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Establishment and Assessment of Pancreas Preservation Using Oxygenated Hypothermic Machine Perfusion

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7 References

A number of conference presentations and publications resulted from the work described in this thesis as listed below:

- French Congress of Urology AFU 2020: Modèle Préclinique de Perfusion Normothermique Ex-Situ de Transplant Pancréatiques.

- Modèle Préclinique de Perfusion Normothermique Ex-Situ de Transplant Pancréatiques (Abstract) 2020. G. Hakim, A. Ogbemudia, J. Hunter, L. Lo Faro, T. Prudhomme, K. Rozenberg, G. Ebeling, F. Dengu, P. Friend, R. Ploeg, J. Branchereau

- British Transplantation Society Poster Presentation 2021: Development of Ex Vivo Normothermic Machine Perfusion as an Innovative Method to Assess Pancreases after Preservation.

- Development of Ex Vivo Normothermic Machine Perfusion as an Innovative Method to Assess Pancreases after Preservation. Transplant International 2021. A. Ogbemudia, G. Hakim, F. Dengu, F. El-Gilani, R. Dumbill, J. Mulvey, K. Sayal, T. Prudhomme, B. Mesnard, K. Rozenberg, L. Lo Faro, T. James, J. Oliver, E. Sharples, S. Mittal, P. Johnson, P. Friend, R. Ploeg, J. Hunter, J. Branchereau.

ABSTRACT

Background: Pancreatic transplantation is the treatment of choice for a selected group of patients with the most severe complications of diabetes. The two main causes of early failure in this transplant are pancreatitis and venous graft thrombosis. Due to its anatomy and physiology, the pancreas is an organ that is particularly sensitive to ischaemia reperfusion injury. The current standard technique for preserving transplants after removal and before transplantation remains static cold storage (SCS). Changes in donor characteristics (older, with more co-morbidities) have led transplant teams to consider for transplant increasingly high-risk pancreases, which are even more susceptible to ischaemia reperfusion injury.

Aim: The objective of this study was to establish the modalities of an innovative technique for the preservation of pancreatic transplants on a pulsatile hypothermic perfusion machine (HMP) and to compare SCS, HMP and Hypothermic Oxygenated Pulsatile Perfusion ($HMPO_2$) preservation methods for pancreas in a Donation after Circulatory Death (DCD) porcine model.

Methods: Pancreases were retrieved after cold preservation solution flush from Sus scrofa domesticus pigs sourced at an abattoir, in a DCD model. Pancreases were cold preserved with Static Cold Storage (n=5), Hypothermic Machine Perfusion (Waves machine) (n=4), or with Hypothermic Oxygenated Pulsatile Perfusion (n=4) (medical air 300ml/min). General injury markers (lactate; lactate dehydrogenase) and exocrine markers (amylase, lipase) were assessed in the perfusate (or preservation solution for SCS) during preservation.

Pancreatic resistance index, pressure and flow were also analysed. Biopsies were collected to assess pancreas oedema (wet to dry weight ratios before and after preservation).

Results: SCS resulted in an oedema scale assessment score of 0 when compared with both HMP groups (score 1), however wet-to-dry weight ratio percentage difference was highest for SCS indicating the presence of oedema. Flow and resistance index were shown to be reciprocal, with the highest flow being achieved in the $HMPO_2$ group. No statistically significant difference was found between $HMPO_2$ and HMP groups for oxygen pressure in the perfusate over time. LDH levels were significantly higher in the SCS group, as well as lactate levels. Amylase and lipase levels were found to be significantly greater in the $HMPO_2$ group. Glucose levels were highest in the $HMPO_2$ group, whilst pH was greatest in the SCS group despite being stable across all three groups HMP, $HMPO_2$ and SCS.

Conclusions: $HMPO_2$ appears to be a promising alternative method to SCS preservation, despite research in the pancreas proving more challenging than in other organs. The flow was higher in the $HMPO_2$ group, while the degree of oedema appeared to be lower alongside lower levels of general tissue injury markers, making $HMPO_2$ a potentially suitable alternative to preservation by static cold storage.

INTRODUCTION

1.0 Diabetes Mellitus

Diabetes mellitus is a multifactorial disease associated with a chronic increase in blood glucose levels: hyperglycaemia. Glycaemia is the level of glucose in the blood and varies between 3.9 and 5.5 mM. Hyperglycaemia develops when the production of insulin by the pancreas is no longer sufficient (Type I diabetes), or when this insulin is no longer effectively used by hepatocytes, myocytes and adipocytes (Type II diabetes). The International Diabetes Federation (IDF) estimates that 629 million patients will be diabetic by 2045. There are two main forms of diabetes: Type I and Type II. Type II diabetes results from deregulation of insulin secretion combined with insulin resistance, while Type I arises from a defect in insulin production. Type I (or insulin-dependent) diabetes accounts for approximately 10-20% of diabetes cases and the number of patients currently living with Type I diabetes is approximately 400,000 in the UK. Its incidence is increasing in the European paediatric population, with an annual increase of 3.5% in children under 15 (1).

Type I diabetes is an autoimmune disease (2) which most often presents before the age of 30 and which combines genetic predispositions and environmental factors. These aetiologies result in an irreversible destruction of the beta cells of the islets of Langerhans and this becomes symptomatic when approximately 80% of beta cells are destroyed. Currently, no effective means are available to stop the destruction of islets of Langerhans before the onset of the disease. Immunological approaches are under development to inhibit the recognition and destruction of beta cells by autoreactive T lymphocytes (3). The destruction of the beta cells affects the endocrine function of the pancreas, with the

consequence of a complete or almost complete absence of insulin secretion, a hormone essential for the regulation of blood sugar and survival. After the discovery of insulin by Banting and Best in 1922, diabetes ceased to be a rapidly fatal disease, and became a chronic condition with long-term complications. The treatment of diabetes requires the use of exogenous insulin to overcome insulinopaenia and to adequately regulate blood glucose levels. However, exogenous insulin does not precisely reflect the physiological need throughout the day. This inability to control the hydrocarbon balance accurately is reflected throughout the course of the disease by the appearance of acute and chronic complications in a high percentage of patients which can endanger the patient's life. With the advent of exogenous insulin therapy by injection, the survival rates of patients with diabetes improved radically, but unfortunately this treatment does not prevent the onset of significant long-term complications, mainly resulting from micro and macroangiopathy: these have, in turn, several consequences, such as end-stage renal disease (ESRD) requiring dialysis or renal transplantation, ischaemia of the lower limbs, multiple cardiac and vascular damage as well as retinopathy (4)(5). Beta cell replacement (solid organ transplantation/islet transplantation) is indicated in a highly selected subgroup of patients with severe complications of diabetes. Beta cell replacement therapy is indicated in patients with Type I diabetes with advanced complications (e.g, renal failure) but who have been assessed as suitable in terms of surgical risk (e.g., cardiovascular and respiratory function). Solid organ pancreas transplantation is also increasingly recognised as an appropriate therapy for selected patients with advanced complications of type II diabetes, with the clear proviso that patients with type II diabetes maybe less suitable as a group because of associated morbidities. The replacement of insulin-producing beta cells by

transplantation is a possible and interesting alternative. The two current alternatives are solid organ pancreas transplantation and islet transplantation. The ongoing shortage of donor organs for transplantation, as well as changes to the demographics of donors, have created a driving pressure to achieve an optimal approach for organ preservation to preserve and improve organ quality. Pancreatic transplantation currently remains the most effective treatment for appropriately selected diabetic patients with the most severe manifestations of diabetes. The sole method for pancreatic preservation to date is static cold storage (SCS), however, alternative methods including hypothermic machine perfusion with oxygenation could challenge this.

1.1 Anatomy & Physiology of the Pancreas

The pancreas is a retroperitoneal organ. It is positioned retroperitoneally, in the epigastric region, between the duodenum and the spleen, and is located behind the stomach. It is 15-20 cm in length, 3 cm in width, and weighs less than 100 g. The pancreas is comprised of three parts: the head, the body and the tail. The head of the pancreas is on the right side of the abdomen and is connected to the duodenum through the pancreatic duct. The tail extends to the left side of the body and is close to the spleen.

The head and neck of the pancreas are supplied by branches from the celiac trunk through the superior pancreaticoduodenal artery, and the superior mesenteric artery through the inferior pancreaticoduodenal artery. The body and tail are supplied by the splenic artery through 8-10 branches. The head and neck of the pancreas are drained by anterior and

posterior venous arcades that form the superior and inferior pancreaticoduodenal veins which follow the corresponding arteries. The body and tail are drained by the splenic vein, a tributary of the portal vein, as shown in Figures 1 and 2 (6).

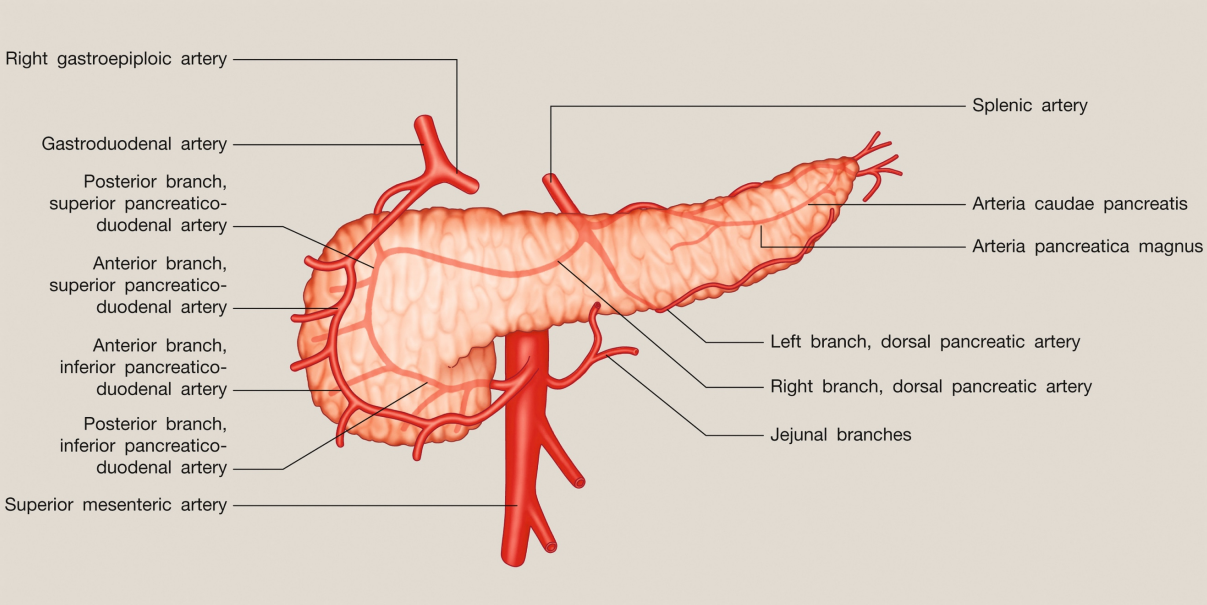


Figure 1: Arterial supply of the pancreas (7).

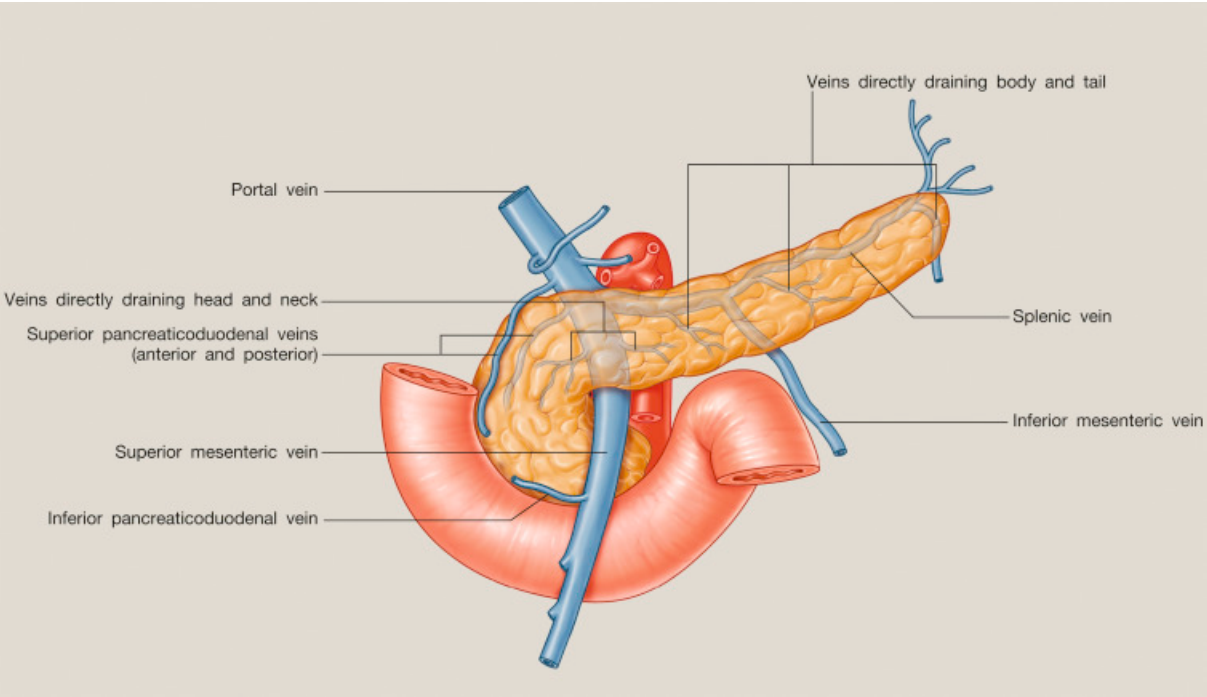


Figure 2: Venous drainage of the pancreas (7).

Physiology

The pancreas has a central role in digestion (exocrine function) and in glycaemic regulation (endocrine function).

Exocrine pancreas

Exocrine tissue in the pancreas is made up of two functional units: acinar cells which secrete digestive enzymes and pancreatic duct cells which secrete electrolytes. It contains 3 key enzymes: amylase for the digestion of carbohydrates, lipase for the digestion of lipids and trypsin for the digestion of proteins. Approximately 90% of the glandular mass of the pancreas is responsible for exocrine secretion. Pancreatic juices are secreted in the secondary pancreatic ducts which join the Wirsung duct to be released into the duodenum. The secretion fluctuates depending on the food intake per day, between 1 and 1.5 L.

Endocrine pancreas

Paul Langerhans described what is now known as the eponymous (pancreatic) islet 150 years ago. The islets of Langerhans, clusters of cells scattered throughout the gland, are responsible for the endocrine secretion in the pancreas. Islets are clusters of cells varying in number between 1 and 5 million and varying in size between 50 and 500 μM in diameter (8)(9). An islet (Figure 3) is composed of beta (β**)** cells (70%), alpha (α**)** cells (20%), and delta (δ**)** cells (5%) (10). The α cells are predominantly at the periphery and the β cells are mainly at the centre of the islets. Each cell type has a specific role in the regulation of blood

glucose and in the feedback signalling of neighbouring cells. Hormones secreted directly into capillaries include insulin, produced by beta cells, glucagon, produced by alpha cells, and somatostatin, produced by delta cells. Insulin has a hypoglycaemic effect; glucagon has a hyperglycaemic effect while somatostatin inhibits the secretion of both insulin and glucagon.

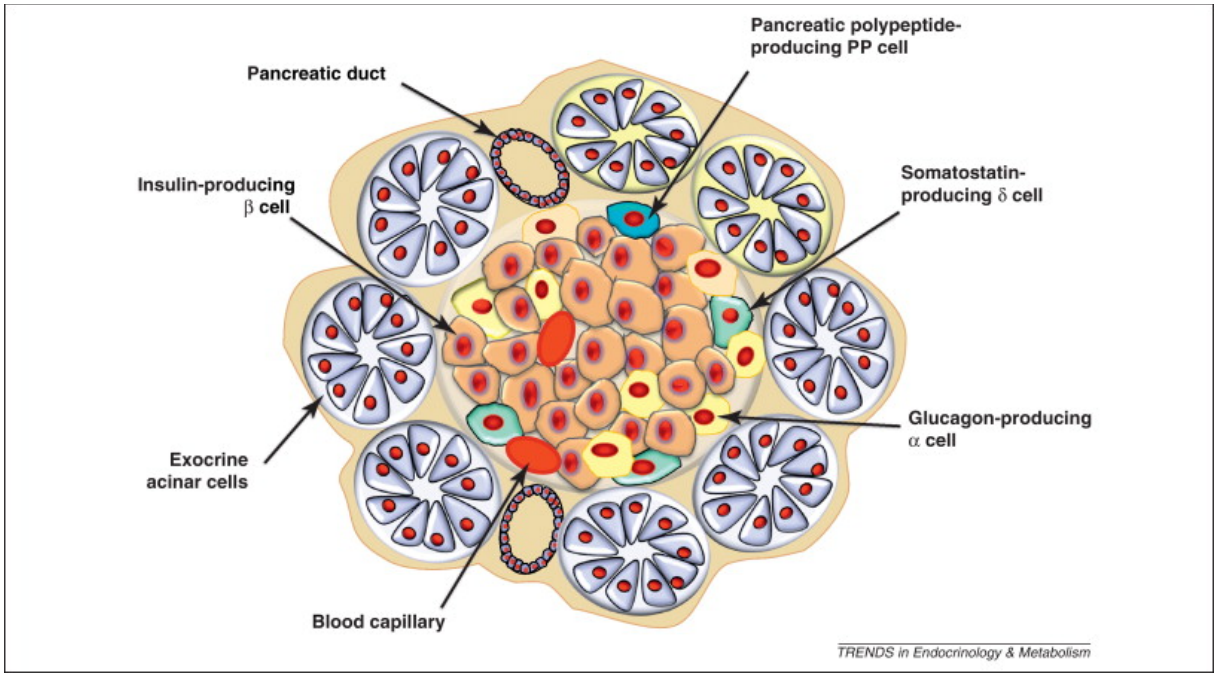


Figure 3: Architecture of a pancreatic islet (11)

1.2 Pancreas Transplantation

Pancreatic transplantation is able to restore endogenous insulin supply and regulation, control carbohydrate metabolism and potentially halt or even prevent the development of acute and chronic degenerative complications (12). There remains a lot of debate about the effect of pancreas transplantation on long-term secondary complications of diabetes.

Although there is evidence of halting progression of renal damage and possibly retinopathy, the evidence of benefit with respect to neuropathy is scarce and most patients are transplanted when the damage is already severe. However, due to the risk of surgery and the risk associated with immunosuppressive therapy, pancreatic transplantation is not indicated in all diabetic patients. Pancreas transplantation is most commonly performed in patients with existing chronic kidney disease, requiring combined pancreas and kidney transplantation (SPK). Isolated pancreas transplants (without a combined kidney transplant) are performed in patients who i) have had a previous kidney transplant, ii) in patients who have lost the functionality of their pancreas following a combined transplant or iii) more exceptionally, in patients with diabetes which is very difficult to control, especially those in whom life-threatening episodes of hyperglycaemia occur without warning. In recent years, the indications for pancreas transplantation have also been extended to include highly-selected Type II diabetic patients with renal failure (13).

Three types of solid organ pancreas transplantation are used at present with the most common being the SPK, used in 83% of cases. This procedure is most commonly used in Type I diabetic patients, presenting very low or absent levels of C-peptide (14). The second method performed in 12% of cases is the pancreas after kidney (PAK) where a pancreas is transplanted in a patient that has previously received a donor kidney. The disadvantage of this procedure is that the organs come from different donors, and it is therefore not possible to use the transplanted kidney as a surrogate for the transplant pancreas in terms of rejection monitoring. This may be the reason for the inferior medium and long-term graft survival (pancreas) of patients undergoing PAK transplantation (the assumption being

that pancreatic rejection is diagnosed later in PAK than SPK). It is important to note that this is no more than a hypothesis and it is not possible to exclude other interactions which result in PAK outcome. However, for patients who have a suitable living kidney donor, there is a good argument to proceed without delay for kidney transplantation, thereby maximising the benefit of kidney transplantation, and carrying out subsequent pancreas transplantation when a suitable donor organ is allocated. The relatively higher proportion of PAK (versus SPK) transplants conducted in the USA compared to the UK may reflect the longer waiting time for SPK in the USA (thereby effectively increasing the timing benefit of the living donor). The third and least common of the three methods, accounting for 5% of cases, is the pancreas transplant alone (PTA). This involves the transplantation of a solitary pancreas in diabetic patients who have normal renal function (15). When performed successfully and early during the course of a patient's diabetic progression, evidence suggests that complications, particularly nephropathy, can be evaded, and that life-threatening hypoglycaemic unawareness is counteracted. However, the benefit achieved with transplantation in these situations is accompanied by the risks associated with long-term immunosuppression (which are not incurred in patients who remain on insulin treatment). SPK is widely regarded as the treatment of choice in patients with diabetes that has led to renal failure and the need for kidney transplantation. Not only is the long-term graft survival superior with the combined transplant, but also this is a cohort of patients who would still undergo kidney transplantation and thereby experience the same long-term implications of immunosuppression.

Venstrom, et al. (16) suggested that the benefits afforded by solitary pancreas transplantation were controversial, reporting a survival disadvantage for PAK and PTA recipients. A follow up study conducted by Gruessner et al. (17) included a more recent patient cohort as well as an extended follow-up period. It was concluded that the mortality at 4 years for solitary pancreas transplantation was equivalent to patients who had remained on the waiting list for the procedure. Despite its obvious benefit in terms of resolving the life-threatening syndrome hypoglycaemia unawareness, the evidence for overall life expectancy benefit is less well proven than in SPK. Pancreas transplantation alone should, therefore, be used very selectively, for those patients in whom the benefits are likely to exceed the risks, particularly of life-threatening or life-restricting hypoglycaemia unawareness.

Technical aspects of pancreatic retrieval and pancreatic transplantation

Pancreatic retrieval and transplantation take place in three main stages: retrieval, preparation of the graft with reconstruction of the vessels, and finally, transplantation.

Technical aspects of pancreatic retrieval and graft preparation

During multi-organ retrieval from brain-dead organ donors, inspection of the pancreas is all that needs to be carried out in the beating heart phase. Palpation of the right edge of the hepatic pedicle reveals an accessory right hepatic artery in 5-10% of cases. This is relevant because preservation of the right hepatic artery is important for the donor liver. An accessory right hepatic artery can often be dissected from behind the pancreas to its origin at the superior mesenteric artery, alternatively, if large, it can be divided at the upper

border of the pancreas and reconnected, e.g., to the gastroduodenal or splenic arterial stumps in order to maintain arterial perfusion of the right lobe of the liver. The posterior omentum cavity (lesser sac) is then opened to enable the assessment of the macroscopic appearance of the pancreas. If the inferior mesenteric vein is cannulated by the liver sampling team, care should be taken to ensure that the cannula has not erred into the splenic or superior mesenteric vein. Any other manipulation of the pancreas should be avoided during this heart beating phase. Cannulation for in-situ cooling and perfusion of the abdominal organs must avoid any compromise to the perfusion of the pancreas: ideally abdominal organ perfusion should therefore be aortic-only, with portal vein flushing of the liver carried out only when the portal vein has been opened following circulatory arrest and cold perfusion.

Cold preservation of the pancreas in usually carried out using University of Wisconsin solution, both in-situ and, later, during further perfusion and cold storage. The duodenal segment attached to the head of the pancreas is separated by the use of a GI stapler immediately upstream of the pylorus and at the level of the first jejunal loop. The root of the mesentery is then stapled and divided at a distance from the organ. The pancreas is then dissected free from left to right, the spleen makes a convenient handle for organ mobilisation; the left adrenal gland marks the plane of separation between the pancreas and the left kidney. The pancreas and liver may be retrieved en-bloc or separately: if the former, then the organs are separated on the back-table, with the organ immersed in ice-cooled preservation solution. The common bile duct and gastroduodenal artery are ligated and divided; the portal vein is transected halfway between the liver and the pancreas

(allowing at least 1cm of length of portal vein from the confluence of the splenic and superior mesenteric veins); the superior mesenteric artery is cut flush with the aorta without a patch; the splenic artery is then divided at its origin and marked with a single stitch. It is essential to remove a common/internal/external iliac intersection from the donor for arterial reconstruction and a length of iliac vein for possible portal vein extension (although this is rarely used).

Back at the hospital, back-table preparation of the pancreas is a major procedure that must be conducted meticulously. During the preparation of the graft, a splenectomy is performed, and its pedicle tied. The duodenum is shortened, and the staple lines are then buried with inverting sutures to reduce the risk of leaks. Arterial reconstruction is a vital step; the donor iliac arterial bifurcation graft is normally used for this. Two end-to-end anastomoses are performed, with Prolene® 5/0, between the internal iliac artery and the splenic artery, and between the external iliac artery and the superior mesenteric (Figure 4).

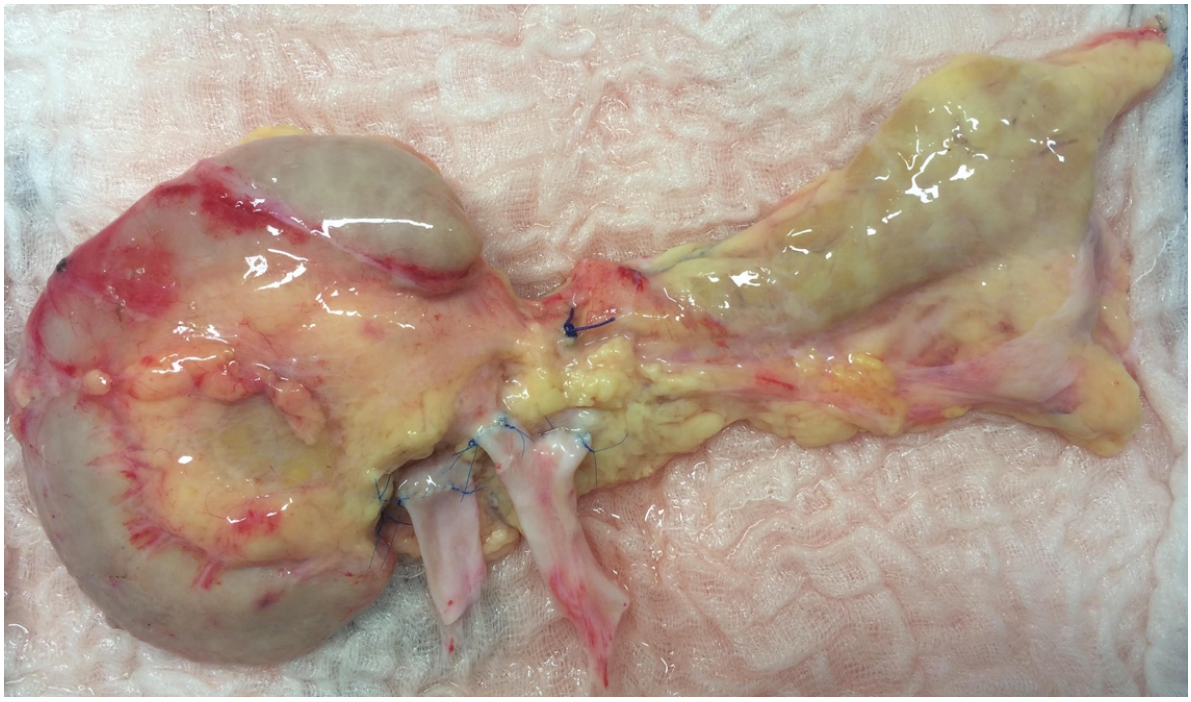


Figure 4: *Human pancreas graft after back table preparation.*

Technical aspects of pancreatic transplantation

The pancreas is transplanted into a location in the posterior abdomen, usually following mobilisation of the right side of the colon and small intestine to provide access to the IVC and iliac vessels. The majority of surgeons anastomose the portal vein of the pancreatic graft to the inferior vena cava (Figure 5) and the graft artery to the right common iliac artery (Figure 6). The most commonly used intestinal drainage is an entero-enteric anastomosis – donor duodenum to recipient jejunum (Figure 7). An older but still sometimes used method of exocrine drainage places the transplant pancreas with the tail cranially, enabling the transplant duodenum to be anastomosed to the dome of the bladder (Figure 8). This has the advantage of allowing urinary amylase to be used as a marker of pancreas function. However, it is associated with frequent and often severe complications including

dehydration, bicarbonate loss and/or cystitis. A high proportion of such bladder drained pancreases require conversion to enteric drainage for these reasons.

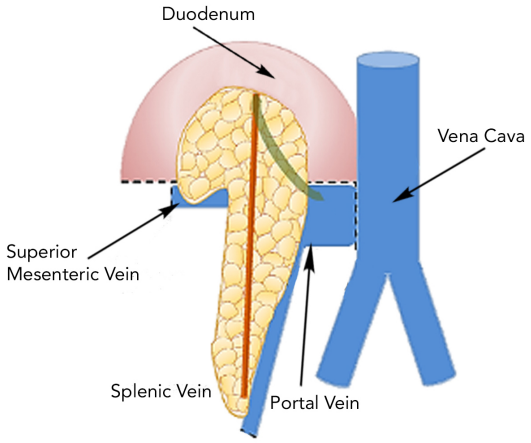


Figure 5: *Porto-caval venous anastomosis of the pancreatic transplant.*

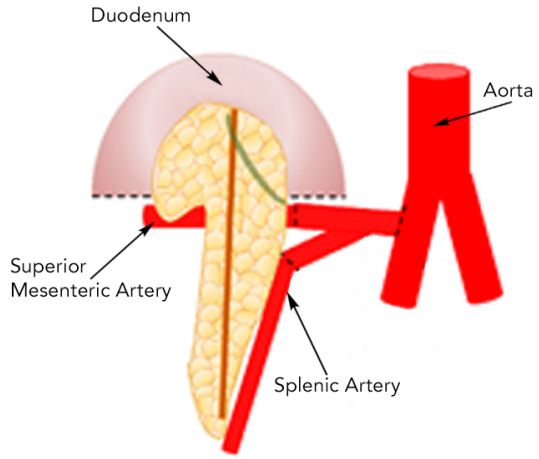


Figure 6: *Arterial anastomosis of the Y-graft on the common iliac artery.*

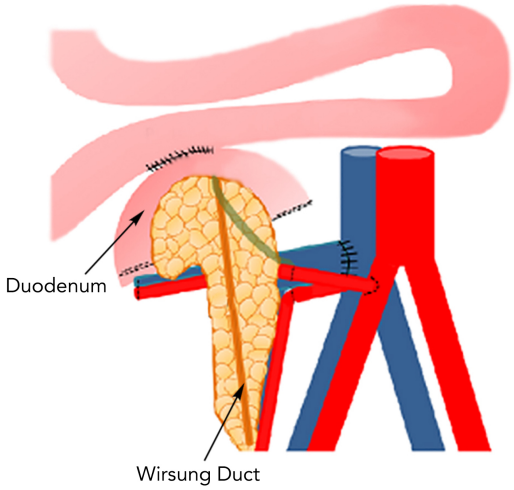


Figure 7: *Enteric anastomosis of the pancreatic transplant.*

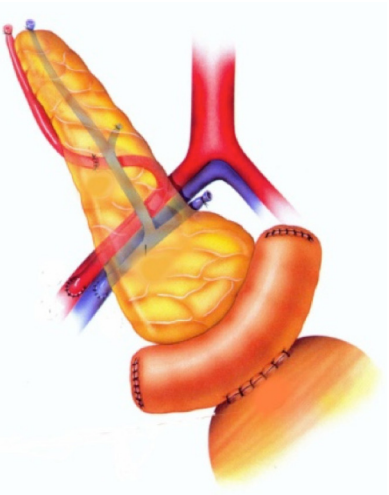


Figure 8: *Whole-organ transplant with systemic vein and bladder exocrine drainage* (18).

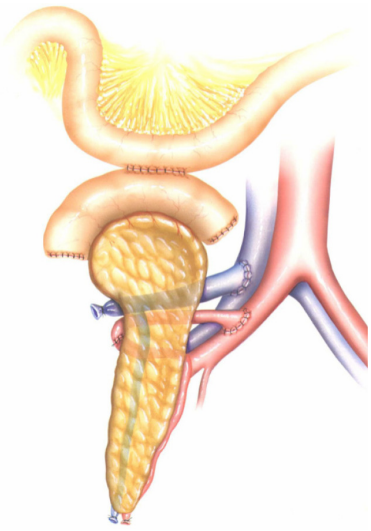


Figure 9: *Whole-organ transplant with systemic vein and enteric exocrine drainage* (18).

History of pancreas transplantation

The first successful pancreatic transplant was performed in 1966 by Lillehei and Kelly in Minneapolis. It was a total pancreas transplant with external drainage of pancreatic exocrine secretions via a jejunostomy. The initial results, discouraging by the seriousness of the surgical complications (in particular secondary to the digestive anastomosis), led very quickly to the abandonment of this method and to the temporary cessation of this type of transplantation. The large doses of steroids used at the time to prevent rejection were the cause of many of the postoperative complications: pancreatic fistulas, pancreatitis and bleeding.

In 1976, J.M. Dubernard and J. Traeger (19) in Lyon introduced a new surgical technique: segmental pancreatic transplantation (pancreatic body and tail only) in order to avoid complications related to transplanting a length of intestine, as well as the complexities of having a dual arterial inflow. The lumen of the pancreatic duct was injected with a synthetic polymer (neoprene) in order to obviate the need for an exocrine drainage procedure. This segmental pancreatic transplantation technique became more widely practiced in a number of centres in the early 1980s. However, surgical complications such as peri-pancreatic collections, the high percentage of venous thromboses and the late functional failure of previously function grafts caused this technique to be largely abandoned by the end of the 1990s.

Since then, surgical techniques have evolved with the return to intestinal drainage, with some variations brought by C.G. Groth in Sweden and by U. Boggi in Pisa (20). The use of more effective newer maintenance immunosuppressive agents (cyclosporine, tacrolimus, mycophenolate), more effective induction immunosuppression (monoclonal and polyclonal antibodies) and reduced reliance on the use of glucocorticoids), allowed a marked reduction in surgical and immunosuppression-related complications.

1.3 Limitation of transplant: Shortage of Donors

Prudent donor selection is essential to ensure satisfactory post-transplant outcomes. 'Ideal' organs come from brain dead donors with criteria which include: age less than 45; body mass index (BMI) less than 30; no history of high alcohol intake and no history of diabetes.

Despite the potential demand for donor organs and increasing rates of organ donation, the pancreas is poorly utilised. The reason that the pancreas is much less well utilised than other abdominal organs relates to its vulnerability (e.g., to surgical trauma) and comorbidities (e.g., obesity), also, the consequence severe ischaemia reperfusion in the pancreas is more severe than is the case in other organs, not infrequently resulting in significant or even life-threatening pancreatitis. There is now better understanding of donor risk factors (21) and this has enabled a more data-driven approach to the use of less-than-ideal organs, including those from donors declared dead by cardiovascular (as opposed to neurological) criteria (DCD). Despite this, however, there remains a decline in the number of pancreas transplants performed (22).

Increase in donor organ supply has been addressed in part by greater use of Donation after circulatory death (DCD) donors, allowing for lengthier warm ischaemia times, and the use of DBD expanded criteria donors (ECD) with increasing age. As a result of this expansion, the effects of ischaemic injury and associated co-morbidities increase organ susceptibility and result in a potentially reduced organ viability. The use of SCS for preservation is not an ideal solution in the context of this greater pool of DCD and ECD organs (23). The advent of machine perfusion, more specifically hypothermic machine perfusion (HMP) and normothermic machine perfusion (NMP) could improve pancreas viability prior to transplantation, as shown for other organs. However, little evidence exists to support the use of either modality of preservation for the pancreas.

1.4 Pancreas Transplantation Complications

Early surgical complications of pancreatic transplantation

The rate of surgical re-exploration following pancreas transplant is up to a third. The four main causes of re-exploration during the first postoperative month include i) haemorrhage, ii) venous thrombosis, iii) enteric leak and (iv) intra-abdominal infections. To a large degree, the latter three are related to the central complication of graft pancreatitis, a manifestation of ischaemia-reperfusion injury. Prevention of technical complications is reliant upon i) a flawless surgical technique of both retrieval and transplantation, ii) a short preservation time, iii) the postoperative maintenance of a stable haemodynamic as well as iv) the use of heparin subcutaneously, followed by long-term aspirin if the transplant is functional. Venous thrombosis generally results in the loss of the transplant as the diagnosis is usually made late and the entire intra-pancreatic venous network is obstructed by clots (Figure

10). This results in an irrecoverable organ despite a few cases of successful recovery being described (24).

Intra-peritoneal infection may be manifested by abdominal pain, fever and the presence of fluid collection on ultrasound. This may be a consequence of pancreatitis or a leak at the enteric anastomosis (itself sometimes a consequence of pancreatitis). Enteric leaks occur at the duodenal staple lines, or, more rarely, at the anastomosis itself. The incidence of this complication varies from 4 to 10%. These leaks can be life-threatening because of the ensuing sepsis which can present late in an immunosuppressed patient.

Postoperative haemorrhage typically presents within 24 hours of surgery and is usually associated with small vessel bleeding from the pancreas itself rather than the vascular anastomoses. It is a frequent indication for surgical re-exploration, which is usually successful. The risk of haemorrhage is greatly increased through the use of peri-operative anticoagulation which is widely used to reduce the risk of thrombosis (haemorrhage being a complication which is more amenable to successful intervention than thrombosis). Patients also receive antiplatelet prophylaxis with aspirin, which is continued indefinitely.

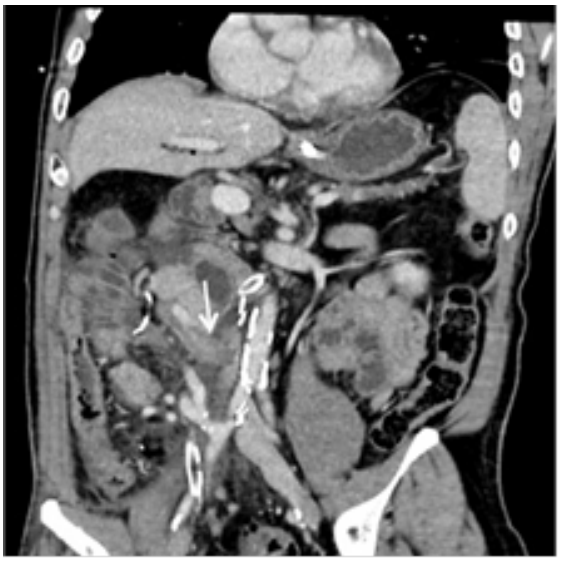


Figure 10: *Abdominal CT Scan of a pancreatic transplant with evidence of complete venous thrombosis* (25).

Late complications of pancreatic transplantation

Despite significant improvements in immunosuppression and graft survival, pancreatic transplant rejection remains a significant clinical problem and is the most common late complication. Pancreatic biopsy is used in a minority of clinical programmes to diagnose and treat pancreatic rejection, however, the reasons for the reluctance of most clinicians includes: (i) the technical challenge of accessing the pancreas in a retroperitoneal location; (ii) the risks of the procedure; and (iii) the interpretation of the histology. When faced with a transplant with deteriorating function, most pancreas transplant clinicians make the diagnosis by exclusion (e.g., CT scanning to show no vascular cause) and may require a biopsy (e.g., of a simultaneously transplanted kidney).

Langerhans islet transplantation: Indications for Langerhans islet transplants

Islet transplantation is used in two situations: (i) type I diabetic patients who have not yet developed significant renal dysfunction, but who have life-threatening episodes of hypoglycaemia without warning (hypoglycaemia-unawareness); (ii) patients with chronic

renal failure who would otherwise qualify for simultaneous pancreas and kidney transplantation, but are medically unsuitable for this much larger operation. and who do not justify an indication for pancreas transplant for reasons of benefit/unfavourable risk (26)(27). In an islet transplant, only large enough numbers of viable islets capable of secreting insulin are injected into the patient. The number of islets isolated from a donor pancreas is not always sufficient to completely correct diabetes. Some recipients are therefore required to receive islets from more than one donor.

Technical aspects of islet transplantation: Isolation of the islets of Langerhans

These islets are isolated from pancreases donated by multi-organ donors. The pancreas is retrieved using the same technique as for a vascularised organ transplant and is then sent as quickly as possible to an isolation laboratory. After dissection of the pancreas, islet isolation begins with the injection of collagenase into the pancreatic duct. The pancreas is then placed in a digestion chamber. The digestion is stopped by diluting the collagenase at 4 °C. The second phase of the isolation consists in purifying the islets from the exocrine parenchyma digested, by density gradient centrifugation. After digestion and purification, the islets are either cultured before transplantation or directly injected. The viability of the islets is tested before being administered to the patient.

Technical aspects of islet transplantation

The islets are conventionally implanted at the hepatic level because of its double vascularisation, as well as the fact that the liver is the main target organ for insulin. The islets are injected vascularly into the pre-sinusoidal venules via the portal vein. In the case

of combined transplantation with another organ, the islets may be surgically injected at the same time of operation, although more commonly as a separate procedure later. In the case of isolated islet transplantation, the injection is carried out in interventional radiology by puncture of the portal vein via a percutaneous approach.

Ischaemia reperfusion injury

Ischaemia-reperfusion is an unavoidable step in any transplant procedure. Ischaemia is defined as the restriction of the blood flow into the organ which causes the organs to be deprived of oxygen and nutrients. This leads to a major decrease in oxygen tension in causing an alteration in cell metabolism. Reperfusion occurs with restoration of the blood flow. This step, by definition essential for any transplant, is frequently associated with an exacerbation of the tissue injury initiated by ischaemia, additionally associated with an acute inflammatory response.

Ischaemia - Energy depletion and intracellular acidosis

Cold ischaemia results in depletion of cellular adenosine triphosphate (ATP). Very quickly (in less than 4 hours) nearly 95% of the amount of ATP disappears due to the decrease in its synthesis vs its persistent consumption (28). ATP is hydrolysed to hypoxanthine. Without oxygen hypoxanthine cannot be metabolised because this process only occurs under aerobic conditions. The absence of oxygen then leads to the decoupling of the respiratory chain, the mitochondria can no longer provide oxidative phosphorylation allowing the production of ATP and the regeneration of cofactors in their oxidised form, necessary for oxidative metabolism.

To maintain some production of ATP, the cell uses the pathway of anaerobic glycolysis. Anaerobic glycolysis is the transformation of glucose to lactate in the absence of or in limited supply of oxygen. However, this residual production is not sufficient to meet the energy needs of the cell. The production of lactate and the hydrolysis of adenosine nucleotides causes the accumulation of protons, causing cellular acidosis (23). The decrease in pH activates proteases and phospholipases resulting in lysosomal instability with the activation of lytic enzymes, which can lead to more or less significant cell damage ranging from a simple increase in cell permeability to lysis and cell death.

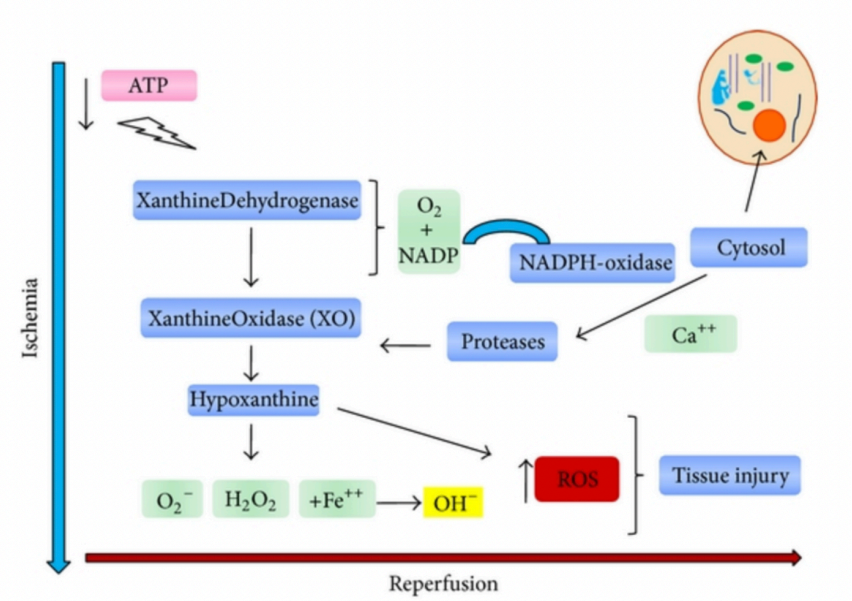


Figure 11: *Impact of ischaemia and production of free radicals* (29).

Calcium overload and cytoskeletal disorganization

During ischaemia, ATP depletion leads to a disruption of the ionic transporters on the cell membrane, normally ensuring the maintenance of the cell membrane potential, and to an alteration of the ionic gradient between extra and intracellular media. The Na^+ / K^+ ATPase

pump, inhibited by the persistence of hypoxia, thus leads to an increase in the cytosolic sodium concentration and to a decrease in the cytosolic potassium concentration. The increase in intracellular sodium causes oedema, cellular disorganisation, and activation of the Na^+ / Ca^{2+} and Na^+ / H^+ exchangers.

There is, additionally, a disorganisation of the cytoskeleton following ischaemia which results in structural modification and further oedema, including that of mitochondria (30). The mitochondria increase in size and present impaired function. At the same time, oedema results in peripheral vascular stasis. The cellular membrane depolarization described above can also cause the opening of voltage-gated calcium channels, resulting in cytosolic calcium accumulation, also exacerbated by inhibition of the Ca^{2+} ATPase pumps (due to lack of ATP). Elevated cytosolic calcium causes the activation of proteolytic enzymes, contributing to disorganisation the cytoskeleton. Increased calcium also leads to increased ROS production by means of activtation of xanthine dehydrogenase and xanthine oxidase.

Mitochondrial dysfunction

The inhibition of oxidative phosphorylation in the mitochondria and the intracellular increase in calcium following ischaemia leads to changes in mitochondrial structure and function. The dysregulation of the mitochondria during ischaemia will not allow normal mitochondrial respiration and thus induce oxidative stress leading to an imbalance in the redox homeostasis of the cell. The slowing of oxidative phosphorylation leads to the accumulation and leakage of electrons to residual oxygen at the level of complexes I and

III of the mitochondrial respiratory chain, inducing the production of free radicals (such as the superoxide anion O_2^-). The enzyme xanthine oxidase, activated by an increase in calcium concentration, is responsible for the production of free radicals under ischemic conditions. These free radicals cause major cell damage and activate cell death through apoptosis or necrosis.

Production of Nitric Oxide

Nitric oxide or nitrogen monoxide (NO) is a free radical synthesized by NO synthases (NOS), enzymes that have three forms. The neuronal form (nNOS) is mainly present in the cytosol of neuronal cells of the central nervous system and has a neuromodulatory role. The inducible form (iNOS) is present in the cytosol of macrophages, is induced by cytokines and endotoxins and has a cytotoxic role. The endothelial form (eNOS) is mainly present in the membrane of cells of the vascular endothelium and has a vasodilatory role. These three isomers are calcium dependent. In hypoxia, the activity of the calcium/calmodulin dependent eNOS decreases, in turn reducing the production of NO. This results in vasoconstriction, particularly unfavourable to the passage of blood or preservation solution during organ flush-out or active perfusion.

Reperfusion

Reperfusion is defined as the restoration of blood circulation and supply of oxygen and nutrients to the pancreas. Paradoxically, this return to a normal physiological situation is deleterious for cells which have undergone an ischaemic phase. The return of oxygen and the return to aerobic metabolism are responsible for a large generation of free radicals.

During reperfusion, the oxidation of hypoxanthine and xanthine, accumulated during ischaemia, to uric acid is accompanied by the production of new free radicals participating in the increase in oxidative stress. The cytosolic calcium concentration also further increases during reperfusion (31)(32). The mitochondrial permeability transition pore (mPTP), a multiprotein assembly, has two configurations: one closed, where the inner mitochondrial membrane is impermeable, the other open, allowing the matrix and cytosol to communicate freely. Calcium and free radical overload result in the opening of the mitochondrial permeability transition pores (mPTP). The opening of these mPTP pores results in a loss of the transmembrane gradient and a release of pro-apoptotic molecules outside the mitochondria in the cytoplasm. These pro-apoptotic molecules can cause cell death and are also pro-inflammatory.

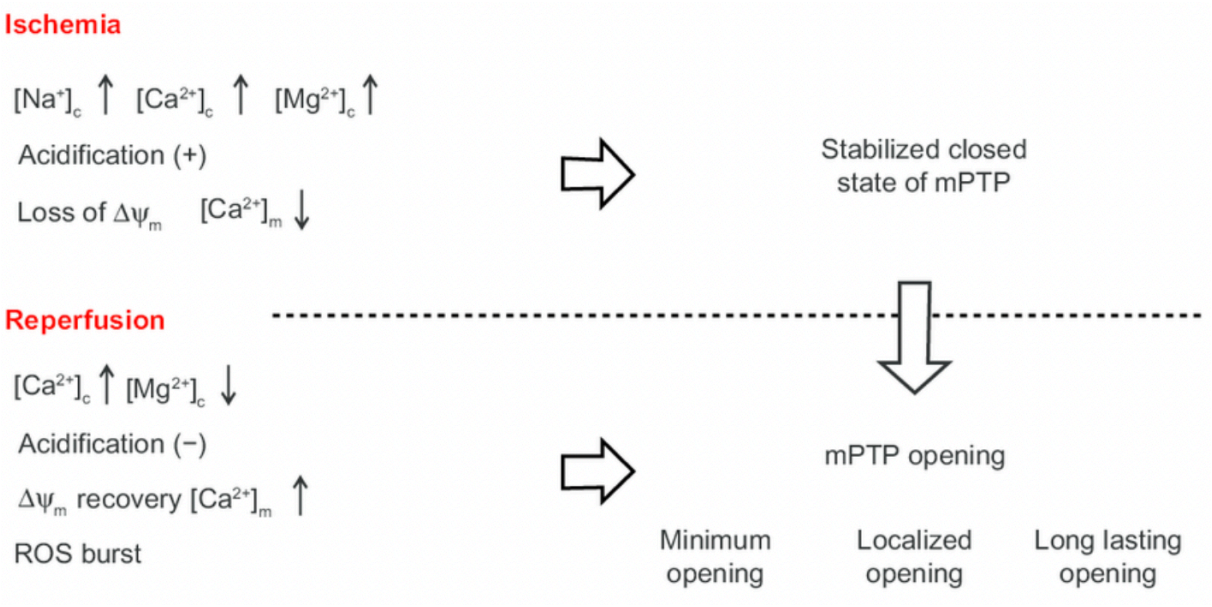


Figure 12: *mPTP opening as a result of reperfusion* (33).

Current methods of pancreas preservation

To date, static cold storage (SCS) is the universal method of pancreas preservation after retrieval due to its simplicity and low cost. Cold ischaemia has a considerable impact on the preservation of the graft and causes major biochemical changes. Hypothermic preservation is based on the principle that cooling an organ reduces the metabolic rate and utilisation of adenosine triphosphate (ATP). Lowering the temperature to 4°C reduces the metabolic rate to about 10%, which in turn minimises ischemic damage due to hypoxia (34). In spite of hypothermia, anaerobic glycolysis persists, causing lactate production. Lactate accumulation can lead to acidosis with very poor energy production efficiency. The cooling of the organs is necessary and essential for a decrease in metabolic activity, however hypothermia has deleterious consequences for the organ: inhibition of the Na^+ / K^+ ATPase pump, which causes cellular and interstitial oedema and disorders of calcium homeostasis activates proteases and promotes proteolytic lesions (35). The pancreas is extremely sensitive to warm and cold ischaemia which have a real impact on storage (36)(37)(38). Warm pancreatic ischaemia is particularly dangerous because islet function is damaged after 30 minutes, impacting beta cell function after transplantation (39)(40). SCS is therefore the current standard preservation method for the pancreas since the first transplantation carried out in 1966 (41).

1.5 Static Cold Storage & Perfusion Solution

The mainstay in solid organ preservation is static cold storage (SCS) and it remains the only method of pancreas preservation used clinically. The multiple stages of transplantation, from retrieval to preservation to implantation are accompanied by cell and tissue changes. Hypothermic conditions limit ischaemic injury by reducing cell metabolism, ATP use as well

as oxygen demand (42). The cooling of organs has enabled effective preservation of pancreases in a cost-effective and simple manner thus far, but there exist several limitations. Metabolic activity is not completely halted, and intracellular toxins coupled with metabolic products accumulate. ATP depletion continues, resulting in a lactic acid build up and subsequent ischaemia-reperfusion injury. (43).

Different preservation fluids are used in combination with cold storage, including Celsior and University of Wisconsin (UW) solution. These solutions contain different components to further limit ischaemia reperfusion injury (e.g. antioxidants, electrolytes, anti-oedema etc). Celsior is an extracellular, low-viscosity preservation solution originally designed for use in heart transplantation but it has also been used for experimental pancreas preservation (44). Its efficacy as an alternative to UW remains rather controversial as reported by Uhlmann et al (45) who found that, using the same pig autotransplant model, the use of Celsior was associated with increased ischaemia-reperfusion injury. This study challenged that of Baldan et al (46) who demonstrated that Celsior was an effective alternative to UW. A more recent study on a pig allotransplant model by Garcia-Gil et al (47) showed that lipid peroxidation after reperfusion of the pancreas preserved in both the aforementioned solutions yielded similar results. Boggi et al (48) also demonstrated that Celsior and UW had similar safety profiles when used in pancreas preservation in a randomised study comparing n=50 UW, to n=50 Celsior – preserved clinical pancreas transplants. Due to the minor differences that have been reported in the preservation solutions, UW remains the gold standard for SCS (49).

The IGL-1 solution, developed by the Georges Lopez Institute, is an extracellular solution containing polyethylene glycol (PEG) as a colloid. IGL-1 has been introduced in abdominal organ transplantation with encouraging results for kidney and pancreas transplants (50)(51)(52)(53)(54). IGL-1 offers similar results to UW in terms of islet isolation and pancreas transplant outcomes for pancreases retrieved from optimal donors.

Composition of UW and IGL-1 preservation solutions

	UW	IGL-1
HES (mmol/L)	0.25	–
PEG-35 (mmol/L)	–	0.03
Lactobionic acid (mmol/L)	100	100
Raffinose (mmol/L)	30	30
$MgSO_4$ (mmol/L)	5	5
KH_2PO_4 (mmol/L)	25	25
Glutathione (mmol/L)	3	3
Adenosine (mmol/L)	5	5
Allopurinol (mmol/L)	1	1
Na^+ (mmol/L)	30	125
K^+ (mmol/L)	125	30
Osmolality (mOsm/Kg)	320	320
pH	7.2-7.4	7.2-7.4

Table 1: Constituents of University of Wisconsin solution and IGL-1 solution (55) .

SCS of organs does not offer opportunities for viability assessment before transplantation whereas HMP enables this to a very limited extent (based on pressure, flow, resistance). There is some evidence for the benefit of biomarkers for injury in cold perfusion e.g., flavin mononucleotide which has been investigated in the context of HMP of the liver grafts (56).

1.6 Oxygenation

SCS effectively does not provide oxygen to the organ. HMP may be combined with oxygenation or not, if there is no active oxygenation of the perfusate then oxygen delivery (or lack thereof) is essentially the same as SCS. Oxygenated HMP delivers oxygen up to the limit of solubility in the perfusate. The emergence of HMP with oxygenation introduces a novel concept, that when used in combination with preliminary cold storage, could enhance donor organ quality via improved preservation.

Optimisation of oxygen delivery during hypothermic machine perfusion is a key focus in organ preservation research. Conventional SCS does not provide sufficient oxygen to the core of solid organs. HMP, a method designed to deliver cold preservation solution into organs via their native vasculature, also has notable reservations as it delivers inadequate oxygen to organs during preservation. The advent of oxygenation to machine perfusion has been shown to be successful in several studies of solid organ preservation. Several methods of oxygenation are currently used, with the main focus being HMP with oxygen. Persufflation (PSF), although a rather restricted area of interest so far, has the potential to provide additional advantages when compared to the SCS or HMP techniques. PSF represents an optimal opportunity to fully oxygenate an organ during preservation.

Two-Layer Method (TLM)

The TLM was first reported at the Kobe University in 1988 as a viable method for pancreas cold storage (57). It was developed in order to reduce ischaemic injury whilst maintaining cellular integrity during solid organ preservation. The method first consisted of a perfluorochemicals (PFC) and Euro-Collins' (EC) solution, with the latter later being

replaced by University of Wisconsin solution. PFC is a colourless, odourless, lipophilic hydrocarbon solution with specific gravity approximately twice that of water. Due to the negligible O_2 binding constant of PFC, release of O_2 into surrounding tissue is more effective than with haemoglobin (58). The high density and lipophilic nature of PFC enables the separation of PFC and EC/UW into layers. This subsequently enables the flotation of the pancreas graft on the oxygenated PFC surrounded by EC/UW. When comparing both the original TLM (PFC/EC) and modified TLM (PFC/UW), the former could preserve canine pancreases for up to 72h, while the latter achieved canine pancreases preservation of up to 96h (59).

The mechanism of TLM reduces cold ischaemic, warm ischaemic and reperfusion injuries (58). During the preservation of pancreases using TLM, the organ is directly oxygenated through PFC and maintains an oxygen tension of 60% in the tissue compared to normal physiologic level. This means that during preservation, ATP is maintained (rather than degraded) and is also rapidly recovered following reperfusion (pancreas grafts continuously generated ATP for up to 96h). ATP levels have also been shown to be enhanced in human pancreases stored using TLM, suggesting the use of TLM to oxygenate the human pancreas might be supported (60). A limiting factor of this method, however, is the low penetration of oxygen. The mass of the organ and limited diffusion of oxygen probably explains the difference in results between the rat studies in which this technology was first tested and the somewhat less convincing data from large animal studies.

An additional study showed that following 90 minutes of warm ischaemic injury, the canine pancreas grafts lost ATP and were no longer viable, however, when preserved and resuscitated by the TLM for between 24 to 48h at 4°C, the grafts regained tissue ATP and became viable (61). Resuscitation by TLM prior to pancreas transplantation was correlated with the pancreas tissue ATP levels post TLM (62). This may be important as ATP might be able to predict the outcome of marginal donor grafts before transplantation which is of crucial importance. There was a difference in effect between pancreases of different sizes, with a potential explanation being that in larger organs, there is failure of the oxygen to diffuse effectively.

Persufflation (PSF)

PSF was first discovered by Rudolf Magnus in 1902 with perfusion of an isolated cat heart with defibrinated blood (63). He illustrated that it was indeed possible to maintain a beating heart in bradycardia during 69 minutes of PSF following several extensive studies designed to elucidate the utility of PSF in preserving cardiac function. His studies were more significantly appreciated in the mid-1950's by a group at McGill University in Montreal where it was discovered that PSF could be used to preserve spinal reflexes in frogs as well as active cardiac skeletal muscle contractions in rabbits (64) (65).

To date, the pancreas and small intestine have not been extensively studied as targets of PSF but the pancreas has attracted more interest in recent years. PSF has been identified as a potential and possible improvement in the current pancreas preservation protocol, particularly prior to islet isolation. A study conducted by Scott et al demonstrated through

nuclear magnetic resonance that oxygen persufflation of the pancreas results in an increase in ATP levels (66). MRI showed that both human and porcine pancreases were preserved using PSF and that pancreatic tissue filled with gas in a homogeneous manner. When comparing TLM with PSF, it was found that when PSF was used, ATP was elevated in human and porcine pancreases to a level achieved by rat pancreases. TLM showed to effectively elevate ATP levels, but not in a porcine model.

Persufflation has several advantages and disadvantages in the context of hypothermic organ preservation techniques. PSF can deliver more oxygen per gram tissue than both SCS and HMP. It is also important to note that gaseous perfusate has lower viscosity and therefore may reach greater regions of the preserved organ and therefore lower the risk of oedema. Preservation time may also be extended as a result and there is potential for resuscitation of marginal quality organs. The monitoring and assessment of viability is facilitated, and implementation of this method is simpler than that of HMP.

Downfalls of this method include the risk of damage to vascular structures. Dependent upon the gaseous oxygen concentration, hyperoxic damage in tissues may be induced, and a risk remains of damaging tissues by desiccation should the gas not be adequately humidified during long term preservation. PSF also shares resemblance to iatrogenic gas embolization, thus challenging clinical dogma. The delivery of nutrients, and removal of waste products is not as efficient as that achieved through liquid perfusion (67).

Oxygen Carriers

The use of oxygen carriers is essential for effective oxygen delivery to organs, at normal physiological temperatures (normothermia). Hemopure (HBOC-201) is a haemoglobin-based oxygen carrier that improves oxygen transport and delivery through promotion of microcirculation. The diffusive components for oxygen transport are increased for multiple reasons; HBOC-201 has a higher P50 (oxygen tension when haemoglobin is 50% saturated with oxygen) compared to native red blood cell haemoglobin, thus the off-loading of oxygen to tissues is increased. Modification to the convective transport achieved by HBOC-201 is threefold; (i) volume expansion encourages efficient organ perfusion, (ii) it has a very low viscosity and thus enhances flow to tissues, and (iii) this method of oxygen delivery in the plasma is insensitive to regulation mechanisms of red blood cell distribution. The reduction of the diffusional barriers for oxygen transport associated with plasma also results in an increase in oxygen transport and delivery, as oxygen is sparingly soluble in the plasma. Solutions of HBOC have been shown to both take up and offload oxygen more efficiently than red blood cell haemoglobin (68).

AQIX-RS-I is another oxygen carrier that supports cellular activity in normothermic temperatures. It is a non-phosphate buffered solution that reflects physiological osmolarity, ionic concentrations and conductivity through its composition. It has potential to be used as an oxygen transport and perfusion medium, as shown by a study where kidneys were able to be flushed and stored for 2 hours using AQIX-RS-I (69).

Hemarina-M101, derived from a marine invertebrate, is an extracellular haemoglobin that has been developed into an oxygen carrier named Hemoxycarrier. Favourable

characteristics of this carrier include its high affinity for oxygen as well as its antioxidant activity. It is also functional within a wide range of temperatures, making it useful in cases of normothermic perfusion (70).

1.7 Hypothermic Machine Perfusion (HMP)

HMP for the pancreas remains underexplored due to the complex differences in anatomy and fragility compared to other solid organs such as the kidney. HMP perfusion technique was originally developed by Carrel and Lindbergh in 1935 (71), and is currently widely used for clinical kidney transplants, but has not yet been implemented in clinical pancreas preservation (72). Attempts to apply HMP to other organs have been largely based on the modifications of renal protocols following successful trials. Such amended protocols, however, cannot be directly applied to the pancreas for numerous reasons such as the low flow and pressure environment. Perfusion could result in damage to the vascular endothelium of the pancreas, resulting in platelet activation and thrombosis on graft reperfusion (73). This was not formally tested in the results described in this thesis, it may be a useful topic for further research.

Early experiments reported by Florack et al (74) using canine pancreatic grafts showed no failure rates at 24 hours and 48 hours when using cold storage. However, when machine perfusion was applied, failure rates were 30% and 40%, at 24 hours and 48 hours, respectively. Therefore, static cold storage (SCS) continues to be the most preferred and widely used method for pancreas preservation until the complexities associated with machine perfusion are overcome. The reasons for significant failure rates were due to the

high pressure and the use of Ringer's solution. Machine perfusion has been adopted once more in later experiments as a result of improved machinery and improved perfusion solutions. There is also now renewed interest in machine perfusion due to shortage of donors, and the need to use more extended criteria donors as a result. Several successful studies in the liver (75) and the kidney (76) are indicative of a possible avenue for perfusion of the pancreas and additional data collected from pancreas preservation prior to islet isolation also supports the use of machine perfusion in the pancreas (37).

A study conducted in France by Branchereau et al (77) used human DBD pancreases. Low pressure perfusion with UW solution was used across 24 hours and viability measures included macroscopic appearance, perfusion dynamics and histology, similar to the Hamaoui study (78). It was concluded that extended duration HMP resulted in minimal macroscopic and cellular oedema, minimal necrosis and positive immunohistostaining for insulin and glucagon in islets. Resistance index during HMP also improved with time. This provides a solid foundation for the incredible potential posed for the use of HMP in pancreas preservation in organs of marginal quality.

A study to test the feasibility and safety of hypothermic machine perfusion on normal pancreas tissue was conducted by Branchereau et al (79) using a baboon model. Non-human primate pancreases were retrieved from baboons in a study approved by the French Research Ministry of Health. In the study, a perfusion procedure including a systolic perfusion pressure of between 15-25mmHg was concluded to be both feasible and safe, and HMP was not found to be deleterious when compared to SCS (79).

Another study by Leemkuil et al (80), conducted in the Netherlands used human DCD and DBD pancreases. Low pressure perfusion with UW solution was again used but for 5 hours and with active oxygenation. Cellular ATP, histology, ROS generation and islet isolation and culture were assessed as viability measures in contrast to the Hamaoui study. The oxygenated HMP resulted in regeneration of cellular ATP levels, but found minimal evidence to suggest ROS generation or apoptosis.

Low pressure and use of UW MPS solution were common components across all three studies, with varying durations of HMP. Where similar viability assessments were measured, the same conclusions were reached demonstrating a positive effect of HMP in pancreatic preservation.

1.8 Previous Studies on Organ Preservation

Lung Preservation

SCS preservation has been used for decades in lung transplantation. Lungs are highly sensitive to CIT, and therefore, lung preservation time is limited (81). As in other organs, SCS reduces organ deterioration by slowing down cell metabolism and by reducing the need for oxygen. During SCS, unlike other organs, the lung is able to maintain aerobic metabolism for some time using the oxygen present in the alveoli (82). The use of a novel extracellular oxygen carrier preservation additive known as M101 (isolated from the marine lugworm Arenicola marina) has been investigated in a porcine model with respect to extending lung preservation time and understanding its mechanism of action. Using M101

led to significantly superior early post-transplant lung function in the case of extended pulmonary preservation times (83).

Small animals

An evaluation of 18 hour lung preservation with oxygenated blood was conducted by Takigami et al (84) in a rabbit model in order to assess optimal oxygen delivery to lung grafts. Eighteen excised rabbit lungs were flushed and stored for 18 hours at 10 degrees. 6 were stored with EC solution, 6 were stored with oxygenated homologous blood and 6 were stored with low-potassium dextran solution. Following evaluation, it was concluded that oxygenated blood may enhance lung preservation when compared to the EC group.

Large animals

A more recent study conducted by Fujiwara et al (85) used donor rat and canine models in order to confirm the effects of CO on ischaemia reperfusion injury of donor lungs using high pressure gas preservation method. Both lung models were preserved in a chamber filled with CO and oxygen, and were ventilated using either air, or a mixture of CO2 and O2. Inflammatory mediator levels and alveolar haemorrhage were significantly lower and weaker in the group ventilated by a mixture of CO2 and O2. Therefore, it was concluded that despite no change in mRNA expression levels of HO-1 across both groups, the system adopted in the high-pressure gas preservation method is effective in suppressing ischaemia reperfusion injury and preserving donor lungs. Although CO is known to be toxic due to its interference with oxygen delivery at high concentrations, the endogenous generation of CO through the catalysis of heme oxygenase has been shown to be a key

mechanism in the maintenance of the integrity of the physiological function of organs. It has been supported that CO functions as a signalling molecule and exerts cytoprotection when at low concentrations. Exogenous delivery of CO mediates protection in injury models due to its anti-inflammatory and vasodilating properties (86).

In a DCD model study using dogs from Kyoto University, Nakajima (87) showed that short-term HMP and ventilation could resuscitate DCD lungs damaged by ischaemia and ameliorate ischaemia reperfusion injury (i.e. a significant decrease in oxidative damage and production of proinflammatory cytokines) compared with SCS.

Clinical Studies

A study conducted by Noda et al (88) compared results of 4 different oxygenation levels (6%, 40%, 60% and 100%), in the perfusate during ex-vivo lung perfusion. The markers evaluated include lung function, compliance, vascular resistance and levels of glucose in the perfusate. Following the perfusion, lung grafts were transplanted, and post-transplant outcomes were compared. Lungs treated with 40% or 60% oxygen showed significantly less inflammation than those treated with 6% and 40%. This was clearly associated with a reduction in pro-inflammatory cytokines messenger RNA levels. However, more oxidative damage was noted following 4 hours of lung perfusion at 100% oxygen. Following transplantation, lungs perfused with 40% O_2 showed the best post-transplant functional outcomes. Therefore, it was concluded that optimising oxygen levels in the perfusate during perfusion improved outcomes in this rat model. When compared with

deoxygenated perfusate models, significantly more inflammation and reduced cellular metabolism were found in the deoxygenated model.

Ex-vivo normothermic lung perfusion (EVLP) has been historically developed to evaluate pulmonary grafts and subsequently, enable an increased lung preservation time (89). EVLP was first used to assess the lung prior to transplantation. Steen et al (90) technique has been brought up to date with the use of transplants from uncontrolled DCD donors (91). Most studies using lung ex situ perfusion now use the normothermic strategy.

Kidney Preservation

Despite the early development of machine perfusion (71), SCS remains the dominant preservation method for clinical kidney preservation in the majority of countries. However, following a large multicentre machine preservation trial the Netherlands introduced HMP as the standard of preservation with all suitable kidneys being placed on an HMP device at the retrieval centre (76).

Oxygen supplementation is mandatory for NMP (92) but has only quite recently been considered during hypothermic preservation. There are many ways to oxygenate the kidney during cold preservation (93): bubbling oxygen into the preservation solution during SCS (94), bubbling oxygen in perfluorocarbon (95)(96)(97), oxygen carriers, oxygenation under increased pressure (98)(99)(100)(101), oxygen persufflation through the renal vasculature or urinary collecting system (102), HMP, and Hypothermic oxygenated machine perfusion, now widely known as 'HOPE' (103). Lemeur et al (104) recently

demonstrated that the addition of an oxygen carrier M101 to preservation solution, was safe with significantly less delayed graft function (DGF) and improved renal function in recipients of the kidneys preserved with M101.

Small animals

Kron and al showed the positive impact of HOPE on rat kidney transplantation models (105). HOPE-treated kidneys showed better function than SCS grafts and less macrophage activation and endothelium damage (106).

Large animals

The interest in active oxygenation during HMP (HOPE) was first developed in preclinical models. A study conducted by Gallinat et al (107) assessed the role of oxygenation during hypothermic machine perfusion, investigating the functional and molecular aspects of active oxygenation using kidney grafts of porcine models from heart beating donors. Following 21 hours of hypothermic machine perfusion, either in an active oxygenation group or a non-oxygenated group, all grafts were auto-transplanted. Renal integrity and function were evaluated both during the perfusion and for 1 week following transplantation. Renal clearance of creatinine was concluded to be significantly improved during the first 2 days following transplantation in the non-oxygenated group. Other molecular expressions such as erythropoietin were unchanged in the oxygenated group. Fractional excretion of sodium, proteinuria and serum levels of lactate dehydrogenase was uniform and similar across both groups. Therefore, it was concluded that the use of active

oxygenation did not result in a significant difference in kidneys from donors with intact circulation.

In contrast, the benefits of active oxygenation during hypothermic machine perfusion were assessed in a study conducted by Thuillier et al (94) using porcine models in a donation after circulatory death (DCD) donor model. Two groups were assessed with cold preservation being performed by either conventional non-oxygenated machine perfusion or oxygenated machine perfusion. Results in the first 2 weeks post-transplant showed oxygenated grafts displaying lower serum creatinine peaks and a more rapid return to homeostatic levels. In a longer follow up, a decreased chronic inflammatory process was demonstrated in the oxygenated grafts. Oxygen delivery during preservation, therefore, appears to enhance the capacity of the graft to withstand stress and may be applicable as a therapeutic tool in marginal quality donor organs.

Conversely, the addition of different oxygen concentrations during hypothermic machine perfusion in porcine models was assessed by Venema et al (108), concluding that the presence of oxygen did not result in a significantly improved renal function. The benefits arose in a reduction of oxidative stress and energy status and showed no detrimental effects. The kidneys were preserved for 24 hours using perfusion solution supplemented with 100% oxygen, 21% or 0% oxygen, with no differences noted across the oxygenated groups (100% and 21%).

Clinical Studies

HMP is now well developed and established in clinical practice (109). After an initial washout of blood, the kidney is connected to a perfusion device, and a solution is pumped continuously through the renal vasculature at low temperatures (approximately 5°C). Moers et al conducted a randomised trial that was the first to demonstrate that HMP (without oxygen) reduced the incidence and duration of DGF, and improved the first-year kidney graft survival when compared with kidneys preserved in SCS (76). An update from the same trial concluded that 3 years after transplantation, the survival of DBD kidneys remained significantly better following machine perfusion when compared to SCS, especially in kidneys from ECD (110). The conclusions of this study were at variants with a smaller study carried out by Watson et al using a different protocol from a multicentred UK consortium (111).

Two clinical trials of HMP with oxygen delivery have been recently reported. The former is the COMPARE trial: an international, double-blinded, randomised, paired phase 3 trial designed to determine the effect on 1-year graft function of continuous oxygenated ($HMPO_2$) vs non-oxygenated hypothermic machine perfusion (HMP) in controlled DCD kidneys from donors aged 50 years or older (112). This first preliminary results reported at ATC 2019 and ESOT 2019 suggest that oxygenation improves 1-year kidney graft function when accounting for the beneficial effect on graft survival (113). Graft failure was also seen to be reduced in the $HMPO_2$ group compared with HMP as well as fewer reported severe complications in the $HMPO_2$ group. The second is the POMP trial (oxygenated hypothermic kidney re-conditioning after cold storage) (114). The objective of this randomised controlled trial was to evaluate pre-implantation reconditioning of donor

kidneys from extended criteria donors (ECD) using oxygenated machine perfusion following cold storage. Results demonstrated no significant differences for delayed graft function, acute rejection and estimated glomerular filtration rate between groups. Therefore, it was concluded that reconditioning of ECD donor kidneys who are DBD through the use of $HMPO_2$ does not improve graft function or survival when compared with SCS alone.

Liver Preservation

SCS has been the mainstay of liver preservation since the first transplantation and remains the standard method for liver preservation. In the early seventies, Brettschneider et al (115) reported the first use of machine perfusion for the preservation of liver allografts in an animal model. Since this first report, several preclinical studies have shown a growing potential for HMP techniques (116)(117)(118)(119).

Small animals

Following this growing recognition of the potential of HMP, active oxygenation during HMP (HOPE) was assessed as an optimisation of donor organ function during the cold perfusion (118)(119)(120)(121). Grafts from DCD donors with oxygenation introduced during hypothermic machine perfusion were evaluated by Lüer et al (122) in male Wistar rats. Livers were subjected to 18 hours of HMP, with preservation solution being equilibrated with 3 groups: 100% oxygen, 20% and 0% oxygen. Oxygenation of the perfusate resulted in a reduced (by 50%) alanine aminotransferase release in a reperfusion

model when compared to the non-oxygenated group. Activation of the AMPK salvage pathway was significantly enhanced in the 100% group, as well as the upstream activation of protein kinase A, when compared to the non-oxygenated group. Enzyme release was reduced by approximately 70% in the 100% oxygen group and 40% in the 20% oxygen group following oxygenation. Therefore, the addition of oxygenation to the perfusate appears to result in improved efficacy in the context of liver preservation.

Large animals

Following this study, oxygenated machine perfusion was assessed in porcine DCD livers by Fondevila et al (123). Donor livers were subjected to either SCS or oxygenated machine perfusion for 4 hours. Livers were then transplanted into recipient pigs and evaluated for up to 5 days. The survival rate following the 5-day evaluation was 0 in the SCS group and 20% in the oxygenated group. However, the oxygenated grafts developed progressive lesions 24-48 hours after reperfusion resulting in death in all but 1. The conclusion was made that despite some advantages in the use of hypothermic oxygenation, there is significant endothelial and Kupffer cell injury that ultimately leads to graft failure.

Clinical Studies

HMP promises to be a beneficial preservation technique for donor livers, especially in the case of ECD. The first clinical case-controlled non-oxygenated HMP liver trial (124) was reported by Guarrera et al. in 2009 (125) and demonstrated significantly lower serum injury markers and mean hospital length of stay in the HMP group.

The first report of HOPE in clinical practice was published by Dutkowski et al (126) in DCD liver transplants. This initial trial demonstrated significantly reduced early allograft dysfunction in the HOPE livers compared with SCS, with a lower rate of biliary complications and a better 1-year graft survival. These positive results were confirmed by another prospective evaluation from the Groningen group (127).

A more recent study conducted by van Rijn et al (75) in 2021 focused on DCD model liver transplantation using hypothermic machine perfusion compared to SCS. The primary endpoint of the study was the incidence of clinically diagnosed nonanastomotic biliary strictures within 6 months following transplantation. Postreperfusion syndrome was seen to have occurred in 12% of HMP livers and 27% for SCS livers, and early allograft dysfunction occurred in 26% of HMP livers compared to 40% for SCS livers. Nonanastomotic biliary strictures was reported to be lower by a factor of 4 following HMP when compared with SCS and it was therefore concluded that HMP resulted in a lower risk of nonanastomotic biliary strictures and therefore demonstrated the benefits of HMP.

Normothermic ex-situ perfusion is also becoming more widely used in liver transplantation. In 2018, Nasralla et al (128) reported results from a randomized trial with 220 liver transplantations comparing NMP to SCS. NMP preservation was associated with a 50% lower level of graft injury, measured by hepatocellular enzyme release, despite a 50% lower rate of organ discard and a 54% longer mean preservation time. Most of the ex-situ sub-normothermic or normothermic liver machine perfusion protocols were developed

using red blood cells (RBC) or other oxygen carriers in animal and preclinical models (129) (130).

Pancreas Preservation

Small animals

The team from Edmonton (131) compared rat pancreas storage for 24 hours using UW versus UW and 95% Oxygen bubbling, utilising the two-layer method. Islet function was evaluated using glucose-stimulated insulin secretion, expressed as the stimulation index. The viable isolated islets were calculated multiplying islet yield by viability. The UW plus oxygen group was a better way to preserve isolated islet yield and function. Hyperbaric oxygenation (HBO) is another oxygenation method which works by increasing the oxygen tension in the fluid. Stiegler et al (132) reported better islet cell viability in a porcine model, if UW solution was pre-oxygenated with a tube delivering 100% oxygen in the solution in a hyperbaric chamber.

Large animals

The first experimental perfusions of pancreas transplants were published more than forty years ago in a canine model. Florack worked on auto-transplantation of segmental pancreases in dogs after HMP, using Ringers solution with a 30mmHg perfusion pressure (74). In 1975, Brynger (133) and Tersigni (73) published their studies looking at dog pancreas allotransplantation. Brynger compared pancreas allotransplantation after 24h of perfusion with albumin versus buffered invert sugar solution (HMP) under hypothermic

conditions. Tersigni compared pancreas allotransplantation after 24h of hypothermic perfusion at different systolic pressures. All perfused pancreases demonstrated severe oedema, haemorrhage and venous congestion after revascularisation. In all these studies, the mean survival time was shorter for recipients transplanted with pancreases preserved under hypothermic perfusion compared to recipients transplanted with SCS. Due to these poor results, most of the teams ceased their studies using HMP for whole pancreas transplantation and focused on islet isolation after HMP (134)(135)(136)(137)(138)(139).

The pancreas is a low flow organ, and therefore, perfusion is challenging. High perfusion pressure such as that used in liver and kidney causes endothelial injury and pancreas oedema, however, if the pressure is too low, perfusion will not be adequate. Despite these limitations there have been some encouraging recent findings in pancreas perfusion. Branchereau et al (77) compared low pressure HMP with static cold storage of discarded human pancreases for a period of 24h. During HMP (Perf-Gen Solution/ Wave machine/pressure of 25mmHG) pancreases did not become oedematous and the resistance index of perfusion initially reduced and then remained stable for up to 12 hours. Prudhomme et al (79) showed using a non-human primate model, that HMP of pancreas was feasible, safe and not deleterious compared to SCS. Leemkuil et al (80) compared HMP and static cold storage of human pancreases from DBD and DCD donors for a period of 6h. No significant differences in histology (oedema formation, acinar cell integrity loss) between HMP and SCS were found. Although amylase, lipase and lactate dehydrogenase levels increased over time in grafts preserved with both HMP and SCS, ATP concentration only increased in the HMP group.

NMP for pancreas preservation has been reported in a limited number of studies and is not sufficiently developed for clinical pancreas preservation (140) (141) (142). One of the issues highlighted by first studies was the injurious effects of the proteolytic enzymes produced by the pancreas. The perfusion solution continuously cycles around the circuit and levels of amylase and lipase increase dramatically throughout perfusion. If it is to be a viable means of preservation, future work on pancreas NMP would need to address the issue of proteolytic enzyme production and auto-digestion in order to advance.

1.9 Graft Function after Pancreas Transplantation

The predominantly genetic and multifactorial disease Type I Diabetes Mellitus is characterised by an absolute insulin deficiency as a result of the destruction of insulin producing **β**-cells in the islet of Langerhans tissue of the endocrine pancreas. Despite successes of exogenous insulin therapy, copious long-term abnormalities develop, including end stage renal disease, neuropathy and retinopathy. With diabetes rapidly becoming one of the most phenomenal diseases, the advent of pancreatic transplantation, particularly the simultaneous pancreas-kidney procedure, has introduced a sustainable means of achieving an insulin-independent state in Type I diabetic patients.

Despite the evidence that pancreas transplantation is an effective treatment for diabetes mellitus, and despite the high incidence of diabetes, pancreases are not as widely used as the kidney or the liver. The reason for this is the higher risk associated with pancreas transplants and resulting poorer graft survival compared to kidney transplants. The

difference in outcomes (as outlined in Figure 13), is very largely manifest over the first 6 months post-transplant and is significantly associated with the occurrence of ischaemia reperfusion injury.

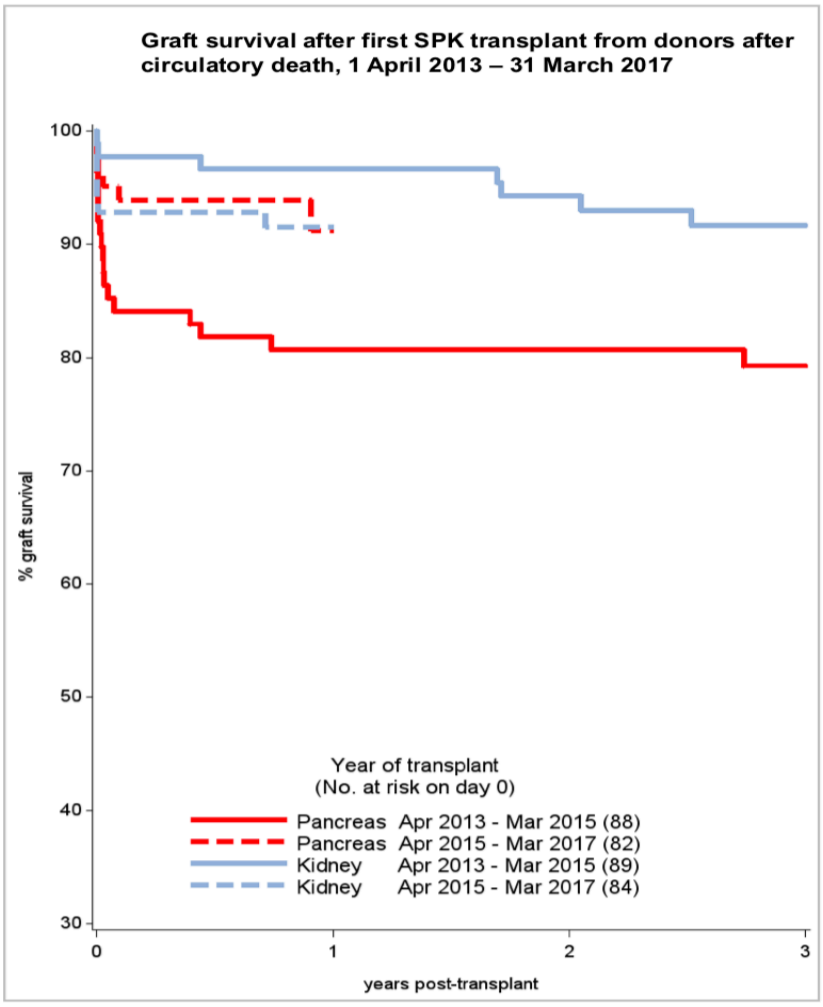


Figure 13: *graft survival after first SPK transplant from DCD, between 1 April 2013 and 31 March 2017 (NHSBT).*

The term ischaemia reperfusion injury describes a cascade of cellular events leading to cellular dysfunction and death, following restoration of blood flow to previously ischaemic tissues. Although reestablishment of blood flow is essential to salvage these tissues,

reperfusion itself paradoxically causes further damage, threatening both the function and viability of the organ. It occurs in all tissues that are subjected to transient ischaemia, including transplanted organs such as the heart, lung, and kidney. Its manifestations involve not only the ischaemic organ itself but may also induce systemic damage to distant organs, potentially leading to multi-system organ failure. Reperfusion injury is a multi-factorial process resulting in extensive tissue destruction.

Ischaemia occurs when the oxygenated blood supply is less than the demand required for normal function, resulting in deficiencies in oxygen, glucose and other substances required for metabolism. All transplant organs suffer from reperfusion injury but when this occurs in the pancreas, it is a lot more serious. For example, when this occurs in the kidney, the patient develops delayed graft function. Kidneys do not function temporarily, but eventually recover. However, in the pancreas, the much more serious complication of pancreatitis occurs. Activation of pancreatic enzymes leads to auto-digestion and a massive inflammatory response. This is a very severe problem in pancreatic transplantation and for this reason, pancreatic transplantation is reserved and restricted to those patients with the most severe complications of diabetes, in whom the risk-benefit ratio is deemed to be favourable.

Figure 14 demonstrates the SPK Pancreas graft function after transplantation, highlighting that early graft failure remains a big problem, despite improvements made in pancreas transplantation. The circled area shows how much survival has improved over the decades since the procedure was first used, but also the substantial early graft loss that persists –

this is to a significant extent a reflection of pancreatitis and its attendant complications (e.g. venous thrombosis). The inset photograph shows the appearance of a pancreas transplant that has been explanted due to venous thrombosis.

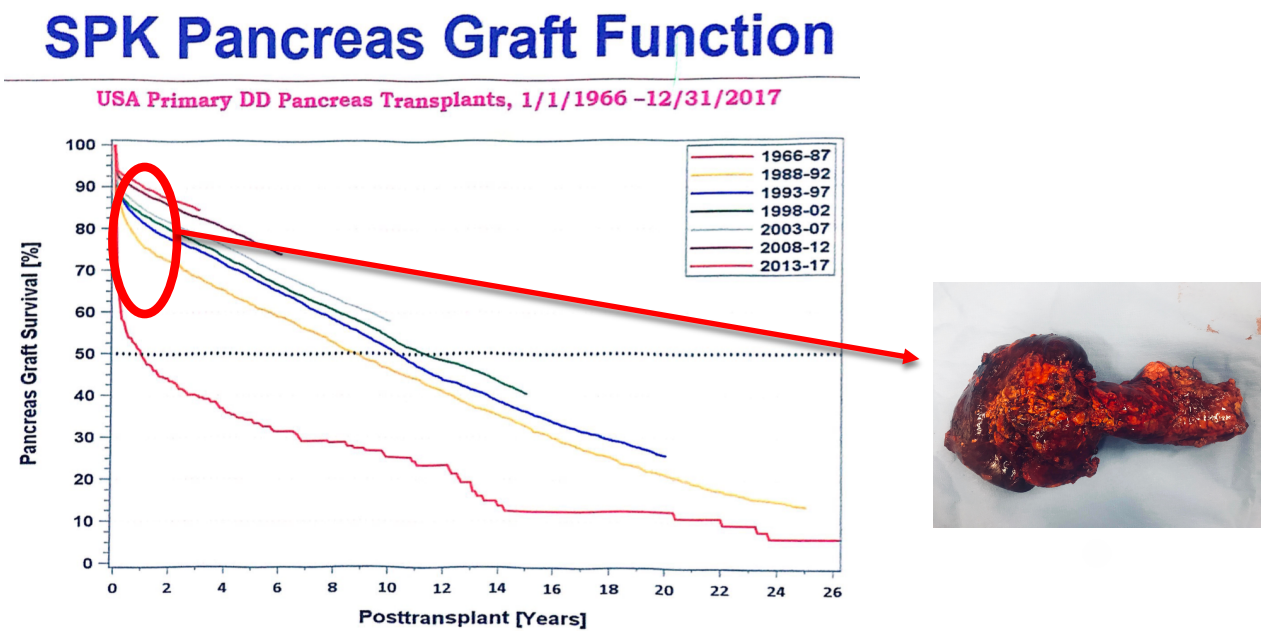


Figure 14: *SPK pancreas graft survival recorded between 1966 and 2017 and associated early graft failure (UNOS registry).*

To date, in clinical pancreas transplantation, SCS is the standard method of preservation. All other options of organ preservation are in the preclinical phase, and this has not changed for over 20 years (143). The research conducted in this study, was centred on the concept that machine perfusion is a method in which the risks of ischaemia-reperfusion, pancreatitis and thrombosis may be improved. The ultimate objective has been to achieve early results to match those of the kidney and the addition of oxygen has been assessed as part of this strategy.

When a pancreas is hypothermic, metabolic activity is decreased significantly, while the activity does not entirely stop, it is heavily reduced. Due to the slowed metabolism during hypothermia, oxygen demand is also reduced which is the basis of hypothermic preservation. In an ischaemic environment, there is an accumulation of succinate in complex II of the electron transport chain. When oxygen is then provided, such as at time of reperfusion the electron transport chain works in reverse, consuming the accumulated succinate, and producing reactive oxygen species at mitochondrial complex 1. The delivery of oxygen during preservation is likely to avoid succinate accumulation by avoiding hypoxia, and this is independent of temperature. The addition of oxygen, therefore, might mitigate one of the important early events in the ischaemia-reperfusion injury cascade.

1.10 Objectives

The work described in this thesis is based on the hypothesis that machine perfusion might improve the success rates of pancreas transplantation, particularly in marginal quality pancreases. The ongoing shortage of donor organs for transplantation as well as changes to the demographics of donors has created a driving pressure to achieve more optimal approaches for organ preservation. The increased use of ECD and DCD donors is driving the need to improve the state (or even recondition) donor pancreases. It is proposed that pancreas perfusion could reduce tissue oedema and ischaemia reperfusion injury.

The objective of this project is to set up and compare different pancreas preservation strategies. We have compared hypothermic, oxygenated machine perfusion ($HMPO_2$)

versus non-oxygenated machine perfusion (HMP) and static cold storage (CS) on whole organ pancreas preservation. The study design comprises an international collaboration based in Oxford, which avoids live animal experimentation. This study is envisaged as the final step before commencing a phase I clinical trial testing the safety, feasibility and impact of the oxygenated hypothermic pulsatile perfusion of the pancreas prior to transplantation.

MATERIALS AND METHODS

Study design

These studies were carried out using porcine pancreases retrieved from an abattoir (see section 2.0). Figure 15 shows the study design for this project. Pancreases (n=13) were retrieved. For each pancreas, there were 25 minutes of warm ischaemia, followed by 2 hours of static cold storage until perfusion was started at the lab for the perfusion groups or static cold storage was continued, for the SCS group.

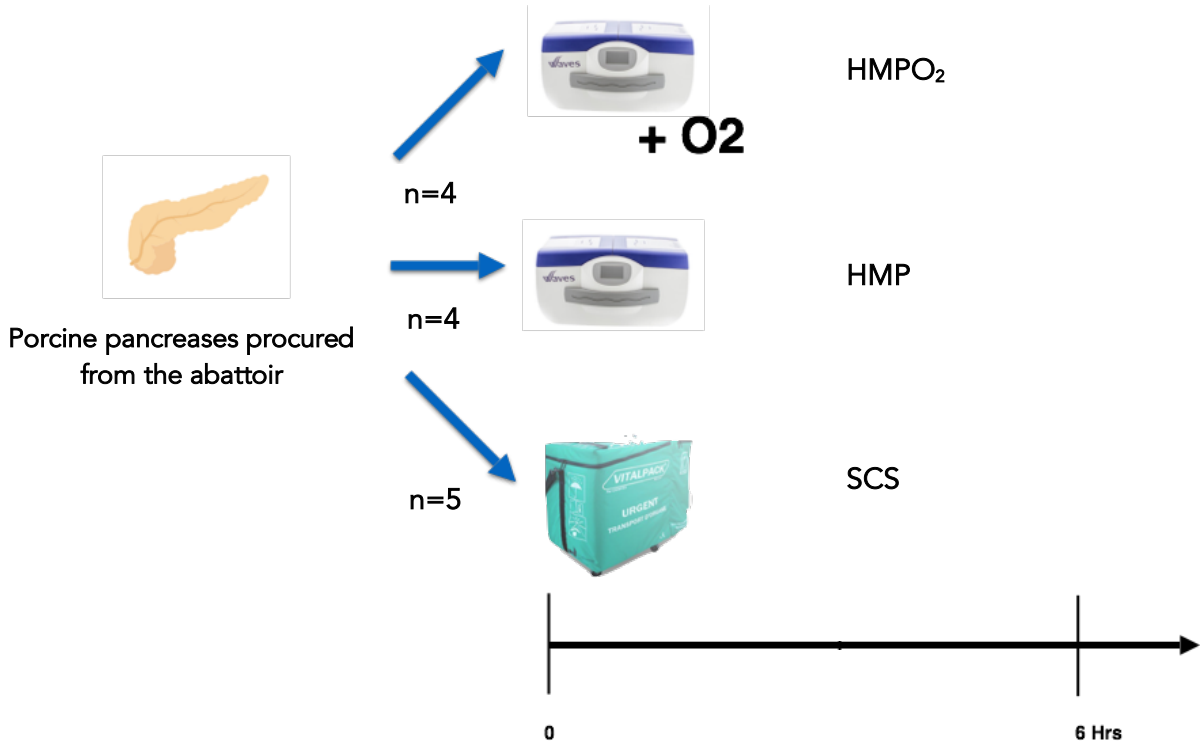


Figure 15: Study design

2.0 Abattoir Organ Procurement Protocol

In order to ensure effective organ procurement, a large, draped table or surface is required. An intravenous fluid delivery stand and giving set were set up in preparation for ex-situ cold perfusion of the organ.

Porcine model

Choice of the porcine model was made after analysis of the literature. J Ferrer et al (144) studied the vascular anatomy of the porcine pancreas. Although the shape of the porcine pancreas is different from the human pancreas, the arterial supply is similar to that of the human pancreas, thus making it possible to achieve the same vascular anastomoses. However, in clinical pancreatic transplantation, due to the associated liver retrieval requiring removal of the common hepatic artery, a vascular anastomosis is performed between the splenic artery and the superior mesenteric artery using a Y-graft segment of the donor iliac artery. This segment of the iliac artery is later used at the time of implantation for anastomosis to the recipient's right common iliac artery.

The small size of the porcine vessels led us to retrieve the entire aorta (and therefore the celiac trunk and the superior mesenteric artery). The perfusion was therefore carried out through the aorta.

Due to the similarity of vascularisation and pancreas morphology, the porcine abattoir model is suitable for long-term translational studies and is ethically, financially and research friendly as the animals are already raised and committed for commercial use.

Animals

Sus scrofa domesticus pigs (50-70kg) are stunned using electric shock and then exsanguinated while unconscious. Incisions to the carotid artery and internal jugular vein are made in line with standard abattoir protocol and in compliance with The Welfare of Animals at the Time of Killing (England) Regulations 2015 (WATOK) and EU regulation 1099/2009. The animal is then cleansed in a hot water bath at 60 degrees Celsius where hair is removed in the process. This portion of the abattoir protocol takes approximately 5-7 minutes.

Organ Retrieval

The animals are then attached and hung from their posterior limbs, in line with local abattoir protocol. A longitudinal midline incision is then made, exposing the abdomen and thorax. A circumferential incision is also made around the anus and rectum. This allows for the organs to be pulled down in continuity with the gastro-intestinal tract. Subsequently, an en-bloc removal of both the abdominal and thoracic organs is conducted. This minimises damage to the organs and facilitates an ex-situ separation of the required organs; i.e. the pancreas.

Multiorgan perfusion

The organs from the animal are then placed on a separate bench in a 'prone' position in order to expose the rear portion of the aorta. This enables the posterior branches to be ligated. The distal portion of the aorta is then identified and clamped, below the renal arteries. Next, the sub-diaphragmatic aorta is identified and transected in order to enable

the insertion of a 21Fr Argyl straight aortic cannula (Cardinal Health, Dublin, Ohio, USA), secured in place by a 0-2/0 Vicryl™ (Ethicon, Livingston, UK) tie. This is then filled with the preservation solution. The multiorgan block is then turned to the anterior side, where the bowel can now be isolated and separated from the hepato-pancreato-renal block. This avoids the wastage of perfusion preservation solution in the splanchnic system.



Figure 16: multiorgan procurement at the abattoir (25 minutes warm ischaemia time)

En bloc abdominal organ procurement

While the cold perfusion flush is running, there is continuity between the small and large bowel and the rectum. This loop is removed by suturing and dividing the distal duodenum. Close attention is paid to the ureters running laterally to the large bowel. Once identified, the ureters are divided approximately 6-7cm from the renal hilum. The gallbladder is then cut, and the bile duct flushed with cold saline. The proximal duodenum is also sutured and divided distal to the pylorus, circumventing the structures of the hepatic hilum. The stomach is dissected by cutting the remaining hepatogastric ligament along the gastric

lesser curve, and the gastrosplenic ligament (including the short gastric arteries) along the greater curve.

The spleen is then easily isolated from the hepatopancreatic block without incurring any damage to the pancreas. The splenic artery and splenic vein are identified approximately 2-3cm from the splenic hilum. This ensures enough distance from potential pancreatic vessels branching out. They are then subsequently and separately tied and divided, respectively.

A portion of the left triangular hepatic ligament links the diaphragm to the left lobe of the liver. This is divided to help expose the aorta. The supradiaphragmatic vena cava can then be identified and sectioned distally to the liver. This enables acquisition of an abundant venous cuff. The right diaphragmatic cupola is also then sectioned around the cuff, freeing the liver from its posterior muscular and tendinous attachments. The inferior vena cava is now exposed and sectioned distally to the left renal vein. The aorta is also sectioned distally from the previously positioned clamp.

Following the completion of the perfusion flush, confirmed by clear fluid flowing from the venous output, the abdominal block consisting of the liver, pancreas and kidneys, is removed from the ice. The block is then placed in a sterile PVC intestinal bag (Bunzl, Leicester, UK) containing 1-1.5L of cooled preservation solution. It is then placed in an ice box and transferred back to the laboratory.

The time from exsanguination to cold perfusion is logged as the warm ischaemia time (WIT) and is approximated to between 12-20 minutes during a typical protocol procedure.



Figure 17: *isolation of the aorta and vena cava with liver, pancreas and kidneys.*

2.1 Isolation of the Pancreas

A major consideration of pancreas isolation is ensuring that the pancreas and the duodenum are rapidly dissected with minimal mobilization of the gland and without disruption to the gland capsule. The mesentery root is stapled at a distance from the pancreas, using a GIA stapler (Medtronic, Minneapolis, USA). The first part of the duodenum (after pylorus) is prepared and stapled, and the fourth part of the duodenum is also mobilised up to the ligament of Treitz and stapled. The pancreas is then mobilized

from its tail. The gland is meticulously prepared from the adjacent bowel, taking every precaution not to damage the pancreas capsule and parenchyma. The splenic artery and vein are ligated to perform a splenectomy. The proper hepatic artery and common bile duct are then divided and ligated. The portal vein is dissected and encircled with an elastic loop. Next, the aorta is dissected on its left lateral face and both renal arteries and veins are isolated and ligated. The celiac artery and the supra-hepatic inferior vena cava are also ligated. This concludes the whole pancreaticoduodenectomy. Pancreas procurement takes approximately 30 minutes.

The pancreas is then flushed with hypothermic preservation solution IGL-2 (4°C) until it becomes pale, and effluent from the portal vein is clear. The pancreas is then stored in static cold storage (SCS) at 4°C until pulsatile perfusion begins in the laboratory (for the HMP and $HMPO_2$ groups). The pancreases were preserved under conventional SCS when in transit to the laboratory from the abattoir. Hypothermic conditions of 4°C were used for 2 hours, in preservation solution.

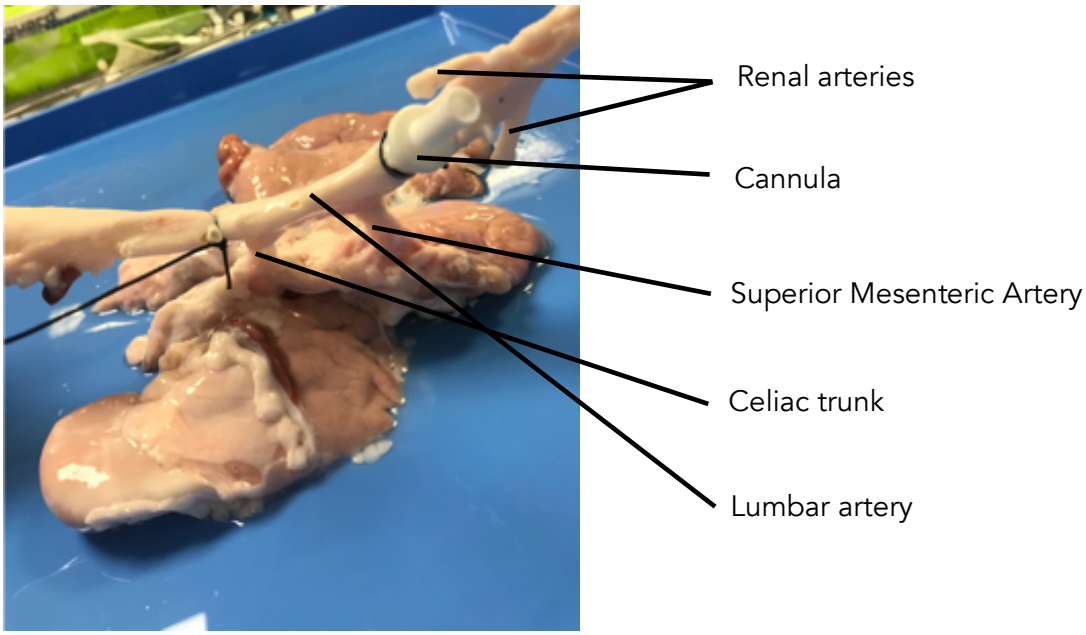


Figure 18: back table preparation prior to machine perfusion. A cannula is inserted into the aorta and the pancreas is flushed through the celiac trunk and superior mesenteric artery.

Laboratory Procedure

Pancreases in the SCS group are kept under conventional static cold storage, while the rest of the organs are prepared for machine perfusion. In the laboratory, lumbar arteries are ligated using a 5/0 non-absorbable monofilament (Prolene). The aortic segment (comprising superior mesenteric artery and celiac trunk) is prepared for perfusion. All small blood and lymphatic vessels around the pancreas are individually ligated. The effectiveness of this preparation was tested by means of aortic injection of preservation solution. All vascular branches sectioned for removal are sutured to replicate conditions similar to those of whole organ transplantation and to avoid leakage during perfusion.

2.2 Waves System

Hypothermic pulsatile perfusion, with greater than atmospheric PO_2, at a temperature of 4 °C was delivered through the use of a Waves machine (Waters Medical System, Rochester, United States of America), using IGL-1 as preservation solution. Waves is a sealed, insulated, transportable preservation system that supports both the transportation and the monitoring of the pancreas. This machine system is designed to provide controlled pulsatile perfusion through the use of oxygenated hypothermic solutions. The interface enables consistent monitoring and display of trends of multiple significant perfusion parameters. These include: perfusate flow, temperature, pressure and resistance.

Waves (IGL company) is a two-part system comprising a 'control unit' and a 'cassette module' as shown in Figure 19. The former is required for perfusion and monitoring of the pancreas, while the latter is a sterile, single-use, disposable part that is used for containing, refrigerating, and circulating perfusate through the entirety of the organ.



Figure 19: Waves system for pulsatile perfusion of the pancreas.

The cassette and the control unit are designed to be automatically aligned, with three latches enabling the security of the cassette in place. 1 litre of perfusion solution (IGL-1) is then added in order to load the perfusate circuit. The hypothermic perfusion is then activated and Waves cools the perfusate to approximately 4°C. Fresh ice is routinely added throughout the perfusion in order to maintain this cold environment that is regulated by the system. A 9mm stainless steel perfusion cannula is inserted into the aorta and the pancreas is subsequently perfused. A disposable sterile drape is placed in order to create an aseptic operating environment. The perfusion pressure is set up and automatically maintained at a set pressure of 15mmHg for 6 hours. It may take approximately 10 minutes following the start of perfusion to reach this pressure which is then subsequently maintained. The perfusion is performed with a set systolic pressure of 15 mmHg and the

machine will automatically adjust the pump parameters in order to obtain this constant. In the $HMPO_2$ group, the perfusion was carried out using an oxygenation membrane actively oxygenating 21% oxygen into the Waves machine, and then the pancreas via the cannula previously inserted in the arterial system. Perfusion data parameters are automatically saved following perfusion and can be visualized on the screen.

2.3 Assessment Markers

Evaluation of pancreas viability and ischaemic injury during SCS and $HMPO_2$

Samples were collected during preservation (SCS, HMP and HMPO2) for further analyses, as detailed below. 10ml of perfusate were taken every hour and immediately frozen at -80 °C. Preservation solution samples were collected from the organ storage bag in the SCS group. Biopsies of the pancreas were taken at the beginning (T0), 4h (T4) and 6h (T6) of perfusion using a 22-mm Bard® biopsy gun in order for several assessments to be made. Some biopsies were snap-frozen in liquid nitrogen and subsequently be stored at -80 °C. Fresh biopsy samples (punch biopsies) were also collected and used for calculating wet to dry weight ratios (as a measure of oedema).

Evaluation of Pancreatic resistance index (PRI) during HMP and $HMPO_2$

Pancreatic resistance index (PRI) is a calculated parameter, used as a marker for transplant organ viability (77). PRI was recorded by the Waves machine © (Waters Medical System, Rochester, United States of America) during hypothermic machine perfusion. As flow and

resistance are reciprocally related, an increase in resistance results in a decrease in flow at any given perfusion pressure.

Oedema Assessment

The assessment of oedema was performed using the Oedema scale (visual scale for assessment of macroscopic oedema). 0: no oedema; 1: slight oedema; 2: moderate oedema; 3: severe oedema (140). A more quantitative measure of oedema was obtained by calculating the wet to dry weight ratios. Fresh biopsies collected at the beginning and end of preservation were weighed and then subsequently dried overnight in an incubator set at 60°C. After drying the biopsies were weighed again and the wet-to-dry weight ratio calculated.

Analysis

Glucose, amylase, lipase, LDH and lactate were all analysed in the perfusate as markers of cell injury.

2.4 Lactate

Lactic acid assay was used for the quantitation of lactic acid (or lactate) in the pancreas perfusate samples. These assays were carried out in the clinical biochemistry lab, John Radcliffe Hospital. Lactate is a by-product of glucose anaerobic metabolism and is produced from pyruvate by the enzyme lactate dehydrogenase. Under normal

physiological conditions, there is a low amount of lactate in the blood, generated in red blood cells, muscle, brain and the gut. Increases in circulating lactate level can cause lactic acidosis and are due to low oxygenation and anaerobic metabolism or inadequate oxygen utilisation by the cells. Therefore, lactate levels are a marker of cellular stress

In this assay, lactic acid is converted to pyruvate and hydrogen peroxide by lactate oxidase. Peroxidase then catalyses the oxidation of a chromogen precursor by hydrogen peroxide to produce a coloured dye. The increase in absorbance at 572nm is directly proportional to the lactic acid concentration in the sample.

2.5 Lactate dehydrogenase (LDH)

LDH is an enzyme found in the cells of many tissues and is responsible for converting lactate into pyruvate (and reverse), an essential step in producing cellular energy. Measurement of the total LDH activity in the perfusate sample is a non-specific marker of tissue injury, as LDH is released by the cells following injury to the plasma membrane.

Lactate dehydrogenase is a hydrogen transfer enzyme that catalyses the oxidation of L-lactate to pyruvate with NAD+ as a hydrogen acceptor. This assay uses the International Federation of Clinical Chemistry (IFCC) recommended forward reaction. A standard assay was used, as performed routinely for clinical use by the clinical biochemistry lab at the John Radcliffe hospital.

2.6 Lipase

Pancreatic lipase in serum and plasma is closely associated with pancreatic diseases. The activity of lipase is an important marker for diagnosing pancreatic diseases and the associated monitoring of therapeutic effects. The test kits currently available include a turbidimetric method using triglyceride as substrate and a colorimetric method using synthetic substrates. These methods, however: 1) lack precision near the normal level; 2) exhibit poor reproducibility; 3) are affected by other enzymes such as esterases. In this study lipase was measured by a colorimetric assay. The enzymatic colour rate assay uses a clear substrate solution of 1,2 diglyceride. The assay is highly sensitive and specific for pancreatic lipase, using colipase and deoxycholate as activators.

Lipase acts on 1,2-diglyceride, to form 2-monoglyceride. This is hydrolysed by monoglyceride lipase into glycerol and free fatty acid. Glycerol kinase acts on glycerol to form glycerol-3-phosphate which is in turn acted on by glycerol-3-phosphate oxidase to generate hydrogen peroxide. A peroxidase converts the hydrogen peroxide produced, 4-aminoantipyrine, and *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS) into a quinone dye. The rate of formation of the dye, measured as an increase in absorbance at 548 nm, is proportional to the lipase concentration in the sample.

2.7 Amylase

Measurement of **α**-amylase activity is of value in diagnosing pancreatitis and other pancreatic disorders which result in elevation of serum and urine **α**-amylase activity.

Normal individuals have low but measurable serum and urine **α**-amylase activity which is produced in the pancreas and parotid glands.

Amylase in this study was measured by a colorimetric spectrophotometric assay. **α**-Amylase hydrolyses 2-chloro-4-nitrophenyl-**α**-*D*-maltotrioside (CNPG3) to release 2-chloro-4-nitrophenol (CPNP) and form 2-chloro-4-nitrophenyl-**α**-*D*-maltoside (CNPG2), maltotriose, and glucose. The rate of formation of the 2-chloro-4-nitrophenol can be detected spectrophotometrically at 404 nm to give a direct measurement of **α**-amylase activity in the sample.

2.8 Blood Gas Analysis

Glucose, pH and pO2 of the perfusate samples were assessed using a bench-top blood gas analyser ABL90 (Radiometer, UK).

2.9 Statistics

Graphpad PRISM was used to statically analyse the investigated parameters. The graphs report the mean with errors bars showing +/- 1 standard error (SEM), averaged over 5 pancreases for SCS and 4 for both $HMPO_2$ and HMP. One and two-way ANOVA, as well as t-tests were used to assess the statistical significance of the results. A p-value of <0.05 was considered statistically significant.

RESULTS

Method optimisation for oxygen delivery

In the initial stages of assessing feasibility of oxygenation, 100% oxygen was used. However, we observed a rise in lactate after 3 hours as well as a rise in glucose (Table 2), potentially associated with injury. Therefore, 21% oxygen (medical air) was used subsequently throughout the perfusions of this study which demonstrated better results with a more stable pO2, and no lactate or glucose produced. (Table 3).

	Time (Hr)			
	30 mins	1	3	4
pO2 (kPa)	26.1	30.1	58.7	92.6
pH	7.19	7.16	7.03	7.03
Lactate (mmol/L)	-	-	1.0	4.0
Glucose (mmol/L)	0.4	0.5	3.7	4.8

Table 2: pO2, pH, lactate, and glucose measurements over time (hr) in the perfusate at 100% oxygen (n=2).

	Time (Hr)					
	0 (-O2)	0	1	2	3	4
pO2 (kPa)	13.5	20.6	20.9	21.7	23.8	23.5
pH	7.178	7.199	7.178	7.186	7.189	7.205
Lactate (mmol/L)	-	-	-	-	-	-
Glucose (mmol/L)	0.0	0.1	0.0	0.0	0.0	0.0

Table 3: pO2, pH, lactate, and glucose measurements over time (hr) in the perfusate at 21% oxygen (n=2).

3.1 Oedema assessment

3.1.1 Macroscopic assessment

The macroscopic assessment of oedema was performed using the Oedema scale (visual scale for assessment of macroscopic oedema) as previously described. 0: no oedema; 1: slight oedema; 2: moderate oedema; 3: severe oedema (140). The oedema assessment was recorded for all three groups: static cold storage (SCS), Hypothermic machine perfusion (HMP) and Hypothermic oxygenated machine perfusion ($HMPO_2$). The SCS group resulted in a score of 0, and both the HMP and $HMPO_2$ groups resulted in a score of 1 at the end of perfusion.

3.1.2 Wet-to-dry weight ratio

Figure 20 shows the wet to dry weight ratio percentage difference between the beginning (T0) and end of preservation (T6) in all three groups: static cold storage (SCS), Hypothermic machine perfusion (HMP) and Hypothermic oxygenated machine perfusion ($HMPO_2$). Within the SCS samples, the highest ratio for T0 was found to be 5.31 and the lowest 2.15 with an average of 3.37. The highest ratio for T6 was 13.2 and the lowest was 1.42 with an average of 5.62. Within the HMP samples, the highest ratio for T0 was found to be 4.8 and the lowest 2 with an average of 3.52. The highest ratio for T6 was 4.8 and the lowest was

2.14 with an average of 3.28. Within the $HMPO_2$ samples, the highest ratio for T0 was found to be 8 and the lowest 1.29 with an average of 5.52. The highest ratio for T6 was 4.6 and the lowest was 1.48 with an average of 3.62. Wet-to-dry weight ratios percentage differences were calculated (T6-T0) and compared between the three group and although HMPO2 presented the lowest levels, the difference was not statistically significant (One-way ANOVA, *(p=0.1035)).*

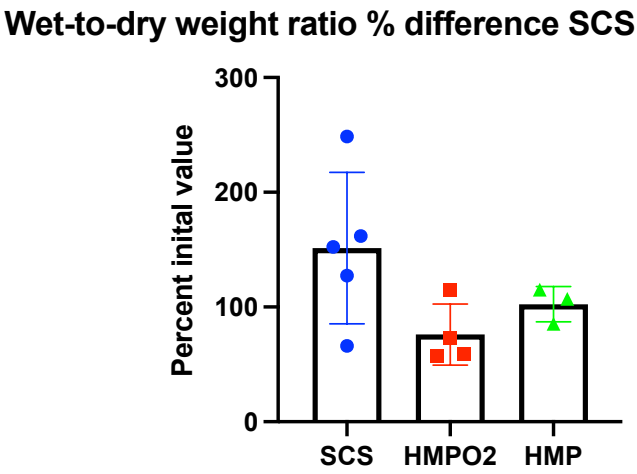


Figure 20: Wet-to-dry weight ratio % difference in the biopsy samples collected across 3 groups; SCS (blue), $HMPO_2$ (red) and HMP (green) for a total of 13 pancreases (n=5 SCS, n=4 $HMPO_2$, n=4 HMP). The data was analysed by one-way ANOVA and no statistically significant difference was found (p=0.1035).

3.2 Perfusion parameters

3.2.1 Resistance index and Flow

Figures 21a and 21b show the intrapancreatic resistance (ru) and flow (mL/min) over perfusion time (hr) in the HMP and $HMPO_2$ groups. Intrapancreatic resistance tends to decrease over time in both HMP and $HMPO_2$ groups, while the flow, subsequently tends

to increase. Within the HMP samples, the highest flow was found to be 39.86 mL/min and the lowest 7.29 mL/min with an average of 23.47 mL/min. The highest resistance within the HMP samples was 2.11 ru, and the lowest 0.13 ru with an average of 0.78 ru. Within the $HMPO_2$ samples, the highest flow was found to be 43.29 mL/min and the lowest 16 mL/min with an average of 29.15 mL/min. The highest resistance within the $HMPO_2$ samples was 0.61 ru, and the lowest 0.31 ru with an average of 0.45 ru. Lowest resistance and higher flows were achieved in the HMPO2 group, however these were not statistically significantly different from HMP (two-way ANOVA, p=0.7373 and p=0.8400, respectively).

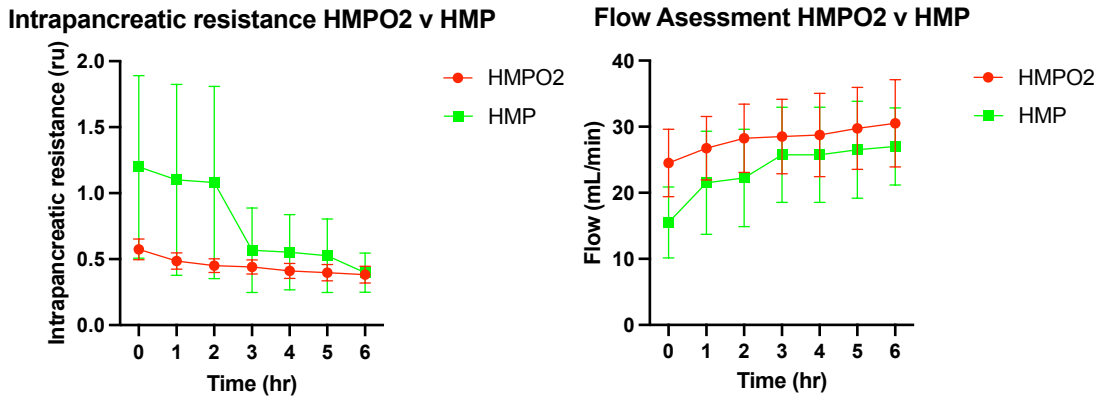


Figure 21a: Intrapancreatic resistance (ru) over time (hr) as monitored during perfusion in the $HMPO_2$ (red) and HMP (green) groups (n=4 $HMPO_2$, n=4 HMP). The perfusate data was analysed using two-way ANOVA and no statistically significant difference was found (p=0.7373). There was no statistical difference across timepoints (p=0.9333) or between treatments (p=0.1003).

Figure 21b: Flow (mL/min) over time (hr) in the perfusion for the $HMPO_2$ (red) and HMP (green) groups (n=4 $HMPO_2$, n=4 HMP). The data was analysed using two-way ANOVA

and no statistically significant difference was found (p=0.8400). There was no statistical difference across timepoints (p=0.9989) or between treatments (p=0.1759).

3.2.2 Oxygen pressure in the perfusate

Figure 22 shows the partial pressure of oxygen PO2 in kPa over time (hr) in SCS, HMP and$HMPO_2$. pO2 was stable overtime across all three groups and no statistically significant difference was found between the groups (Two-way ANOVA, p>0.05)., HMP yielded a mean average PO2 of 23.88 kPa, followed by $HMPO_2$ with 22.37 kPa and finally SCS with 19.52 kPa.

The differences in oxygen pressure for $HMPO_2$ and HMP were found to be indistinguishable and henceforth, have been combined and the implications have been outlined in the discussion.

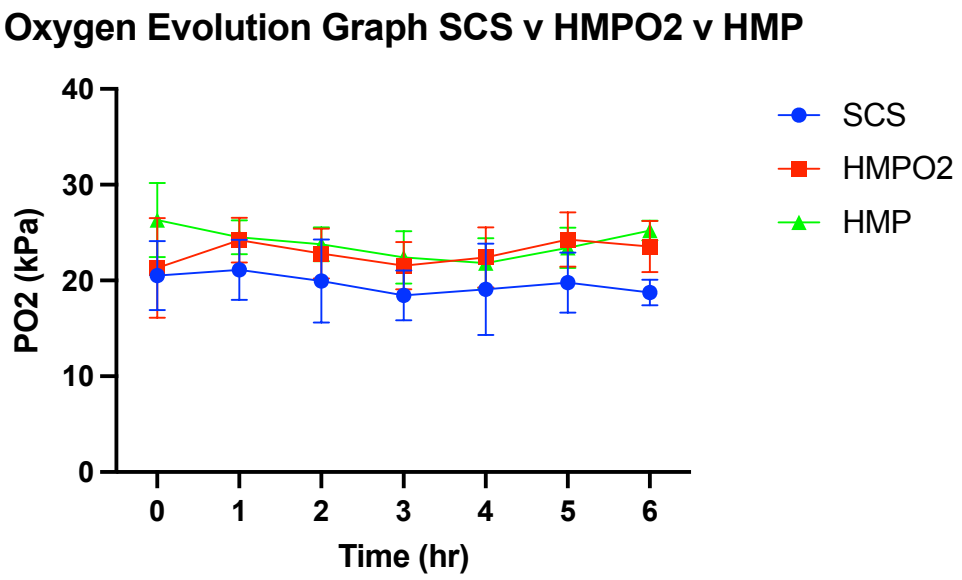


Figure 22: Evolution of oxygen (kPa) over time (hr) in the perfusate samples collected across 3 groups; SCS (blue), $HMPO_2$ (red) and HMP (green) for a total of 13 pancreases (n=5 SCS, n=4 $HMPO_2$, n=4 HMP). Perfusate data was analysed using two-way ANOVA, no statistically significant difference was found (p>0.05).

3.3 General injury assessment

3.3.1 LDH

Figure 23 shows the LDH (U/L) levels over time (hr) in the static cold storage (SCS) and Hypothermic oxygenated machine perfusion ($HMPO_2$) groups. Due to the restricted time in the laboratory as a result of Covid, only two groups were assessed. The SCS group saw analysis of 4 pancreases due to laboratory constraints. In the $HMPO_2$ group, the LDH levels increased with time until 5h (T5) where levels peaked and then decreased to 187.8 U/L at 6h of perfusion (T6). In the SCS group, the LDH levels were significantly higher than in the $HMPO_2$ group (t-test, p=0.0027) and decreased over time with final mean value of 238.2 U/L at T6.

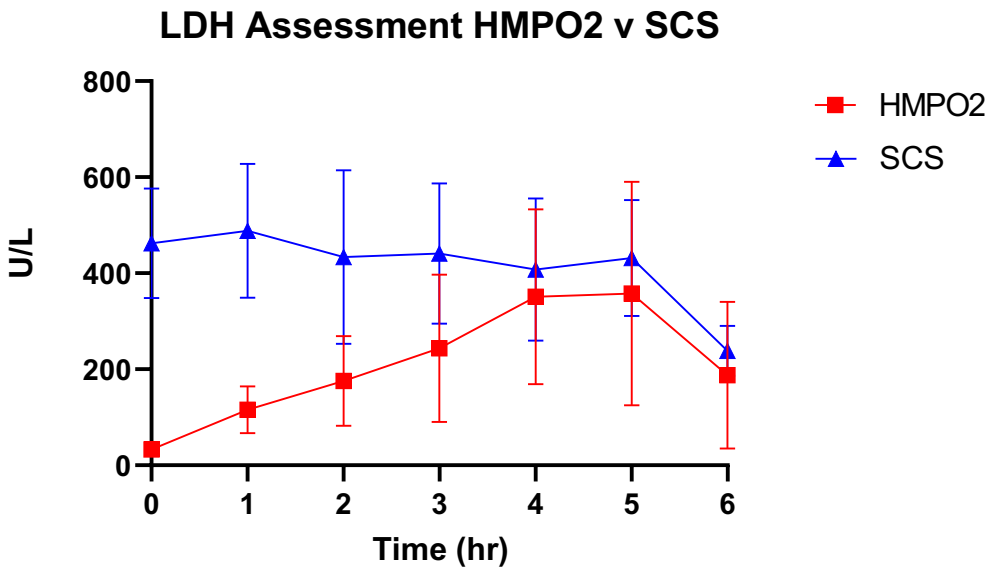


Figure 23: LDH (U/L) levels over time (hr) in the perfusate samples collected across 2 groups; SCS (blue) and $HMPO_2$ (red) for a total of 8 pancreases (n=4 SCS, n=4 $HMPO_2$). Perfusate data was analysed using a t-test, a statistically significant difference was found (p=0.0027).

3.3.2. Lactate

Figure 24 shows the lactate (mmol/L) levels over time (hr) in the static cold storage (SCS) and Hypothermic oxygenated machine perfusion ($HMPO_2$) groups. Due to the restricted time in the laboratory as a result of Covid, only two groups were assessed. The SCS group saw analysis of 4 pancreases due to laboratory constraints. Both groups saw an increase in lactate levels over time, with peaks of 1.04 and 1.54 mmol/L at the end of preservation (6h) in $HMPO_2$ and SCS respectively. Lactate levels were statistically significantly higher in SCS compared to HMPO2 (t-test, p=0.0404).

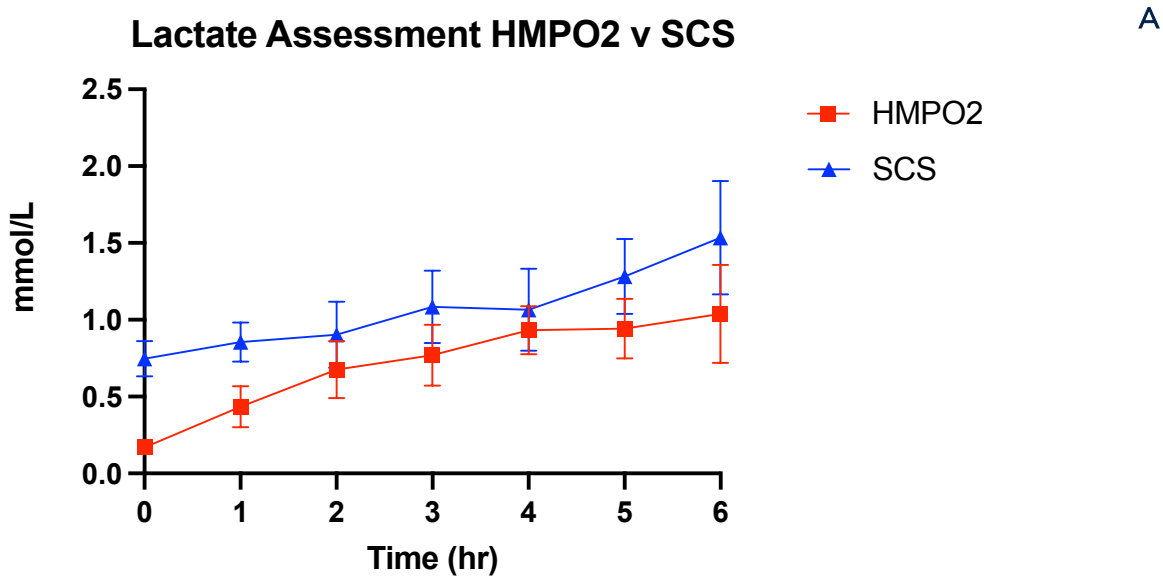


Figure 24a: Lactate (mmol/L) levels over time (hr) in the perfusate samples collected in SCS (blue) and $HMPO_2$ (red) for a total of 8 pancreases (n=4 SCS, n=4 $HMPO_2$). Perfusate data was analysed using a t-test, a statistically significant difference was found (p=0.0404).

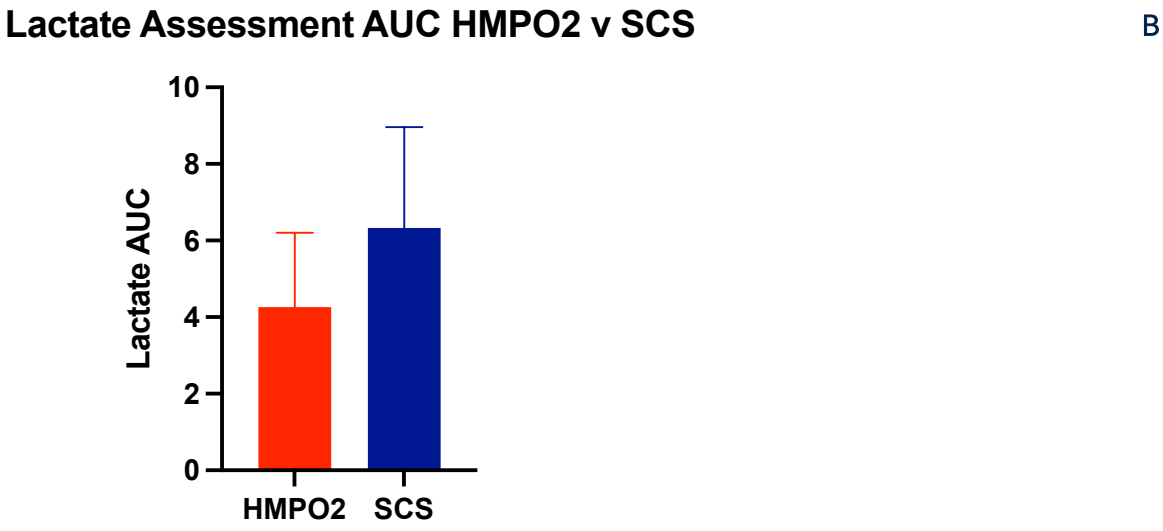


Figure 24b: Lactate area under the curve between the two groups of SCS (blue) and $HMPO_2$ (red) for a total of 8 pancreases (n=4 SCS, n=4 $HMPO_2$). Perfusate data was analysed using a t-test, a statistically significant difference was not found (p=0.2533).

3.4 Exocrine assessment

3.4.1 Amylase

Figure 25 shows the amylase (U/L) levels over time (hr) across two groups: static cold storage (SCS) and Hypothermic oxygenated machine perfusion ($HMPO_2$). Due to the restricted time in the laboratory as a result of Covid, only two groups were assessed. The SCS group saw analysis of 4 pancreases due to laboratory constraints. Both groups saw an increase in amylase levels, with a peak of 69582.75 and 48661.3 U/L at 6h in $HMPO_2$ and

SCS respectively. Amylase levels were significantly higher in the HMPO2 group compared to SCS (T-test, p=0.0494).

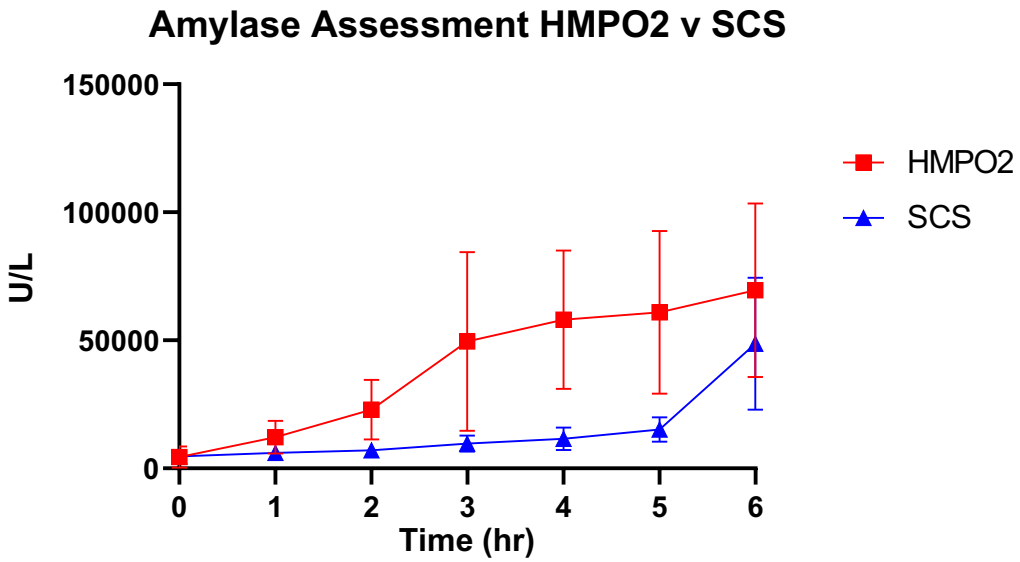


Figure 25: Amylase (U/L) levels over time (hr) in the perfusate samples collected in SCS (blue) and HMPO$_2$ (red) for a total of 8 pancreases (n=4 SCS, n=4 HMPO$_2$). Perfusate data was analysed using a t-test, a statistically significant difference was found (p=0.0494).

3.4.2 Lipase

Figure 26 shows the lipase (U/L) levels over time (hr) in static cold storage (SCS) and HMPO$_2$. Both groups saw an increase in amylase levels, with a value of 4958.95 and 1464.38 U/L at 6h preservation in HMPO2 and SCS groups respectively. Due to the restricted time in the laboratory as a result of Covid, only two groups were assessed. The SCS group saw analysis of 4 pancreases due to laboratory constraints. No statistical difference was found between the two groups.

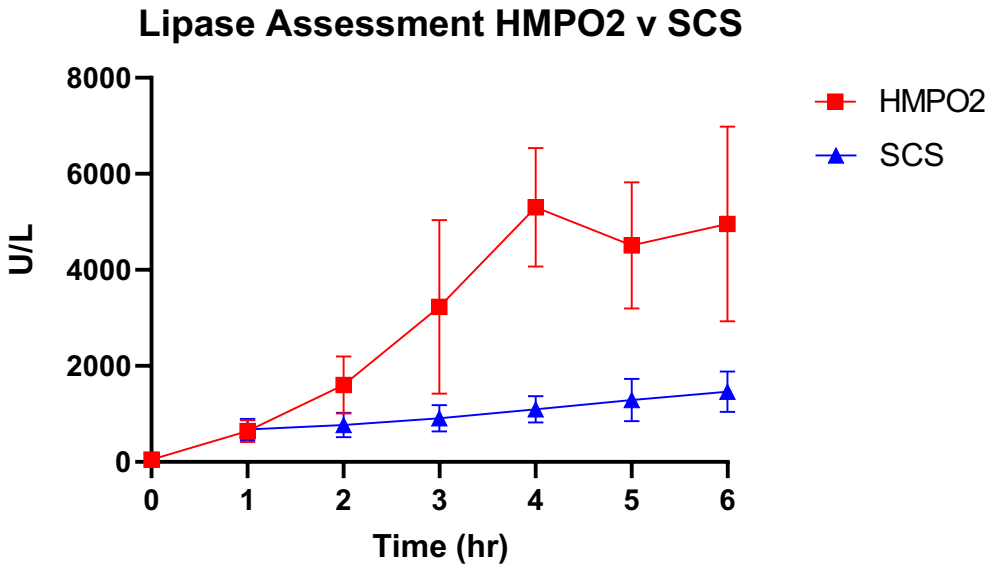


Figure 26: Lipase (U/L) levels over time (hr) in the perfusate samples collected across 2 groups; SCS (blue) and $HMPO_2$ (red) for a total of 8 pancreases (n=4 SCS, n=4 $HMPO_2$). Perfusate data was analysed using a t-test, no statistically significant difference was found (p=0.1721).

3.5 Endocrine assessment

3.5.1 Glucose

Figure 27 shows perfusate glucose (mmol/L) over time (hr) in static cold storage (SCS), Hypothermic machine perfusion (HMP) and Hypothermic oxygenated machine perfusion ($HMPO_2$). The highest glucose levels were found in $HMPO_2$ with a value of 10.87 mmol/L, followed by SCS with a value of 1.52 mmol/L and finally HMP with a value of 0.19 mmol/L. Glucose levels appeared to be different already at the beginning of perfusion (static preservation in the case of SCS) and remained mostly stable throughout

perfusion/preservation, except for a slight increase in the SCS group. However, no statistically significant difference was found between the groups.

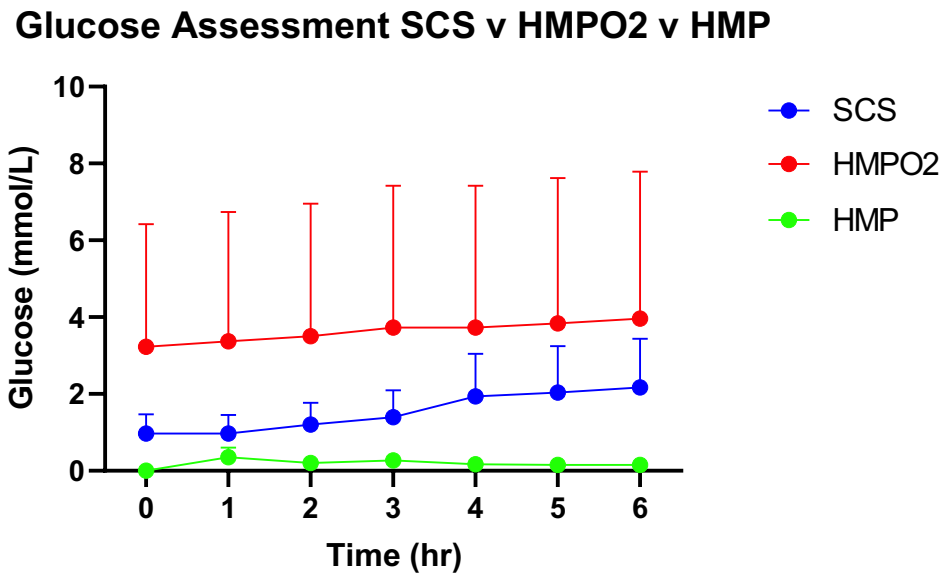


Figure 27: *Glucose (mmol/L) levels over time (hr) in the perfusate samples collected across 3 groups; SCS (blue), HMPO₂ (red) and HMP (green) (n=5 SCS, n=4 HMPO₂, n=4 HMP). The perfusate data was analysed using two-way ANOVA and no statistically significant difference was found (p=0.5408). There was no statistical difference across timepoints (p=0.2137) or between treatments (p=0.6980).*

3.6 Metabolic assessment

3.6.1 pH

Figure 28 shows the perfusate pH over time (hr) across all three groups: static cold storage (SCS), Hypothermic machine perfusion (HMP) and Hypothermic oxygenated machine perfusion ($HMPO_2$). The average values were 7.49, 7.2 and 7.21 respectively. The pH remained mostly stable throughout perfusion/preservation. There was a statistically significant difference between the groups (p=0.0175, two-way ANOVA).

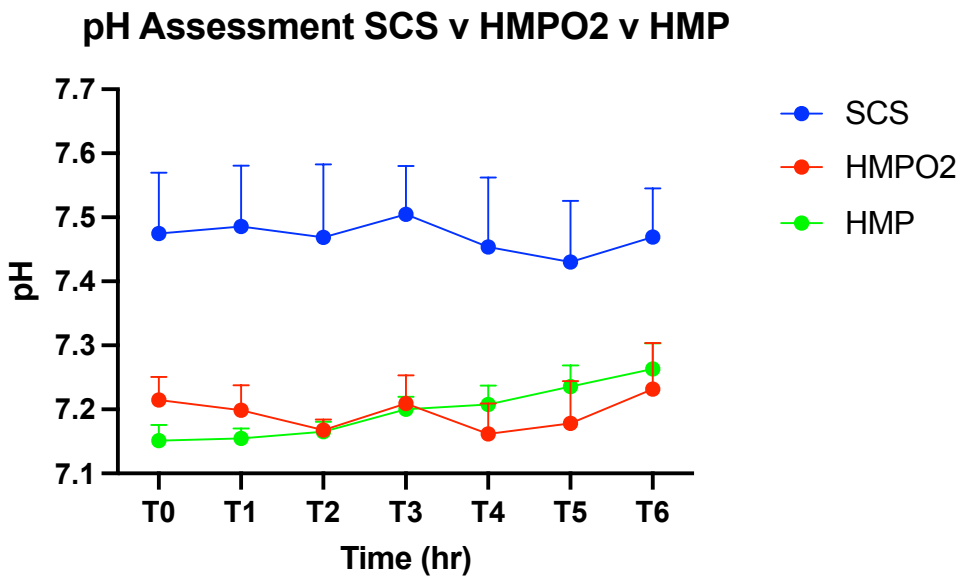


Figure 28: *pH in the perfusate samples over time (hr) collected across 3 groups; SCS (blue), $HMPO_2$ (red) and HMP (green) (n=5 SCS, n=4 $HMPO_2$, n=4 HMP). The perfusate data was analysed using two-way ANOVA and the difference was found to be statistically significant (p=0.0175). There was no statistical difference across timepoints (p=0.3926) but there was between treatments (p=0.0175). The change of pH over time for each group is not statistically different (p=0.3506).*

DISCUSSION

The results from this study indicate some beneficial differences between oxygenated hypothermic machine perfusion compared with static cold storage, demonstrating that $HMPO_2$ could be used an alternative method for pancreas preservation. The markers and features assessed included oedema, pancreatic resistance index, pressure, flow, exocrine markers and general injury markers. The objective of this study was to assess pancreas preservation on an oxygenated pulsatile perfusion machine and to compare the results to HMP and SCS. The results obtained and described above confirm the potential for HOPE in the pancreas.

Assessment of the pancreas using perfusion might be necessary due to the increasing number of DCD organs, as well as the lack of an early thrombosis predictive factor. The requirement for reconditioning the pancreas in order to improve the presence of oedema and to reduce cellular and endothelial ischaemia reperfusion injury was a focal point of this study.

The proposed mechanisms of HMP include: i) improving oxygen and nutrient transport to the organ, ii) removing metabolic wastes and toxins caused by ischaemia, iii) maintaining vascular and endothelial patency through the persistence of pulsatile flow as well as iv) the evaluation of viability through resistance indices. Use of HMP in other solid organs as a method for preservation has been shown to be successful and therefore, was the basis upon which this study was designed for the pancreas.

4.1 Analysis of Results

Oedema Assessment

For a stronger and more quantitative assessment of oedema, wet-to-dry weight ratios were calculated across all three groups of SCS, $HMPO_2$ and HMP. Results showed higher values (thus higher weight gain) for the SCS group compared to that of $HMPO_2$, with HMP results being slightly higher than that of $HMPO_2$. This suggests that perfusion of the pancreas, with the conditions used in this study, may cause less oedematous damage to the organ than SCS.

Historically, the first perfusions conducted by Florack et al (74) demonstrated that SCS with osmolar silica gel filtered plasma was superior to HMP as oedema occurred during pulsatile machine perfusions at a pressure of 30 mmHg in the pancreas models, and concluded that there was sufficient time during SCS to enable the logistics of clinical pancreas transplantation from deceased donors. The preservation failure rates with machine perfusion were found to be between 30-40% at 24 to 48 hours, compared to 0% in those preserved with SCS and silica gel. These results contrast with the results in this study showing that machine perfusion did not cause oedema. A possible reason for this difference includes the use of PEG in the perfusion solution and the lower perfusion pressure used in the present study.

A study conducted by Branchereau et al (77) evaluated the feasibility of hypothermic machine perfusion on 11 human pancreases that were unsuitable for clinical transplantation due to several reasons such as advanced age and alcoholism. Preservation lasted 24 hours at 25mmHg and the main finding was the complete absence of oedema at all time points analysed, 0, 6, 12 and 24 hours respectively. Limitations of this study however, included the lack of molecular and functional markers tested, as well as a small sample size. Nonetheless, as these organs were of marginal transplantability and the results positive, the study is very valuable and supports the hypothesis that the intervention would have been more effective on pancreases of better quality.

Further to this study, Prudhomme et al (145) focussed on a non-human primate model which confirmed the results of the human discarded pancreases. SCS and hypothermic machine perfusion were compared across three different perfusion pressures of 15, 20 and 25 mmHg and results showed that a perfusion pressure of between 15-20 mmHg did not result in oedema or pathological injury of the organs.

The perfusion model we used in this study was adapted from the aforementioned studies and PEG solution was also added, due to its osmotic properties, as a component of the IGL-2 preservation solution used in the study. A study conducted by Bejaoui et al assessed the use of a higher concentration of PEG in male rats that were subjected to an hour of 70% ischaemia followed by 2 hours of reperfusion at 2 and 10mg/kg. The results were shown to protect rat livers against IRI at 10mg/kg through assessment of mitochondrial membrane polarisation and a decrease in transaminase levels (146). A further study

focussed on assessing the effects of PEG following 24 hours of cold ischaemia as well as after 2 hours of reperfusion. The results also demonstrated that the intravenous administration of 10mg/kg improved liver injury as well as protecting mitochondria (147).. The pancreases in the present study were perfused with a pressure of 15 mmHg and with IGL-2 containing PEG solution at a concentration three times that of the standard. PEG was shown to be used for all successful transplantations in several studies but was not used in the Florack study, and therefore it can be suggested that PEG may be playing a beneficial role in preventing oedema. The primary endpoints of these studies remains a matter of some conjecture, it is not clear whether oedema is the most useful marker of the composite quality of preservation or whether a more basic measure, for example ATP/ADP ratio may be more consistent and objective.

Resistance Index and Flow

When assessing the intrapancreatic resistance and flow, the results showed a decrease of the resistance index and an increase in the flow as expected. This decrease in resistance and increase in flow was seen in both of the hypothermic machine perfusion groups across 6 hours. The flow, however, was found to be marginally greater in the oxygenated group, showing that the pancreases were better perfused with the addition of oxygen. These results directly replicate those obtained by Branchereau et al (77) and Prudhomme et al (79). Overall a decrease in the resistance index is suggestive of machine perfusion being a superior method of organ preservation, as low resistance indices have been associated with increased organ quality (77).

Oxygen Pressure in The Perfusate

At 4 degrees Celsius, the enzymatic metabolic activity is heavily reduced but there remains approximately 10% of activity. Without sufficient oxygen to supply this low metabolic activity, anaerobic glycolysis can occur, resulting in production of general injury markers such as lactate, and a decreased pH.

The results obtained from this study showed a small but marked difference between SCS and $HMPO_2$ groups in reference to oxygenation in the perfusate, with the former yielding a mean average PO_2 of 19.52 kPa and the latter a mean average of 22.37 kPa. At the end of the 6 hours, more oxygen is found in the preservation solution in the $HMPO_2$ group, which may enable superior metabolic function.

Our findings are further corroborated with studies from the team in Edmonton (131) as well as by Stiegler et al (132). The former compared pancreas storage in a rat model with UW versus UW and 95% oxygen bubbling and demonstrated the oxygenated group to be a superior method for preserving the isolated islet function and yield. Stiegler et al also showed better islet cell viability in a porcine model with the oxygenated group delivering 100% oxygen in solution in a hyperbaric chamber. Whereas the Edmonton study focussed on the improvement of beta cell function, our study was focussed on general and exocrine markers for improved assessment of the pancreas during preservation.

Lactate and LDH Assessment

When assessing the levels of lactate release in the perfusate over time, higher values were found in the SCS group when compared with $HMPO_2$, with a peak, at 6h, of 1.54 and 1.04 mmol/L, respectively. The reason for this difference could be due to the activation of anaerobic glycolysis during SCS as mentioned above. Less lactate in $HMPO_2$ suggests it might be a superior method for organ preservation and it might be better at supporting tissue metabolic requirements, as anaerobic glycolysis is not an effective source of energy for the cells.

Glycolysis occurs in the cytoplasm of cells where one molecule of glucose is oxidised and generates two molecules of pyruvate. The presence or absence of both oxygen and mitochondria in cells then dictates the fate of pyruvate and the subsequent generation of ATP in the mitochondria. In anaerobic conditions, the pyruvate is reduced to lactate as NADH is reoxidised to NAD+ by lactate dehydrogenase. This process results in the production of ATP essential for cell survival (148). Through the anaerobic route, though, only 2 moles of ATP can be produced, compared to 32 moles of ATP under aerobic conditions. Therefore, the lower levels of lactate suggest that the use of hypothermic oxygenated machine perfusion is a possible method for supporting cell metabolism, reducing cell injury and improving the quality of the organ.

It is important to note that not only injury markers but also functional markers are important in predicting graft survival or failure. A possible limitation of the measurement of lactate is that while it can be demonstrative of metabolism in the tissue, it is not a direct indicator

of injury. The need for a measure of organ functionality as opposed to injury sustained is an essential part of the development of machine perfusion.

LDH release was measured over time in the two groups and the levels were greatest at the beginning of preservation for the SCS group with near to no damage in the $HMPO_2$ group. LDH levels remained fairly stable across the SCS group until 5h, after which the levels fell. In contrast, the levels rose in the $HMPO_2$ group until 4h before falling. However, the levels in SCS were higher than $HMPO_2$ throughout the preservation period.

LDH is released when cell membranes are compromised or ruptured following injury. It is a large protein, normally found in the cytosol without the capability of crossing the membrane except in circumstances of cell damage. As a result, this release takes time and therefore LDH can be considered a 'lagging marker'. The high levels of LDH release in the SCS group at T0 therefore demonstrate a greater cell injury than that of the $HMPO_2$ group. This study measured the total release over time; however, a possible limitation could be that the time period used to measure may not have been enough time to accurately represent the true release and therefore extent of injury.

Exocrine Assessment: Amylase and Lipase

Amylase and lipase release reflect the exocrine part of the pancreas. If levels are high, the organ has encountered injury, and this could lead to pancreatitis and subsequent graft thrombosis and loss. Amylase and lipase levels were both greater in the $HMPO_2$ group than the SCS group. These results are further corroborated by Leemkuil et al (80). Although

these levels could reflect injury of the pancreas, they may also be due to the fact that HMP is oxygenated and therefore the organ is more metabolically active when compared to SCS.

Lipase is released in order to process triglycerides and break these down into free fatty acids and glycerol. Amylase functions to hydrolyse glycosidic bonds in starch molecules in order to convert complex carbohydrates to simple sugars. Their release as inactive forms occurs under standard normal conditions by the pancreas but are both dependent on ATP and therefore, their release would require oxygen. The active flushing and oxygenation of the organs in oxygenated machine perfusion is a further possible reason for our results in relation to the release of these proteins. A plateau is reached after 4 hours, which might mean that the proteins have been washed out of the pancreas and the organ is no longer suffering injury. However, in SCS values continue to rise. A potential limitation, however, could be the lack of sampling in the HMP group. If the release were due to metabolism alone, there would be an evident difference in results when comparing the HMP to the $HMPO_2$ group. However, if the results are due to purely the mechanics of the perfusion alone, then the same results would occur when comparing HMP with $HMPO_2$. These enzymes are normally released into the duodenum as digestive enzymes and the presence in the perfusate (i.e., blood in transplantation) beyond a certain level is reflective of injury to the exocrine part of the pancreas.

Glucose

The glucose levels were found to be stable in the hypothermic machine perfusion groups, however an increase in glucose was observed in the SCS group. Glucose levels in the perfusate start at different levels at T0, however there is a trend of an increase in the SCS group as compared to the HMP and $HMPO_2$ which seem to be relatively stable. Differences between the groups are not statistically significant but better insight could be achieved by performing statistical analyses into the specific groups and the changes from baseline values.

pH

The pH levels obtained were stable across all groups with the highest pH in the SCS group. The pH of the preservation solution in SCS is 7.4, and as the sampling is performed in static conditions in this group, it is well reflected in our measurements. The absence or low O2 presence could cause a switch to anaerobic glycolysis with lactate production which could contribute to a lower pH.

The baseline pH levels differ across the three groups already at the start but this could be attributed to a difference in sampling, as none of the fluid collected in the SCS groups has been filtered through the pancreas, so the fluid is collected from the pancreas bathing in perfusion liquid as opposed to an active and continuous flush. The reason for low pH in the HMP groups might be due to active flush-out of acidic substances (or high CO_2) which would indicate metabolic acidosis. However, it is worth noting that the pH remains stable and tends to rise rather than fall. The combination of high lactate and high pH appears

paradoxical but may be a function of anaerobic metabolism (generating lactate) in the context of the buffering capacity perseveration solution.

4.2 Future Work

Pancreas assessment using normothermic machine perfusion

Renal hypothermic pulsatile perfusion has shown increasing evidence of benefit, in terms of transplant survival, ischaemia-reperfusion, and transplants from ECD. The survival of pancreases and clinical consequences of ischaemia-reperfusion injury and associated vascular thrombosis and pancreatitis, could possibly be improved through the use of pulsatile hypothermic machine perfusion. The evaluation of resistance indices of perfusions might also provide valuable information to assist evaluation of the organ.

The next phase of this study would be to use a more clinically-relevant functional outcome endpoint: this might be achieved using normothermic machine reperfusion as a surrogate for transplantation, or experimental transplantation itself – which would be the only means of properly assessing the effect of $HMPO_2$ on pancreatitis.

Undoubtedly, there are well known challenges and a few published experiences and therefore this progression must be done in phases. The first of which, would include developing normothermic machine perfusion of pancreases in order to enable organ assessment. A study of this calibre would enable assessment of any appreciable differences between hypothermic pancreas preservation and normothermic pancreas preservation.

Introduction of an Oxygen Carrier

Another possible phase of study would be to assess whether the use of an oxygen carrier could provide a better and more effective method of oxygen delivery throughout the pancreas. As discussed previously, Hemopure has been demonstrated to both take up and offload oxygen more efficiently than haemoglobin in red blood cells and therefore could be an option to explore within the pancreas. Hemarina-M101 is also a suitable oxygen carrier with favourable characteristics including a high affinity for oxygen and antioxidant activity (149). It is also functional across a wide range of temperatures so could prove useful in normothermic assessment following hypothermic machine perfusion as well. A preclinical study by Lemarie et al saw a decrease in oxidative stress, necrosis as well as improved function when M101 was injected into rat pancreases. In addition, when human pancreases had M101 injected, insulin secretion was upregulated as well as an increase in mitochondrial activity (150). Another study, namely the OXYgen carrier for Organ Preservation (OXYOP), compared M101 with preservation solution in one of the two kidneys from the same donor (104). Secondary endpoints of the study showed less delayed graft function as well as improved renal function in the M101 group when compared with the contralateral kidneys. Therefore, introducing an oxygen carrier into further studies may prove a beneficial next step.

HMP as a platform for treatment prior to transplantation

A potential next development to this study may be the use of machine perfusion as a way of delivering drugs to the pancreas in order to prepare it for transplantation, these could include the use of anti-inflammatory agents to protect the pancreas from pancreatitis and could prove to be beneficial in the conditioning of the organ before being transplanted into the recipient (151). There has been some indication that some agents can be used in cold temperatures, as used in HMP for the kidney, and therefore it could be a potential area for study in order to improve the protocol (152).

Islet cell extraction and transplantation

The isolation of islet cells following machine perfusion for a set period of time might result in an increased yield of islet cells. The yield of islets is a major rate limiting step in the use of this treatment. A major potential benefit of HMP would be an improvement to the yield and function of isolated islets and an improved yield and potentially viability could prove beneficial for the success of islet transplantation. A study conducted by Taylor et al demonstrated that 24hr HMP results in fluid accumulation in the organ that is uniform and thus results in an extracellular space with beneficial effects for islet isolation. The method, although developed for a juvenile porcine model, can be applied in an adult porcine model (153). This could be an endpoint for future studies following the research carried out in this thesis.

4.3 Limitations

A possible limitation of the study is the abattoir model. Clearly there is a significant degree of tissue injury associated with the model: we did not quantify this and histological and immunohistochemical evidence might have given a better estimate of the degree of this. We attempted to use pigs with similar characteristics for each perfusion, however, as the pancreases were chosen according to availability of the pigs on the day, it was not possible to ensure accuracy of size, weight and other limiting factors. A potential way to improve on this would be to manually choose and weigh every pig to ensure that the pancreases were all approximately the same. Other than the focus on possible weight discrepancy, another limitation could be the extent of injury to the pancreases that is a function of the abattoir process: the combination of warm ischaemia and the hot-wash. However, it is important to mention the benefits of this model including the reduced cost and the animal welfare.

A second limitation includes the cold ischaemia time, the time between flushing the pancreas on ice and then the time taken to transport the organ back to the laboratory to be put on the Waves perfusion device. The average time from the point of killing to start of perfusion was approximately 2 hours and could have potentially resulted in dampening effects of perfusion. Attempting to reduce this cold ischaemic time during transport of the pancreas might lead to better results in improving the quality of the organ.

CONCLUSIONS

There has been a renewed interest in the application of hypothermic machine perfusion in the preservation of the pancreas. With a growing shortage of ideal pancreas donors, the criteria for donation have been extended in order to supply the demand for pancreases. The pancreas poses a significant problem in that it is a physiologically low-flow organ, with highly delicate vasculature and can easily be injured. Therefore, due to its fragility, it is necessary to optimise the pancreas during procurement as well as preservation.

This study has shown that undergoing 6 hours of hypothermic machine perfusion with a low-pressure perfusion protocol results in stable perfusion dynamics, optimal assessment marker results and minimal oedema when compared to static cold storage alone. This study also provides evidence that $HMPO_2$ could be tested in a clinical trial of pancreas preservation as an alternative to SCS. This pre-clinical work is encouraging and translation towards clinical studies may be timely and beneficial. The application of perfusion technology in this way might enable further expansion of pancreas transplantation, with improved utilisation of donor organs without compromise to the outcomes.

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