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Original article

PANCREAS *EX-SITU* PRESERVATION AND EVALUATION. DEVELOPMENT OF A NORMOTHERMIC MACHINE PERFUSION SYSTEM

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Summary

Simultaneous kidney-pancreas transplantation remains the gold standard treatment for patients with type I diabetes and end-stage renal disease. The evolution of donor demographics requires the use of marginal donor organs including donors with extended criteria and donation after circulatory death; donor organs which are particularly sensitive to ischemia-reperfusion injury. *Ex-situ* normothermic perfusion (NP) is currently established in other solid organs to preserve, assess and rejuvenate donor grafts prior to transplantation. We describe our NP system based on extracorporeal machine oxygenation as a reproducible and affordable strategy to enable pre-clinical experiments in both small animal and human grafts to enable *ex-situ* assessment. Our NP system is established using modified paediatric extracorporeal machine oxygenation system and using low circuit perfusate volumes to allow prolonged perfusions. This system has reliably supported pancreas grafts for 6 hours maintaining normal macroscopic appearances and characteristics in human (n = 3), porcine (n = 8) and primate non-human pancreases (n = 2). This system mimics the early reperfusion period in transplantation however is readily adaptable to investigate other research objectives in *ex-situ* pancreas studies.

Key words: pancreas transplantation, organ preservation, perfusion

Abbreviations

DBD: Donation after Brain Death; DCD: Donation after Circulatory Death; ECMO: ExtraCorporeal Machine Oxygenation; NP: Normothermic Perfusion

INTRODUCTION

Simultaneous kidney-pancreas transplantation is the gold standard treatment for patients with type I diabetes and end-stage renal disease. It restores physiological insulin secretion and avoids hypo- or hyperglycaemia peaks respectively responsible for threatening coma and micro or macro vascular complications. Whole pancreas transplantation is currently considered as a treatment

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that can improve the quality but also the duration of life [1-4]. The pancreas is a particularly sensitive organ to ischemia-reperfusion injury. Restrictive donor selection for pancreatic transplantation is necessary to achieve optimal safety with the least number of complications (venous thrombosis, graft pancreatitis) and the best long-term function of the transplanted graft. Allograft availability is limited, due to the evolution of donor characteristics which is increasingly older, with higher body mass index and increased co-morbidities requiring the increased consideration of marginal organs, specifically from donors with extended criteria or after donation after circulatory death (DCD).

Ex-situ organ machine perfusion technologies is regaining popularity as it provides solutions to donor organ scarcity. By prolonging preservation times, allowing assessment and organ resuscitation more organs that might have been discarded may be recruited for transplantation.

Machine perfusion strategies are classified by a few definitions with one of the most popular being by temperature: hypothermic (0-10°C), subnormothermic (20-33°C) and normothermic (33-38°C).

Ex-situ normothermic perfusion (NP) mimics physiological conditions by the supply of oxygen and nutrients at normal temperature. It is a technology already clinically established, in France, normothermic regional perfusion is mandatory in DCD organ procurement with the first pancreas transplantation now performed post this [5].

NP has proven to be effective in kidney [6-7], liver [8-10], lung [11] and heart [12] transplantation with published clinical studies. The exciting possibilities of NP are being expanded with multiple groups exploring *ex-situ* injection of drugs and or cellular therapies with the goal of organ resuscitation prior to transplantation [9].

Data on NP in pancreatic transplantation are few published animal and non-transplanted human graft studies with all being preclinical [10].

Our transplantation research group has been focused on establishing NP for both experimental and clinical uses. There are a few known NP systems on the market particularly for liver, heart and lung transplantations however these are expensive systems due to price of the consumables and expertise required. These considerations limit use of these systems for preclinical studies where knowledge gained from repeated and frequent use of these systems are invaluable. Here we share our NP system based on extracorporeal machine oxygenation (ECMO) system to be reproducible, low cost and adaptable to pre-clinical experiments for *ex-situ* pancreas graft assessments.

MATERIAL AND METHODS

Description of the normothermic machine perfusion

Our normothermic perfusion machine is based on ECMO system well established in cardiothoracic surgery (Figure 1) provides a schematic of the normothermic perfusion system and Figure 2 the photograph. The system is composed of a centrifugal pump (Revolution® centrifugal blood pump, Sorin Group, LivaNova, London, United Kingdom), oxygenator (D100 oxygenator, Sorin Group, LivaNova, London, United Kingdom) and heat exchanger (Heater unit HU 35, Getinge, Sweden) set to 37°C. The

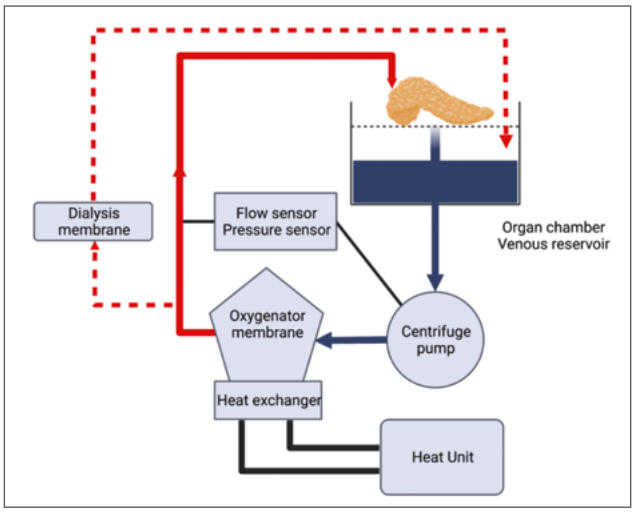

Figure 1. Normothermic perfusion machine derived from a pediatric extracorporeal machine oxygenation.

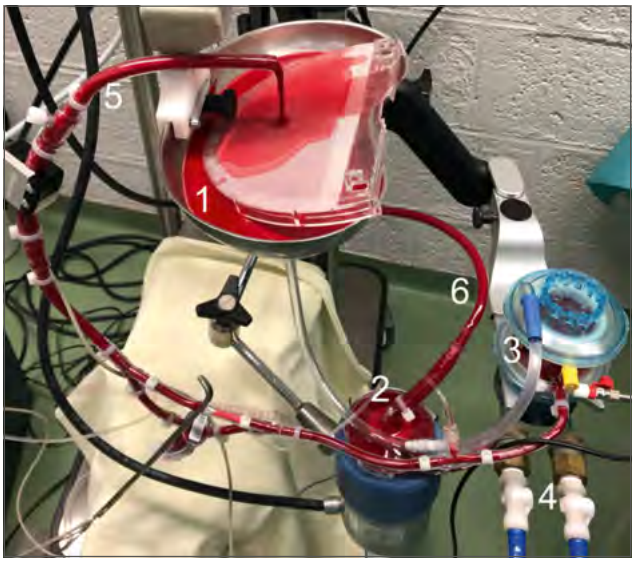

Figure 2. Normothermic perfusion machine set up for pre-clinical study in animals. Normothermic perfusion machine derived from a paediatric extracorporeal membrane oxygenation (SCP system, Sorin Group, LivaNova, London, United Kingdom). 1: Organ chamber; 2: Centrifugal pump; 3: Oxygenation membrane; 4: Heat exchanger; 5: Arterial line; 6: Venous return.

system is supplied by an air/oxygen mixer (3500 series, Sechrist industries, USA). An arterial line filter that comes with the oxygenator is included to capture debris, thrombi and air bubbles. Perfusion monitoring is provided by arterial flow and pressure sensors that are incorporated in the centrifugal pump; both are adjustable to produce desired parameters in this flow-controlled system. Deliberate steps taken to minimise circuit priming and eventual circuit perfusate volume included minimising circuit tubing by eliminating a portal venous line and allowing free drainage from the graft into the organ chamber that also serves as the perfusate reservoir.

Although there is no dialysis membrane incorporated in this design it can be easily added into the circuit in line to the circuit and just after the oxygenator. One may consider incorporating a dialysis membrane dialysis in prolonged perfusions to maintain electrolyte and acid base homeostasis to potentially reduce organ oedema.

Pre-clinical animal and human models

To assess our normothermic perfusion machine, we first performed NP in a pre-clinical porcine model, then in a pre-clinical non-human primate model, and finally in a human model.

For animal models, all experiments were in accordance with ARRIVE guidelines 2.0 and was carried out according to EU Directive 2010/63/EU for animal experiments.

Porcine pancreas model: the study protocol was approved by the French Research Ministry (APAFiS# 31507). Animal house male pigs "large white" Sus scrofa pig with an average weight of 80 kg were used. Pancreas procurement was performed according to a DCD protocol. Under general anaesthesia procedure (ventilation with a mixture of isoflurane (2%), nitrous oxide (49%) and oxygen (49%), laparotomy is made through a xypho-pubic incision, to expose retroperitoneal vessels which are cannulated after a 300 IU/kg dose of heparin. The thoracic aorta and suprahepatic inferior vena cava were then clamped to simulate DCD with 30 minutes of warm ischemia. During the warm ischemia, an exsanguination at the thoracic level was performed to collect one litre of autologous blood to be used in the NP solution. The blood was collected in collection systems with leukoreduction filters (Leucoflex LXT, Macopharma, Mouvaux, France). After 30 minutes of warm ischemia, the abdominal viscera and pancreas are flushed with cold IGL-1© solution (Institut Georges Lopez, Lissieu, France). Crushed ice was added into the abdominal cavity to facilitate additional cooling. The pancreas and duodenum were then separated from the spleen, intestines, stomach, and liver and are then removed en-bloc with the aorta. On the back table, a cannula was inserted into the aorta, a short tube is placed into the portal vein to splint it open and the lumbar arteries were ligated.

Primate non-human pancreas model: nonhuman primate pancreases were procured from male cynomolgus macaques weighing 5 kg in our laboratory. All procedures were performed on animals after euthanasia in a study previously approved by the French Research Ministry (APAFiS# 23409). After euthanasia, laparotomy is performed the retroperitoneal vessels were cannulated, and the blood collection was performed through a large incision of the thoracic vessels. Following this, the procedure was identical to that performed on the pig with an en bloc procurement of the abdominal aorta to permit NP through it.

Non-transplanted human pancreas model: the study protocol was approved by the French national agency – Agence de Biomédecine (PFS08-07). Consent was taken from donors' family to procure human pancreases solely for scientific purposes. Pancreas procurement was similar to that performed for whole pancreas transplantation. The splenic artery was divided after its separation from the celiac trunk and the superior mesenteric artery was also divided just after its origin from the aorta. The portal vein was divided between the liver and the pancreas. The pancreas was extracted en bloc with the spleen. The iliac bifurcation was also collected to perform an arterial Ygraft-plasty between the splenic and the superior mesenteric artery. The blood was collected during the organ flush of the abdominal organs. The first litre from the venous discharge was collected and immediately stored in leukoreduction bags. Full Blood count and ionogram performed on the first litre of blood to establish electrolyte values.

Then the pancreas was prepared on the back table. The spleen was separated from the tail of the pancreas. An arterial Y graft-plasty between the superior mesenteric artery and the splenic artery was performed with the iliac bifurcation and any leaks were noted ligated prior to perfusion. A venous cannula is introduced to into the portal vein. A cannula or catheter is inserted into the duodenum before being fixed to collect pancreatic and duodenal secretions.

Perfusion solution and pancreas perfusion

The perfusion solution used for the development of the system was almost exclusively composed of leukocyte-depleted autologous blood (red blood cell + plasma). For one litre of leukocyte-depleted whole blood, 5000 units of heparin, 10 mg of nicardipine, and 1 gm of co-amoxiclav (antibiotic) were added. NP was performed at a pressure target of 40 mmHg. Oxygenation delivered was with 95% oxygen plus 5% carbon dioxide at a flow rate of 2 litres/minute via the oxygenation membrane. This oxygenation resulted in a pO2 level around 650 mmHg in the arterial line during perfusion. The heater was set for a target temperature of 37°C. During NP, the oxygenation level and acid-base balance were monitored at the start of NP

and then every 30 minutes (EPOC Blood Analysis system, Siemens, Erlangen, Germany). Perfusate acidosis was corrected with the addition of sodium bicarbonate.

RESULTS

Porcine model: 8 porcine pancreases were perfused on NP over 2 hours. All pancreas grafts maintained normal macroscopic appearances of both the parenchyma and duodenum post reperfusion and showed no evidence of macroscopic tissue necrosis. Oxygen consumption by the organ could be visually appreciated by the darker deoxygenated colouration of the portal venous outflow compared to the brighter colouration of the arterial inflow (Fig. 3). This arteriovenous difference was demonstrated on serial blood gas analyses. At a fixed 40-mmHg perfusion pressure, blood flow initially increased during the first 30 minutes and then stabilised until the end of the perfusion (mean flow of 40 mL/min) (Fig. 4). An increase in mean venous lactate was observed throughout the NP associated with an acidosis that required bicarbonate compensation. Amylase and lipase levels increased progressively during the NP.

Primate non-human model: 2 primate non-human pancreases were maintained on NP over 6 hours. In both cases, NP was delivered with a total volume of 300 mL leukocyte-depleted whole blood. The pancreatic tissue and duodenum maintained macroscopically normal appearances for 6 hours without necrosis (Fig. 5). Similar trends observed in the porcine were noted in this model. Blood flow was stable over the period of 6 hours. Steady increases in lipase, amylase and blood lactate were observed throughout the perfusion. However, after 2 hours of NP and in contrast to the porcine model, there was a production of pancreatic secretions collected via the duodenal catheter.

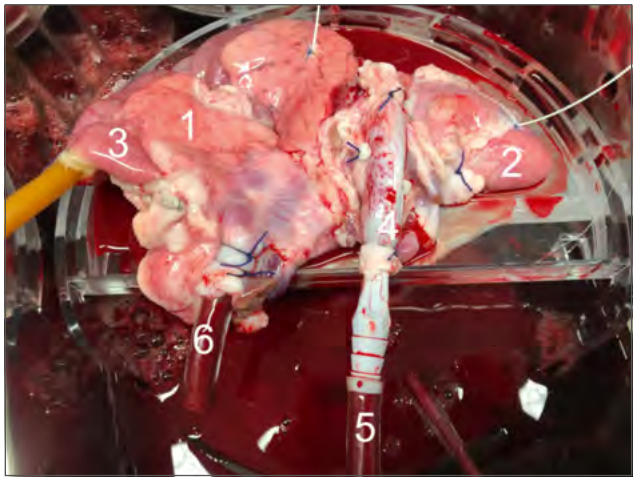

Figure 3. Normothermic perfusion of a porcine pancreas. Macroscopic oxygen consumption. 1: Head of the pancreas; 2: Tail of the pancreas; 3: Duodenum; 4: Aorta; 5: Arterial line; 6: Venous return through the portal vein.

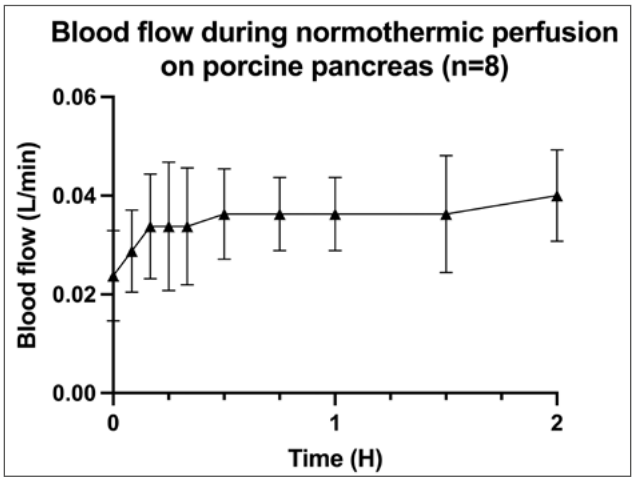

Figure 4. Blood flow during normothermic perfusion on porcine pancreas (n = 8). Perfusion pressure at 40 mmHg. Values expressed in mean +/- sd.

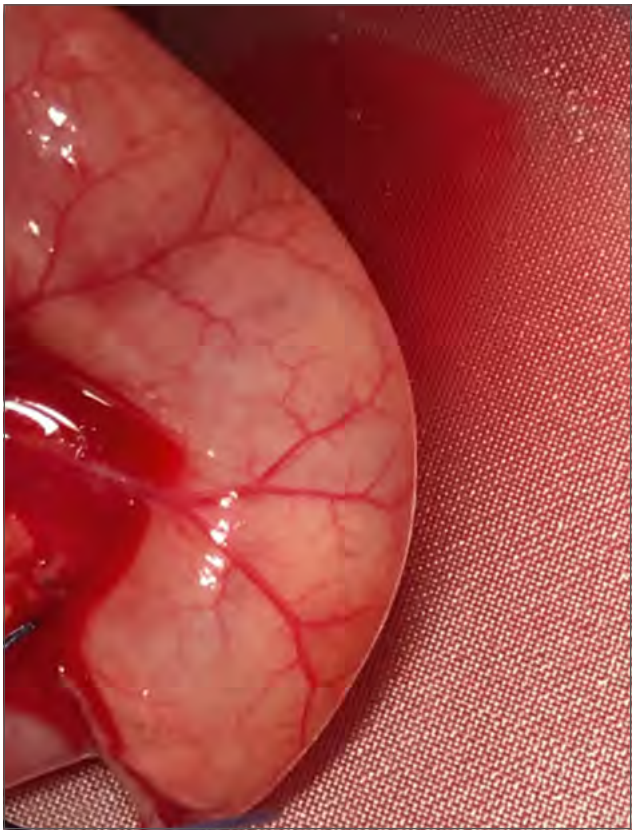

Figure 5. Macroscopic assessment of the duodenum after 3 hours of perfusion in a primate non-human model.

Human model: 3 human pancreases were perfused for a period of 6 hours. Pancreas #1 was procured from a 20-year-old male DBD donor with no medical history. Cold ischemia time was 18 hours (preservation solution: IGL-1). Pancreas #2: was procured from a 43-year-old male DCD donor with a history of alcohol excess. Cold ischemia time was 18 hours (preservation solution: IGL-1). Pancreas #3 was retrieved from a 50-year-old male DCD donor with a background of alcohol excess. Cold ischemia time was 4 hours (preservation solution: IGL-1). Serum amylase and lipase were noted to be normal for all the donors prior to organ procurement. During NP The pancreatic tissue and duodenum maintained normal macroscopic appearances for 6 hours without necrosis (Fig. 6). Blood flow was stable over the period of 6 hours. NP was associated with large production of duodenal and pancreatic secretions. After 4 and 5 hours of NP, respectively, pancreases #2 and #3 the duodenal effluent was noted to appear blood stained and similar in appearance to the perfusate. This phenomenon was not observed during NP of pancreas #1 during the 6 hours. The rate of increase of amylase and lipase levels differed between pancreases. Pancreas #1 showed a slower increase in these markers compared to pancreases #2 and #3 (Fig. 7).

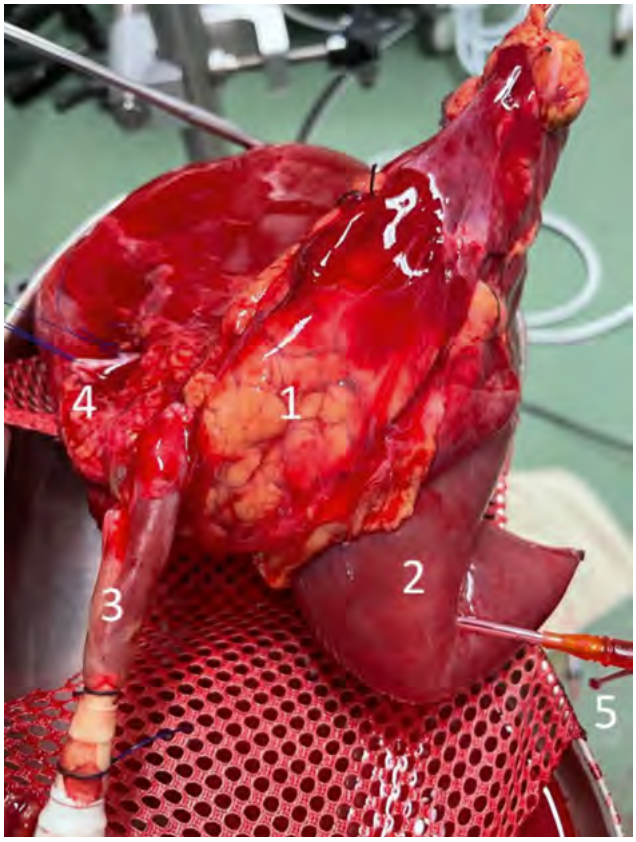

Figure 6. Normothermic perfusion of a human pancreas. Macroscopic assessment after 4 hours of perfusion 1: Tail of the pancreas; 2: Duodenum; 3: Y-iliac plasty; 4: Portal Vein; 5: Collection of pancreatic secretions.

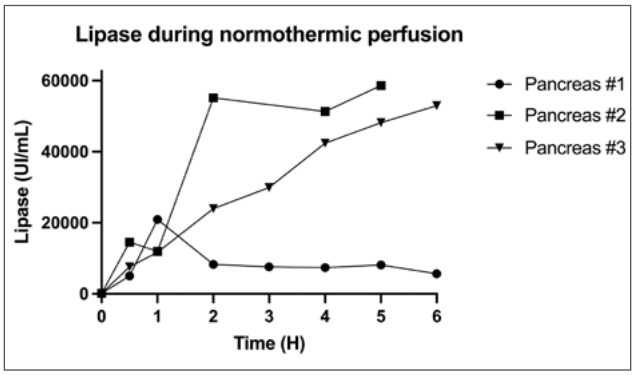

Figure 7. Lipase level during normothermic perfusion in a human model. Pancreas #1 was retrieved from a 20-year-old man with no comorbidities during a DBD procedure. Cold ischemia time was 18 hours (preservation solution: IGL-1). Pancreas #2: was retrieved from a 43-year-old man with alcohol-dependence. Cold ischemia time was 18 hours (preservation solution: IGL-1). Pancreas #3 was retrieved from a 50-year-old man with alcohol-dependence. Cold ischemia time was 4 hours (preservation solution: IGL-1).

DISCUSSION

In this paper we share our design and experience of an affordable and efficient NP system specific for pancreas grafts to enable pre-clinical studies in both animal or non-transplanted human models. This model in our hands reliably supports *ex-situ* pancreas grafts from 2 to 6 hours while maintaining normal macroscopic appearances expected in viable grafts.

In the series of 3 human donor grafts with differing donor characteristics NP analysis appeared to differentiate perfusion performance with more favourable measures (slower increase of pancreatic enzymes and no haemorrhagic duodenal effluent) observed in pancreas #1. Better reperfusion characteristics is expected in Pancreas #1 as fits the criteria for an ideal donor for pancreas transplantation however what was of interest to us was that these favourable characteristics were detected by the NP system demonstrating its assessment capacity.

The pancreas is a low flow organ and therefore venous outflow is of particularly low volume therefore direct connection to the circuit pump may produce injurious negative pressure to the graft vasculature therefore the venous outflow is left on free drainage into the organ chamber/reservoir.

A lot of care is taken during pancreas back bench preparation to reduce leaks which further reduces venous outflow.

To minimise perfusion volumes to an average of 300 mls per NP a number of steps were undertaken. We ensured we used a small volume, a paediatric oxygenator. Additionally, we modified the NP system to utilise the short length of tubing further reduce perfusate volumes.
To ensure ease, affordability, and reproducibility of use the NP system was designed to allow recycling of 80% of its components (all except the oxygenator following manufacturers guidelines).
Experience with this system has highlighted that when using with human and non-human primate grafts a strategy must be in place to replenish the NP perfusate as there are losses in the duodenum during perfusion, this observation was not observed in the porcine models. A duodenal catheter should be placed to allow drainage and prevent pressure necrosis. The duodenal draining catheter prevents recirculation of concentrated effluent that contains concentrated proteolytic pancreatic enzymes.
Perfusate composition is key. Perfusate should promote cellular ionic balance, proper oncotic pressure, and nutrition to prevent oedema and support metabolism. This model uses autologous, leucocyte-depleted whole blood. Using autologous blood is an affordable source of an oxygen carrier that does not rely on precious blood bank resources. The perfusate solution also contains anticoagulant, heparin, a vasodilator, and antibiotic. Other pre-clinical studies have cited other perfusate compositions, to limit oedema, some groups have proposed adding different components to the perfusion solution. The addition of gelofusin [13], albumin [14] or STEEN solution [15] has been proposed. By using autologous whole blood we have maintained normal presumed oncotic pressure for the model.
Our perfusate composition solution nearly mimics the reperfusion period in transplantation to enable organ assessment, we are aware the ideal scenario would be transplantation, also it is not clear what the role of leucocytes are in an *ex-situ* reperfusion model but our suspicion is it might be injurious. This model is not yet robust for longer preservation (> 6 hours) or *ex-situ* reconditioning of pancreas grafts but provides a good foundation for these goals.
Some other NP models mention the use of a dialysis membrane in their experiences, something we did not include yet in our design. We agree that it appears essential for prolonged NP with the Toronto group, Mazilescu et al reporting an absence of oedema and ionic imbalance after 6 hours of NP by incorporating this [15]. This group notably reported the addition of aprotinin to their perfusate reduce the inflammatory response and reduce diffuse micro-thromboses [15].
A dialysis membrane is easily added to our system, we ensured to monitor serial electrolyte levels with an ionogram and correct when required.
40 mmHg is a low pressure suitable for the pancreas graft and found by us to be enough to provide a perfusion and not to be injurious to the microcirculation over a prolonged period of perfusion. Our observation is in keeping with the available studies that low perfusion pressures are associated with viable pancreas grafts during machine perfusion [10]. The ideal pressure level has not yet been established but a U.K group, Kumar et al. proposed an even lower perfusion pressure of 25 mmHG reporting excellent preservation of parenchyma while maintaining good blood flow [16].
This system uses continuous flow delivered by a centrifugal pump. The alternative system is pulsatile flow that claims to replicate physiological flow and may provide beneficial effects for the vasculature. Continuous flow is potentially less injurious to red cells leading to less hemolysis. Despite the benefits cited for pulsatile perfusion mostly in hypothermic perfusions [17], there is no definitive evidence to date supporting overall benefit of either.
In this model oxygen concentration delivered was 95% supplemented with 5% carbon dioxide.
This is a common concentration cited in other NP studies of both pancreas and other solid organs. This concentration corresponds to hyperoxia of the perfusate with blood gas analysis showing a to a partial pressure of oxygen at 650 mmHg. We have concerns for the implications of hyperbaric pressures in the pancreas tissue, whether is propagates the production of injurious free radicals during reperfusion plus other underappreciated insults, this is a research focus that is worth investigating.
Several parameters can be measured during NP to evaluate the pancreas graft. Vascular resistance index, arterial flow and tissue temperature are parameters we used and appear to be useful. We assessed degree of exocrine injury by measuring the levels of lipase and amylase in the perfusate and duodenal effluent. Duodenal effluent was also assessed by measuring volume, biochemistry and fluid appearance (whether it was blood stained). In this model it is possible to perform glucose-stimulated insulin secretion studies to assess endocrine function which we have done in earlier work [18].
In this paper we share our experience and design of a NP system that provides *ex-situ* reperfusion of pancreases and enables graft assessments.
We have evaluated this use of this system in porcine, non-human primate and human pancreas graft models and found it can support graft viability for 2 to 6 hours.
Our *ex-situ* normothermic perfusion system is an affordable, reproducible and easily adaptable design made to enable preclinical studies interested in *ex-situ* pancreatic graft research.

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Conflict of interest statement

The Authors declare no conflict of interest.

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Author contributions

BM: conceptualization, investigation, writing-original draft; DK: conceptualization; investigation, data curation, writing-original draft; TP: conceptualization, investigation, writing-original draft; IC: investigation and data curation SLB-B: methodology, project administration; SB: methodology, supervision; DM: data curation; JH: data curation; JR: supervision; LB: supervision; GB: supervision, project administration; LML: supervision, project administration; EO: conceptualization, investigation, supervision; JB: conceptualization, investigation, supervision, project administration

Ethical consideration

Porcine model: the study was approved by the French Research Ministry (APAFiS# 31507).
Primate non-human model: the study was approved by the French Research Ministry (APAFiS# 23409).
Human model: the study was approved by the French national agency - Agence de Biomédecine (PFS08-07).

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