**Use of *ex vivo* normothermic perfusion for quality assessment of discarded human donor pancreases**

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*Running title:* EVNP of discarded human pancreases

**Abbreviations**
ATP, adenosine triphosphate; DBD, donation after brain death; DCD, donation after cardiac death; EVNP, ex vivo normothermic perfusion

**Abstract**

A significant number of pancreases procured for transplantation are deemed unsuitable due to concerns about graft quality and the associated risk of complications. However, this decision is subjective and some declined grafts may be suitable for transplantation. *Ex vivo* normothermic perfusion prior to transplantation may allow a more objective assessment of graft quality and reduce discard rates. We report *ex vivo* normothermic perfusion of human pancreases procured but declined for transplantation, with ABO-compatible warm oxygenated packed red blood cells for 1-2 hours. Five declined human pancreases were assessed using this technique after a median cold ischemia time of 13h19mins. One pancreas, with cold ischemia over 30 hours did not appear viable and was excluded. In the remaining pancreases blood flow and pH were maintained throughout perfusion. Insulin secretion was observed in all four pancreases, but was lowest in an older donation after cardiac death pancreas. Amylase levels were highest in a gland with significant fat infiltration. This is the first study to assess the perfusion, injury, as measured by amylase, and exocrine function of human pancreases using EVNP and demonstrates the feasibility of the approach, although further refinements are required.

## Introduction

Pancreas transplantation is a potentially curative treatment for type I diabetes, but is severely limited by the lack of availability of suitable organs. Approximately 30% of pancreases procured from deceased donors in the United States (1) and 50% in the United Kingdom (UK) (2) are deemed unsuitable for transplantation and are discarded, most commonly because of concerns about major post-operative complications associated with use of suboptimal grafts. However, this decision is often subjective. In addition, a number of potentially suitable pancreases are never considered for transplantation as the donor does not fulfil agreed acceptance criteria, such as age.

Furthermore, despite advances in operative technique and post-operative management, pancreas transplantation remains a high-risk procedure with a complication rate of 25-50% (3-5) and a re-laparotomy rate of approximately 20% (6). Graft pancreatitis arising from ischemia reperfusion injury is a major contributing factor to many of these complications and may result in peri-pancreatic fluid collections (7), vascular thrombosis (8) and bleeding necessitating further surgery, in addition to the risk of graft loss or recipient death. Factors thought to increase the risk of graft pancreatitis include increased cold preservation time (9), donor obesity (8,10) and donor age (10,11).

Despite the significant adverse consequences of ischemia reperfusion injury in pancreas transplantation, there have been little or no advances in pancreas preservation techniques over recent years (12) and cold static storage remains the only technique in clinical use.

*Ex vivo* normothermic perfusion (EVNP) of organs prior to transplantation has the potential to improve preservation, abrogate the effects of ischemia reperfusion injury, facilitate the administration of therapeutic interventions and allow more objective selection of grafts for transplantation. It has already been introduced into clinical practice by members of our group for the preservation of kidneys prior to transplantation (13,14) and by others for lungs (15). EVNP has also been shown to be feasible for viability testing of discarded human donor livers (16) and kidneys (17). To date, the only reports of EVNP of pancreases have been restricted to experimental animal models (18-21). Here we report the first use of EVNP to assess the function and viability of discarded human pancreases.

## Methods

Five human pancreases procured for transplantation but deemed unsuitable for transplantation by all UK pancreas transplant centres were included in the study between February 2014 and November 2014. The study was approved by the local research ethics committee and NHS Blood and Transplant. Consent to use the pancreas for research was obtained from donor families.

*Procurement and back bench preparation*

The pancreases were all procured by one of the UK national organ procurement teams, using *in situ* perfusion with ice cold University of Wisconsin solution (CoStorSol®; Bridge To Life, London, UK). Organs were packed and stored in preservation fluid and transported on ice. On arrival at the study centre, back bench preparation was performed by an experienced member of the transplant team as for clinical transplantation. The spleen was removed and splenic vessels ligated; arterial reconstruction was performed with a donor iliac Y graft to the splenic and superior mesenteric arteries using 5-0 polypropylene sutures. An arterial cannula was secured in the common iliac portion of the Y graft. The small bowel mesentery was over-sewn and excess peri-pancreatic tissue removed. A wide-bore catheter was inserted into the distal duodenum and secured with a purse-string suture. The pancreas was weighed on completion of back-bench preparation. Prior to EVNP the pancreas was flushed with cold Gelofusine (B. Braun, Sheffield, UK) and any obvious leaking vessels ligated with absorbable ligatures. The portal vein was left to drain freely back into the reservoir.

*Perfusion circuit*

The perfusion circuit was developed by members of our group (MLN, SAH) for EVNP of the kidney, and is now in clinical use (13,14). It comprises customised paediatric cardiopulmonary bypass technology (Medtronic UK, Watford, UK) and is made up of an organ chamber, venous reservoir, Biopump 560 centrifugal blood pump, Affinity hollow fiber membrane oxygenator, ¼ inch PVC tubing and a Hirtz heat exchanger (Chalice Medical, Nottinghamshire, UK) (figure 1). The hardware includes a speed controller, TX50P flow transducer and a temperature probe (Cole-Palmer, London, UK). The system is pressure controlled, with variable flow due to auto-regulation by the organ. Pressure was set at 50-55 mmHg and temperature maintained at 37°C. A 95% $O_2$/5% $CO_2$ gas mix flowing at 0.1L/min was used to oxygenate the perfusion solution.

*Perfusion solution*

The perfusate was blood-based; donor ABO-compatible packed red cells were obtained from the hospital blood bank. This was diluted with a matched volume of Gelofusine (B. Braun, Sheffield, UK) to obtain a hematocrit of around 20%. 8.4% sodium bicarbonate was added to normalize pH. Other additives were 20 ml of 10% mannitol (Baxter Healthcare, Thetford, UK), 5 ml of 5% glucose (Baxter Healthcare) and 2,500 iu of heparin (CP Pharmaceuticals, Wrexham, UK).

*Outcome measures*

Prior to placing the organ on the circuit samples were taken for blood gas analysis (pH, $pCO_2$, $pO_2$, base excess, $tCO_2$, and $HCO_3$) and immediately analyzed (Opti CCA-TS Blood Gas System, Una Healthcare, Stoke on Trent, UK). Samples were also taken for biochemical assays (amylase, lipase, lactate dehydrogenase, insulin), placed in lithium heparin tubes on ice, centrifuged (10 minutes at 2000 rpm at 4°C) within 30 minutes of collection and stored at -80°C.

During EVNP, blood flow, pressure, pump speed and temperature were regularly recorded. For the first two pancreases, samples for blood gas analysis and biochemistry were taken at 30 minutes and then hourly. For the second two pancreases additional samples for biochemistry were taken every 5 minutes for the first 30 minutes of perfusion. At the end of perfusion insulin stimulation tests were performed. Firstly, 20mls of 5% glucose (Baxter Healthcare) was added as a bolus to the arterial limb of the circuit and samples taken for insulin at 1, 3 and 6 minutes following infusion. For the latter two pancreases, this was followed by a bolus of 250mg arginine (Sigma Aldrich, Gillingham, UK), with samples for insulin at 1, 3 and 6 minutes following infusion. At the end of perfusion the pancreas was weighed and the volume of duodenal drainage was also noted.

Insulin levels were measured using a one-step chemiluminescence immunoassay on a Diasorin Liaison® XL automated immunoassay analyzer (Diasorin S.p.A, Saluggia, Italy). Amylase and lipase levels were determined using a colorimetric method on a Siemens Dimension RXL autoanalyzer (Siemens Healthcare, Camberley, UK).

*Histopathology*

On completion of EVNP mesenteric fat, vessels, duodenum and spleen were dissected from the pancreas and the gland sectioned at 1cm intervals. Representative sections from the head, body and tail were formalin-fixed and paraffin-embedded. Slides were stained with haematoxylin and eosin and were assessed for both pre-existing pathological changes and features of acute ischaemic/preservation injury by an experienced histopathologist blinded to any donor details or outcome measures. None of the glands demonstrated any significant, pre-existing underlying pathology.

*Statistical analysis*

Differences in amylase, lipase and insulin levels were analyzed by one-way analysis of variance, with post-test analysis using Bonferroni's multiple comparison test as appropriate. All tests were two-tailed and $p<0.05$ was taken as significant. All analysis was performed using GraphPad Prism (GraphPad, La Jolla, CA).

**Results**

Five human pancreases procured but deemed unsuitable for transplantation underwent EVNP. Due to the logistics of transport and availability of study members to carry out EVNP, the final pancreas had accrued a cold ischemic time of over 30 hours by the time normothermic perfusion commenced. Macroscopically both the pancreas and particularly the duodenum appeared frankly ischaemic during EVNP. It was therefore felt that this pancreas would not provide meaningful data and was excluded from further analysis. The characteristics of the remaining four pancreases are shown in table 1.

*Perfusion parameters*

Macroscopically all pancreases appeared well perfused during EVNP (figure 2). Peristalsis was observed in the duodenum of the first graft. Pancreatic blood flow remained stable throughout perfusion with a mean arterial flow of 35±2.8 mls/min/100g (figure 3). Blood flow was highest in the pancreas from the 27-year old DBD donor (table 2). Perfusion of the fourth pancreas had to be terminated after 60 minutes because of low perfusate volumes in the circuit as a result of excessive drainage from the duodenal catheter.

*Biochemical parameters*

Perfusate amylase and lipase increased in all pancreases during the course of perfusion (figure 4 and 5). The increase in perfusate amylase was most marked in the pancreas with fatty infiltration from the 46-year old donor, although there were no statistically significant differences in mean perfusate amylase ($p=0.1011$) or mean perfusate lipase ($p=0.9088$) levels between pancreases.

There were significant differences in mean insulin levels between pancreases ($p=0.0199$) Basal perfusate insulin levels were highest in the pancreas from the 27-year old DBD donor, and this was the only pancreas in which insulin secretion was stimulated by a glucose challenge (figure 6). Although the pancreases from the 14-year old and 46-year old DBD donors did not respond to glucose stimulation, perfusate insulin levels did increase following an arginine challenge. The pancreas from the 51-year old DCD donor had the lowest basal and glucose-stimulated insulin levels, and mean insulin level was significantly lower than that from the 27-year old DBD pancreas (202±30 vs. 2815±839 pmol/L/100g, $p<0.05$).

The more in-depth time course of perfusate insulin performed in the second two pancreases demonstrated a peak in insulin levels immediately following perfusion, with stable levels following this in non-stimulated conditions (figure 7).

Arterial pH remained within normal limits during the course of perfusion without the need for administration of additional bicarbonate (figure 8).

*Histopathological analysis*

Histology from the pancreas from the 27-year old DBD donor post-EVNP showed only very focal acinar cell necrosis of the body and tail. The pancreas from the 14-year old DBD showed very focal to patchy acinar and fat necrosis distributed throughout the gland. The gland from the DCD donor demonstrated similar changes also throughout the gland. Finally, the pancreas from the 46-yr old donor showed more extensive parenchymal and fat necrosis throughout the gland (figure 9).

## Discussion

This is the first report of the use of *ex vivo* normothermic perfusion to assess the function and quality of the human pancreas prior to transplantation. This study demonstrates that the technique is technically feasible and that endocrine function is maintained during a short period of EVNP. The method allows assessment of blood flow, cellular injury, exocrine and endocrine function. The preparation of the pancreas described for EVNP would not compromise any implant procedure as the portal vein is not cannulated and the distal portion of the iliac Y graft used to secure the arterial cannula can be excised leaving sufficient length for anastomosis. The cannulated distal duodenum can also be excised.

At present no objective means to assess pancreas graft quality prior to transplantation is available. Axelrod *et al* have reported a pancreas donor risk index which utilizes 10 donor and one transplant characteristic to provide an estimated 1-year pancreas allograft survival (22). However, this tool is subject to the limitations of all registry analyses. It is derived predominantly from low-risk grafts and only provides a crude estimate of potential graft outcome with no reports of the predictive value of the model. It also does not differentiate between potential causes of graft loss. Other studies have reported the use of hypothermic machine perfusion to assess the viability of canine segmental pancreatic autografts. Raised perfusate amylase levels and lower oxygen extraction were shown to correlate with worse pancreas function post transplant (23). Hypothermic machine perfusion has been associated with edema and congestion, which increases the risk of early venous thrombosis and graft failure (24,25). As such, there is reluctance for its use in clinical pancreas transplantation. Tissue level of ATP at the end of cold preservation has also been shown to predict graft viability in canine segmental pancreatic autografts (26,27), although the complexity of the ATP assays reported limits the clinical utility of these findings.

Clearly it is inappropriate to analyze correlations between donor factors and outcome measures during EVNP with such a small sample size. However, there are some interesting observations that can be made from the outcome data. Subjectively, the lowest quality pancreas was the fatty gland from the 46-year old DBD donor. This pancreas had the highest perfusate amylase levels, a marker of cellular injury, and the lowest insulin levels, a marker of endocrine function. Furthermore, histology following EVNP demonstrated extensive fat and parenchymal necrosis consistent with severe ischemia reperfusion injury. Conversely, the pancreas with the highest blood flow and perfusate insulin levels, alongside the least severe histological changes, was from the young 27-year old DBD donor. Perhaps unexpectedly, the lowest perfusate amylase level was in the pancreas from a DCD donor. However, this pancreas was significantly fibrotic and this may have been associated with underlying acinar cell loss or dysfunction. It is also surprising that the pancreas from the 14-year old donor did not have better endocrine function. Interestingly, this donor was taking high-dose risperidone and such antipsychotics have been associated with the development of diabetes (28).

An initial peak in perfusate insulin was noted immediately after commencement of EVNP in the latter two pancreases. This may be the result of insulin diffusing out of β cells during cold storage, which is then flushed out of the gland on reperfusion. An insulin surge such as this immediately following reperfusion is seen as a marker of injury in clinical pancreas transplantation. As such, basal insulin levels are unlikely to be a reliable marker of pancreas viability during EVNP. Of more relevance is the insulin response to stimulation as this requires active secretion from β cells. It is interesting that two pancreases in this study did not respond to glucose stimulation, but did demonstrate insulin secretion in response to additional arginine administration, which is known to be a potent stimulant of insulin release in the presence of glucose (29). Glucose stimulated insulin secretion is mediated via membrane depolarization due to closure of $K^+$/ATP channels, whereas arginine depolarizes the cell by a different mechanism independent of ATP. Therefore, one explanation for the findings is that ATP levels are not sufficient for glucose to initiate adequate membrane depolarization for insulin secretion, and that the added depolarization of arginine is required. This warrants further investigation.

There are some concerns about the use of EVNP to assess pancreases prior to transplantation. Any organ quality assessment technique should not confer additional damage beyond that already sustained during procurement and cold storage. Active pancreatic

enzymes released from damaged acinar cells are not cleared and will recirculate in the perfusate, which may propagate additional pancreatic injury. This damage could potentially be limited by the addition of a protease inhibitor such as aprotonin to the perfusate. Moreover, much further work is required to determine the most relevant outcome measures. As ischemia reperfusion injury and graft pancreatitis, rather than endocrine function, are the clinically significant sequelae of using marginal pancreases this should arguably be the focus of further investigation. It is of note that this model utilized packed red cells rather than whole blood in the perfusate. Therefore many of the mediators of ischemia reperfusion injury, such as leukocytes and complement are absent, and the degree of injury seen during EVNP may underestimate that which would occur with clinical transplantation. However, it is clearly important that EVNP does not confer any additional injury to the gland; indeed, it would be ideal if the technique can be used for organ 'reconditioning' as well as quality assessment. In this context, comparison of markers of injury between pancreases perfused with whole blood or packed red cells may be informative.

Whether parameters during EVNP are reliable predictors that can discriminate suitable from unsuitable pancreas grafts will require study of a much larger cohort of organs, work that is ongoing in this unit. It is noteworthy that due to logistical constraints, all the pancreases had cold ischemic times longer than would be acceptable for clinical transplantation. Such durations of cold ischemia are known to potentiate ischemia reperfusion injury (9), and this may explain why a degree of necrosis was seen after EVNP in all four pancreases. It is not clear the degree to which this necrosis is due to cold ischemic damage or due to damage sustained during EVNP. Further studies of EVNP after shorter cold ischemia are required to corroborate this.

A significant amount of further work is required before EVNP of the pancreas can be moved into the clinical arena. The technique requires optimization to limit any additional injury during EVNP, outcome measures during EVNP need to be validated against clinical outcomes and the safety of EVNP of the pancreas prior to transplantation established. We are currently conducting a series of controlled experiments using EVNP of pig pancreases. Clinical translation of these findings will likely also require studies in large animals comparing pancreas transplant outcomes following EVNP versus conventional cold storage.

In summary, this is the first report to demonstrate that EVNP of discarded human donor pancreases is technically feasible and allows assessment of cellular injury and function. Nevertheless, significant refinements to the technique are required before it can be considered for clinical use. However, this has the potential to improve the selection of pancreases for transplantation, decrease the number of organs that are discarded and improve the availability of organs for transplantation. Through organ conditioning and the application of cellular and pharmacological interventions it also has the potential to help improve outcomes for pancreas transplant recipients.

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**Disclosure**

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

**Figure legends**

**Figure 1.** Schematic diagram of circuit used for ex vivo normothermic perfusion.

**Figure 2.** Pancreas 3 (14-year old DBD) following 30 minutes of EVNP. The duodenal contusion is visible. The cannulated iliac Y graft can be seen to the left and the duodenal catheter to the bottom right.

**Figure 3.** Pancreatic blood flow (mls/min/100g) during EVNP. Data are presented as mean ± SEM.

**Figure 4.** Individual perfusate amylase (U/L) measured from portal vein of pancreas during EVNP.

**Figure 5.** Individual perfusate lipase (U/L) measured from portal vein of pancreas during EVNP.

**Figure 6.** Individual perfusate insulin (pmol/L/100g) measured from portal vein of pancreas during EVNP under basal, glucose (1g) and arginine (250mg) stimulation.

**Figure 7.** Time-course of perfusate insulin levels (pmol/L/100g) measured from portal vein of pancreas 3 and pancreas 4 during EVNP.

**Figure 8.** Arterial pH during EVNP. Data are presented as mean ± SEM.

**Figure 9.** Hematoxylin and eosin stains of pancreas sections following EVNP, showing varying degrees of damage. (A) Pancreas 1: 27yr old DBD (very focal acinar cell necrosis); (B) Pancreas 2: 51yr old DCD (very focal to patchy acinar cell and fat necrosis); (C) Pancreas 3: 14yr old DBD (very focal to patchy acinar cell and fat necrosis); (D) Pancreas 4: 46yr old DBD (extensive parenchymal and fat necrosis).

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| | Pancreas 1 | Pancreas 2 | Pancreas 3 | Pancreas 4 |
|---|---|---|---|---|
| **Donor type** | DBD | DCD | DBD | DBD |
| **Age (years)** | 27 | 51 | 14 | 46 |
| **Sex** | Female | Female | Male | Male |
| **Height (cm)** | 172 | 164 | 184 | 175 |
| **Weight (kg)** | 60 | 63 | 65 | 95 |
| **BMI** | 20.3 | 23.4 | 19.2 | 31.0 |
| **Glucose (mmol/L)** | 8.4 | 4.9 | 8.5 | 8 |
| **Amylase (iU/L)** | 40 | 322 | 147 | 104 |
| **Cause of death** | Hypoxic brain damage | Intra-cerebral hemorrhage | Traumatic brain injury | ICH; grade 4 glioblastoma |
| **Length of hospital stay prior to procurement (hrs)** | 47 | 44 | 79 | 22 |
| **Smoker** | Yes | Yes | Yes | No |
| **Excess alcohol use** | No | Yes | No | No |
| **Family history of diabetes** | No | Yes | No | No |
| **Reason not transplanted** | Parenchymal injury | Pancreas fibrotic | Duodenal contusion | Fatty infiltration and calcification |
| **Time between withdrawal of treatment and cardiac death (mins)** | N/A | 24 | N/A | N/A |
| **Time between cardiac death and cold perfusion (mins)** | 0 | 14 | 0 | 0 |
| **Cold Ischemic time** | 16 hrs 35 mins | 18 hrs 34 mins | 13 hrs 23 mins | 13 hrs 14 mins |
| **Preservation solution for initial in-situ cold perfusion** | University of Wisconsin | University of Wisconsin | University of Wisconsin | University of Wisconsin |
| **EVNP duration (mins)** | 120 | 120 | 120 | 60 |

**Table 1.** Donor characteristics of discarded human pancreases undergoing EVNP. DBD, donation after brain death; DCD, donation after cardiac death.

| | **Pancreas 1** | **Pancreas 2** | **Pancreas 3** | **Pancreas 4** |
|---|---|---|---|---|
| Mean bloodflow (mls/min/100g) | 50 ±6.3 | 32 ±2.6 | 28 ±2.4 | 33 ±3.5 |
| Mean perfusate amylase (iU/L) | 582 ±127.2 | 185 ±52.2 | 198 ±31.1 | 1525 ±457 |
| AUC perfusate amylase (iU/L) | 1748 | 455 | 844 | 4680 |
| Mean perfusate lipase (iU/L) | 6352 ±2092 | 2168 ±704.5 | 6199 ±645.5 | 7960 |
| AUC perfusate lipase (iU/L) | 8490 | 2908 | 8981 | |
| Mean pH | No data | 7.36 ±0.04 | 7.45 ±0.02 | 7.31 ±0.11 |
| Perfusate insulin/100g (pmol/L)<br>*Basal*<br>*Glucose-stimulated*<br>*Arginine-stimulated* | <br>1976<br>3654<br>No data | <br>172<br>232<br>No data | <br>1029<br>940<br>1777 | <br>892<br>750<br>1306 |
| Duodenal drainage (ml) | 80 | 85 | 100 | 390 |

**Table 2.** Outcome measures during EVNP of discarded human pancreases. Data are presented as mean±SEM unless otherwise indicated.

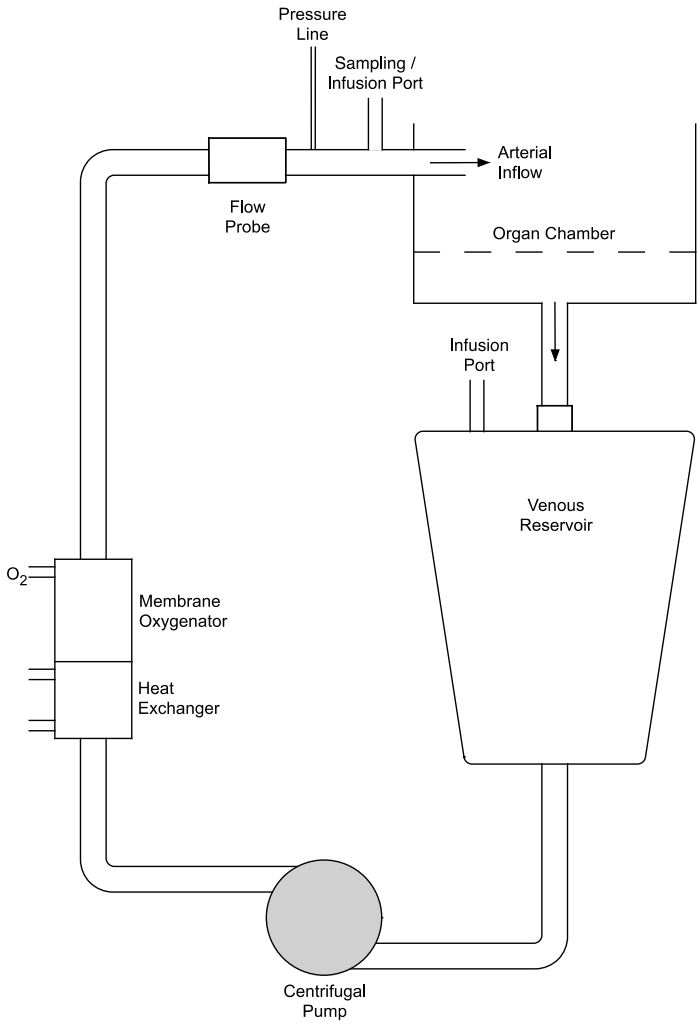
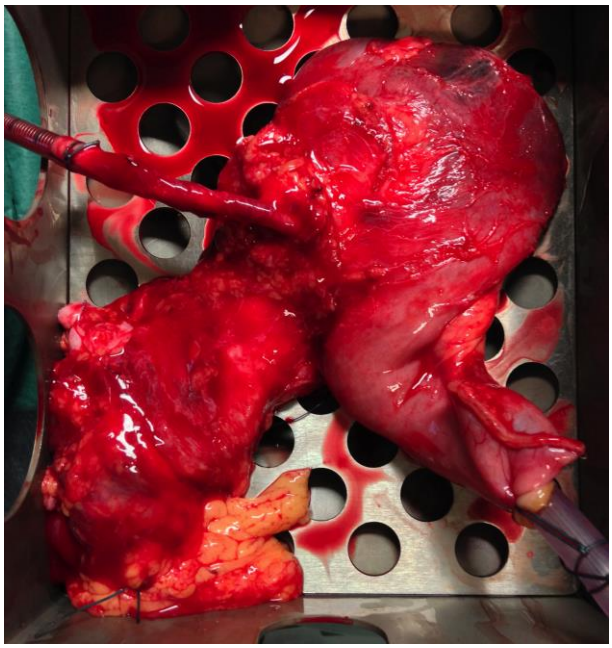



**Figure 1.** Schematic diagram of circuit used for ex vivo normothermic perfusion.



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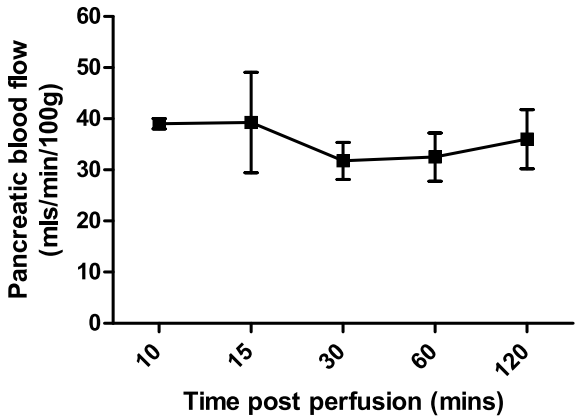


**Figure 3.** Pancreatic blood flow (mls/min/100g) during EVNP. Data are presented as mean ± SEM.

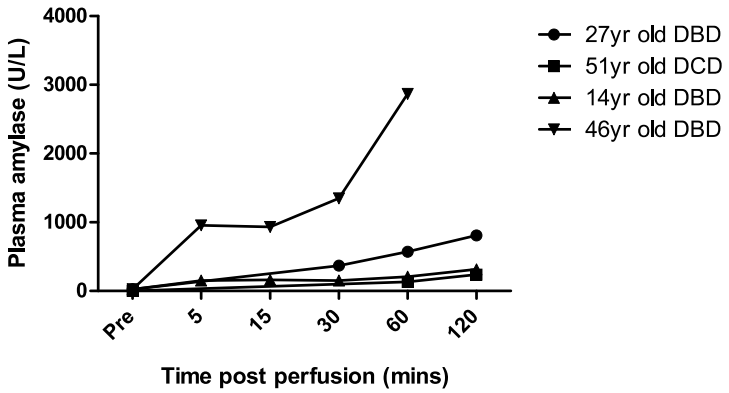


**Figure 4.** Individual perfusate amylase (U/L) measured from portal vein of pancreas during EVNP.

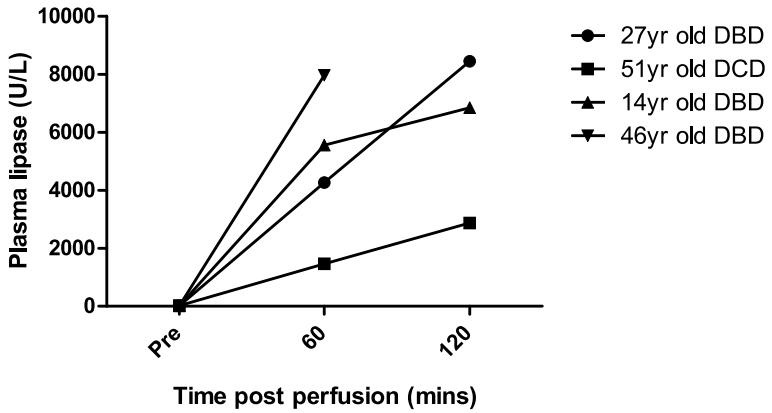


**Figure 5.** Individual perfusate lipase (U/L) measured from portal vein of pancreas during EVNP.

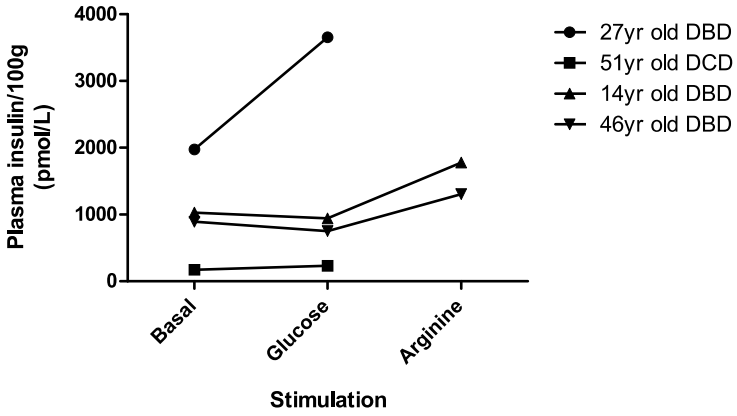


**Figure 6.** Individual perfusate insulin (pmol/L/100g) measured from portal vein of pancreas during EVNP under basal, glucose (1g) and arginine (250mg) stimulation.

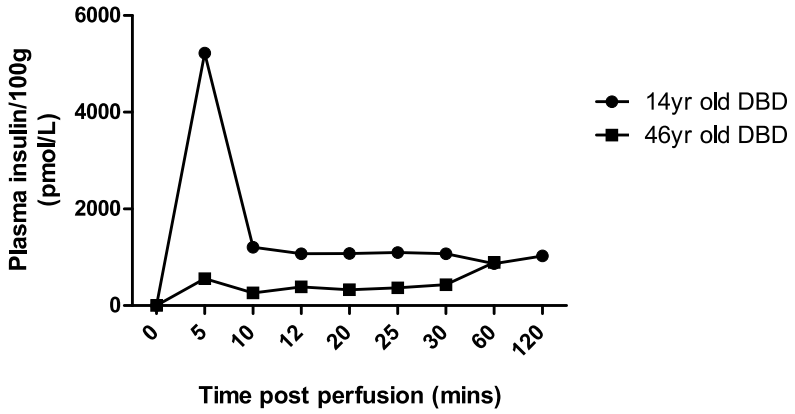


**Figure 7.** Time-course of perfusate insulin levels (pmol/L/100g) measured from portal vein of pancreas 3 and pancreas 4 during EVNP.

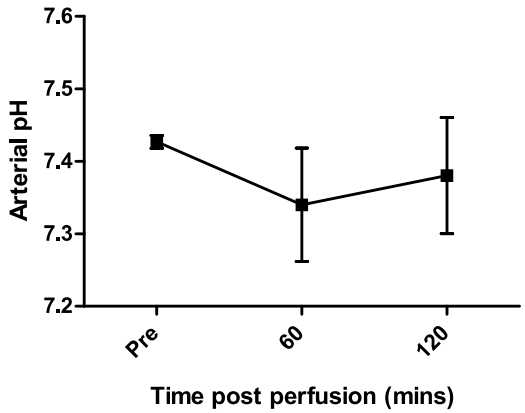


**Figure 8.** Arterial pH during EVNP. Data are presented as mean ± SEM.

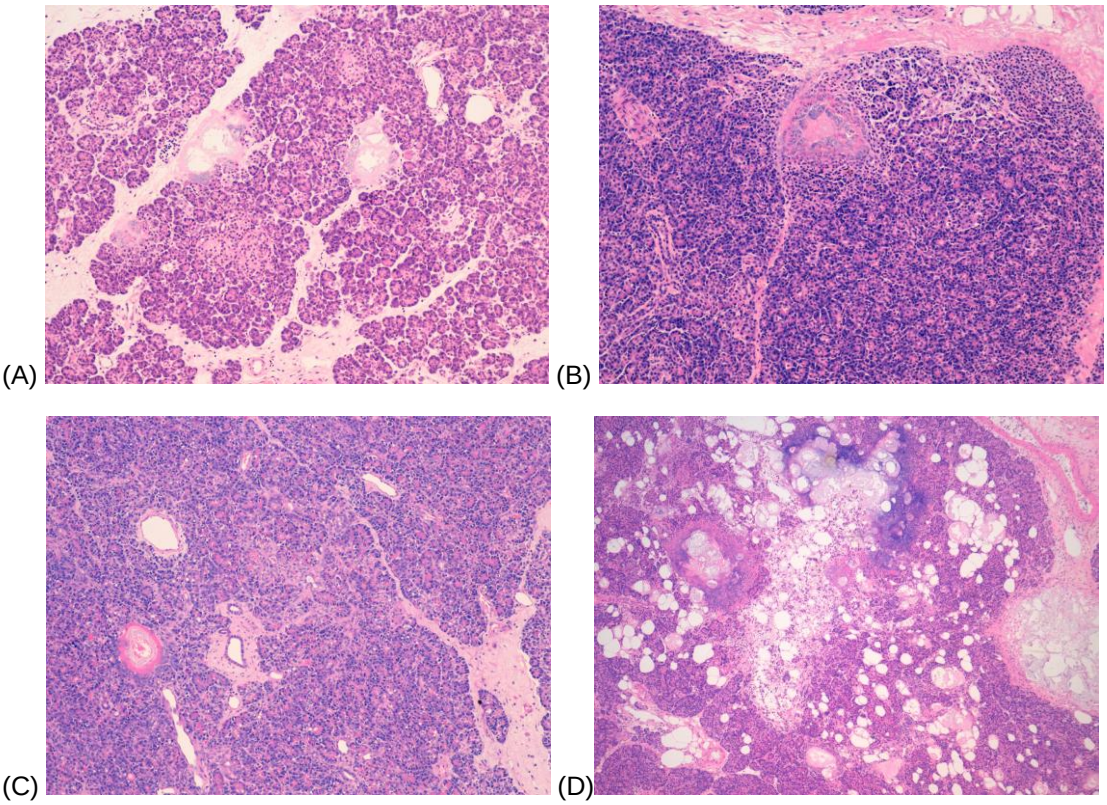


**Figure 9.** Hematoxylin and eosin stains of pancreas sections following EVNP, showing varying degrees of damage. (A) Pancreas 1: 27yr old DBD (very focal acinar cell necrosis); (B) Pancreas 2: 51yr old DCD (very focal to patchy acinar cell and fat necrosis); (C) Pancreas 3: 14yr old DBD (very focal to patchy acinar cell and fat necrosis); (D) Pancreas 4: 46yr old DBD (extensive parenchymal and fat necrosis).