 'spare' within the liver perfusion kit by the Cardiovascular Division (Europe) from Medtronic Inc., Minneapolis, Minnesota, USA. The length was cut to size prior to each organ retrieval.

During one porcine pancreas retrieval, the aortic cannula was temporarily displaced and although this was noticed and corrected within a few seconds, it caused unnecessary interruption to the retrieval process and resulted in extra-luminal loss of the ice-cold preservation solution. During another retrieval the rough surface of the distal end of the plastic tubing introduced into the descending aorta, inadvertently 'sheared' the aortic endothelium (Figure 2B) which resulted in an 'aortic dissection'. The true lumen of the dissection was still in continuity with the coeliac and superior mesenteric axes, which in turn was contiguous with the aortic cannula. Fortunately, this resulted in successful delivery of the preservation solution and serendipitously the false lumen served only to occluded all the posterior lumbar spinal artery branches, which were routinely ligated and divided as the final step in every retrieval. As a consequence on this occasion the retrieval and graft perfusion was successful although these two experiments clearly demonstrated that the primitive tubular 'cut to size' cannula would either repeatedly continue to fall out and/or the rough edges would repeatedly result in further dissections leading to wasteful porcine models.

After discussion with our perfusion kit supplier, a bespoke aortic cannula was designed with a distal ‘cuff’ and the tip was ‘bevelled’ to facilitate laminar blood flow (Figure 3).

Figure – 2A (Left) and Figure – 2B (Right). Preliminary experiments made use of spare plastic conduit tubing, cut to size for aortic cannulation. Often this resulted in 'rough' edges (arrow). This was identified after one experiment resulted in an 'aortic dissection' and this provided the impetus to design a bespoke cannula.



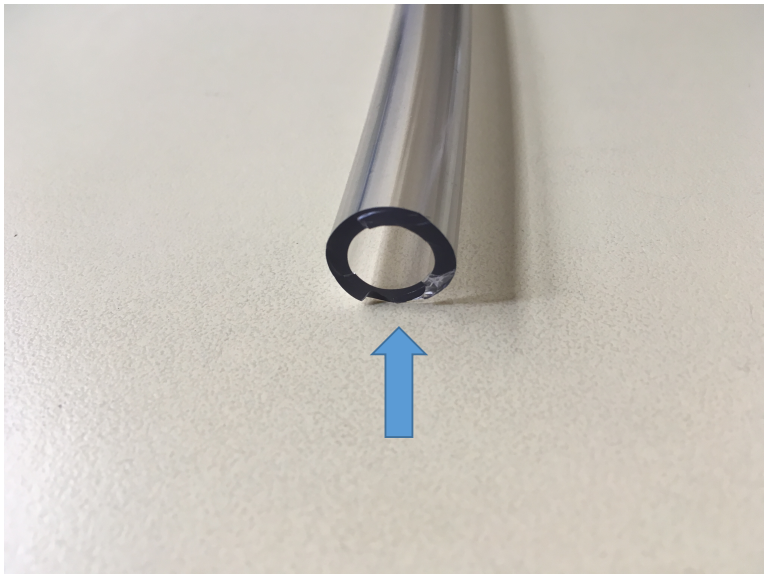
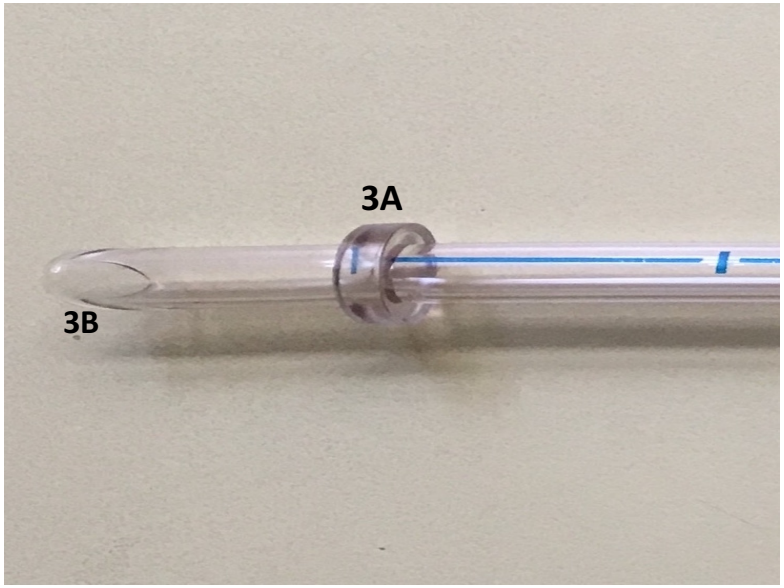


Figure 3 – The Bespoke Aortic Cannula, complete with a 'cuff' (3A) which reduced the risk of proximal slippage within the aorta following suture ligature. The 'bevelled' distal end aimed to improve laminar flow and thus minimise the risk of graft thrombosis (3B).



2.4 PERFUSION CIRCUIT MODIFICATIONS

Our laboratory has previously established *ex vivo* perfusion models of kidney, liver and liver with kidney (simultaneous). However, work on *ex vivo* porcine pancreas perfusion had never been previously conducted and in addition as discussed in Chapter 1, the worldwide literature on porcine pancreas perfusion is limited. Preliminary porcine pancreas perfusion models were initially attempted by replicating our liver specific custom perfusion pack (Figure 4).

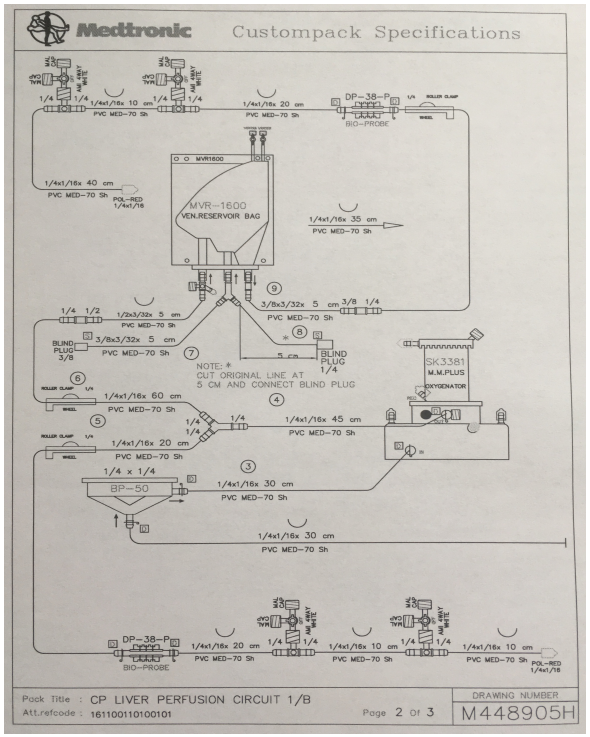
The porcine liver has two inflow vessels, similar to humans with the main perfusion via the portal vein and again as in man the second via the hepatic artery. The liver specific circuit reflected this *in vivo* anatomical inflow system with two outflows from the centrifugal pump and each of which was incorporated independent pressure and biosensor probes. *In vivo,* the portal vein delivers blood from the gut at a lower pressure into the liver with concomitant lower oxygen saturations while the hepatic artery, a branch of the coeliac axis derives oxygenated blood at a higher (systemic arterial) pressure. To reduce the pressure within the portal inflow the liver circuit perfusion kit contained a reservoir to pool blood and the portal blood flow from the reservoir to liver was gravity dependent while the additional biosensor enable independent continuous pressure monitoring. Meanwhile, the hepatic arterial inflow enabled the delivery of the higher pressure flow straight from the centrifugal pump to the liver again, with independent biosensor pressure monitoring.

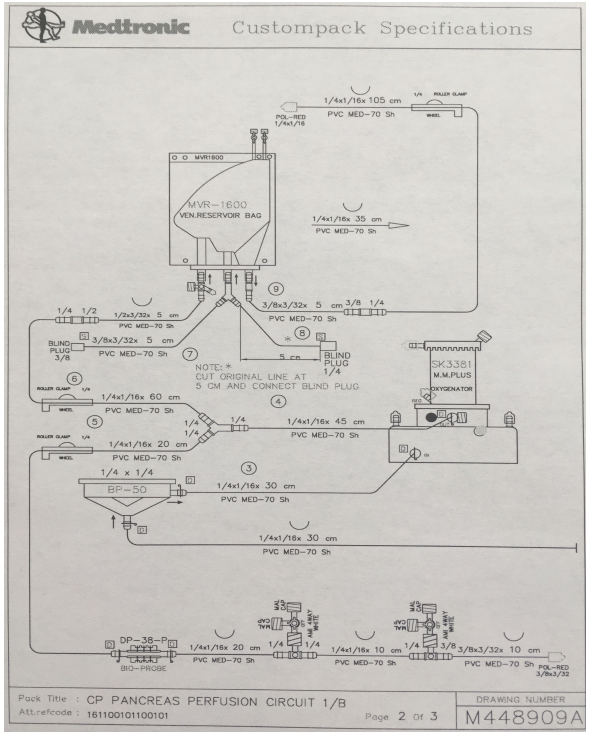
Two differential pressure inflow channels were deemed unnecessary for pancreas perfusion. By using the liver perfusion kit, it caused pooling of blood and potential cooling within the reservoir system making thermoregulation difficult. The reservoir also increased issues with haemostasis and coagulation because the gravity dependent nature produced a low flow. Of note, Poiseuille's law describes the flow of fluids through an intravenous catheter. It states that

the flow of fluid is related to a number of factors: the viscosity of the fluid, the pressure gradient across the tubing, and the length and diameter of the tubing. Thus, an increased length of unnecessary tubing confers an increased systemic vascular resistance throughout the circuit which meant an increased work load for the centrifugal pump. To avoid this problem the circuit was optimised with modifications specific for pancreas perfusion with the help of designers from the Cardiovascular Division (Europe) representatives in Medtronic Inc., Minneapolis, Minnesota, USA (Figure 5).

Figure – 4 (Left) Technical drawing of the liver perfusion circuit. There are two outflow systems with independent pressure / bio-senor monitoring and the necessity for a blood reservoir

Figure – 5 (Right) Technical drawing of the pancreas perfusion circuit, following discussions with Medtronic, the liver circuit was modified. An independent second outflow with its own pressure / biosensor was unrequired. Additionally the reservoir was adapted such that it could be removed entirely from the circuit.





2.5 REDUCTION OF GRAFT THROMBOSIS

Primary non-function rates following pancreas transplantation remain substantial and are often due to graft thrombosis and severe inflammation (pancreatitis) [91, 92]. Thrombosis was an issue with the preliminary models initially suspected at the time of retrieval with a sudden reduction in outflow of clear preservation solution from the portal circulation. In the laboratory, graft thrombosis was signalled by low flow rates through the organ despite an adequate perfusion pressure. Following a failed perfusion, the pancreas was divided and the parenchyma of the pancreas was macroscopically inspected confirming haemorrhagic and thromboses internally, which confirmed the diagnosis.

In order to reduce the rate of graft thrombosis, I initially considered administering heparin prior to animal death. Unfortunately this would have meant a lengthy ethical application process evaluating the potential harm, pain and suffering to the animal from the subcutaneous injection. This issue was averted by the addition of heparin directly into the ice cold preservation solution allowing for timely anti-coagulation therapy to the graft via the aorta. This method was successful in preventing any immediate, overt and major graft thrombosis.

2.6 PERFUSION PRESSURE

There are two types of machine perfusion pump. One type delivers pulsatile flow utilising either a 'roller' or oscillatory pump and the alternative approach delivers continuous flow using a centrifugal pump. Pulsatile flow is believed by some workers to induce genes which induce an environment favorable to the endothelium (e.g. Kruppel – like factor 2). Kruppel – like factor 2 protects the vascular endothelium by inhibition of the proinflammatory response during perfusion. However, these studies were recently systematically reviewed and the evidence supporting the use of pulsatile or continuous perfusion pressure remains equivocal.

Additionally, all these studies were based on the kidney and liver systems with no studies comparing the two modalities of perfusion in the context of pancreas perfusion [52]. Furthermore, given that the pancreas is a low pressure organ and highly susceptible to shear stress [14] data from kidney and/or liver perfusion studies cannot be directly extrapolated to the pancreas model. This is especially true when considering the use of either a pulsatile or continuous flow and it is likely that the absolute pressure during the perfusion period is more relevant [52]. Porcine *in vivo* data suggests a mean arterial pressure of 90 mmHg [79] and while there is a paucity of physiology studies on anaesthetised large landrace species in the literature, the reports that exist describe an aortic systolic pressure of 111.7 (+/- 13.6) mmHg with diastolic aortic pressure of 79.0 (+/- 12.03), equating to a mean aortic pressure of 89.9 mmHg [93, 94].

The first pressure chosen for the pancreas perfusion was 80 mmHg in order to reflect our best current understanding from the limited literature available. It was chosen to be lower than an *in vivo* pressure of 90 mmHg in order to compensate for a 'low pressure' pancreas system. However, the first graft became oedematous at 40 minutes, resulting in graft failure and consequently over the next three sequential models, the pressure was reduced incrementally in 10 mmHg steps from 70 mmHg to 50 mmHg. The reduction in perfusion pressure resulted in a progressive increase in perfusion times before graft failure. At 50 mmHg pressure the graft could be perfusable for up to four hours. 50 mmHg was the final continuous perfusion pressure described in the SOP in Chapter 3.

2.7 OPERATIVE STEPS

During the preliminary experimental model retrieval, the operative steps evolved with important lessons being learnt during the preliminary phase of the perfusion experiments.

In particular, reducing the direct handling of the pancreas was felt to be important in minimising damage to the pancreas. Initially the splenic artery was clipped, ligated and divided in one step. However, this was modified to ligation only with the division at a later stage in the retrieval to reduce direct handling of the graft. The early ligation of the splenic hilum in combination with a late division facilitated vascular control resulting in less preservative solution being wasted from the distal pancreas. Simultaneously a late division allowed for traction and an improved purchase on the distal pancreas to assist with dissection.

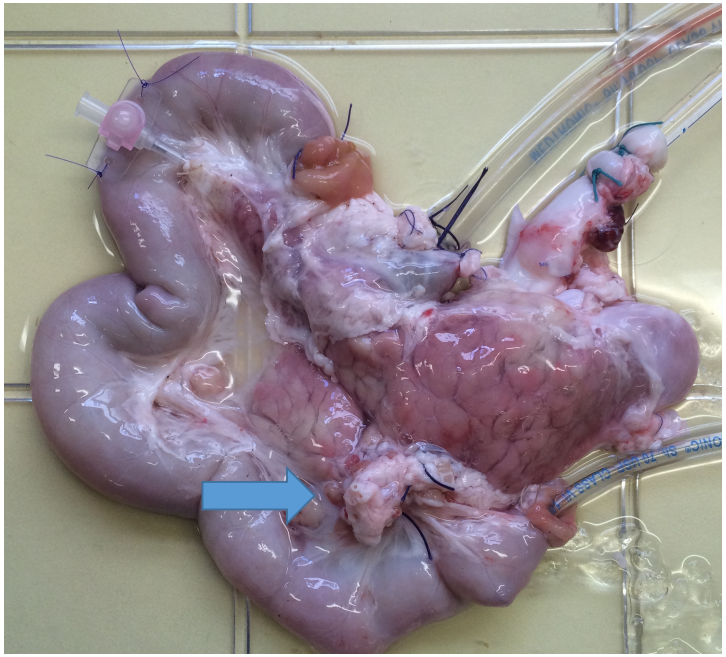
Identification of the duodenal suspensory ligament and the superior mesenteric vessels was an important step which required close attention to surgical technique. This step was achieved by division of the congenital adhesions between the ileum, duodenum, transverse colon and mesocolon. The ligament was then clamped and divided and the ends were inspected to confirm the presence of the superior mesenteric vessels which were then ligated.

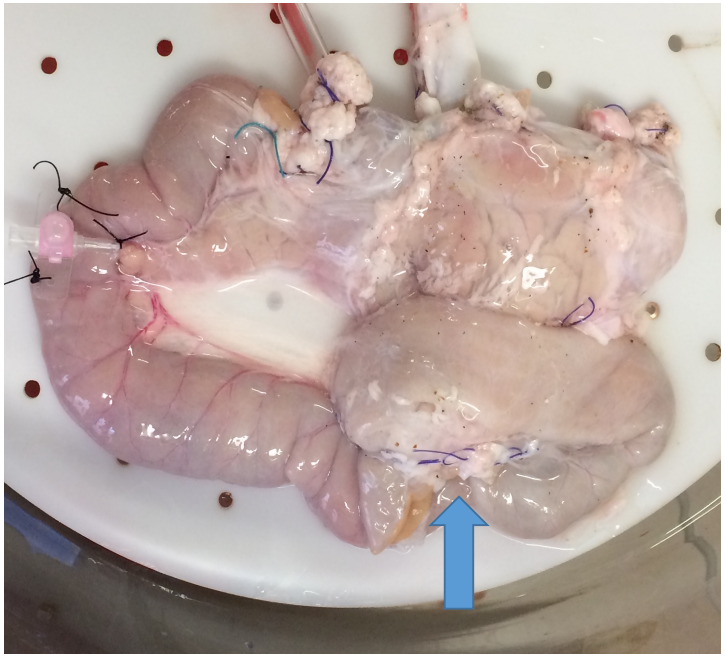
This final method incorporated all the lessons from the preliminary models and during one such retrieval experiment, the connecting lobe of the pancreas was inadvertently completely transected. This resulted in an extremely high 'flow' rate due to the greatly reduced vascular resistance of the organ. This particular failure in adequate perfusion served to improve my understanding of the operative anatomy of the porcine pancreas. The anatomy of the porcine pancreas has been previously described comprising three designated lobes; splenic, duodenal and a connecting lobe [78]. However operatively the anatomical relationships of the connecting lobe with respect to the suspensory ligament of duodenum and superior mesenteric vessels was often difficult to clearly trace because of dense congenital adhesions. The transverse colon not infrequently obscured the view of the area and on occasions, the duodenum was found to be entirely retroperitoneal, whilst other times it was only the distal duodenum which was retroperitoneal. With experience and attention to detail, the entire duodenum distal to the

pylorus could be carefully and methodically traced in a stepwise fashion. Even if the retrieval process slowed down at this stage, having a clearly identified and dissected pancreas was always the focus over and above the extra time taken.

Two porcine pancreas experiments demonstrated a 'hidden' connecting lobe of the pancreas (Figure 6). The preliminary experiment, which subsequently failed because of complete transection of the connecting lobe of the pancreas allowed for an improved anatomincal understanding.

Figure – 6 Dissection of the suspensory duodenal ligament with ligation of the superior mesenteric vessels, on one occasion resulted in complete division of the connecting lobe of the pancreas, resulting in poor perfusion. These are two examples demonstrating the dense adhesions often encountered between duodenum, suspensory duodenal ligament and connecting lobe of the porcine pancreas (marked by arrows).





2.8 HISTOLOGICAL ANALYSES

A variety of immunohistological stains were used to confirm cellular viability from the biopsies of the pancreata ranging from the standard haemotoxylin and eosin (H&E) stain to markers of caspase-dependent pathways.

The two patterns of cellular death are known as apoptosis and necrosis. Apoptosis is a programmed cell death process, executed by an active process which may be intercepted by interfering with intracellular signalling pathways [95]. The presence of caspase (*c*ysteinyl *asp*artate-specific protein*ases*) enzyme activation during apoptosis was considered a unique feature of programmed cell death. Necrosis is defined as a non-programmed chaotic process, characterised by cell membrane breakdown and cellular swelling with water. In necrosis there is an inflammatory tissue response with uncontrolled release of cell components [95].
However, this classic dichotomy when considering the two types of cell death has more recently been considered an oversimplication of a highly complex process and apoptosis has been shown to occur in caspase-independent pathways [95]. Following a noxious stimulus, cells may enter into fatal pathways with apoptosis or necrosis at the two extremes. Between these two extremes there are a number of defined entities which constitute a spectrum including mitotic catastrophe, slow cellular death, paraptosis and autophagy. Further, a continuum between the two extremes is now known as necrosis-like programmed cell death (without chromatin condensation) and apoptosis-like programmed cell death (with chromatin condensation) [95]. The presence of this continuum between apoptosis and necrosis helps to justify the use of classic apoptotic caspase markers in order to quantify the pancreas graft cellular damage sustained during the graft retrieval and perfusion process, irrespective of whether the graft damage is attributable to a specific classification of apoptosis or necrosis.

Mammalian cells contain a cytoskeleton composed of three types of filament; microfilament, microtubule and intermediate filament. Cytokeratins are the major component of intermediate filaments found within epithelial cells and in hepatocytes cytokeratin 18 is protective against cellular apoptosis and necrosis. During a cellular insult effector cytoplasmic caspases (caspases 3, 6, 7 and 9) are released which mediate specific proteolysis pathways and cause the cleavage of intermediate filaments such as cytokeratin 18. Caspase cleaved cytokeratin 18 along with other cytokeratin fragments are then released into the blood during the presence of glandular epithelial tissue injury. Caspase cleaved cytokeratin 18 is now a recognised biomarker of aopotosis and necrosis in hepatocytes and cholangiocytes, in clinical practice [96]. Beyond this, cytokeratin 18 has also been shown to be a useful serum biomarker for epithelial-derived carcinomata [97, 98] and their use as a biomarker in pancreas ductal adenocarcinoma has been demonstrated [99].

Caspase cleaved cytokeratin 18 can be identified in various preparations by a commercially available monoclonal antibody called M30 [96]. Biopsy sections were stained with M30 CytoDEATH (Sigma-Aldrich Company Ltd., UK) for quantification of irreversible cellular damage. Polyclonal antibody against the active form of cleaved caspase 3; anti-Caspase 3 antibody (Sigma-Aldrich Company Ltd., UK) was used to objectively identify the presence of effector caspase 3 in the cell death process. The marker for caspase 3 was chosen because it has been shown to play a central role in cellular death pathways that have been conserved throughout the evolution of multicellular eukaryotic cells and is important in the intitiation of irreversible cell death [100, 101].

The usefulness of H&E staining is reliant upon the differential staining of cellular components on a spectrum from blue to red. Structures such as nuclei are blue, whilst the cytoplasm stains varying shades of pink. Parenchymal viability assessment using H&E staining is dependent upon

the evaluation of morphology which of course may be subjective. In other areas of clinical practice such as upper gastrointestinal surgery, the Sydney System for classification of gastritis has been developed using H&E stain. The Sydney system endeavours to remove subjectivity by taking into account the three domains of aetiology, topography and morphology to define and classify the severity of gastritis objectively. Morphology is further categorised into graded and non-graded variables with grade variables defined as inflammation, activity, atrophy, intestinal metaplasia and the presence of infection *H. pylori* [102, 103].

Similarly, in order to reduce the subjectivity that may have arisen, a strict grading system was developed for the porcine pancreata. Topographic classification from the preliminary experiments proved difficult as taking biopsies from varied locations such as the centre of the duodenal or splenic lobe proper, often led to uncontrollable haemorrhage. Haemorrhage within the centre of the graft could not be controlled easily and suture ligation often increased haemorrhage and interferred with the perfusion. It was decided to routinely take samples from just the very distal part of the splenic lobe. Additionally, the choice of very distal part of the splenic lobe is useful as it represents a vascular 'watershed' area, as discussed in the previous chapter. In the case of haemorrhage occuring at the distal splenic lobe following biopsy, suture ligation controlled it without compromising the perfusion to the rest of the graft.

Other ways to reduce subjectivity with immunohistochemical quanitification include using a method of blinding the observer to both the experiment biopsy time and the perfusion parameter variable. Blinding could also be combine with making use of both an inter and intra observer error in the counts. Then the errors could be statistically analysed for the level of agreement (e.g. using a Cohen's kappa coefficient test). All the counts in this thesis were fully blinded during the assessment. However, due to the constraints of time and availability, intra-observer counts were not possible and this represents a limitation in the study.

The morphological descriptors of biopsy sections were graded I, II, III and IV (Table 4) and examples demonstrated (Figures 7A, 7B, 8).

Table 4 Grading System for Pancreas Morphology Assessment

Grade	Glandular / Parenchymal assessment	Nuclear apoptotic bodies	Intra-parenchymal Haemorrhage
I	Well preserved acinar /parenchymal structure representing more than 95% of the section area	Minimal nuclear apoptotic bodies in acinar cells representing less than < 5% of section area	Minimal, intra-parenchymal haemorrhage representing less than < 5% of section area
II	Loss of acinar parenchymal structure representing < 25 % of the section area	Apoptotic bodies in acinar cells representing > 5% but < 25% of the section area	Sparse, focal, intra-parenchymal haemorrhage representing > 5% but < 25% of the section area
III	Loss of acinar parenchymal structure representing < 50 % of the section area	Apoptotic bodies in acinar cells representing > 25% but < 50% of the section area	Intra-parenchymal haemorrhage representing > 25% but < 50% of the section area
IV	Loss of acinar parenchymal structure representing > 50 % of the section area	Apoptotic bodies in acinar cells representing > 50%	Diffuse intra-parenchymal haemorrhage representing > 50% of the section area

Figure – 7A Haematoxylin & Eosin Grading system. Grade I (left) Grade II (right). Well preserved acinar parenchyma (white arrow). Apoptotic bodies (blue arrow)

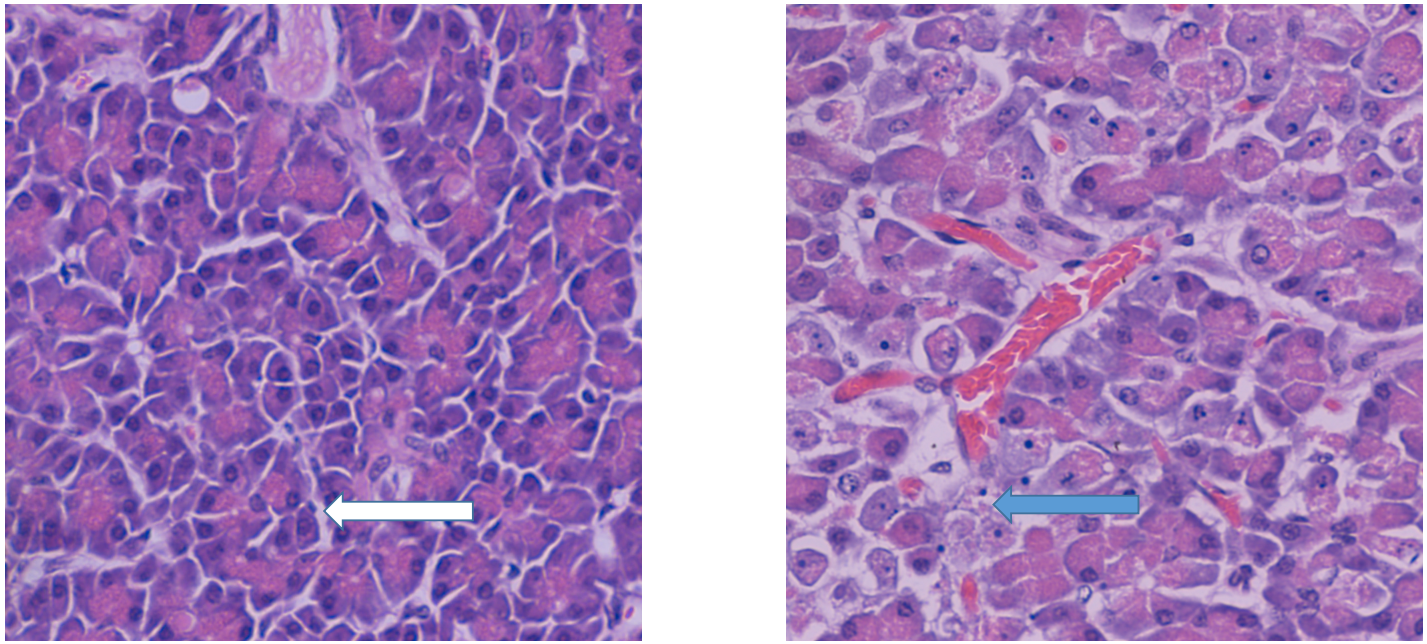


Figure – 7B Haematoxylin & Eosin Grading system. Grade III (left) Grade IV (right). Loss of acinar parenchymal integrity representing less than 50% of the total section (white arrow). Diffuse intraparenchymal haemorrhage representing greater than 50% of the total section (blue arrow).

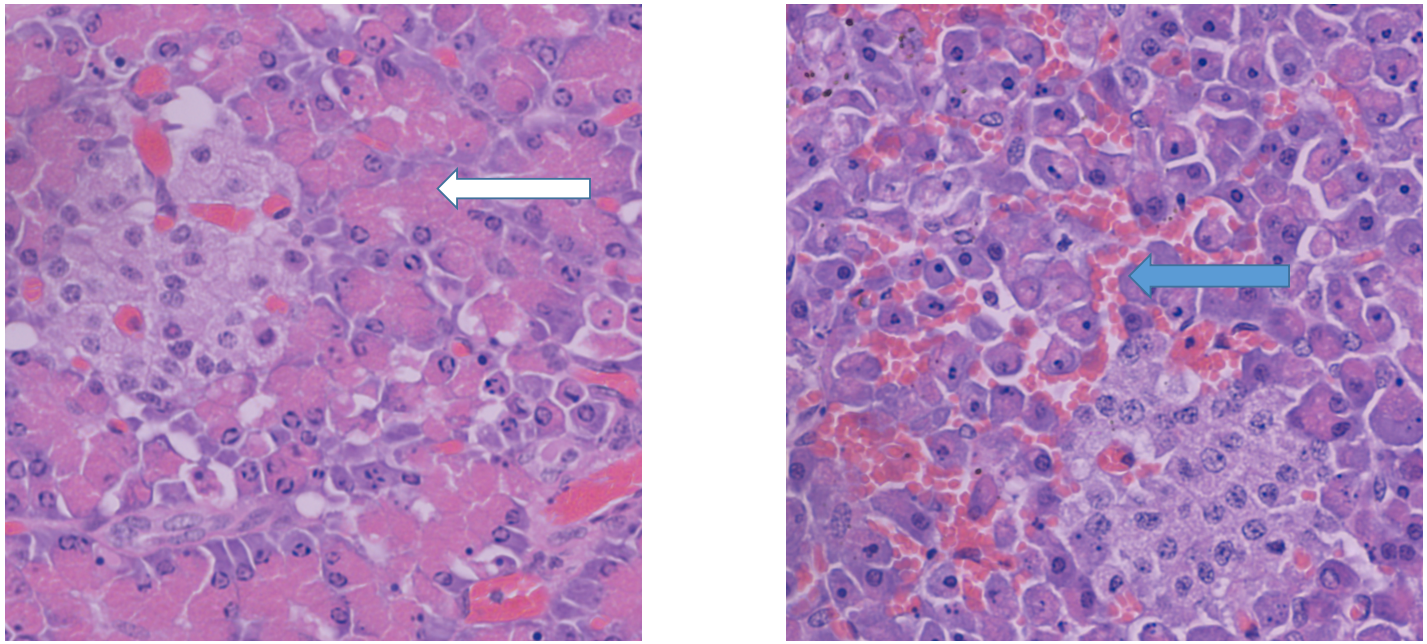
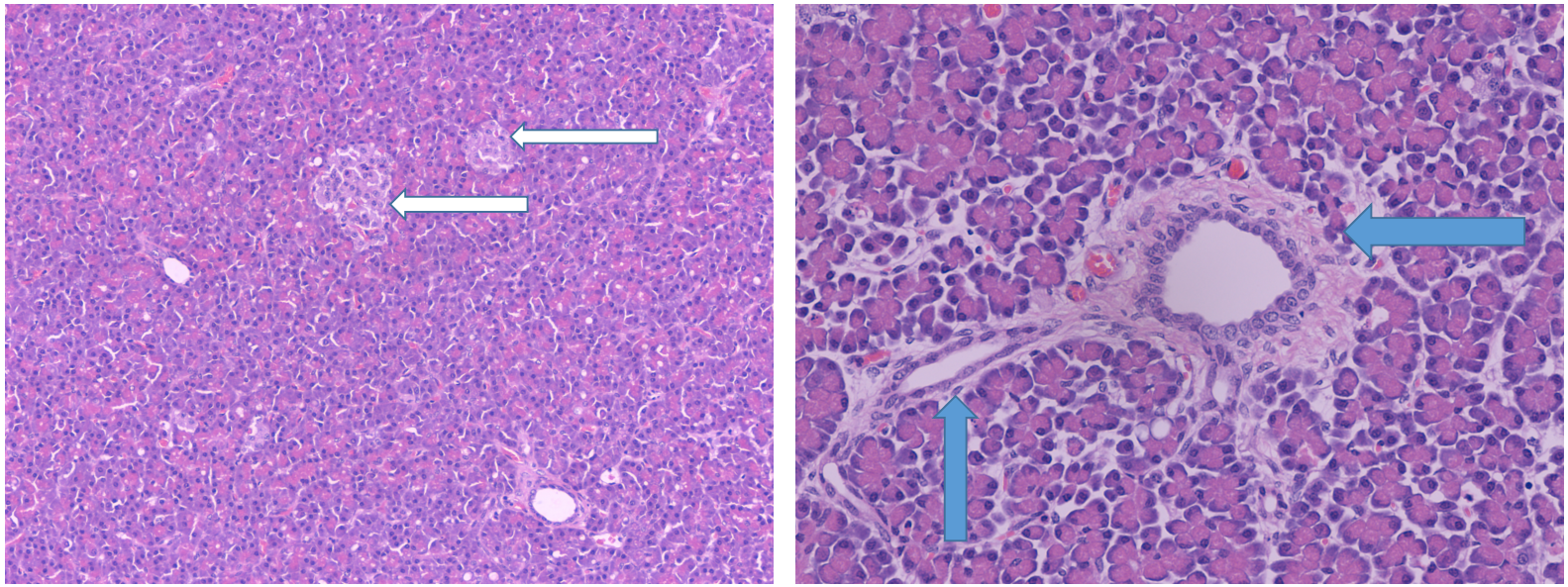


Figure – 8 An example of preserved islets (white arrow) with surrounding parenchyma grade II, 10 x magnification (left). An example of preserved exocrine ducts (blue arrow) with surrounding parenchyma grade II, 20 x magnification (right).



2.9 CONCLUSION

Eight porcine pancreata were retrieved and analysed. Failures, were logically investigated and addressed and lessons learn incorporated into subsequent successful perfusion experiments in an organ which is technically challenging and associated with the greatest risk profile and complication rates within the entire field of transplantation surgery.

Using an inductive research methodology with the few number of iterations described in this chapter could be argued as limited. Inductive research is based upon an asumption of 'uniformity'. That is to say, one particular observation, in isolation, is considered representative of *all* further iterations. This may not be the true reality and it does not account for events due to 'random chance'. It also relies upon the process of observation for the collection of all the information. The process of observation is also limited to the ability of the observer to understand the nuances and decifer all data into a useable and useful form.

Furthermore, the use of any explantory principles is based upon an asumption of 'best rationalisation' or parsimony (e.g. Occam's razor) and the uniformity may then fall foul during hypothesis testing and statistical analysis. But in the absence of an already established porcine pancreas model available in the literature, jumping straight to a deductive research methodology would have been impossible for this thesis.

Thus, using an iterative approach the perfusion circuitry and adjuncts were optimised and modified specifically for the porcine pancreas. The difficult operative steps were improved and WI damage was successfully reduced to a routinely acceptable and reproducible time. The risk of major graft thrombosis was also minimised by the appropriate use of anticoagulants.

Table 3 The Kolb Experiential Learning Model applied to each iteration of the preliminary porcine perfusion models.

Preliminary Porcine Model (Concrete experience)	Reflective observation (Thinking / identify problems)	Abstract Conceptualisation (Apply theory to resolve problems encountered)	Active Experimentation (plan / changes made / trying out)
Porcine model 1	Coeliac axis – difficult access due to retroperitoneal adhesions and also coeliac access difficult due to a vaso-constriction in a non-compliant narrow calibre vessel.	Principle of Poiseuille's law applied to minimise (or be able to control) warm ischaemic time.	Aortic cannulation chosen in preference to coeliac ostium.
Porcine model 2	Aortic cannulation from an abdominal approach difficult because of retroperitoneal attachments and interference with diaphragm, crural and lumbar muscles.	Principle of Poiseuille's law applied to minimise (or be able to control) warm ischaemic time "top down" approach access to abdominal aorta from the thorax.	Aortic cannulation from the thoracic aorta was easier and quicker for delivery of 'ice cold' preservation solution.
Porcine model 3	Tip of aortic cannula advanced beyond the coeliac ostium resulting in a thrombosed, primary non-functional graft.	A "top down" approach to access the abdominal aorta, principle to address the warm ischaemic time.	Aortic cannulation to distal diaphragm but not beyond the coeliac access. Modification to bring the distal end of the aortic cannula proximal to coeliac ostium.
Porcine model 4	Profound graft thrombosis (a recognised complication of pancreas transplantation).	Delivery of anticoagulation therapy to minimise graft thrombosis.	Heparin added directly into the ice cold preservation solution.
Porcine models 1 to 4	Minimisation of graft oedema (stepwise reduction in perfusion pressure from 80mmHg to 50mmHg).	Literature review revealed that pancreas is a 'low pressure' organ. Relevant information from porcine physiology studies evaluated [93, 94].	The mean porcine arterial (aortic) pressure *in vivo* is circa 90mmHg, therefore stepwise reduction per model to finalise upon a continuous perfusion pressure of 50mmHg, resulting in upto four hour perfusion.
Porcine model 5	Aortic cannula temporarily displaced despite being secured with ligature surrounding the thoracic aorta.	Discussions with the cardiovascular division (Europe) Medtronic Inc., Minneapolis, Minnesota, USA.	Design and manufacture of our bespoke aortic cannula (Figure 3).
Porcine model 6	Aortic cannula sheered the endothelium to create a false lumen (Figure 2)	Discussions with the cardiovascular division (Europe) Medtronic Inc., Minneapolis, Minnesota, USA.	Design and manufacture of our bespoke aortic cannula (Figure 3).
Porcine model 6	Unrequired double organ inflow and biosensors to pancreas as compared to the liver The liver specific circuitry (Figure 4) was modified.	Discussions with the cardiovascular division (Europe) Medtronic Inc., Minneapolis, Minnesota, USA.	Design and manufacture of a perfusion circuit specific to pancreas perfusion instead of liver specific perfusion circuitry (Figure 5).
Porcine models 1 to 7	Centrally taken biopsies tended to cause uncontrollable haemorrhage and interfered with perfusion. Cellular stain protocol developed to confirm cellular viability using a variety of stains	Biopsy time points and sample location were standardised across models. Cellular immunohistochemical stains to confirm absence of cellular death as well as the presence of viability.	Location of biopsy finalised to the 'watershed' area at the tail of the splenic lobe. Development of a reproducible Haemotoxilin and Eosin grading system analogous to the Sydney gastritis classification [102, 103].
Porcine models 1 to 7	Division of connecting lobe in error resulting in failure to perfuse the graft.	Pancreatic anatomy understanding gained from available studies defining specifically porcine anatomy e.g. [78].	Attention to detail when dissecting the pancreatico-duodenal segment from the suspensory ligament of duodenum and superior mesenteric vessels and slow purposeful dissection when dealing with congenital adhesions in the region (Figure 6).
Porcine model 8	Reflections and active experimentation from models 1 to 7.	Finalised protocol trialled successfully.	Detailed standard operating protocol described in Chapter 3, Material and Methods.

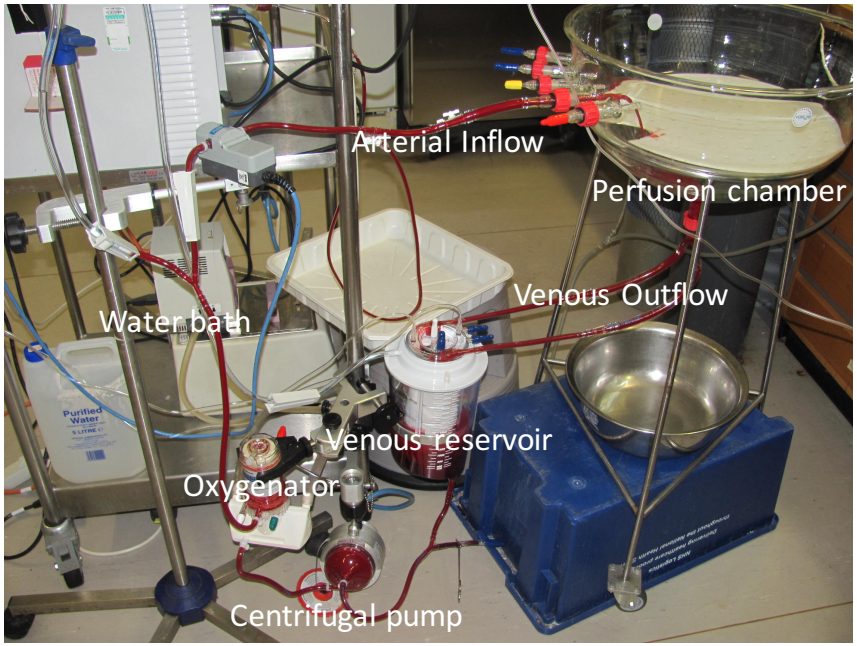
CHAPTER 3: MATERIALS & METHODS

3.1 ORGAN PERFUSION CIRCUIT

The e*x vivo* perfusion of porcine pancreata was achieved with bespoke perfusion circuitry. The circuit was connected to a Bio-Console® 560 Extracorporeal Blood Pumping Console for the electronic monitoring of an Affinity® CP Centrifugal Blood Pump. Oxygen delivery was achieved with a Minimax® Plus Oxygenation System connected in series with this pump (Figure 9). All of this equipment was supplied by the Cardiovascular Division (Europe) of Medtronic Inc., Minneapolis, Minnesota, USA.

Normothermia of autologous blood was achieved with a thermostatic water based heat exchanger unit with a degree of accuracy of 0.1°C and it was set at 37°C for normothermia. The circuit was set up and primed with 1000mL of Normal Saline (Baxter Healthcare Ltd, Hospital Equipment and Supplies, Northampton, UK), to confirm correct continuity of tube connections and identify any electrical problems prior to retrieval.

Figure – 9 Perfusion Circuit, Centrifugal Pump, Oxygenator with heat exchanger via the Water Bath. The circuit has been primed with autologous blood.



3.2 AUTOLOGOUS BLOOD RETRIEVAL

Porcine animal care and final exsanguinations were all compliant with Local Authority Law. Under the Home Office Scientific Procedures Act (1986) Schedule 1 methodology, large white female Landrace Yorkshire pigs weighing between 40 – 50 Kg were sacrificed. Sacrifice occurred via induction of cerebral death by electrocution and was performed humanely by the holder of a valid license granted under the Welfare of Animals (Slaughter or Killing) Regulations of 1995 Act [104]. Following electrocution, exsanguination was achieved with division of the major neck vessels and autologous blood was collected in a sterile, non-pyogenic container. 1.5L of blood was collected and 5000 U of heparin (Multiparin®, CP Pharmaceuticals, Wrexham, UK) immediately added in order to prevent coagulation.

Pancreata (Chapters 4 & 5) were retrieved from animals sourced from the School of Veterinary Medicine and Science, The University of Nottingham, UK. Alternatively, pancreata for establishment of protocols (Chapter 2 & 6) were retrieved from a local, fully EC licensed abattoir. These animals were, and remained, a part of the abattoir meat production chain. Removal of the pancreas did not interfere with the meat production.

Once the blood was collected, the animal was transferred to the operating table for organ retrieval in a peri-mortem state. Organ access was afforded via median sternotomy and laparotomy. The heart was universally found to be in pulseless electrical activity, secondary to hypovolaemia. This porcine model was representative of DCD transplant retrieval surgery.

3.3 PANCREAS RETRIEVAL

The pancreas retrievals were carried out with the help of a surgical trainee to assist in the application of counter traction for dissection; these two colleagues have been mentioned in the Acknowledgements (vide supra).

Each cupola of the diaphragm was divided anterior to posterior down towards each respective muscular crus. The diaphragmatic attachments to the parietal pleura were then divided, allowing improved access to the mediastinum. The supra-hepatic inferior vena cava and oesophagus were clamped, divided and ligated. This allowed the thoracic (descending) aorta to be accessed for cannulation. Cannulation of the thoracic (descending) aorta was achieved by division between haemostats and insertion of a bespoke aortic cannula. The tip of the cannula was carefully advanced to the level of the abdominal aorta, taking care not to go beyond the coeliac ostium. It was then secured in place with Vicryl™ ligatures (Polyglactin 910, Ethicon, Johnson and Johnson). 1000mL of ice-cold preservative solution (Soltran® kidney perfusion fluid, Baxter Healthcare Ltd, Renal Division, Hospital Equipment and Supplies, Northampton, UK) was immediately delivered to the pancreas and other abdominal viscera, via the coeliac and superior mesenteric arterial vessels. The ice-cold preservation solution was flushed under manual pressure and contained 5000 U of heparin (Multiparin®, CP Pharmaceuticals, Wrexham, UK) in order to improve visceral delivery and to reduce the risk of organ coagulation. This step marked the end of WI and the commencement of CI.

The abdominal viscera were retracted medially allowing access to the retroperitoneum and visualisation of the left renal hilum. The aorta was clamped and ligated at the level of the left renal artery. This reduced the volume of ice-cold preservative solution being unnecessarily delivered to the peripheral circulation (e.g. lower limbs). The splenic hilum was then identified and the splenic vessels clamped, and ligated. The suspensory ligament of the duodenum and the superior mesenteric vessels were then identified. This was achieved by division of the congenital adhesions between the ileum, duodenum, transverse colon and mesocolon. The duodenum was traced from the level of the gastric pylorus to the level of the suspensory ligament and further adhesions cleared. The ligament was then clamped and divided. The ends

were inspected to confirm the presence of the superior mesenteric vessels which were then ligated.

The portal vein was identified and skeletonised at the level of the porta hepatis and then clamped and divided. The portal vein was then cannulated with a bespoke portal vein cannula. This cannula was ligated and secured in place with Vicryl™ (Polyglactin 910, Ethicon, Johnson and Johnson). The presence of clear ice-cold preservative solution was seen to flow into the portal vein cannula. The proximal duodenum was divided at the level of the gastric pylorus and the right gastric artery ligated and divided. The hepatic artery was identified at the level of the porta hepatis and ligated and divided. Thus, in this way, the vascular supply derived from both the coeliac and superior mesenteric arteries supplying the pancreas and duodenum were isolated.

The proximal aorta was then freed from the retroperitoneum by careful ligation of the lumbar artery branches (usually three to four pairs) followed by adhesiolysis of the crural lumbar muscles from the adventitia of the aorta. The splenic hilum previously ligated was then divided. Once the aortic segment was freed from the retroperitoneum; the pancreas, duodenum (in continuity), coeliac artery, superior mesenteric vessels and portal vein, were carefully isolated *en masse*. The pancreas graft was then placed, submerged in ice-cold preservation solution (Soltran® kidney perfusion fluid, Baxter Healthcare Ltd, Renal Division, Hospital Equipment and Supplies, Northampton, UK). The organ was transported by car to the laboratory bench side.

3.4 PRIMING OF ORGAN PERFUSION CIRCUIT

Upon return to the laboratory, the entire volume of the 1000mL of normal saline that had been used to prime the circuit was discarded. The systemic venous reservoir was then filled with the

1500mL of retrieved autologous blood. Cefuroxime sodium antibiotic (750mg; GlaxoSmithKline plc., Middlesex, UK) diluted in 20mL of normal saline was added as a bolus. The choice to use antibiotics was based on advice taken from previous research students who had successfully perfused porcine liver and kidney organs. Additionally, a 20mL bolus of a prostacyclin solution for vasodilation was administered to the circuit. The choice of administering a vasodilator was in order to counteract the vasoconstriction that was often encountered in the allografts after CS. This was a 20mL aliquot of a solution of 500µg of epoprostenol sodium made up in a 200mL volume of normal saline.

3.5 BENCH SIDE PREPARATION OF THE PANCREAS FOR PERFUSION

The pancreas was removed from CS and placed into a working bench preparation tray. The ice-cold preservative solution was discarded and the pancreas was flushed with 1000mL of normal saline, via the aortic segment cannula. The mesentery of the duodenum, and the major arterial supply to the pancreas and the lumbar branches of the segmental aorta were carefully inspected. Any compromise to arterial integrity was evidenced by leakage of normal saline. This was repaired with fine (usually size 5-0) Prolene™ (Polypropylene, Ethicon, Johnson and Johnson) sutures (Figure 10A).

The major pancreatic duct was identified and dissected out at its insertion into the duodenum, taking care not to devitalise the duodenal mesenteric vessels and the gastroduodenal artery. A 16G venous cannula (BD Venflon™ Becton, Dickinson & Co., NJ, USA) was then introduced into the pancreatic duct. The cannula was secured into place to overlie the duodenum (Figure 10B) with prolene (Polypropylene, Ethicon, Johnson and Johnson), seromuscular sutures. Discarded plastic perfusion tubing was inserted into the distal end of the duodenum and secured with

Vicryl™ (Polyglactin 910, Ethicon, Johnson and Johnson) suture. This allowed any intra-luminal contents to be evacuated and discarded via the tube, without contamination of the perfusion circuit (Figure 10B).

Figure – 10A Bench Preparation of pancreas. Mesentry is carefully inspected and any vessels repaired, saline is flushed via the aortic cannula and pancreatic duct is palpated and identified.

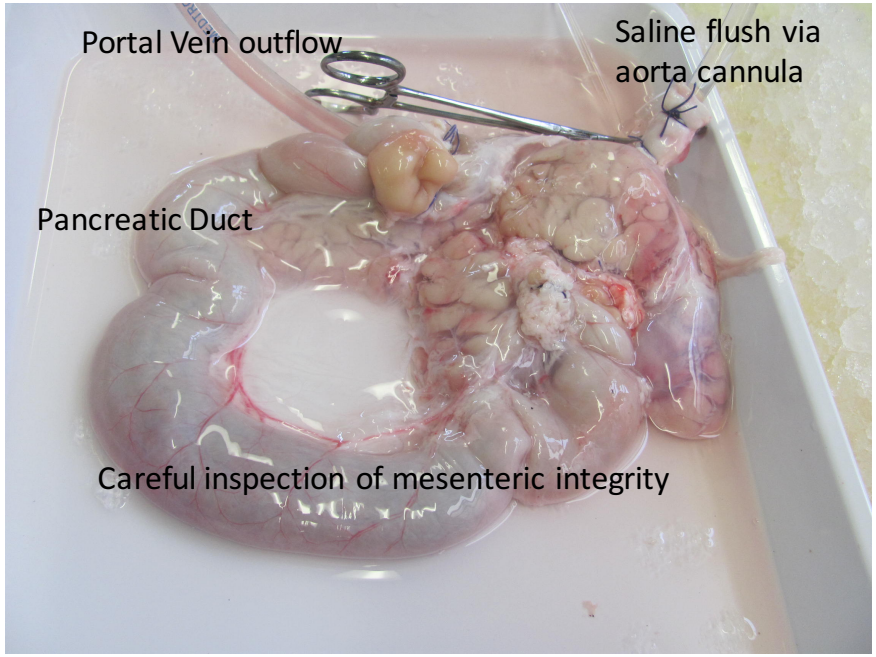
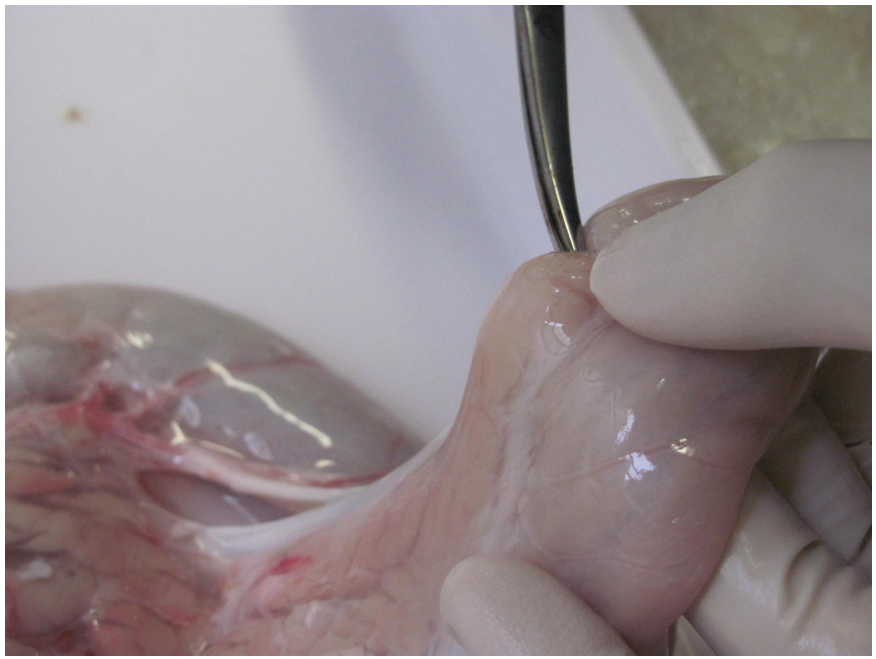


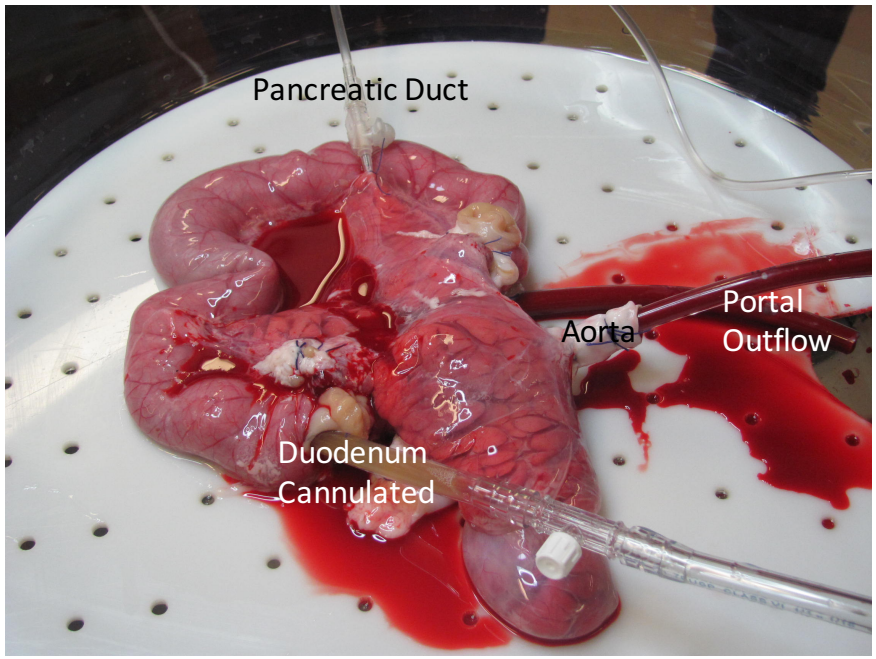
Figure – 10B Pancreatic duct is dissected out with careful attention to preventing disruption to the underlying gastro-duodenal artery



Once the circuit had been primed for 15 minutes the *ex vivo* pancreas was then connected to the circuit via the aortic cannula (Figure 11). During the first 15 minutes of autologous blood perfusion, the baseline blood samples at time point 15 minutes were taken, immediately prior to organ perfusion. Routine biochemistry samples were taken hourly.

Each *ex vivo* normothermic perfusion experiment was aimed to continue for four hours. However, if a precipitous drop in graft blood flow despite a constant (continuous) blood pressure occurred; then the experiment was electively terminated. The experience from preliminary models demonstrated that a sudden fall in graft blood flow was invariably associated with overwhelming graft thrombosis, oedema and / or venous congestion. These changes within the graft cause an increased vascular resistance which manifested as a sudden reduction in graft blood flow.

Figure – 11 *Ex Vivo* Normothermic Pancreas Perfusion, complete with arterial inflow, portal venous outflow. The pancreatic duct has been cannulated to collect pancreatic juice. The duodenal contents have been controlled with plastic tubing to prevent autologous blood contamination.



3.6 DATA COLLECTION

The physiological parameters of blood perfusion pressure and blood flow delivery into the organ were collected with respect to time. Similarly, blood samples were collected regularly during perfusion. Blood gas analysis for oxygen partial pressures, pH and lactate levels flowing into the organ (via the aorta) were compared against portal venous outflow samples, after organ perfusion. All these samples were collected at 15 minutes, (immediately prior to graft connection to serve as a baseline) then at time points 1, 2, 3 and 4 hours.

Haematological and biochemical parameters, haemoglobin, standard electrolytes, glucose and amylase were serially collected as baseline at time 15 minutes (prior to organ collection) and then at each of the 1, 2, 3, and 4 hourly time points until experiments were completed. Exocrine function was assessed by the rate of pancreatic juice produced with the hourly volume produced recorded at each time point.

Endocrine function was confirmed with Enzyme Linked Immuno-Sorbent Assay (ELISA) for insulin measurements from blood samples taken pre and post stimulation with glucose. The glucose stimulation was via a bolus dissolved into 20mL of sterile water added to the venous reservoir. Blood samples confirmed that adequate hyperglycaemia had been achieved following the bolus injection. The glucose bolus was administered at 125 minutes after the beginning of the perfusion. Serum samples for insulin were collected at one hour, two hours, 135 minutes, three hours and a final measurement at four hours. The relative formula mass of dextrose is 120 and using Avogadro's constant, one mole therefore has a mass of 120 grams. In 1.5 litres of blood, 3.5 grams of neat glucose dissolved into 1.5 litres of blood equates to a rise in glucose concentration of 20 millimoles. An assessment of endocrine function was made

by correlation of the glucose concentration against ELISA quantification of insulin concentrations pre and post bolus delivery.

The pancreas was biopsied regularly at 15 minutes (i.e. immediately at the beginning of the reperfusion / connection), and then at each hour time point following the beginning of the perfusion. Biopsies were routinely taken from the tail of the pancreas chosen as it represents the end arterial region of the organ and any haemorrhage at this location was controllable with suture ligation without compromising the remainder of the organ perfusion experiment (vide supra).

3.7 IMMUNOHISTOCHEMICAL STAINING

The pancreatic biopsies were immediately preserved in 10% formaldehyde for 48 hours. After this, the samples were embedded into paraffin wax blocks. The staining protocols are described below.

It is important to mention that all the processing of paraffin wax blocks and staining was carried out by Ms Angie Gilles in the Robert Kilpatrick Clinical Sciences Building. The analysis and interpretation of all slides and sections however, was carried out by myself (following guidance and teaching) to an agreed protocol from a Professor of Histopathology, Kevin West. Additionally, Professor West independently verified the staining counts and grading as accurate and true. All descriptive histological analyses (e.g. morphology for haematoxylin and eosin grading system) were agreed and discussed with Professor West prior to analysis. The data was presented to him to confirm the accuracy of counts and grading. Any queries that arose were clarified and agreed contemporaneously during analyses.

The paraffin-embedded sections were sectioned into 3 – 5 µm slices with a microtome. These were then mounted onto glass slides, available as a kit from Novolink™ Polymer Detection Systems (Leica Biosystems Newcastle LTD, UK). The stains were then carried out in the following way:

3.7.1 HAEMATOXYLIN AND EOSIN

Sections were heated at 65 °C for 5 minutes. They were de-waxed / deparaffinised and rehydrated by passage through a series of staining dishes which contained xylene and graded denatured alcohols, in the form of industrial methylated spirits (IMS) from 99% to 95%.

The sections were rinsed in tap water and then immersed in Mayer's Haematoxylin for 5 mins. They were then rinsed in tap water, counterstained with 1% Eosin for 3 minutes and then rinsed in tap water.

The sections were then dehydrated by the passage through a series of staining dishes: 95% IMS for 15 seconds, 99% IMS for 1 minute, 99% IMS for 1 minute, xylene for 3 minutes and then immediately mounted onto slides.

Slides were mounted with synthetic resin (dibutyl phathalate xylene) placed onto each coverslip using a pipette and then lowering the slide onto coverslip. The coverslip was pressed gently to remove air bubbles. The sections were dried overnight.

3.7.2 IMMUNOHISTOCHEMISTRY FOR CYTODEATH M30 AND ANTI-CASPASE 3 ACTIVITY

Two immunohistochemical stains M30 CytoDEATH and anti-Caspase 3 antibody were used as markers of irreversible cellular damage.

Sections were heated at 65 °C for 5 minutes. They were de-waxed / deparaffinised and rehydrated by passage through a series of staining dishes which contained xylene and graded denatured alcohols, in the form of industrial methylated spirits (IMS) from 99% to 95%.

Antigen retrieval was performed by immersion of the sections into a solution of tris buffered saline (TBS), rinsed for 5 minutes and then repeated. The TBS was used both as a wash and primary antibody diluent. A peroxidase block was used for 5 minutes to neutralise the endogenous peroxidase activity. Following this, sections were washed in TBS for 5 minutes and then repeated. A protein block was then used for 5 minutes. Incubation then occurred with the diluted primary antibody of choice.

A dilution of 1:600 was used for the anti-Caspase 3 antibody. A dilution of 1:100 was used for the M30 CytoDEATH antibody. Incubation with the primary antibody was overnight with the slide rack kept in a fridge set at 4°C.

The sections were washed twice in TBS for 5 minutes at a time. They were then incubated with post primary block for 30 minutes. TBS wash was repeated twice, each for 5 minutes. Incubation with the Novolink^{TM} polymer for 30 minutes was undertaken. The sections were then washed twice in TBS for 5 minutes, this time with gentle agitation. The development of peroxidase activity with diamino benzidine working solution for 5 minutes was followed by a rinse with distilled water. A counterstain with Mayer's Haematoxylin for 30 seconds was applied and then sections were rinsed in tap water. The sections were then dehydrated, mounted and dried overnight.

In order to confirm the success with the staining protocol, a negative control was used. The negative controls were randomly chosen, duplicate slide sections that were incubated both in the presence and absence of primary antibody. The absence of staining in the absent primary antibody slide and the presence of staining in the concomitant slide confirmed the immunostaining process.

3.7.3 ANTI ADENOSINE TRIPHOSPHATE SYNTHASE COMPLEX V STAINING IMMUNOHISTOCHEMISTRY

Adenosine Triphosphate (ATP) synthase complex V (ATP Synthetase) is an enzyme located within the inner mitochondrial membrane. It is responsible for producing ATP from the reduced form, adenosine diphosphate. This occurs in the presence of an electrochemical gradient of protons during oxidative phosphorylation. An antibody stain for the presence of the active form of Adenosine Triphosphate Synthetase Complex V was chosen as a surrogate marker to provide evidence for active ATP production at the cellular level. The immunohistochemical staining protocol was the same as the above section. A dilution of 1:5000 was used for the anti-ATP Synthetase antibody. Incubation was overnight with the slide rack kept in a fridge set at 4°C.

3.7.4 MICROSCOPIC HISTOLOGICAL ANALYSES

H&E staining grades were assigned to porcine pancreas sections. The grade system was from I to IV as previously described, based on evaluation by light microscopy. The sections were scored blinded to the experiment. At least twenty-five fields, viewed throughout the entire section were visualised and then an overall grade assigned.

Quantitative analysis of cellular death was evaluated by light microscopy of the cytoplasm. Only whole cytoplasmic stains were counted and any light granular stain was ignored, as artefact. An eye-piece graticule, containing 100 squares, was used to help keep count of positive and negative cells. The entire section was viewed under magnification at high power field (x40). The total number of positive cells were counted per 100 square field. The total number of negative cells were counted per 100 square field and this process was continued until a total of 3000 cells had been counted per section slide.

Quantitative analysis for the presence of ATP synthetase activity was also evaluated with light microscopy. Given that the majority of sections stained positive for the presence of anti-ATP synthetase Complex V, it was decided to quantify the *absence of the stain* rather than the presence. The entire section was therefore visualised, using the eye-piece graticule, to quantify the proportion of the total area that was negative for ATP Synthetase activity.

3.8 STATISTICAL ANALYSIS

Data was analysed with a commercially available statistical package, GraphPad Prism version 6.0h, October 16, 2015 for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com).

Descriptive statistical analysis of mean and standard deviation were used for continuous parametric variables, whilst median and range was reserved for non-parametric variables. Frequency was used for categorical variables. Comparisons of haemodynamic, endocrine and exocrine function parameters with respect to time and for repeated experiments were subject to normality test. Following this, statistical analysis with Student's *t*-test and analysis of variance were applied for parametric data variables in two or more group comparisons, respectively. Alternatively, statistical analysis with Mann Whitney *U* test and Kruskal-Wallis were applied for non-parametric data variables in two or more group comparisons, respectively. Probability values of less than 0.05 (designated, $p< 0.05$) were considered to be statistically significant. If the p value was not significant, it was designated as p=NS.

Chapter 4: A PHYSIOLOGICAL *EX VIVO* PORCINE PANCREAS

4.1 INTRODUCTION

The primary aim of the thesis was to investigate and answer the following research question:

Is it feasible to establish a physiological ex vivo porcine pancreas model that is both reproducible and reliable?

In this chapter, an argument is made attesting to the physiological and reproducible nature of the *ex vivo* porcine pancreas model. Data are described and taken from nine porcine pancreata, which were retrieved and perfused by following all the SOP described in Chapter 3, Material and Methods.

All nine pancreata were viable for two to four hours with a median time of three hours. Additionally, all nine duodenal segments in continuity with the retrieved pancreas graft, demonstrated evidence of visible peristalsis during the experiments. The biochemical data presented in this chapter show that both endocrine and exocrine functionality have been preserved. Additionally, the immunohistochemical data analysed the grafts and confirmed viability at a cellular level.

4.2 GRAFT RETRIEVAL ISCHAEMIC TIMES

The WI time was defined as the time taken to deliver the ice-cold preservative solution via the cannulated aorta, from the arrest of cardiac circulation (confirmed by visualisation of a non-beating heart). The WI time was recorded in minutes to the nearest 15 seconds. The median

WI time was 4 minutes. The range of values was between 3 minutes and 30 seconds to 7 minutes and 15 seconds (Figure 12).

The CI time was defined as the time from delivery of ice-cold preservative solution into the graft via the cannulated aorta to the commencement of graft perfusion. The CI time was recorded to the nearest minute. The median CI time was 127 minutes. The range of values were 112 minutes to 145 minutes (Figure 12).

Figure 12 – Box and whisker plots demonstrating the frequency distribution of warm ischaemic time (right) & cold ischaemic time (left).

4.3 PERFUSION PARAMETERS

Pancreatic graft blood flow and blood pressure remained stable throughout each perfusion experiment (Figure 13). The mean arterial blood flow was 141.3 (95% confidence interval 122.4 to 160 $mL.min^{-1}$). This did not change significantly between repeated porcine models (p=NS). The mean continuous arterial blood pressure was 50.5 (95% confidence interval 50.2 to 50.8) mmHg. This did not change significantly between repeated porcine models (p=NS).

All pancreas grafts demonstrated oxygen consumption confirmed by blood gas analyses (Figure 14). There was a mean differential between arterial and venous partial pressures of oxygen of 60.3kPa. The partial pressure of oxygen in the arterial blood inflow was measured from the aortic inflow segment with mean value of 73.3 (95% confidence interval 69.1 to 77.4) kPa. The partial pressure of oxygen in the venous blood outflow was measured from the portal vein with mean value of 13.0 (95% confidence interval 11.1 to 14.9) kPa. This arterial to venous oxygen differential reached statistical significance, $p < 0.0001$.

Figure 13 – Mean Arterial Blood Flow (+/- standard deviation) in $mL.min^{-1}$ & Continuous (centrifugal) Arterial Blood Pressure in mmHg. Both plotted with respect to Time of perfusion in minutes. Repeated measures across each perfusion experiment did not demonstrate any significant difference (p=NS).

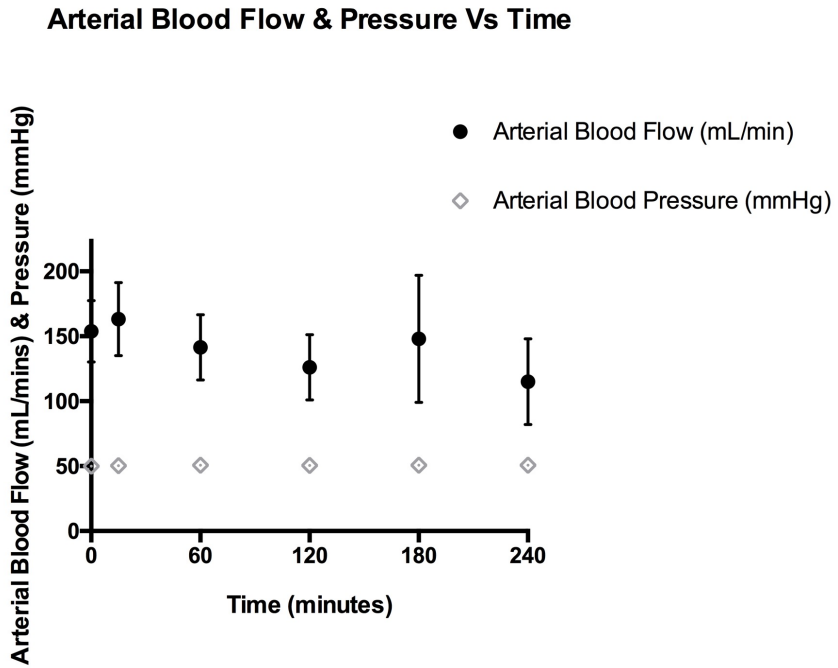
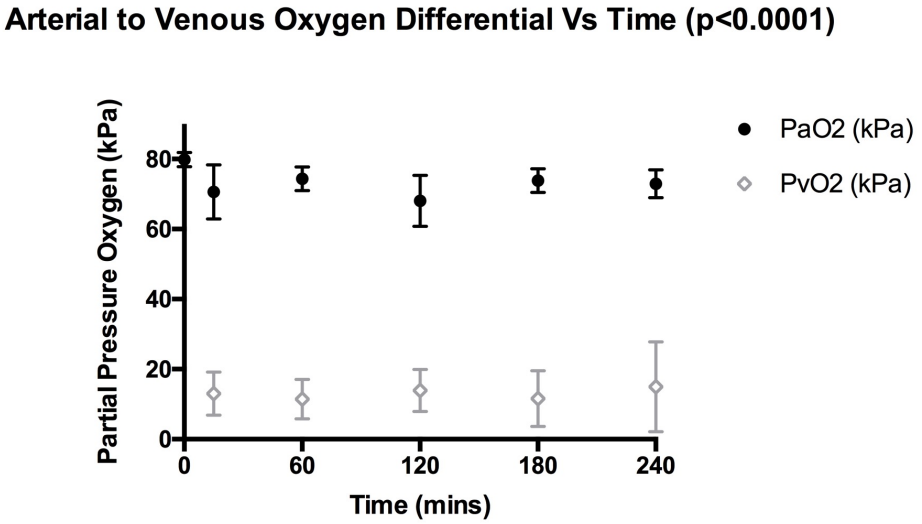


Figure 14 – Partial pressures of Oxygen sampled from the arterial inflow at the aortic segment (PaO_2) and the venous outflow, sampled at the portal vein (PvO_2). Error bars represent the 95% confidence interval at each time point during the perfusion. The arterial to venous oxygen differential reached statistical significance.



4.4 ACID-BASE (pH), LACTATE AND ELECTROLYTES

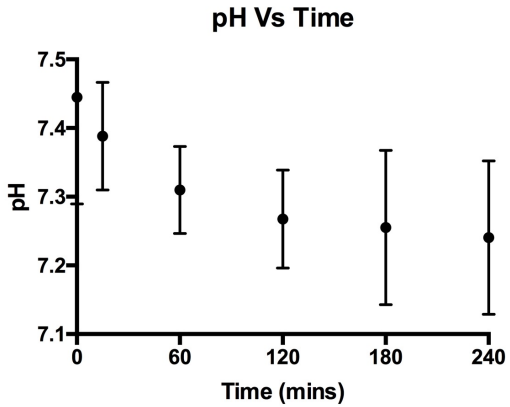
As the time of perfusion progressed, all pancreatic grafts demonstrated an increase in blood perfusate acidity, evaluated by a decreased arterial blood gas pH (Figure 15). There was an increase in both the accumulation of blood perfusate lactate anion and potassium cation concentrations (Figure 15). In contrast, the sodium and chloride ions were relatively stable during perfusion and similar to normal range, *in vivo* (Figure 16). Although these were the trends observed during perfusion, none of the repeated measures were associated with statistical significance, p=NS, (Table 5).

The mean arterial pH was 7.32 (95% confidence interval 7.23 to 7.40). The mean arterial lactate concentration was 6.85 (95% confidence interval 4.83 to 8.87) mM. The mean potassium concentration was 8.2 (95% confidence interval 7.24 to 9.15) mM. The mean sodium concentration was 137 (95% confidence interval 136 to 139) mM. The mean chloride concentration was 115 (95% confidence interval 114 to 116) mM.

4.5 EXOCRINE FUNCTION

The exocrine component was assessed with the collection of serum amylase and the volume of pancreatic juice collected per hour (Table 4). The mean serum amylase was 8692 (95% confidence interval 2385 to 15000) $U.L^{-1}$. The mean serum amylase at each time point demonstrated an increase trend from 3500 $U.L^{-1}$ to 18300 $U.L^{-1}$ (p=NS). The mean rate of pancreatic juice production rate was 4.5 (range 1 to 17) $mLs.hr^{-1}$.

Figure 15 – The pH decreased with increased time of perfusion, indicating an increase in acidity (left). This decrease in pH of the blood perfusate was associated with an increase in lactate anion concentration (right). Error bars represent the 95% confidence interval at each time point during the perfusion. The repeated measures did not reached statistical significance, p=NS.



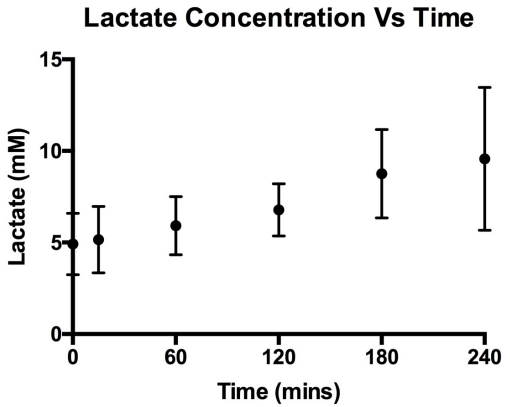
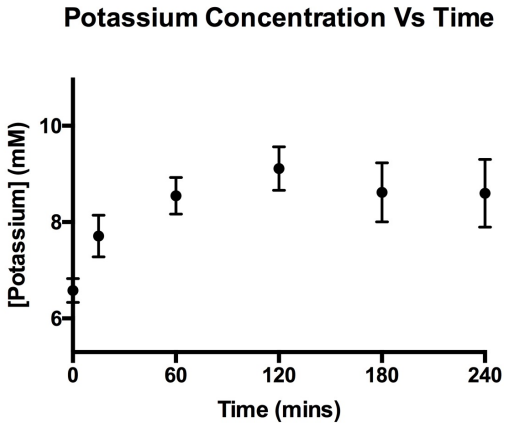


Figure 16 – The potassium cation concentration increased with increased time of perfusion (left), this was associated with an increase in acidity and lactate concentration. The sodium and chloride concentrations remained relatively stable in contrast (right). Error bars represent the +/- SD at each time point during the perfusion. The repeated measures did not reached statistical significance, p=NS.



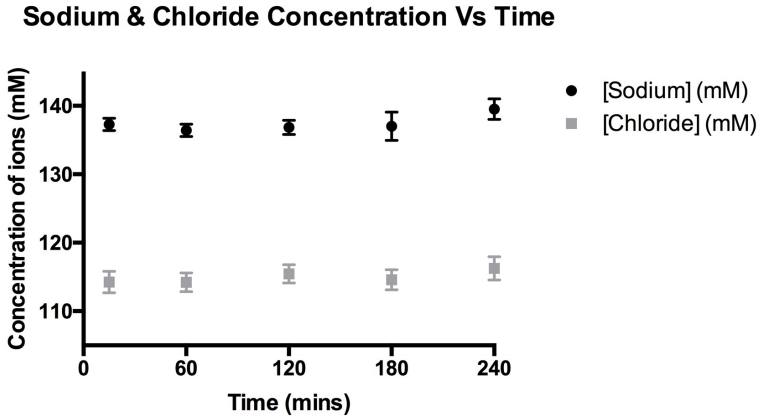


Table 5 – Biochemical parameters during perfusion with respect to time. All values are mean values with one standard deviation in parentheses.

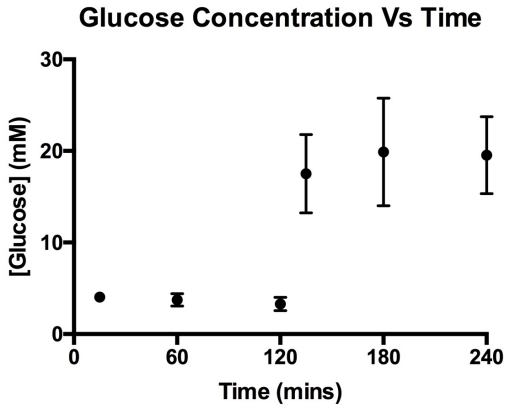
Time (mins)	*pH ($-\log_{10}[H^+]$)*	*[Lactate] (mM)*	*$[K^+]$ (mM)*	*$[Na^+]$ (mM)*	*$[Cl^-]$ (mM)*	*[Amylase] ($U.L^{-1}$)*	*Pancreatic Juice rate ($mL.hr^{-1}$)*
0	7.45 (0.07)	4.91 (2.0)	6.6 (0.7)	139 (1.9)	111 (4.6)	-	-
15	7.39 (0.08)	5.16 (2.2)	7.7 (1.3)	137 (2.4)	114 (4.4)	3500 (300)	-
60	7.31 (0.06)	5.92 (2.1)	8.5 (1.1)	136 (2.4)	114 (4.1)	7100 (700)	2.7 (1.1)
120	7.27 (0.07)	6.78 (1.9)	9.1 (1.4)	137 (2.7)	115 (3.9)	9500 (1200)	3.6 (3.3)
180	7.26 (0.07)	8.76 (1.9)	8.6 (1.4)	137 (3.6)	115 (3.3)	12000 (2000)	6.3 (7.2)
240	7.24 (0.07)	9.58 (2.5)	8.6 (1.4)	139 (2.1)	116 (3.4)	18000 (500)	5.6 (5.5)

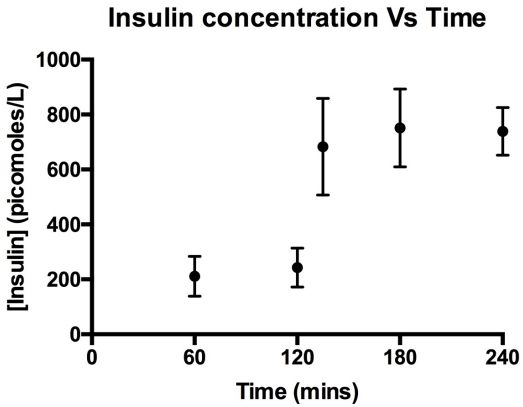
4.6 ENDOCRINE FUNCTION

The functionality of the endocrine component of the grafts was assessed by quantification of insulin production in response to an appropriate stimulation following a glucose bolus (Figure 17). The mean 'steady state' glucose concentration was 3.7 (95% confidence interval 3.4 to 4.0) mM. Following delivery of a glucose bolus (administered at 125 minutes) the mean concentration of glucose rose to 19.0 (95% confidence interval 14.0 to 16.8) mM. This was a statistically significant increase in glucose concentration, ($p < 0.0001$).

The baseline concentration of insulin in the perfusate was a mean of 227 (95% confidence interval 182 to 272) picomoles per Litre. Following graft stimulation with glucose bolus, the mean insulin concentration in the perfusate rose to 798 (95% confidence interval 665 to 794) picomoles per Litre. This was a statistically significant increase in insulin production, ($p < 0.0001$).

Figure 17 – The pancreata demonstrated evidence of endocrine functionality. Following glucose bolus stimulation from a mean concentration of 3.7 mM to 19.0 mM ($p < 0.0001$); the insulin concentration in the perfusate increased from a mean of 227 to 798 picomoles per Litre ($p < 0.0001$). The error bars are 95% confidence intervals.





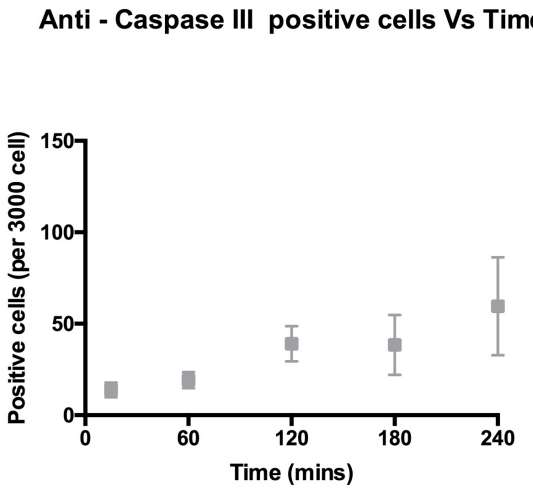
4.7 IMMUNOHISTOCHEMICAL ASSESSMENT OF CELLULAR VIABILITY

All pancreata were subject to immunohistochemical analysis to assess for viability at the cellular level. Anti-Caspase 3 (i.e. active form) and M30 CytoDEATH antibodies were used to quantify the presence of cellular death. H&E grades were assigned to each section. These grades ranged from I (best) to IV (worst), as previously described. The activity of ATP synthetase was observed and graded, as previously described. Grade I (best), represented the presence of > 95 % of the slide section area staining positive for ATP synthetase, grade II (>90 but < = 95) %, and grade III (>85 <=90) %.

The mean number of cells staining positive for anti-Caspase 3 was 34 (95% confidence interval 11 to 57) per 3000 cells counted per section. The mean number of cells staining positive for M30 CytoDEATH was 45 (95% confidence interval 7 to 84). Both these stains demonstrated an increase in the mean number of positive cells with increased perfusion time (Table 5 & Figure 18).

For both H&E and ATP Synthetase staining, the median score grade assigned to the biopsy sections increased with an increased perfusion time (Table 5 & Figure 19).

Figure 18 – The pancreata demonstrated evidence of cellular viability. An increase in number of cells staining positive for Anti-Caspase III and M30 CytoDeath was noted with increase in perfusion time. The mean number of cells (+/- 1SD) are plotted with respect to time.



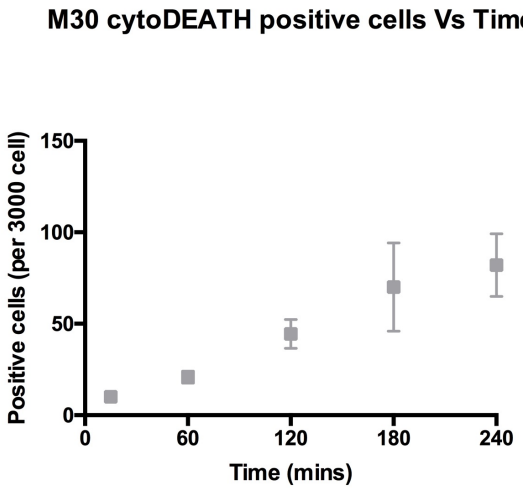
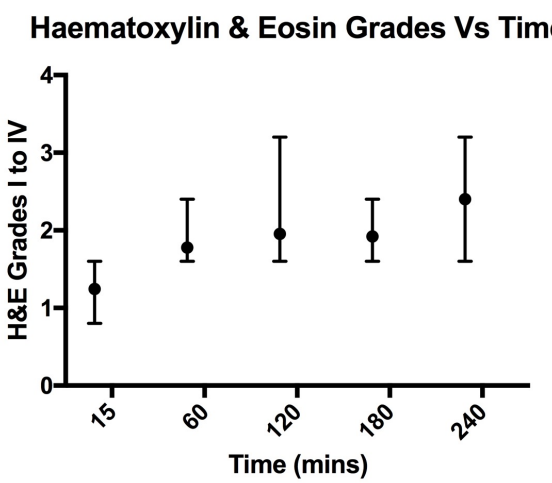


Figure 19 – The pancreata demonstrated evidence of cellular viability. A deterioration in immunohistochemical grade for both H&E stain and anti - ATP Synthetase Complex V stain were noted with an increase in perfusion time. The median grade with the error bars representing the inter-quartile range are plotted with respect to time.



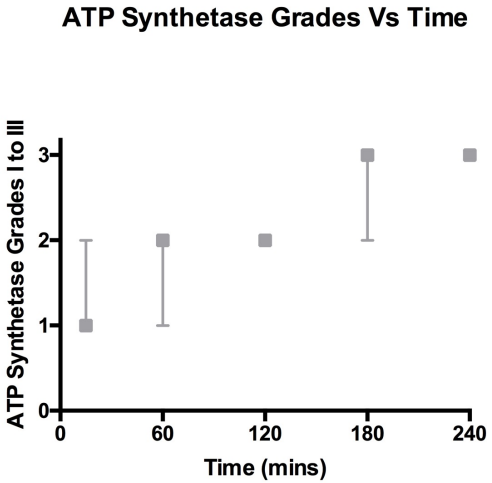


Table 6 – Immunohistochemistry biopsy scores during perfusion with respect to time. Anti-Caspase 3 and M30 CytoDEATH values are mean positive cells per 3000 cells counted, with one standard deviation in parentheses. H&E and ATP Synthetase values are median scored grade, with the range in parentheses.

Time (mins)	*Anti-Caspase 3 (+ve Cells / 3000)*	*M30 CytoDEATH (+ve Cells / 3000)*	*H&E (I - IV)*	*ATP Synthetase (I - III)*
15	14 (12)	10 (6)	II (I - II)	I (I - II)
60	19 (13)	21 (10)	II (II - III)	II (I - II)
120	39 (29)	44 (24)	II (II - IV)	II (II - III)
180	38 (37)	70 (54)	II (II - III)	III (II - III)
240	60 (54)	82 (34)	III (II - IV)	III (III - III)

4.8 DISCUSSION

4.8.1 *EX VIVO* NORMOTHERMIC PORCINE PANCREAS PERFUSION AS A MODEL

A physiological porcine pancreas perfusion model has been established to investigate techniques in whole organ pancreas preservation. The focus of this chapter was to establish an *ex vivo* normothermic porcine pancreas (ENPP) perfusion model. It was hoped that it should be physiological, both in terms of endocrine and exocrine pancreas function, in addition to being viable histologically for several hours *post mortem*.

This is the first report of a successful ENPP perfusion model. For up to four hours, the ENPP model is functional with both exocrine and endocrine components preserved. This is within the context of cellular viability confirmed with immunohistochemistry. Furthermore, this technique is feasible and reproducible with statistical reliability.

All pancreata were viable macroscopically and the duodenal segment showed peristalsis. All grafts received normothermic organ blood flow and had a statistically significant arterial to venous oxygen partial pressure differential, confirming graft oxygen uptake. All pancreata produced pancreatic juice, representative of exocrine function. Endocrine function was confirmed by a statistically significant rise in insulin in the portal venous flow following a statistically significant increase in glucose concentration within the perfusate. Insulin secretion following pancreatic stimulation with glucose occurs via ATP energy dependent channels in β islets of Langerhans, which is a further argument for a metabolically active graft.

Histology of all biopsy samples demonstrated minimal physiological cellular death with relevant immunohistochemical stains with the degree of cellular death increasing with length of

perfusion. However, all the absolute values are only within the order of tens of cells that were counted as positive from 3000 cells counted. The biopsy sections demonstrated the widespread presence of ATP Synthetase activity. Even the 'worst' slides showed that over 85% of the section area was positive for the active enzyme.

Considering that all grafts demonstrated an arterial to venous oxygen differential and in the presence of positive cells stained for active form of an enzyme involved with ATP production it is reasonable to argue that the oxygen uptake by grafts was being utilised at a cellular level, adding further support to the belief that this is a viable and physiological graft.

4.8.2 LIMITATIONS OF THE ENPP PERFUSION MODEL

The mean serum amylase ranged from 3500 to 18300 $U.L^{-1}$. In humans, amylase levels that are greater than the upper limit of normal range are considered diagnostic for pancreatitis although paradoxically the absolute value does not predict the severity of an episode of acute pancreatitis. Instead, well-established physiological scoring systems are used to determine severity (e.g. Glasgow score and Ranson's Criteria).

In pancreatic transplantation, higher amylase levels may be related to poor outcomes in donor grafts [105, 106]. In ENPP, high amylase levels may also be representative of graft dysfunction, but this has to be interpreted with care. Given that all pancreata demonstrated insulin response to glucose stimulation and were histologically viable, a high amylase level cannot simply be solely attributable to pancreatic damage.

Potentially, any acinar damage sustained during operative retrieval and CS may have been responsible for the initially raised amylase levels. Given that ENPP perfusion occurs within a closed circuit, amylase along with other exocrine enzymes are free to circulate and cause further graft (proteolytic) damage. Graft proteolysis would then lead to progressively increased

amylase levels with time. Therefore, one explanation may be that the initial absolute amylase level may help to quantify the level of acinar graft damage sustained during operative retrieval and CS? However, further rises in amylase have to be interpreted with caution, given that this is a closed circuit.

The addition of a porcine kidney in parallel to our pancreas circuit, may confer the advantage of stabilising not only the biochemical parameters but also allow for excretion of urinary amylase. Serial urinary amylase levels in our model may then become meaningful and quantitative of pancreas acinar damage. Furthermore, the addition of a kidney would not be dissimilar to current clinical transplantation practice whereby patients with type I diabetes and end stage renal failure undergo a simultaneous pancreas and kidney transplant as curative treatment.

Another limitation encountered was the practical difficulty of accurate measurement of the pancreatic graft mass. In ENPP the pancreatic graft was retrieved '*en masse*' along with the duodenal segment, its vascular pedicle (aortic segment) and the portal vein. Weighing the pancreas in isolation prior to perfusion was not possible. Following the termination of each ENPP perfusion experiment, measuring the weight of the pancreas would have been inaccurate due to fluid shifts and particularly oedema during the perfusion. This increasing oedema and/or haemorrhage by the end of perfusion, therefore precludes the estimation of an accurate "dry" weight. This meant that in the ENPP model it has been impossible to express blood flow and oxygen consumption parameters as values per unit mass of pancreas.

The use of an *ex vivo* perfusion model is limited to the lifespan of the organ which is usually hours rather than days. The limited lifespan of porcine *ex vivo* organs may be improved with the use of commercially available perfusion kits. Previously Chung *et al.*, and Weegman *et al.*,

have demonstrated that other porcine *ex vivo* organs can remain viable for up to 24 hours [71, 76]. However, the fragile nature of the pancreas gland may *per se* prevent viability for 24 hours *post mortem* although the present ENPP model is progress in this direction.

An interaction of autologous blood with the inert, non-biological surface of a commercially available MP pump creates a systemic response. Therefore, the interpretation of absolute data must be taken with this borne in mind. With increased iterations of the same experiment, the effects of the systemic response are negated because they should be the same from experiment to experiment. Repeated *ex vivo* experimental runs allow relative comparisons between preservation methodology, solutions or temperatures to be meaningful and amenable to statistical analysis. Several runs of the same experiment are inexpensive relatively easy to perform in an *ex vivo* porcine model when compared with *in vivo*, live models.

4.8.3 CONCLUSION

With the exception of the pancreas, there are several models in the literature, which argue that *ex vivo* porcine organ models are a suitable tool for DCD transplantation research [107]. *Ex vivo* porcine organ studies have investigated preservation methods comparing CS with HMP or NMP, or they have endeavoured to optimise MP perfusion parameters in other porcine organs [31, 57, 58, 64, 69, 76, 77]. These have led to an incremental understanding relevant to translational transplantation research.

The physiological porcine pancreas presented in this chapter is now one such model for further optimisation and for utilisation to investigate whole organ pancreas and islet preservation techniques.

CHAPTER 5: A COMPARISON OF PERFUSION PRESSURES USING THE *EX VIVO* NORMOTHERMIC PORCINE PANCREAS PRESERVATION MODEL

5.1 INTRODUCTION

In the previous chapter, a physiological *ex vivo* porcine pancreas was established. The model was demonstrated to be 'feasible' because several successful iterations were repeated and data presented supporting a physiological and viable model. In this chapter an argument for the statistically 'reproducible and reliable' nature of the model is to be made by testing the null hypothesis:

During ex vivo normothermic porcine pancreas machine perfusion organ viability is no different when the porcine pancreas undergoes perfusion with normotensive pressure compared with a low pressure system.

This chapter describes data from a further four porcine perfusion models, perfused at a lower continuous pressure of 20 mmHg. This group has been defined as the 'low pressure' group. The data from the low pressure group was compared with the data derived from the nine porcine perfusions described in Chapter 4. Those grafts were perfused at a continuous 'normotensive' pressure of 50 mmHg. The nine perfusion experiments from Chapter 4 have been defined as the 'control' group in this Chapter 5.

All four low pressure porcine pancreata were perfused for the full four hours' duration. Again, all four duodenal segments in continuity with the retrieved pancreas graft, demonstrated evidence of visible peristalsis during the experiments. The data presented for the low pressure system again demonstrated that both the endocrine and exocrine function has been preserved.

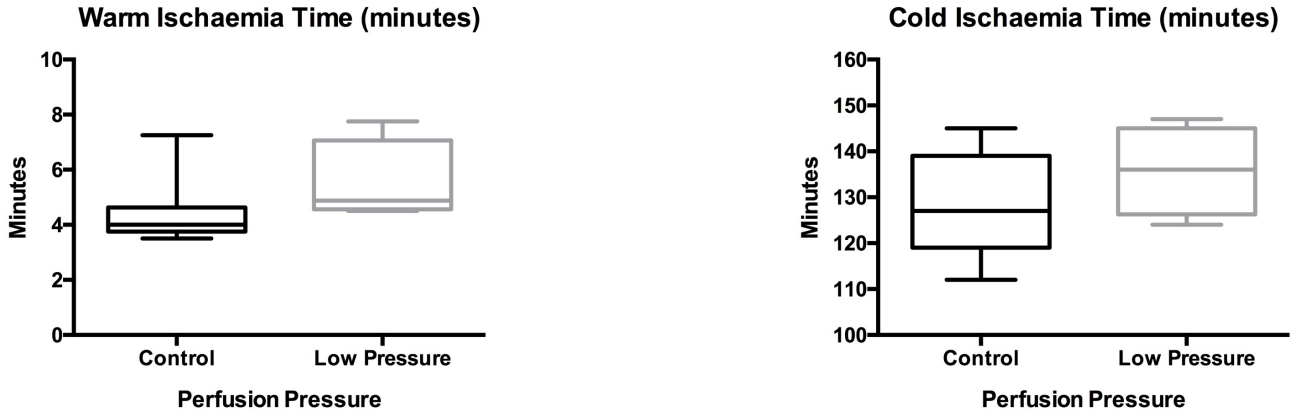
Furthermore, the immunohistochemical data analysed confirmed the grafts to have been viable at a cellular level.

5.2 GRAFT RETRIEVAL ISCHAEMIC TIMES

The warm and cold ischaemic times were defined previously. The WI time was recorded in minutes to the nearest 15 seconds and the median WI time for the low pressure system was 5 minutes. The range of values was from 4 minutes and 30 seconds to 7 minutes and 45 seconds (Figure 20). When the control group of models was compared to the low pressure system, there was no significant difference between the WI times (p=NS).

The CI time was recorded to the nearest minute. The median CI time for the low pressure system was 136 minutes. The range of values was 124 minutes to 147 minutes (Figure 20). When the control group of models was compared to the low pressure system, there was no significant difference.

Figure 20 – Box and whisker plots demonstrating the frequency distribution of warm ischaemic time (right) & cold ischaemic time (left). There was no significant difference between the control and low pressure system.



5.3 PERFUSION PARAMETERS

Pancreatic graft blood flow and blood pressure remained stable throughout each of the four low pressure perfusion experiments (Figure 21). The mean arterial blood flow to the graft was 40 (95% confidence interval 31 to 48) $mL.min^{-1}$. The lower pressure system had a significantly lower blood flow in comparison with the control grafts ($p < 0.01$) (Figure 21). The mean continuous arterial blood pressure for the low pressure system was controlled by the centrifugal pump and the mean value was 21.1 (95% confidence interval 20.5 to 21.7) mmHg (Figure 21). This did not change significantly between repeated porcine models (p=NS).

All low pressure system pancreas grafts demonstrated oxygen consumption using blood gas analysis (Figure 22). There was a mean arterial to venous partial pressure of oxygen differential of 70.4 kPa. The partial pressure of oxygen in the arterial blood inflow was measured from the aortic inflow segment with a mean value of 76.7 (95% confidence interval 69.7 to 83.6) kPa. The partial pressure of oxygen in the venous blood outflow was measured from the portal vein with a mean value of 6.2 (95% confidence interval 5.4 to 7.1) kPa. This arterial to venous oxygen differential reached statistical significance, $p < 0.0001$. The arterial to venous oxygen differential did not significantly differ when the control group was compared with the low pressure system, (p=NS) (Figure 22).

Figure 21 – Mean Arterial Blood Flow (+/- standard deviation) in mL.min^{-1} for the low pressure system perfused at a continuous pressure of 20mmHg. Both blood flow and pressure have been plotted with respect to time of perfusion in minutes (left).

The arterial blood flow (mL.min^{-1}) in the control grafts was compared with the low pressure group (right). The arterial blood flow was higher in the control grafts when compared with the low pressure perfusion system ($p < 0.01$).

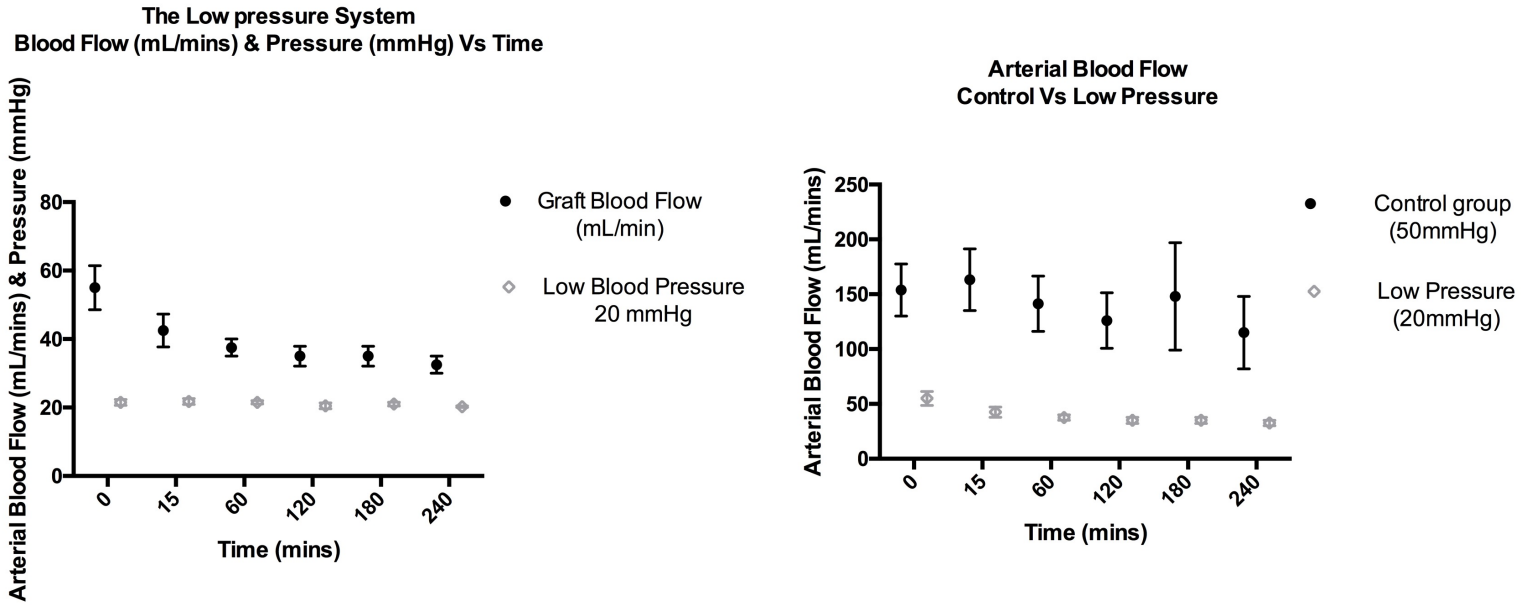
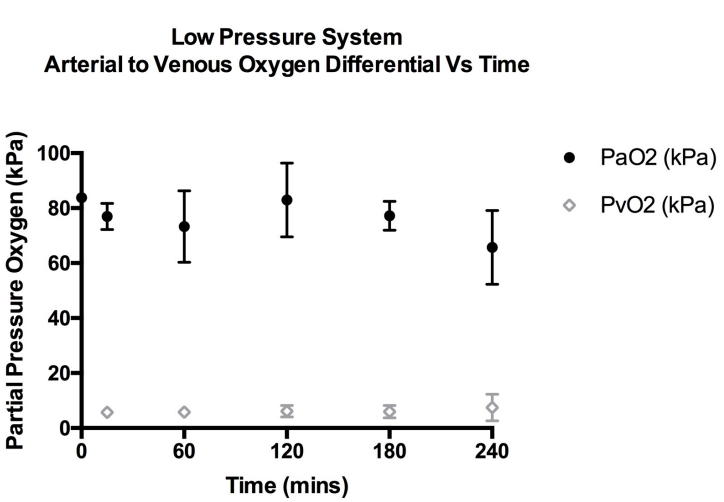
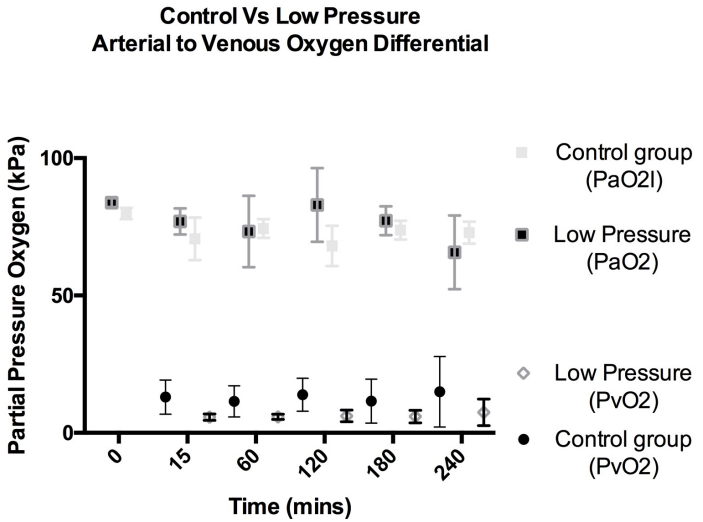


Figure 22 – Partial pressures of Oxygen sampled from the arterial inflow at the aortic segment (PaO_2) and the venous outflow, sampled at the portal vein (PvO_2). Error bars represent the 95% confidence interval at each time point during the perfusion. The low pressure system demonstrated a significant arterial to venous oxygen differential ($p < 0.0001$). The arterial to venous differential did not significantly differ when the control group was compared with the low pressure system (right).





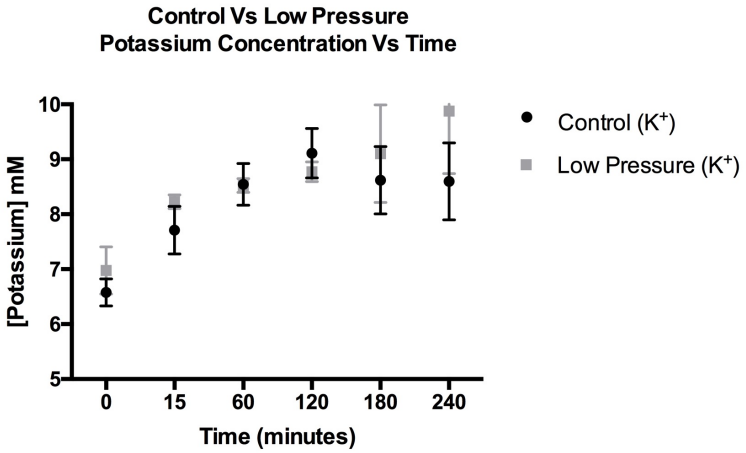
5.4 ACID-BASE (pH), LACTATE AND ELECTROLYTES

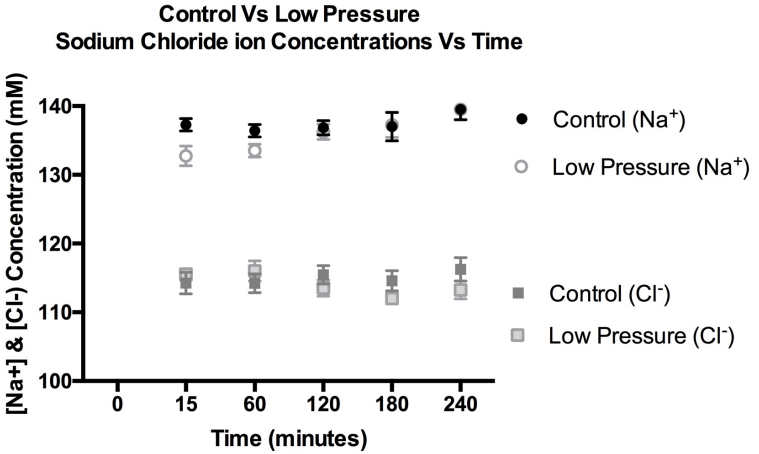
As the time of perfusion progressed, all four low pressure pancreatic grafts demonstrated an increase in blood perfusate acidity, demonstrated by an arterial blood gas pH decrease (Figure 23). There was an increase in both the accumulation of blood perfusate lactate anion and potassium cation concentrations (Figure 24). In contrast, again the sodium and chloride were relatively stable during perfusion and similar to normal range, *in vivo* (Figure 24). Although these were the trends observed during perfusion, none of the repeated measures were associated with statistical significance (p=NS).

The mean arterial pH was 7.32 (95% confidence interval 7.20 to 7.43). The mean arterial lactate concentration was 7.4 (95% confidence interval 5.4 to 9.4) mM.

The pH, lactate anion, potassium, sodium and chloride ion concentrations with respect to time of perfusion did not differ significantly between the control group and the low pressure system. The mean potassium concentration for the low pressure system was 8.7 (95% confidence interval 8.0 to 9.5) mM. The mean sodium concentration for the low pressure system was 136 (95% confidence interval 132 to 139) mM. The mean chloride concentration was 114 (95% confidence interval 112 to 116) mM

Figure 24 – The potassium cation concentration increased with increased time of perfusion, there was no significant difference when the control was compared to the low pressure system ($p > 0.05$) (left). The sodium and chloride concentrations remained relatively stable in contrast (right) for both the controls and low pressure system, there was no significant difference ($p > 0.05$). Error bars represent the +/- 1SD at each time point during the perfusion.





5.5 EXOCRINE FUNCTION

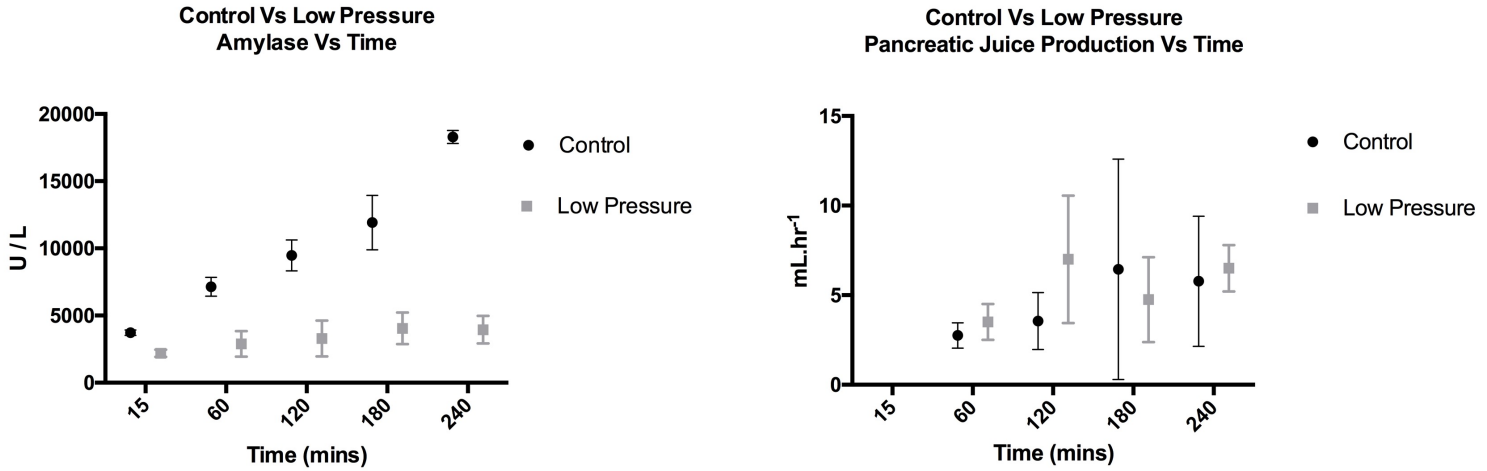
The exocrine component was assessed following the collection of serum amylase and the volume of pancreatic juice collected per hour. The values of the control group were compared with the low pressure system values (Table 7 & Figure 25).

Amylase levels were significantly lower in the low pressure system in comparison with the control group ($p < 0.03$). The mean amylase level was 3300 (95% confidence interval 2300 to 4200) $U.L^{-1}$. The low pressure had mean rate of pancreatic juice production rate of 5.4 (range 3 to 12) $mL.hr^{-1}$. However, the rate of pancreatic juice production was not significantly different in the control group when compared with the low pressure system (p=NS).

Table 7 – The control exocrine parameters have been compared with the low pressure system during perfusion with respect to time. All values are means with value of one standard deviation in parentheses.

Time (mins)	*[Amylase] ($U.L^{-1}$)*	*[Amylase] ($U.L^{-1}$)*	*Pancreatic Juice rate ($mL.hr^{-1}$)*	*Pancreatic Juice rate ($mL.hr^{-1}$)*
	Control	*Low Pressure*	*Control*	*Low Pressure*
15	3500 (300)	2200 (500)	-	-
60	7100 (700)	2900 (1900)	2.7 (1.1)	3.5 (1.0)
120	9500 (1200)	3300 (2700)	3.6 (3.3)	7.0 (3.6)
180	12000 (2000)	4000 (2300)	6.3 (7.2)	4.8 (2.4)
240	18000 (500)	4000 (2000)	5.6 (5.5)	6.5 (1.3)

Figure 25 – The exocrine function of the control group was compared against the low pressure models. The amylase levels were higher in the control group ($p < 0.03$) (left). The rate of pancreatic juice production in millilitre per hour was not significantly different when the control group was compared with the low pressure models ($p > 0.4$) (right). The error bars are (+/- 1 SD).



5.6 ENDOCRINE FUNCTION

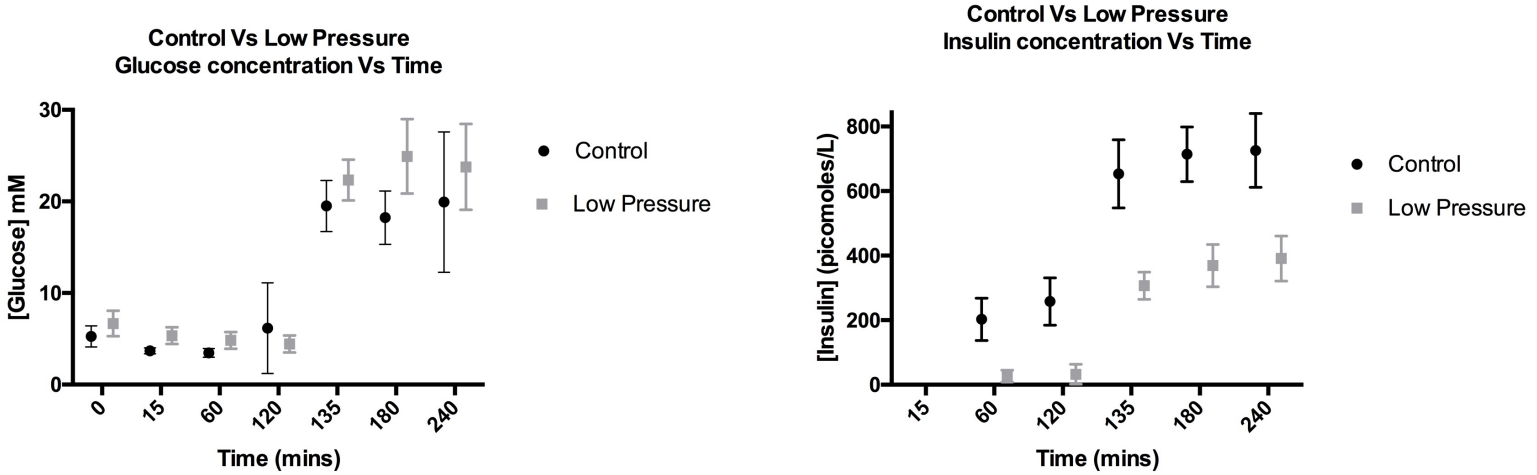
A comparison of the endocrine functionality between the control and low pressure grafts was again assessed by the quantification of the insulin production in response to an appropriate stimulus following delivery of glucose bolus (Figure 26).

The mean 'steady state' glucose concentration was 4.9 (95% confidence interval 4.3 to 5.3) mM. Following the delivery of a glucose bolus administered at 125 minutes, the mean concentration of glucose rose to 23.2 (95% confidence interval 21.0 to 25.6) mM and this was a statistically significant increase in glucose concentration, ($p < 0.0001$). When the glucose stimulation increases from 'steady state' levels were compared between the control group and low pressure models, there was no significant difference in the glucose increase following bolus administration in either system (p=NS). Therefore, it is arguable that the glucose bolus administration in the control group was 'equivalent' to the glucose stimulation in the low pressure graft experiments.

However, during the 'steady state' of glucose concentration, the mean concentration of insulin in the low pressure system was lower at 29 (95% confidence interval 17 to 42) in comparison with 227 (95% confidence interval 182 to 272) picomoles per litre in the control group.

Following the glucose bolus stimulation, at 125 minutes, the mean insulin concentration in the low pressure models increased significantly to 356 (95% confidence interval 324 to 388) picomoles per litre ($p < 0.0001$). This increase was noted to be lower than the control group which had previously risen to 798 (95% confidence interval 665 to 794) picomoles per litre following glucose stimulation. The control group, in comparison with the lower pressure model, demonstrated both a higher baseline and post glucose stimulation insulin level during perfusion ($p < 0.03$).

Figure 26 – Both the control group and low pressure models demonstrated significant insulin response to an appropriate glucose stimulation (right). The glucose stimulation was administered at 125 minutes (left). Both the 'steady state' and the increased glucose levels during stimulation were statistically comparable in both the control and low pressure models. However both the mean 'steady state' and stimulated state insulin levels were higher in the control group. The error bars are 95% confidence intervals.



5.7 IMMUNOHISTOCHEMISTRY ASSESSMENT OF CELLULAR VIABILITY

All pancreata were subject to immunohistochemical analysis to assess for viability at the cellular level. Anti-Caspase 3 and M30 CytoDEATH antibodies were used to quantify the presence of cellular death (Table 8 & Figure 27A&B). These stains demonstrated an improved cell death profile in the low pressure models. The mean number of Anti-Caspase 3 positive cells in the low pressure group was 6 (95% confidence interval 3 to 9) compared with 34 (95% confidence interval 11 to 57) cells per 3000 cells ($p < 0.008$). The mean number of M30 CytoDEATH positive cells in the low pressure group were 5 (95% confidence interval 3 to 8) compared with 45 (95% confidence interval 7 to 84) cells per 3000 cells ($p < 0.02$).

The activity of ATP synthetase was observed and graded, as previously described (Table 7 & Figure 28). Grade I (best), represented the presence of > 95 % of the slide section area staining positive for ATP synthetase complex V, grade II (>90 but < = 95) %, and grade III (>85 <=90) %. The scores assigned to the low pressure models reflected an improved profile for ATP Synthetase activity in comparison with the control group ($p < 0.016$).

H&E grades were assigned to each section (Table 8 & Figure 28) with the potential grades ranged from I (best) to IV (worst), as previously described. The scores assigned were similar when the control was compared against the low pressure models (p=NS).

Figure 27A – The low pressure pancreata also demonstrated evidence of cellular viability. An increase in number of cells staining positive for Anti-Caspase 3 (left) and M30 CytoDEATH (right) was noted with increase in perfusion time in both low pressure and control groups. However, the low pressure models had a lower mean number of cells staining positive for anti-caspase III ($p < 0.008$) and M30 CytoDEATH ($p < 0.02$). The mean number of cells (+/- 1SD) are plotted with respect to time.

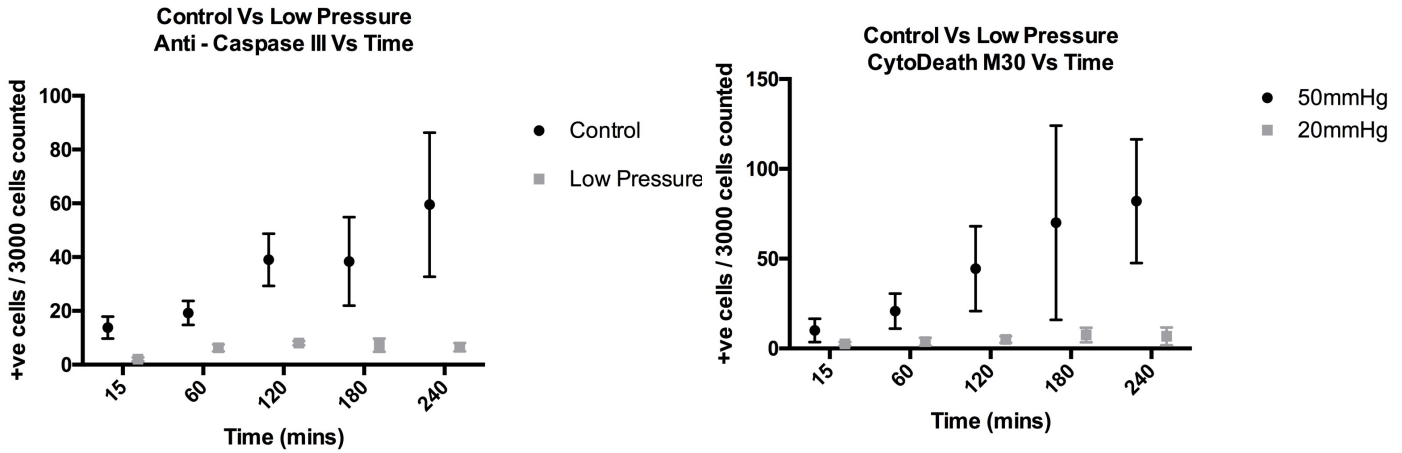


Figure 27B – Examples of M30 CytoDEATH stain to demonstrate quantification in preservation. These images are taken at 2 hours of perfusion. An example of a well perfused low pressure graft (left) is compared with a less well perfused control graft (right). The blue arrow mark a positive cell with the cytoplasm stained brown. Power magnification x 10.

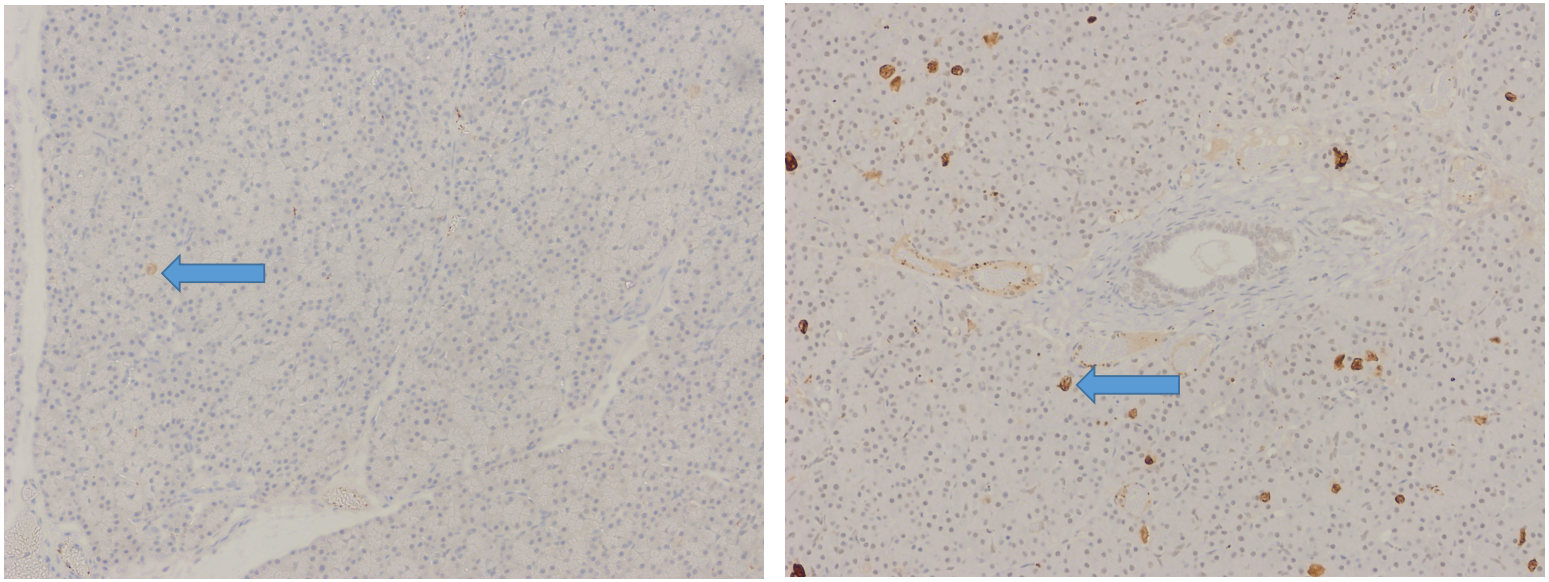
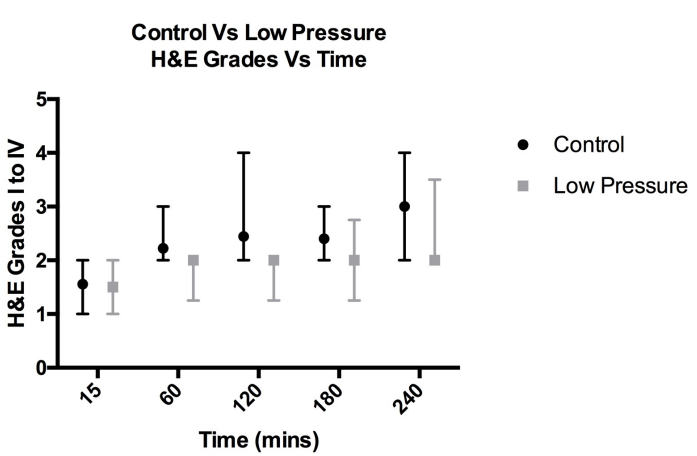


Figure 28 – The low pressure pancreata demonstrated evidence of cellular viability with similar score grades for the Haematoxylin and eosin staining ($p > 0.28$) (left); however an improved score grade for ATP Synthetase Complex V stain ($p < 0.016$) (right). The median grade with the error bars representing the inter-quartile range are plotted with respect to time.



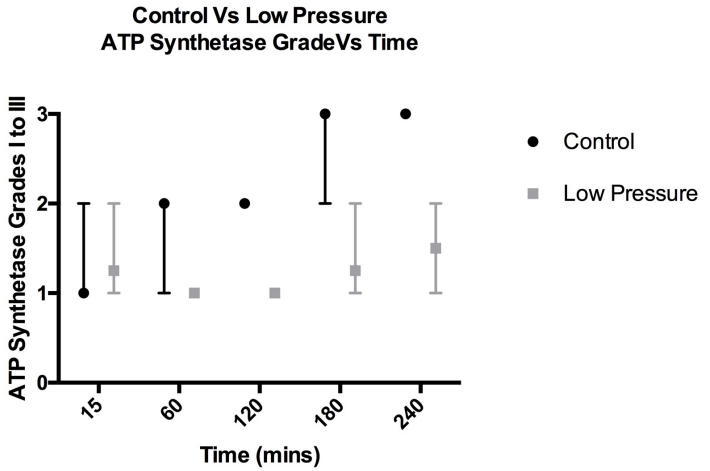


Table 8 Comparative immunohistochemistry biopsy scores during perfusion with respect to time. Anti-Caspase 3 and M30 CytoDEATH values are mean positive cells per 3000 cells counted, with one standard deviation in parentheses. H&E and ATP Synthetase values are median scored grade, with the range in parentheses.

Time (mins)	*Anti-Caspase 3*	*Anti-Caspase 3*	*M30 CytoDEATH*	*M30 CytoDEATH*	*H&E*	*H&E*	*ATP Synthetase*	*ATP Synthetase*
	(+ve Cells / 3000)	*(+ve Cells / 3000)*	*(+ve Cells / 3000)*	*(+ve Cells / 3000)*	*(I - IV)*	*(I - IV)*	*(I - III)*	*(I - III)*
	Control	*Low Pressure*	*Control*	*Low Pressure*	*Control*	*Low Pressure*	*Control*	*Low Pressure*
15	14 (12)	2 (1)	10 (6)	3 (1)	II (I - II)	I (I – II)	I (I - II)	I (I – II)
60	19 (13)	6 (3)	21 (10)	4 (2)	II (II - III)	II (I – II)	II (I - II)	I (I – I)
120	39 (29)	8 (2)	44 (24)	5 (2)	II (II - IV)	II (I – II)	II (II - III)	I (I – II)
180	38 (37)	7 (5)	70 (54)	8 (4)	II (II - III)	II (I – III)	III (II - III)	I (I – II)
240	60 (54)	7 (3)	82 (34)	7 (5)	III (II - IV)	II (II – IV)	III (III - III)	II (I – II)

5.8 DISCUSSION

In the previous chapter, a physiological *ex vivo* porcine pancreas perfusion model was established and shown to be feasible. In this chapter, the aim was to use this model to investigate one perfusion parameter, blood pressure (independent variable) and its effect on graft viability (dependent variable).

Data from the control group were compared with a further four models perfused at a lower pressure. Following statistical analysis outcome parameters demonstrated significant differences between the two groups (e.g. blood flow to graft, exocrine assessment with amylase levels, the magnitude of insulin production and immunohistochemistry staining). These differences in the data findings, *per se*, make it possible to argue that evidence has been provided to reject the null hypothesis.

Arguably, a potential limitation of ENPP would be the small numbers in each group. A prospective power calculation was impossible because this is the first study of its kind and an accurate estimation of the 'size of effects' between the groups unknown. Following the literature review (Chapter 1 and summarised Table 2), universally all porcine *ex vivo* studies, hitherto, contain very few numbers in each group. This is in stark contrast with the numbers used in population studies.

A power calculation is important in avoiding a type II error. However, given that most of the thesis probability values are highly significant rather than borderline; it makes the chances of a type II error highly unlikely.

Nevertheless, in order to satisfy a fuller understanding, a retrospective power calculation has been provided using the insulin data as a worked example. The 'size of effect' may be approximated using a Glass's delta test: difference in the means between the low pressure and control grafts would be 'delta' (285.6 picomoles per litre) divided by the SD of the control group

(79.4 picomoles per litre). Using a significance level set at 0.05 with number of replicates (n=9), this gave an adequate power, calculated at 0.967.

Notwithstanding the statistical discussion above, the ability to have tested a research null hypothesis successfully, *per se*, validates the pancreas perfusion system as a 'reproducible and reliable' model.

5.8.1 *EX VIVO* NORMOTHERMIC PORCINE PANCREAS PERFUSION AS A MODEL TO INVESTIGATE GRAFT PRESERVATION

Blood pressure was the independent variable that was investigated in the context of the 'low pressure' pancreas graft model. The low pressure was 20 mmHg and these grafts were compared with the control group, which had been perfused at a higher pressure of 50 mmHg. The low pressure experiments followed the identical protocols that were used for the control group. The WI and CI times, acidity (pH), lactate, and electrolytes were similar in both sets of experiments. The only perfusion parameter that was altered was the continuous perfusion pressure. This reduction in blood pressure, led to a reduction in the mean graft organ blood flow from 141 ml.min^{-1} to a mean of 40 mL.min^{-1} ($p < 0.01$) which was an expected observation. In vascular physiology, blood flow is dependent upon the dot product of the pressure difference and inverse of vascular resistance. Therefore, given that the resistance throughout the *ex vivo* circuitry remained unchanged between the two groups, a reduction in arterial blood pressure from 50 to 20 mmHg translated into a reduced blood flow in the closed system.

A rough estimation of the graft resistance is possible by calculating it from the pressure divided by flow. Taking mean values: for low pressure grafts, 20 mmHg / 40 mL.min^{-1} = 0.5 mmHg.min.mL^{-1}. For the control grafts, 50 mmHg / 140 mL.min^{-1} = 0.35 mmHg.min.mL^{-1}. Perhaps thrombosis at a microvascular level within the graft could be one explanation for this apparent increase in graft resistance seen with perfusion at low pressure.

However, irrespective of potential differences in resistance within the grafts between low and high pressure; It is important to highlight that this reduction in graft blood flow was not associated with a change in the arterial to venous oxygen differential when compared to the control experiments.

The remainder of the perfusion parameters such as acidity, lactate and electrolytes did not vary significantly. The mean values of lactate and potassium are outside the normal range *in* vivo, and these may therefore represent an area that needs further optimisation. However, given that the two groups had statistically similar values any differences observed in the graft preservation parameters are meaningfully interpretable. The immunohistochemical stains for markers of cell death also demonstrated an improvement in addition to an increase in the presence of ATP synthetase in the low pressure models when compared to controls.

All four pancreata at low pressure were viable macroscopically for four hours and produced pancreatic juice, representative of exocrine function. The volume of pancreatic juice was not significantly different in comparison with controls however, the amylase level was significantly lower at all time points during the lower pressure perfusion in comparison with controls ($p < 0.03$). As previously argued, the absolute value of amylase may only be of relevance at the beginning of the perfusion process, given that this is a closed system. Therefore, a reduction in the amylase levels in the low pressure models may represent an improvement in graft preservation and reduction in overall parenchymal and acinar damage.

The endocrine function was also confirmed by a statistically significant rise in insulin in the portal venous flow following a statistically significant increase in glucose concentration an observation that was also seen in the low pressure system. Given that both the controls and low pressure models had similar levels of glucose increase from 'steady state' to 'stimulated

state' (p=NS) it may have been expected for both sets of experiments to also exhibit similar levels of insulin secretion before and after glucose stimulation? However, following stimulation the absolute value of insulin production in the low pressure models was lower in comparison with the controls ($p < 0.0001$).

Previously, it was argued that the pancreas is a low pressure organ and perhaps the use of a perfusion pressure of 20 mmHg in comparison with 50 mmHg may have led to a reduction in 'shear stress' to the grafts. A reduction in shear forces would have led to a reduction in acinar and parenchymal damage. A reduction in acinar and parenchymal damage would account not only for the reduction in amylase levels but also the lower insulin levels observed. The observation that there was a statistically significant rise in insulin following similar glucose stimulation argues favourably for an intact endocrine feedback system in both the controls and low pressure models. There are a number of possible explanations for these findings and the lower insulin secretion values may represent an additional reduction in islet damage from 'shear stress' in the low pressure models. However, when islets are damaged they de-granulate and release insulin and it is not possible to differentiate between these two mechanisms in the early phase.

Important differences in the susceptibility of acinae when compared with islets from the ischaemia sustained during retrieval and reperfusion injury may also account for some of these observations? These findings are also reflected in clinical transplantation, where there appear to be differences in susceptibility to damage sustained between the exocrine (acinar) and endocrine (islet) components of the allograft. This is reflected by a universal acceptance that there will be some element of donor allograft pancreatitis with high amylase levels being considered to be of diminished value for diagnosis whilst islet function is seemingly well preserved in comparison even in the most difficult of transplants [51, 91].

5.8.2 CONCLUSION

The interpretation of a reduction in acinar and parenchymal damage in the low pressure models from the amylase and insulin data, taken in conjunction with the improvements that were observed in immunohistochemical staining, supports the premise that the lower pressure model may represent grafts with an improved organ preservation profile in terms of viability. The null hypothesis was therefore rejected.

CHAPTER 6: PANCREAS DIGESTION AND ISLET ISOLATION

6.1 INTRODUCTION

The principle of islet isolation involves the enzymatic dissociation of the pancreas parenchyma into a suspension. The enzyme is collagenase and it is usually delivered via the pancreatic duct. This intra-ductal delivery of collagenase is important in digestion of the pancreas because it directly exposes the acinae to the effects of the enzyme and allows the exocrine tissue component of the pancreas to be separated from islets. Too little exposure causes the islets to remain surrounded by redundant exocrine tissue and conversely, too much exposure causes the islets to fragment. Porcine islets are more irregular and have an increased susceptibility to fragmentation in comparison with humans and therefore great care must be employed in porcine islet isolation in order to reduce the risk of a poor yield or high proportion of non-viable islets [108, 109].

Chapter six aimed to answer the following research question:

Is it feasible to digest porcine pancreata followed by the isolation and quantification of viable islets?

6.2 MATERIAL & METHODS FOR PANCREAS DIGESTION AND ISLET ISOLATION

Five Pancreata were retrieved and transported to the laboratory in ice cold preservative solution, stowed in an ice box, as previously described (Chapter 3).

6.2.1 PANCREAS PREPARATORY BENCH-WORK

The integrity of the pancreas capsule was maintained throughout. An intact capsule was of paramount importance to create sufficient gland distension during collagenase infiltration.

With this is mind, the duodenum was carefully dissected away and discarded and extraneous fat and lymphoid tissue was then removed. The pancreas gland mass was then recorded and a wide bore venous cannula (BD Venflon™ Becton, Dickinson & Co., NJ, USA) was introduced into the pancreatic duct and secured with suture ligation. Further suture ligations were placed either side of the duodenal and connecting lobe, isolating the splenic lobe. All ligatures were Vicryl™ (Polyglactin 910, Ethicon, Johnson and Johnson).

6.2.2 PANCREAS DIGESTION

NB 8 GMP grade collagenase was used and coupled with neutral protease NB Grade specific for porcine pancreas digestion available commercially as a kit (SERVA Electrophoresis, Heidelberg, Germany). The concentrations of NB 8 GMP grade collagenase and neutral protease NB grade were 6 PZ units and 1.5 DMC units (Wünsch), respectively.

A volume of 2mL of NB 8 GMP grade collagenase solution was used for every unit gram mass of pancreas to achieve glandular distension. The pancreas was then placed into a 1L plastic tub with any remaining collagenase and the digestion was then allowed to proceed by static incubation in a water bath set at 37°C until the pancreata became 'loose' and the parenchyma was easy to 'slough' with a pair of tweezers. This usually took between 30 to 60 minutes and the glands were then mechanically 'morsel-ised' by hand and the tissue was passed through a metallic filter mesh with netting holes of 500 µm diameter. The fine tissue effluent pushed through the mesh was collected in cold Hanks Balanced Salt Solution (HBSS) made up with 10% Foetal bovine Serum (FBS). HBSS and 10% FBS solution was stored ready made in a 4°C fridge, both supplied by Sigma-Aldrich Company Ltd, UK. They were used directly from the fridge, with minimal environmental exposure at room temperature, to prevent unnecessary warming. The digested pancreas tissue suspended in the cold solution of HBSS with 10% FBS was then decanted into 250mL conical, plastic tubes. These conical tubes were then centrifuged at 100G

for 2 minutes. Centrifugation resulted in a 'tissue pellet' of pancreas digest material in each conical tube. These were re-suspended in Minimal Essential Medium (MEM) (Sigma-Aldrich Company Ltd, UK) and pooled together into a total volume of 50mL.

6.2.3 ISLET QUANTIFICATION

The re-suspended islet solution of 50mL was gently agitated by inversion and 5 x 200 µL aliquots were sampled. These five samples were then diluted by a factor of 10, being made up in MEM to a volume of 2mL total. From these 5 x 2mL samples, 2 x 100 µL aliquots were sampled leading to 10 total samples, each having a dilution factor of 5000 to the original 50mL final digest volume. The 10 samples of 100 µL were 'streaked' across a Petri dish and to this 100 µL of dithizone stain (Sigma-Aldrich Company Ltd, UK) were added to mark the islets and aid counting (Figure 29). Dithizone is an organic sulphur containing compound which acts a ligand for heavy metal cations. The stain is useful for binding to the zinc ions for the zinc present in beta (insulin producing) islets. The islets were counted under a light microscope at 40 x magnification.

The quantification of the islet yield was calculated in the following way, using the sample means from all 10 of the 100 µL streaks:

100 µL sample to 50mL pancreas digest solution has a dilution factor of 1:5000

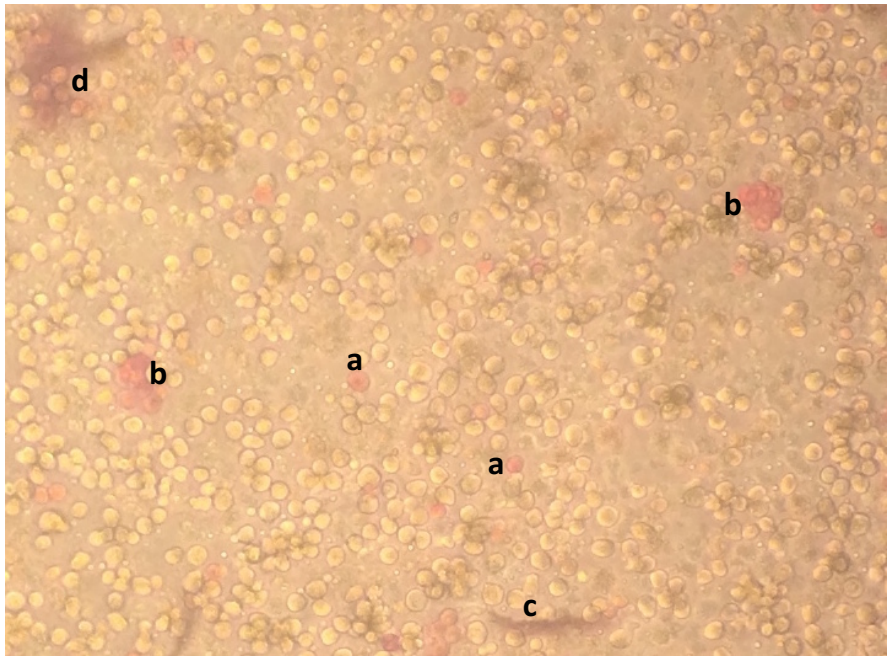
Mean islet count from ten 100 µL samples x 5000 = ∑ number of islets.

Yield of Islets = ∑ number of islets / mass of pancreas = Islet per gram)

6.2.4 CLEAVAGE INDEX

An assessment of cleavage index was possible during the determination of islet yield. A single islet in completed isolation from exocrine tissue was defined as cleaved (Figure 29). The total number of cleaved islets was divided by the total number of islets, the cleavage index

Figure 29 – Light microscopy of pancreas digest suspended in MEM solution, imaged at 10 x magnification. Beta Islets have been stained with dithizone (red – pink). A single islet has been marked (a); a cluster of islets marked (b); redundant exocrine tissue marked (c); a cluster of islets, still in association with exocrine tissue 'uncleaved' (d).



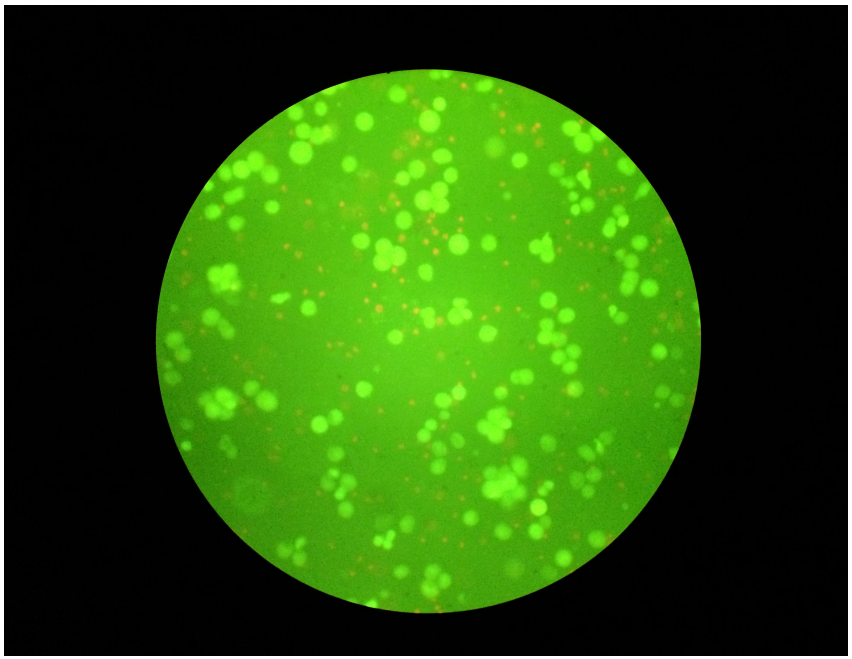
6.2.5 ISLET VIABILTY STAIN

Fluorescein diacetate (FDA) is an esterase that permeates the cell membrane. An intact cell membrane is a requirement for FDA to be activated because it must be retained within the cell to be exposed to hydrolysis by intracellular esterases. This process only occurs in viable cells and as a result, the stain fluoresces green, under UV light microscopy while non-viable cells stain in red colour.

In order to quantify islet viability, approximately 100 µL of the pancreas digest solution was diluted in MEM and made up to 1 mL. Using a 1.5mL Eppendorf tube 10 µL of FDA were added to 500 µL of ethidium bromide solution, both supplied by Sigma-Aldrich Company Ltd, UK. 200 µL of this solution was added to the islet solution. This was protected from the light and left for 5 minutes to develop and then poured into a Petri dish and swirled manually, in order to 'centre' the islets. The Petri dish was angulated and excess medium aspirated and discarded using a pipette.

The islet sample was then inspected under fluorescence UV light microscopy and cells staining green counted as viable whilst red stained islets deemed non-viable. The proportion of viable islets was recorded as a percentage.

Figure 30 – Fluorescent UV light microscopy image of islets isolates having been stained with fluorescein diacetate and ethidium bromide stain in order to assess viability. Green islets are viable under fluorescent light, whilst those staining red are non-viable and often islet fragments.



6.3 DISCUSSION

Since 1991 the Leicester group has been involved in clinical and experimental human islet isolation and implantation. Particularly in islet auto-transplantation, Leicester enjoys the third largest series in the world-wide literature.

This chapter presented an islet isolation SOP, that has been developed iteratively based on five porcine experiments. Some of the technical aspects were adapted from human isolation practice protocols in conjunction with discussion and guidance from previous work carried out by a doctorate student, who had experience in rat islet isolation. This helped to finalise the concentration of digestive reagents and it allowed for improved staining with dithizone. The technical aspect for the quantification and assessment of viability in porcine islets is based on advice from the Oxford Consortium for Islet Transplantation. This expert advice was delivered through tutorials and learning via personal communication from Professor Paul Johnson, Dr Heide Brandhorst and Dr Stephen Hughes. Their recommendations for using ethidium bromide instead of propidium bromide for the FDA stain had allowed for a major improvement in islet viability staining.

This is a reproducible, SOP for porcine islet isolation without purification. The SOP is reliably able to derive relevant outcome measures of interest, islet yield being expressible as a function of graft mass and quantification of islet viability. Islet purification is not a step in this protocol and therefore, the inclusion of a cleavage index outcome measure may be considered moot. However, it has been included for completeness and it may help quantify over (or under) exposure to collagenase. The SOP is ready to be used as an outcome measure to assess porcine islet preservation techniques.

CHAPTER 7: DISCUSSION, LIMITATIONS & FUTURE WORK

7.1. INTRODUCTION

This chapter has aimed to summarise the experimental work and contextualise it with respect to the literature review. Limitations of the physiological pancreas perfusion model were previously discussed. Suggestions have been offered here for further study to both improve these limitations and to advance the field.

7.2 A SUMMARY OF PERFUSION PRESSURE EXPERIMENTAL WORK

A porcine pancreas physiological perfusion model has been developed. The model has demonstrated the capacity to reach beyond simply a 'feasibility' study. The model has been used to investigate and test a null hypothesis in a reliable and reproducible way, acquiescent to statistical analysis.

For a sufficient length of time *post mortem,* the model was viable at a cellular level with evidence of preserved endocrine and exocrine function. Some of these outcome parameters were arguably improved in grafts that were perfused at a low pressure. The low pressure perfusion models, translated to a lower graft flow rate. A potentially improved preservation of grafts with a low pressure / low flow is within the realm of current understanding that the pancreas is a low flow organ. This understanding was reflected in the choice of a continuous perfusion pressure of 50 mmHg, following the preliminary study. 50 mmHg was a much lower pressure in comparison to mean aortic pressure of circa 90 mmHg, taken from physiological study *in vivo*.

7.3 THE 'OPTIMAL' PERFUSION PRESSURE

In order to contextualise this, within the field of pancreas transplantation, the British Transplantation Society have published guidelines on the acceptability of donor pancreata following DCD retrieval. Currently their recommendations are in favour of discarding the pancreas if the functional WI time (defined as mean arterial perfusion pressure has fallen below <50 mmHg systolic with oxygen saturations of < 70%) has been for 30 minutes or greater [110]. Yet, in contrast, the *ex vivo* perfusion model showed a potential improvement in graft preservation with a significantly lower perfusion pressure of 20 mmHg. Given that both systems had evidence of an arterial to venous oxygen differential and evidence of cellular ATP synthetase activity, it is arguable that pressures of 20 mmHg were also 'adequate' at achieving microvascular and capillary bed circulation.

Animal pancreatic blood flow studies have shown evidence for a differential blood flow to exist within the pancreas. A higher flow is demonstrated for the endocrine component while lower blood flow is reserved for the exocrine parenchyma. Furthermore, islets have a very rich vascular supply and have been shown to be able to support their own internal milieu by autonomous regulation from interactions between the nervous system, metabolites, endothelium derived mediators and hormones [111-113].

Comparative anatomical studies in rat, dog and Guinea pig have confirmed the presence of three types of end arterioles; some ending directly at capillary glomeruli of islets, whilst some terminate at the capillaries around the acinar unit and the remainder to the ductal system [114]. Both control and low pressure porcine pancreata produced insulin in response to glucose stimulation and the only difference between the two groups was the absolute levels of insulin production. A similar mechanism of autoregulation and or differential functional blood supply at the islet level within the porcine grafts may potentially explain these differences in insulin

production in these experiments. Whether these findings translate to humans remains to be seen. Although the limited anatomical studies in humans have shown evidence of a differential termination of end arterioles at the islets – acinar level that go beyond the confines of a functional 'one vessel to one lobe' anatomy [115, 116].

Alternatively, the differences between low pressure and control grafts, may be explained by intrinsic differences between fluid flow dynamics in continuous and pulsatile flow. The exact optimum machine perfusion pressure for use with porcine pancreas grafts remains unknown. The choice was based on the understanding that *in vivo*, the mean arterial pressure within a pulsatile porcine aorta is a mean of around 90 mmHg. The choice of 50 mmHg worked well for the control experiments and it was suitably low in comparison with the *in vivo* pressure data and it was appropriately justified for a 'low flow, low pressure' organ system. Further rationalisation was based on optimisation during the preliminary experimental work.

The *ex vivo* model perfused porcine pancreata grafts with a continuous pressure delivered via a short aortic segment, instead of a full length and pulsatile aorta. All vessels have a fundamental ('natural') frequency, which in part is dictated by length. When the cardiac pulse matches this fundamental frequency, the longitudinal delivery of blood is efficient, with optimal Windkessel phenomenon established for longitudinal energy (blood flow) transmission [117]. Therefore, in analogy, the perfusion of the graft with a lower continuous pressure of 20 mmHg, might have been better suited to the natural frequency of a shorter aortic segment graft. This may have resulted in an improved microvascular perfusion at the parenchymal level?

Tortuosity in arterial blood vessels are commonly found in the human body. They are associated with hypertension. Blood vessel studies have shown that tortuosity develops as a result of hypertension; as a defence mechanism to 'dampen' the arterial pressure waveform to reduce the effects of hypertension on the end organ. Porcine carotid vessel studies have

demonstrated evidence to support the development of tortuosity in hypertension [118]. In humans, the splenic artery is naturally tortuous and it supplies the dorsal pancreatic artery. The natural tendency of the splenic artery to be tortuous, may have evolved to dampen the high blood pressure immediately off from the aorta, coeliac trunk, in preparation for low pressure organs such as the pancreas and spleen.

Thus in conclusion, the apparently very low perfusion pressure of 20 mmHg may actually have been better suited to perfuse grafts in our model. A very low pressure may have negated the differences in fluid flow dynamic that exist between a pulsatile and continuous setting. Furthermore, a lower pressure may be a better reflection of the true pressure within an *in vivo* pancreas from the knowledge that the splenic artery is naturally tortuous and it may have been better matched for the differential vascular supply for islet to acinar anatomy.

In the field of machine organ perfusion, the few limited studies in the literature have shown that the difference between continuous and pulsatile blood flow may not be important within the context of other organs. However, the choice of continuous or pulsatile perfusion may be more important for the pancreas as it is usually recovered on a short aortic segment graft. Furthermore, a very low perfusion pressure may be adequate to achieve optimal delivery of nutrients to the donor pancreas. With further evidence, the perfusion pressure defining the current guidelines for tolerable functional WI times for donor pancreas in the clinical setting may change.

7.4 THE EXOCRINE SYSTEM

As discussed previously the significance of raised amylase levels must be interpreted with some caution. The absolute initial value may represent acinar damage, attributable to the retrieval process, WI, CI and reperfusion injury. A reduction in amylase levels at the commencement of

perfusion may reflect an improvement in acinar damage. The further rise in amylase during a closed circuit perfusion was arguably to be expected and may represent autolysis with further glandular enzyme damage. Within the field of pancreas transplantation, high amylase levels are not considered to be an absolute contraindication for pancreas donation [110]. Although some have argued that higher serum amylase levels in the donor are related to poorer outcomes post transplantation [105, 106].

The *ex vivo* model is limited in its full evaluation of the exocrine system in this sense, because serum lipase levels were not measured. The addition of lipase collection to the experimental design was considered but it was discarded because of practical difficulties involving an off-site processing system. The model may be thus optimised further by considering this as adjunct to assess exocrine function more fully.

7.5 THE MODEL AS A REPRESENTATION OF THE DCD SETTING

The WI time is a limitation in the *ex vivo* model with the median WI was 4 to 5 minutes across all models with range of 3 minutes and 30 seconds to 7 minutes and 45 seconds. The WI time in this porcine DCD model was short in comparison with human pancreas DCD transplantation scenario. Data from the University of Wisconsin which has been collected over 29 years showed that the mean WI time for a pancreas transplantation was 20.8 minutes (+/- 10.9) with an outlier at 64 minutes [30].

In order to make the model more representative of the DCD setting, a longer WI time could be easily implemented. By allowing more time to elapse between the visualisation of circulatory arrest and the cold flush of the aorta, during retrieval the WI could be prolonged to better reflect current clinical practice.

The 'mode of dying' in ENPP is different when compared with clinical practice and this may represent a potential limitation for arguing a similarity with a DCD setting, viz. During the

agonal phase immediately prior to death, the animal may release vasoconstrictor catecholamines in response to the hypovolaemia and pulseless electrical activity that occurs from the current method of pancreas retrieval in this thesis. In contrast with current clinical practice, organs may be recovered in a 'controlled' DCD setting with the withdrawal of care from a donor suffering with failed / futile intensive care management. Although, on the intensive care unit, some of these differences in neuro-humoral responses between ENPP and the clinical practice may be offset in donors that have received high levels of inotrope and vasopressor infusions.

7.6 THE PERFUSATE, BLOOD GASES AND ION IMBALANCES

The perfusate in ENPP was autologous blood with the addition of heparin to reduce coagulation. Citrate to chelate the calcium ions or thrombolytic agents (e.g. urokinase, streptokinase) are potential alternatives that may have been considered. The choice to use heparin was based on ease of availability and cost.

The perfusate could be further optimised and made representative of clinical practice by using 'packed red cells' only (i.e. exclude the platelets and leukocytes), which would lead to a potential reduction in graft thrombosis. However, within the current constraints of the laboratory set up, isolating 'packed red cells' would require an expensive cell salvage machine. Interestingly, during the H&E immunohistochemistry staining analysis, none of the sections demonstrated the presence of (inflammatory) white cells. Perhaps, one explanation for this is that the leukocytes had 'marginated' secondary to laminar flow within the circuitry? Thus the removal of leukocytes with an expensive device may not therefore be an imperative step after all. This explanation for the 'margination' of white cells would also help to account for the absence of leukocytes from the H&E stained sections.

The perfusate milieu became increasingly acidotic with an increase in lactate and potassium ionic concentration in both control and low pressure models. This did not differ significantly between the two groups. However, the addition of a kidney will only serve to ameliorate the acid base imbalances. Amylase removal from the circuit would require a plasmapheresis machine in order to remove large protein molecules, effectively 'opening up' the closed circuit.

It may also be worth considering the use of a different preservation solution. The preservation solution used in these experiments was Soltran® kidney perfusion fluid, (Baxter Healthcare Ltd, Renal Division, Hospital Equipment and Supplies, Northampton, UK) and the potassium in this solution is composed with citrate. UW is the 'gold standard' for CS in pancreas preservation and the potassium is instead conjugated with lactobionate. These differences may have contributed to potassium accumulation within the closed circuit.

The choice of using Soltran® was based on price and availability. It was relatively inexpensive and easy to obtain especially considering that Leicester specialises in renal transplantation and is a tertiary referral centre for kidney dialysis.

7.7 THE ISLET ISOLATION PROTOCOL

The work undertaken has resulted in a reproducible protocol for porcine islet isolation, with the ability to measure the yield and viability. In the two available *ex vivo* models investigating islet isolation, one study demonstrated that acellular HMP may improve islet yield but the study did not reach significance [71]. Another study indicated that the oedema associated with MP pancreata may be beneficial for islet isolation [70].

Further work should focus on combining the model with the islet isolation protocol to investigate the effect(s) that a short period of graft perfusion has on islet isolation, yield and viability.

7.8 CONCLUSION

Ex vivo perfusion of the pancreas has been a technical challenge, but it has been possible to establish a suitably physiology, reliable and reproducible model and SOPs. In comparison with live animal studies, it is inexpensive, ethical and facilitates the controlled manipulation of isolated variables. It provides a suitable platform to study whole organ pancreas preservation and salvage and islet preservation techniques potentially improving the clinical outcomes in both groups.

CHAPTER 8: BIBLIOGRAPHY

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