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# Comparison of “Continuous” and “End-ischemic” Approaches of Oxygenated Hypothermic Machine Perfusion for Pancreas Preservation

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**Background.** Pancreas transplantation can successfully restore physiological insulin production, but it comes with a potentially significant morbidity burden. Pancreas grafts are extremely vulnerable to ischemia/reperfusion injury. Investigating technologies that aim to mitigate ischemia/reperfusion injury, such as oxygenated hypothermic machine perfusion (HMPO<sub>2</sub>) for pancreas preservation and their modes of application, is crucial to inform clinical translation. **Methods.** A circulatory death porcine model was used to compare different HMPO<sub>2</sub> modes for pancreas preservation. Eighteen porcine pancreases were allocated to 3 experimental groups: static cold storage (SCS), continuous HMPO<sub>2</sub>, and end-ischemic HMPO<sub>2</sub>. Normothermic reperfusion (NR) was used for assessment. **Results.** The ischemic times and wet:dry ratios were comparable among the 3 groups. Perfusate flow increased throughout NR and was highest at the end of NR across all groups, with no significant difference between the groups. Amylase, lipase, lactate dehydrogenase, and cell-free DNA showed no significant differences in the pattern of change among the groups. Red blood cells were present consistently in vessels and islet capillaries at the end of NR in all pancreases. The continuous HMPO<sub>2</sub> group showed significantly higher biphasic insulin secretion in response to glucose stimulation ( $P = 0.01$ ). **Conclusions.** Continuous HMPO<sub>2</sub> is a superior method for preserving beta-cell function compared with SCS and end-ischemic HMPO<sub>2</sub> in a porcine model of circulatory death when assessed by NR with whole blood as a surrogate for transplantation. As end-ischemic HMPO<sub>2</sub> is also feasible and potentially superior to SCS, testing its safety would be an excellent first step in translating this technology into clinical practice.

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## INTRODUCTION

### Diabetes Mellitus and Pancreas Transplantation

Diabetes mellitus significantly reduces both quality of life and life expectancy. The current standard of care does not prevent long-term complications in many patients, including nephropathy, neuropathy, limb loss, and cardiovascular disease.<sup>1</sup> The occurrence and progression of long-term secondary complications of diabetes correlate with the quality of blood glucose control.<sup>2</sup> Pancreas transplantation is the only method approved for clinical practice, which can consistently reestablish physiological insulin production.<sup>3</sup>

Despite substantial improvements in pancreas graft and recipient outcomes, the proportion of pancreases retrieved that result in a transplant, termed “utilization,” is the lowest among solid abdominal organs. In the United Kingdom, >50% of pancreases retrieved for the purpose of transplantation are not implanted.<sup>4</sup> The reason for this is primarily clinician uncertainty about organ quality and concern over potential postoperative complications, namely graft pancreatitis.

### Pancreas Vulnerability During Preservation

The pancreas is extremely vulnerable to ischemia/reperfusion injury (IRI), which is a key factor in the pathophysiology of early posttransplant morbidity and mortality, particularly graft pancreatitis. This has important clinical implications, such as reoperation after pancreas transplant, which occurs in about 20%–25% of patients, and pancreatitis remains 1 of the main causes of early graft loss.<sup>5,6</sup>

The current standard for storage and transport of donor pancreas grafts is static cold storage (SCS). During SCS, ATP is depleted, and the toxic byproducts of anaerobic respiration, such as succinate and lactate, accumulate in the tissue. When the pancreas is transplanted and reperfused with recipient blood, IRI occurs, accompanied by free radical production, and the severity of this cold ischemic injury is unmasked.<sup>7</sup>

### Oxygenated Hypothermic Machine Perfusion for Organ Preservation

Oxygenated hypothermic machine perfusion (HMPO<sub>2</sub>) has been successfully used to mitigate the effects of IRI in clinical liver<sup>8</sup> and kidney<sup>9</sup> transplantation. HMPO<sub>2</sub> delivers oxygenated perfusion solution through the organ under hypothermic conditions (<12 °C). This combines the safety of hypothermia with the benefits of dynamic machine perfusion, delivering oxygen to support mitochondrial function, reducing IRI by flushing out toxic metabolic byproducts, and avoiding the accumulation of mitochondrial succinate and subsequent production of reactive oxygen species.<sup>7</sup>

Clinically, HMPO<sub>2</sub> for the pancreas is due to be assessed in a safety and feasibility clinical trial, which is yet to start recruitment.<sup>10</sup> It has been investigated in various preclinical models of porcine, nonhuman primate, and human pancreas grafts and assessed using multiple perfusion parameters, perfusate composition, and methods of oxygenation, showing feasibility and potential benefit.<sup>11–17</sup> There is no consensus on the mode required for improved pancreas preservation, whether from the start of hypothermic preservation, the continuous mode, which has shown benefit in kidney preservation<sup>9,18</sup> or if it is sufficient to oxygenate the pancreas for a period after a small number of hours in SCS before transplantation, the end-ischemic mode, which has shown benefit in liver preservation.<sup>8</sup>

### Aim and Research Questions

Our aim was to assess and compare different modes of preservation for porcine pancreas grafts obtained after circulatory death using HMPO<sub>2</sub>. Specifically, to answer the following questions:

1. Is HMPO<sub>2</sub> superior to SCS in the preservation of pancreases?
2. Is there a benefit from starting the HMPO<sub>2</sub> of the pancreas at retrieval (continuous HMPO<sub>2</sub>) versus after transport in SCS to the implant site (end-ischemic HMPO<sub>2</sub>)?

### MATERIALS AND METHODS

Porcine pancreases (n = 18) were retrieved from domestic pigs with a weight between 45 and 72 kg at a UK abattoir. Animal death was complied with the Welfare of Animals at the Time of Killing (England) Regulations 2015 and the retained EU Regulation 1099/2009; therefore, no further ethical approval or license was required to perform this research.

### Procurement and Surgical Preparation

After regulated stunning and exsanguination, 1 L of whole blood was collected and anticoagulated with 10 000 IU of unfractionated heparin sodium (1000 units per 1 mL, Wockhardt UK Ltd). The surgical technique of pancreas retrieval previously published by our group was performed, using 1 L of University of Wisconsin Cold Storage Solution (Carnamedica, Poland).<sup>14,19</sup> Refinements to the retrieval technique included (1) cannulating the superior mesenteric artery and celiac axis, instead of an aortic tube, using 10 Fr cannulas for organ perfusion (Carnamedica, Poland) and (2) not using a stapling device but applying a vascular clamp to the root of mesentery and controlling it with a running 0 PDS Plus (Johnson & Johnson Medical N.V., Belgium) suture.

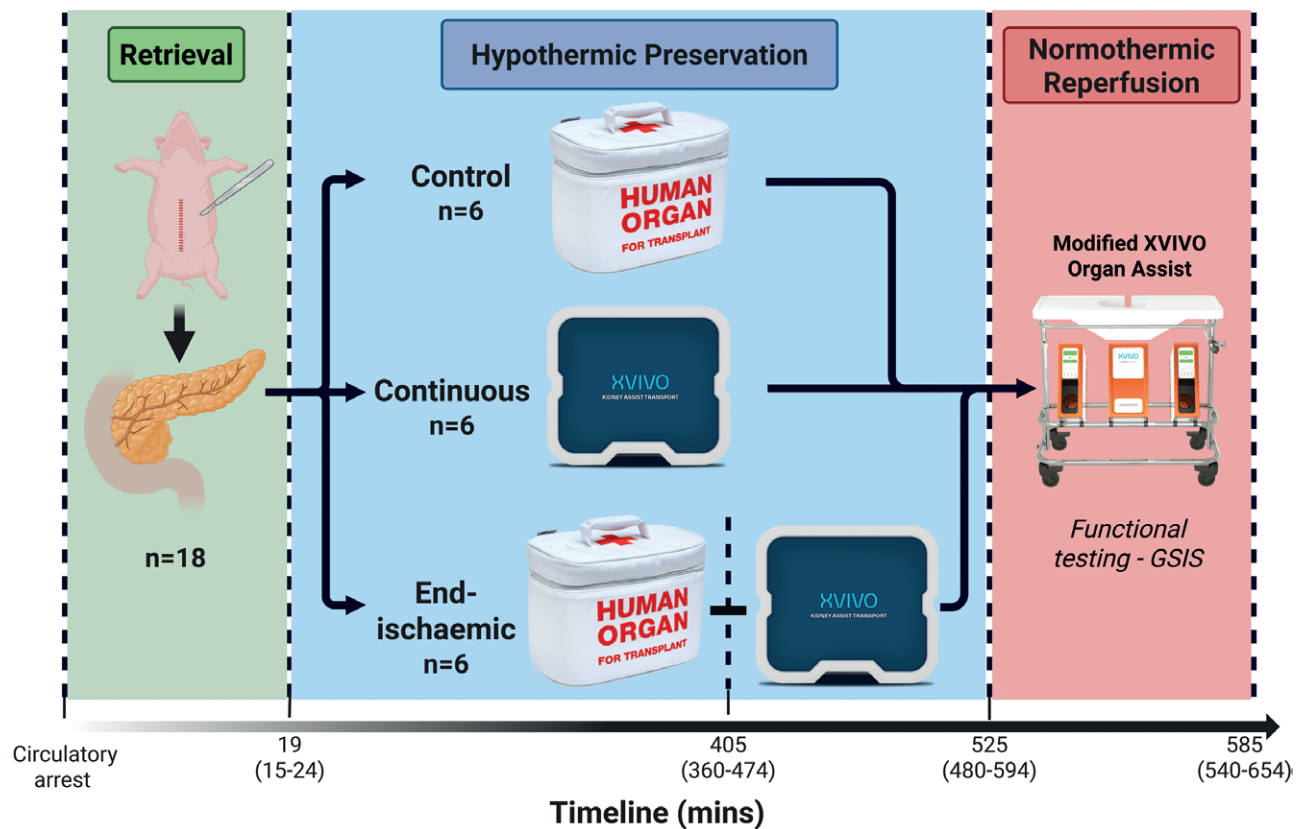
After surgical preparation, 6 pancreases were allocated to each of the 3 experimental groups for 8 h of hypothermic preservation:

1. Control group: SCS for the totality of the hypothermic preservation time.
2. Continuous group: HMPO<sub>2</sub> for the totality of the hypothermic preservation time.
3. End-ischemic group: SCS followed by HMPO<sub>2</sub> for the last 2 h of the hypothermic preservation time.

After hypothermic preservation, all 18 pancreases underwent normothermic reperfusion (NR) with autologous whole blood for 60 min (Figure 1).

### Oxygenated Hypothermic Machine Perfusion

Pressure-controlled pulsatile perfusion was delivered via the superior mesenteric artery and celiac axis cannulas at a set systolic pressure of 25 mm Hg with a temperature range of 4–8 °C on a modified XVIVO Kidney Assist Transporter device using University of Wisconsin Machine Perfusion Solution (Carnamedica, Poland). This device was selected to accommodate modifications designed to suit the pancreas and to optimally deliver oxygen. These include an in-house 3D-printed organ chamber and a polymethylpentene membrane oxygenator (MEDOS HILITE 2800 LT) with oxygen delivered via a medical-grade oxygen cylinder (BOC, Linde Group, Surrey, United Kingdom) at a flow rate of 0.5 L/min. This resulted



**FIGURE 1.** Flowchart of experimental design. The 3 groups in the study are depicted with the duration of each preservation period. The control (SCS) group was preserved in SCS for the entire hypothermic preservation time. Continuous HMPO<sub>2</sub> group was preserved using HMPO<sub>2</sub> for the entire hypothermic preservation period. End-ischaemic HMPO<sub>2</sub> group was preserved using SCS, then HMPO<sub>2</sub> for the last 2 h of hypothermic preservation. Each group then underwent 60 min of normothermic reperfusion, including functional testing with glucose-stimulated insulin secretion (GSIS). HMPO<sub>2</sub>, oxygenated hypothermic machine perfusion; SCS, static cold storage.

in a perfusate partial pressure of oxygen (pO<sub>2</sub>) ≥150 mmHg (~20 kPa). The portal vein drained passively into the reservoir at the bottom of the organ chamber, and the perfusate was then recirculated.

### Normothermic Reperfusion

As a surrogate for transplantation, we used autologous heparinized whole blood to perfuse pancreases after the hypothermic preservation phase. NR was set up using a modified XVIVO Kidney Assist device, based on our previous work.<sup>14</sup> The modifications included using the same 3D-printed organ chamber used for HMPO<sub>2</sub>, adding a bypass, and a flow aim of 100 mL/min with a maximum pressure setting of 60 mmHg for 1 h.

The NR circuit was primed with 900 mL of heparinized whole blood with verapamil and 0.9% saline to achieve a hematocrit of 0.35. Oxygenation with 21% oxygen, 5% carbon dioxide, and 74% nitrogen at a flow rate of 0.5 L/min was delivered to the polymethylpentene membrane oxygenator (MEDOS HILITE 2800 LT), which is integrated with a bubble trap to enable gas exchange and provides the surface area to keep the perfusate at 37 °C via connection to the heat exchanger unit of the device.

### Sample Collection and Analyses

#### Perfusate

Perfusate samples were collected at baseline, at the end of hypothermic preservation, and end of NR. During NR, the

collected perfusate samples were centrifuged at 1800g, to separate the red blood cell (RBC) component, and the supernatant was sampled. Perfusate samples were stored at -80 °C, then thawed and analyzed as a batch to investigate markers of tissue damage. Amylase, lipase, and lactate dehydrogenase (LDH) were analyzed in the ARCHITECT c Systems (Abbott GmbH & Co. KG, Germany) using the following inserts: Amylase2 (Abbott Ireland, Ireland), lactate dehydrogenase2 (Abbott Ireland, Ireland), and lipase (Abbott GmbH & Co. KG, Germany). Circulating cell-free DNA (cfDNA) was isolated using the QIAamp Circulating Nucleic Acid kit (Qiagen, Venlo, Limburg, Netherlands) following manufacturer's instructions. The isolated cfDNA was then quantified by the Circulating DNA Quantification Kit (Abcam Ltd, Cambridge, United Kingdom) following manufacturer's instructions.

#### Biopsies

Wedge tissue samples were removed at baseline, at the end of hypothermic preservation, and at the end of NR. Tissue samples were used fresh to determine wet:dry weight ratio, which is a measure of tissue fluid content to indirectly assess parenchymal edema, as described in.<sup>14</sup> Wedge tissue samples for histology were removed at the same timepoints, fixed in 10% formal saline, and stored at 4 °C before wax embedding. 5-µm sections were stained using hematoxylin and eosin to assess histological appearance, tissue architecture, and the presence of RBCs in vasculature by 2 blinded assessors. Insulin retention in islet beta cells was determined by immunolabeling

for insulin using an “in-house” anti-insulin antibody raised in a guinea pig, fully cross-reactive with porcine insulin, previously validated and used by our group<sup>20,21</sup> and visualized with peroxidase/3,3'-diaminobenzidine. To characterize other islet hormones, sections were simultaneously immunolabeled for insulin (antibody as above), glucagon (mouse anti-glucagon, SIGMA), and somatostatin (rabbit anti-somatostatin, DAKO, Agilent, United Kingdom) and fluorescently-labeled secondary antibodies: anti-guinea pig 633, anti-mouse 568, and anti-rabbit 633 (Invitrogen, MA). These labeled slides were examined with a Radiance 2000 confocal microscope (Bio-Rad, CA) to illustrate porcine islet structure.

### Glucose-stimulated Insulin Secretion

Perfusate samples were taken, processed, and stored as described above. A 20mM glucose bolus was administered on the arterial side of the circuit 30 min after the start of NR. Baseline samples were collected at 5 min, 3 min, and immediately before administering the bolus. Samples were then taken at 1-min intervals for the first 5 min after bolus administration, then at 5-min intervals for the following 15 min. The venous effluent was collected and sampled or discarded for the 20 min after bolus administration, and therefore not recirculated. Insulin secretion was measured using ELISA for human insulin (Merckodia, Uppsala, Sweden;  $n = 3$  in each experimental group), following manufacturer's instructions. Glucose was measured using a Bayer Contour Next device (Bayer, Germany).

### Statistical Analysis Plan

Data are expressed as mean  $\pm$  SEM unless otherwise specified. Mixed-effects analysis with a Geisser-Greenhouse correction was used to determine differences between the groups for reporting, amylase, lipase, and lactate dehydrogenase to handle a maximum of 2 missing values per outcome. One-way ANOVA was used to determine the difference between wet:dry ratios normalized to baseline of each experiment. Mixed-effects analysis with the Geisser-Greenhouse correction was used to compare perfusate flow during NR. Two-way ANOVA with a Geisser-Greenhouse correction and a post hoc Tukey test was used to determine differences between the groups for cfDNA values normalized to the baseline of each experiment and for insulin secretion values during glucose-stimulated insulin secretion (GSIS) normalized to the mean of the 3 basal measurements (minutes  $-5$ ,  $-3$ , and 0) for each experiment. All statistical tests were performed using Prism version 10.2.1 (GraphPad, CA).

## RESULTS

### Perfusion Parameters

Median warm ischemia time (WIT), defined as the time between the start of exsanguination and the time of cold perfusion with University of Wisconsin Cold Storage Solution, was 19 min in all 3 groups. WIT ranged between 15 and 22 min in the continuous group, 18 and 20 min in the end-ischemic group, and 15 and 24 min in the SCS group. The median (range) hypothermic preservation time was 493 (480–532) min in the continuous group, 532.5 (505–570) min in the end-ischemic group, and 543 (490–594) min in the SCS group (Kruskal-Wallis,  $P = 0.09$ ; illustrated in Table 1).

**TABLE 1.**  
WIT and HP times

Experimental group	WIT	HP
Continuous HMPO <sub>2</sub> (n = 6)	19 (15–22)	493 (480–532)
End-ischemic HMPO <sub>2</sub> (n = 6)	19 (18–20)	532.5 (505–570)
Control (SCS) (n = 6)	19 (15–24)	543 (490–594)

WIT during retrieval and HP in the 3 experimental groups. Illustrated as median and range in minutes.

HP, hypothermic preservation; SCS, static cold storage; WIT, warm ischemia time.

During continuous HMPO<sub>2</sub> arterial perfusate flow gradually increased through perfusion from a mean of 10.59 mL/min/100 g of graft weight to a mean of 15.23 mL/min/100 g of graft weight (Figure 2A), but not during the shorter HMPO<sub>2</sub> in the end-ischemic group which started with a mean of 7.26 mL/min/100 g of graft weight and ended with a mean of 7.33 mL/min/100 g of graft weight. The perfusate flow at the end of NR across all groups was higher compared with the start of NR (mixed-effects analysis,  $P < 0.01$ ), but there was no significant difference between the groups ( $P = 0.19$ ; Figure 2B).

### Markers of Edema and Damage

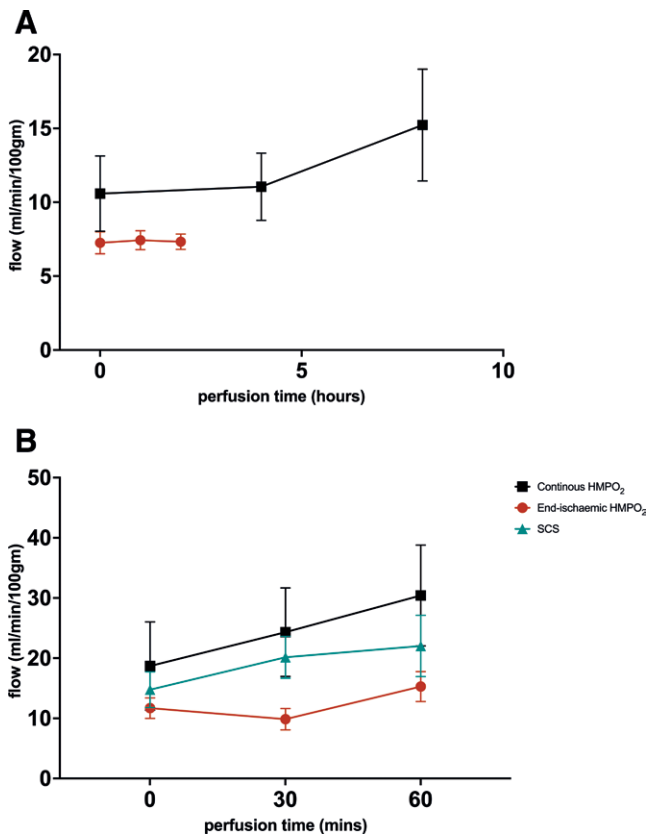
Amylase and lipase were not significantly different between the groups (mixed-effects analysis,  $P = 0.47$  and  $P = 0.7$ , respectively; Figure 3A and B). All pancreases showed a statistically significant increase in these markers by the end of NR compared with baseline (mixed-effects analysis,  $P = 0.0011$ ,  $0.0017$ , and  $0.0006$ , respectively), peaking at mid NR in the SCS group (Figure 3A and B). Lactate dehydrogenase (LDH) showed significantly different overall levels between the groups (mixed-effects analysis,  $P = 0.03$ ) and change over time ( $P = 0.0006$ ), but no significant differences were noted in the pattern of change, that is, increase, among the 3 groups ( $P = 0.13$ ; Figure 3C).

The levels of circulating cfDNA (Figure 4) were significantly different between individual pancreases (2-way ANOVA,  $P = 0.008$ ), but no significance was noted in the mean differences between groups, over time, or in the pattern of change between the 3 groups (2-way ANOVA,  $P = 0.24$ ,  $0.27$ , and  $0.08$ , respectively). On multiple comparisons, there was a statistically significant decrease between start and end of cold preservation in the continuous HMPO<sub>2</sub> group (Tukey's post hoc,  $P = 0.006$ ) and start of cold preservation and end of NR in the SCS group (Tukey's post hoc,  $P = 0.0003$ ).

There were no significant differences in parenchymal edema between the 3 groups as assessed by tissue wet:dry ratio (1-way ANOVA,  $P = 0.09$ ). All groups demonstrated a gradual increase in parenchymal edema during the 8 h of hypothermic preservation and 60 min of NR.

### Histological Assessment

Histological assessment of hematoxylin and eosin-stained sections from all pancreases by 2 blinded assessors (Table 2) showed no RBCs in vessels or capillaries at the start of hypothermic preservation in all pancreases (Figure 5A). RBCs were present consistently in vessels and islet capillaries at the end of NR in all 3 groups (Figure 5B). All pancreases showed autolysis and exocrine damage at the end of NR, which was graded as more severe in the SCS group (Figure 5C).

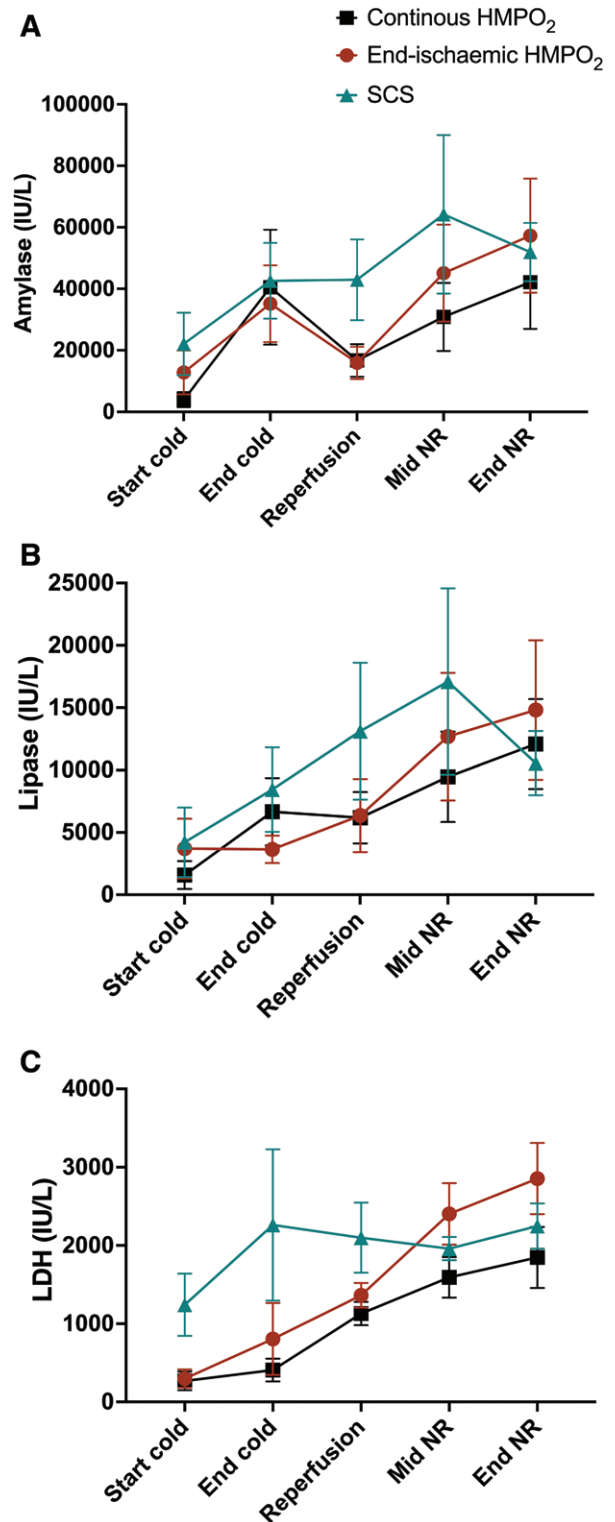


**FIGURE 2.** Arterial perfusate flow. A, Arterial perfusate flow (mL/min/100g of graft weight) during continuous HMPO<sub>2</sub> and end-ischaemic HMPO<sub>2</sub>. B, Arterial perfusate flow (mL/min/100g of graft weight) at the start, midpoint, and endpoint of NR. Values displayed as mean  $\pm$  SEM. There was a significant increase in flow during NR in all 3 groups over time (mixed-effects analysis,  $P < 0.01$ ), with no significant difference between groups. HMPO<sub>2</sub>, oxygenated hypothermic machine perfusion; NR, normothermic reperfusion; SCS, static cold storage.

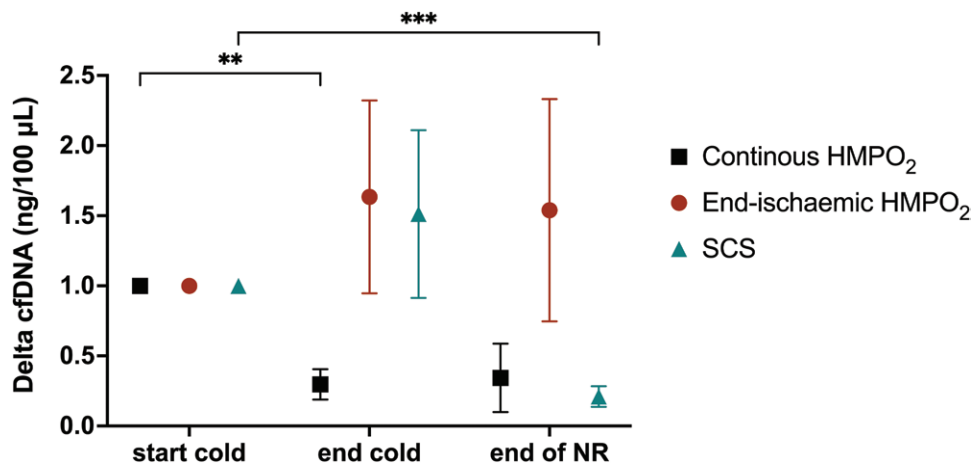
Immunolabeling for insulin demonstrated the presence of large islets and many smaller clusters of beta cells in all groups (Figure 5D–F). Simultaneous labeling of insulin, glucagon, and somatostatin using fluorescence microscopy (Figure 5G) demonstrated preservation of all islet-cell hormone content with no cross-reactivity.

### Functional Assessment

Portal venous effluent samples showed a similarly sharp and sustained rise in glucose levels across all pancreases, ascertaining the comparability of all experiments (Figure 6A). The contemporaneous insulin levels in the effluent demonstrated typical pancreatic islet insulin secretion as a biphasic pattern only in the continuous HMPO<sub>2</sub> group and were significantly higher than in the end-ischaemic HMPO<sub>2</sub> group (2-way ANOVA, Tukey's test post hoc,  $P < 0.02$ ; Figure 6B). Insulin secretion was highly variable and not synchronized to glucose levels in either the SCS or end-ischaemic group (Figure 6B). The initial phase refers to the peak 1 min after glucose administration, and the second phase, sustained insulin secretion, lasted from 5 to 20 min in response to sustained glucose stimulation. Overall, continuous HMPO<sub>2</sub> promoted significantly higher cumulative insulin secretion than end-ischaemic HMPO<sub>2</sub> in the initial phase (unpaired  $t$  test,  $P = 0.01$ ).



**FIGURE 3.** Analysis of biochemical injury markers, amylase (A), lipase (B), and LDH (C), in each group, measured at the start of cold preservation, end of cold preservation, then at start, midpoint, and end of NR. The 3 markers increased during perfusion, and there was a statistically significant difference between the 3 groups for LDH but not for amylase or lipase (mixed-effects analysis with a Geisser-Greenhouse correction,  $P = 0.03$ ,  $P = 0.47$ , and  $P = 0.7$ , respectively). All values are displayed as mean  $\pm$  SEM. “Start cold” is the start of cold preservation and “end cold” is the end of cold preservation. HMPO<sub>2</sub>, oxygenated hypothermic machine perfusion; LDH, lactate dehydrogenase; NR, normothermic reperfusion; SCS, static cold storage.



**FIGURE 4.** cfDNA levels in perfusate samples. cfDNA levels in each group measured at the start of cold preservation, end of cold preservation, and then end of NR. The change (delta) in cfDNA is presented as mean  $\pm$  SEM for each group and has been normalized to the baseline value of each pancreas. \*Levels of cfDNA were significantly different among individual pancreases (2-way ANOVA,  $P = 0.008$ ). Multiple comparisons with post hoc correction by the Tukey test demonstrated a statistically significant decrease between start and end of cold preservation in the continuous HMPO<sub>2</sub> group (\*\* $P = 0.006$ ) and a statistically significant decrease between start of cold preservation and end of NR in the SCS group (\*\* $P = 0.0003$ ). cfDNA, cell-free DNA; HMPO<sub>2</sub>, oxygenated hypothermic machine perfusion; NR, normothermic reperfusion; SCS, static cold storage.

**TABLE 2.**

#### Histological assessment

Experimental group	Timepoint	RBCs in capillaries	RBCs in arterioles/venules	Necrosis	Architecture
Continuous HMPO <sub>2</sub>	Start cold	1	0	0.5	3
	End cold	0.5	0	1	2
	End NR	2	3	3	1
End-ischaemic HMPO <sub>2</sub>	Start cold	0.5	0	0	3
	End cold	0	0	2	1.5
	End NR	3	2	2	1
SCS	Start cold	0	0	1	2
	End cold	0.5	0	1	2
	End NR	3	3	3	0.5

Scores of hematoxylin and eosin–stained biopsies were taken at baseline, end of hypothermic preservation, and end of normothermic reperfusion. Each pancreas was scored 0 (none), 1, 2, or 3 (maximum) for necrosis, normal architecture, presence of RBCs in capillaries and arterioles/venules. The scores are illustrated as medians of each group at each timepoint. HMPO<sub>2</sub>, oxygenated hypothermic machine perfusion; NR, normothermic reperfusion; RBC, red blood cell; SCS, static cold storage.

## DISCUSSION

This study suggests that continuous HMPO<sub>2</sub> is a superior method for preserving beta-cell function compared with SCS and end-ischaemic HMPO<sub>2</sub> in a porcine model of circulatory death, assessed by NR with whole blood as a surrogate for transplantation.

### Perfusion and Assessment Models

The porcine abattoir model for solid organ normothermic perfusion has been successfully used by our group to establish protocols for translational clinical trials in kidney<sup>22</sup> and liver<sup>23</sup> perfusion studies. Assessment of the reconditioning potential of HMPO<sub>2</sub> on porcine pancreases using this normothermic perfusion model was chosen because it results in consistent and sufficient organ injury to elicit significant differences. NR is a technique that uses machine perfusion under normothermic conditions using whole blood as a surrogate for transplantation. This allows assessment of the organ morphology and function *ex vivo*.<sup>14,18,24</sup>

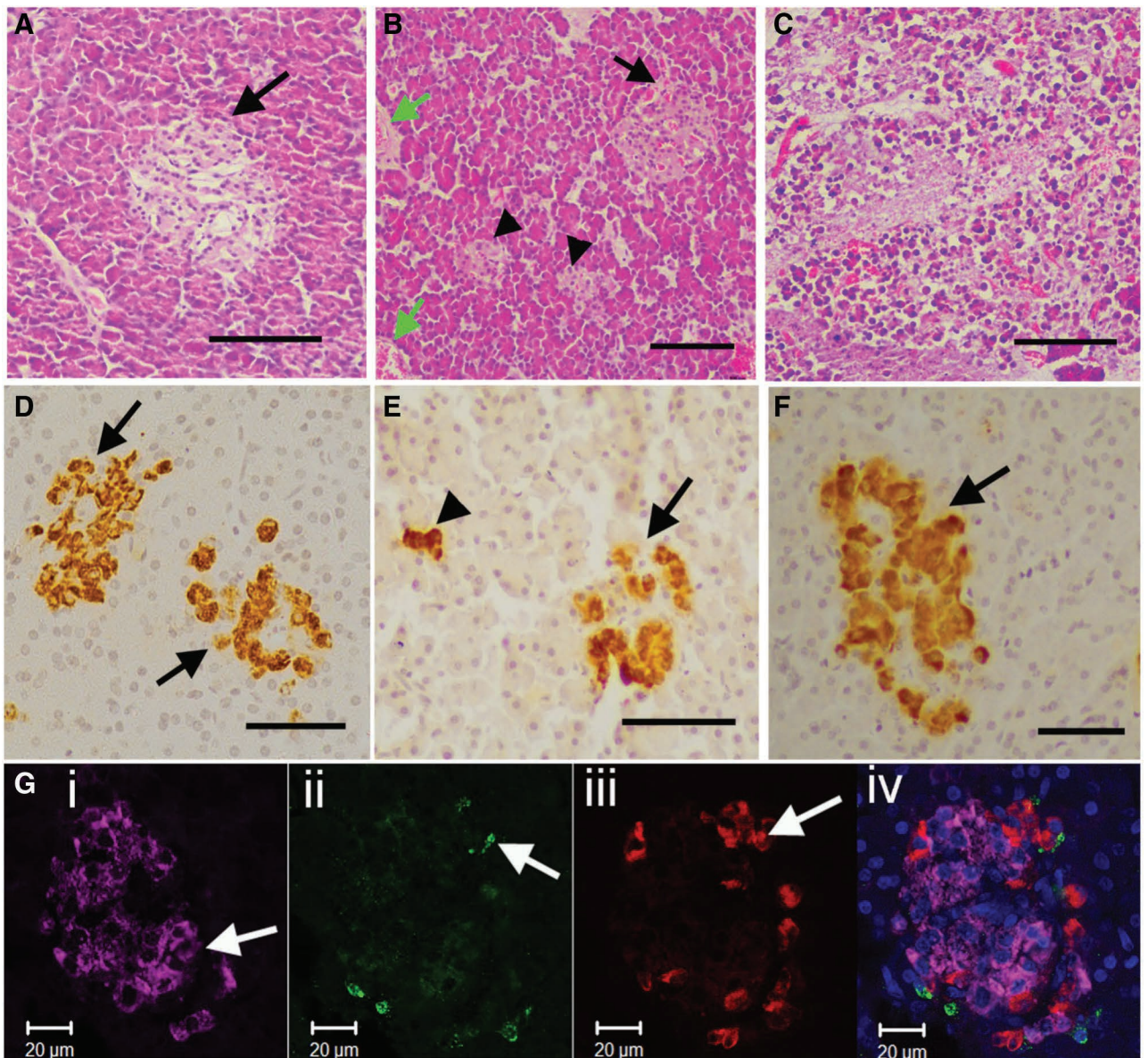
### HMPO<sub>2</sub> and NR Model Development and Feasibility

HMPO<sub>2</sub> is a superior modality for other solid organ preservation such as heart, kidney, and liver compared with SCS and

has shown promise in multiple preclinical models for pancreas preservation. Previous work on HMPO<sub>2</sub> of human pancreases resulted in homogeneous perfusion, confirmed by Acridin Orange staining, and an increase in ATP concentration, supporting the hypothesis that oxygen delivery restores cellular energy stores under hypothermic conditions. Tissue analysis showed no evidence of cellular injury, edema, or increased reactive oxygen species after 6 h of perfusion. In that study, 2 pancreases underwent islet isolation and *in vitro* assessment to confirm islet viability and function.<sup>11</sup> Allotransplantation of pancreases in a diabetic porcine model after 6 h of hypothermic machine perfusion (HMP) after minimal WIT was successful, demonstrating no vascular damage or thrombosis postmortem.<sup>15</sup>

### HMPO<sub>2</sub> Durations are Clinically Relevant

The 2 durations of HMPO<sub>2</sub> were selected on the basis of evidence from clinical trials in liver and kidney transplantation. Two-hour end-ischaemic HMPO<sub>2</sub> has shown improvement in biliary outcomes in liver preservation,<sup>8,25-27</sup> and continuous HMPO<sub>2</sub> has shown a lower incidence of rejection and better 1-y graft function in kidney grafts.<sup>9</sup> In addition, preclinical work published by our group showed that 2-h end-ischaemic HMPO<sub>2</sub> was beneficial over SCS in pancreas preservation.<sup>14</sup>



**FIGURE 5.** Histological assessment of pancreas wedge biopsies. A, Representative hematoxylin and eosin (H&E) staining of pancreas at the start of hypothermic preservation. Islet (arrow) does not contain any red blood cells. Scale bar 50  $\mu\text{m}$ . B, Representative Pancreas from the continuous HMPO<sub>2</sub> group at the end of NR. Red blood cells in islet capillaries (black arrow) and in larger blood vessels (green arrows). Small endocrine clusters (arrowheads) were typical of porcine pancreas. Scale bar 50  $\mu\text{m}$ . C, Representative pancreas from the SCS group at the end of NR showing severe autolysis and exocrine damage. Scale bar 100  $\mu\text{m}$ . D–F, Islets immunolabeled for insulin from pancreases in continuous, end-ischemic HMPO<sub>2</sub>, and SCS groups, respectively. Arrowhead in (E) shows a small cluster of endocrine cells; these contained only beta cells. Scale bars 25  $\mu\text{m}$ . G, Fluorescent immunolabeling for (i) insulin: violet (arrow); (ii) somatostatin: green (arrow); (iii) glucagon: red (arrow) using confocal microscopy; and (iv) merged images with nuclear DAPI labeling. Scale bars 20  $\mu\text{m}$ . DAPI, 4',6-diamidino-2-phenylindole; HMPO<sub>2</sub>, oxygenated hypothermic machine perfusion; NR, normothermic reperfusion; SCS, static cold storage.

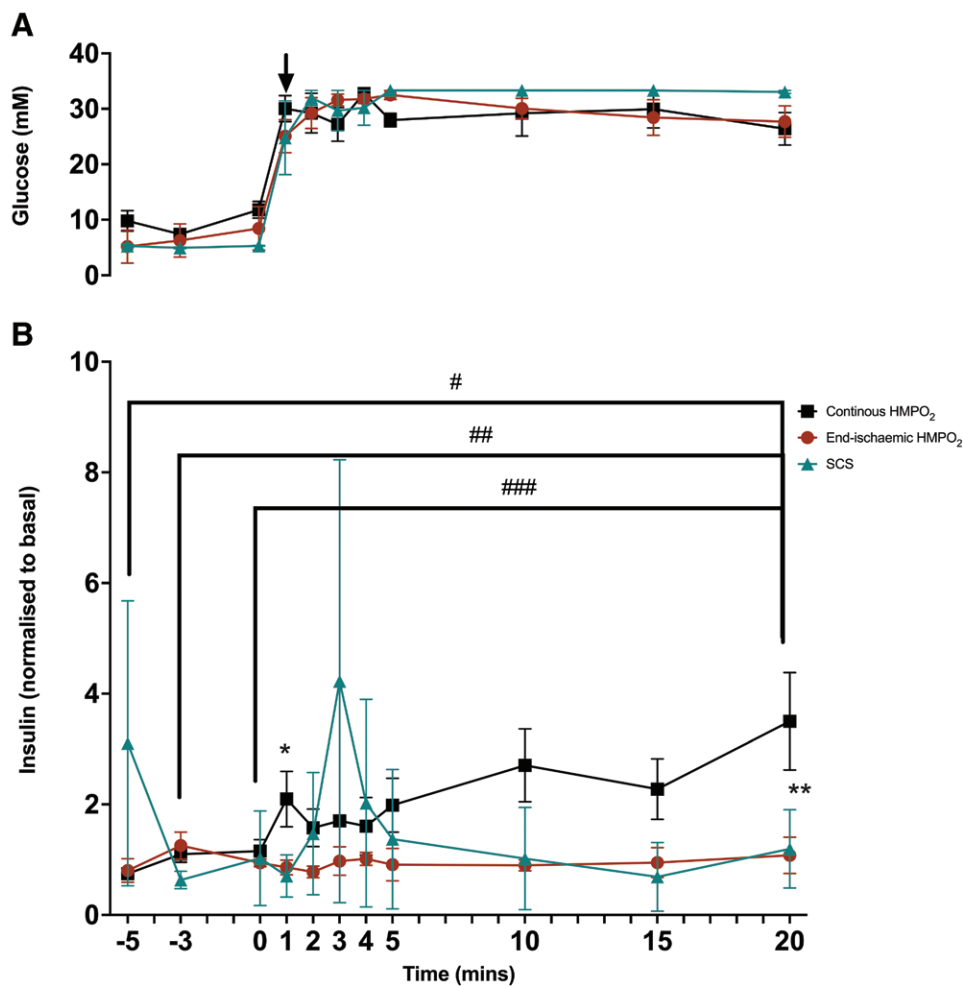
The 2 HMPO<sub>2</sub> interventions ( $n = 12$ ) of continuous and end-ischemic preservation, as well as all NR assessments ( $n = 18$ ), were feasible with the described protocol.

#### Validity and Advantages of Applied Perfusion Models

The 3 groups had comparable WIT and cold ischemic time (CIT), which closely translates to that seen in the clinical setting. The continuous HMPO<sub>2</sub> group had a longer duration of HMPO<sub>2</sub>, which resulted in an increase in perfusate flow compared with the shorter HMPO<sub>2</sub> duration in the end-ischemic group; however, during NR, there were no significant

differences in perfusion parameters. The presence of RBCs in the capillaries in all the biopsies ( $n = 18$ ) taken at the end of NR, but not at the start of hypothermic preservation, was evidence of capillary-level perfusion. This affirms the validity of our reperfusion model.

The ethical and financial advantages of using abattoir organs come with variation in the degree of tissue injury sustained during the retrieval process, which is inevitable in this setting but may also reflect biological variability. This model also uses young, healthy animals without cerebral injury, immunosuppression, or alloantigen exposure, which makes it an excellent preclinical option but limits direct translatability.



**FIGURE 6.** Glucose measurements and glucose-stimulated insulin secretion during normothermic reperfusion after continuous HMPO<sub>2</sub> (n = 3), end-ischaemic HMPO<sub>2</sub> (n = 3), and SCS (n = 3): The black arrow signifies the administration of glucose. A, Glucose levels in portal venous samples are increased and sustained in all groups from minute 1 onward. B, Insulin secretion (contemporaneous) in all groups, which is significantly higher in the continuous HMPO<sub>2</sub> group, compared with the end-ischaemic HMPO<sub>2</sub> group (2-way ANOVA with post hoc Tukey test [ $P < 0.02$ ]; difference at minute 1 [ $*P = 0.02$ ] and at minute 20 [ $**P < 0.001$ ]). Insulin secretion is shown in mU/L as mean  $\pm$  SEM. In the continuous HMPO<sub>2</sub> group, the baseline samples were significantly different from the end of the test: #minute -5 to minute 20 ( $P < 0.001$ ), ##minute -3 to minute 20 ( $P = 0.007$ ), and ###minute 0 to minute 20 ( $P = 0.0011$ ). Cont, continuous HMPO<sub>2</sub> group. End, end-ischaemic HMPO<sub>2</sub> group. HMPO<sub>2</sub>, oxygenated hypothermic machine perfusion; SCS, static cold storage

### Perfusate Biomarkers

Amylase, lipase, and LDH increased over time in all pancreases, but no statistically significant difference was noted between the groups, which is consistent with results seen in previous porcine and nonhuman primate pancreas preclinical HMP and HMPO<sub>2</sub> studies.<sup>14-16</sup> Interestingly, the increase of these markers slowed during NR after continuous HMPO<sub>2</sub>.

cfDNA has been shown to reflect degree of injury during ex vivo preservation of lungs<sup>28</sup> and we investigated it as a reflection of cell death, necrosis, and apoptosis. Although the levels of circulating cfDNA were significantly different between individual pancreases, there was no significant difference in the mean levels between the 3 groups.

When comparing cfDNA within each group at different timepoints, there was a statistically significant decrease from the start to the end of cold preservation in the continuous HMPO<sub>2</sub> group, which is the only marker of damage during hypothermia to correlate with functional assessment.

In the SCS group, we observed a significant decrease between the start of cold preservation and the end of NR, not

cold preservation. As the results were normalized to the baseline value (at the start of cold preservation) for each pancreas, a high starting level in the SCS group may explain this result.

### Lack of Correlation Between Biomarkers and Functional Assessment

The lack of correlation between the measured biomarkers and endocrine function could be explained by the physiological differential microvascular vulnerability between exocrine and endocrine pancreatic tissue, by the distinctly different pharmacokinetics of each biomarker, or by the challenge of interpreting their levels in an ex vivo isolated organ perfusion circuit.

Our amylase, lipase, and LDH results are in line with previously published work in pancreas normothermic perfusion models.<sup>14,29,30</sup> Pancreas normothermic perfusion, particularly an ex vivo reperfusion model, is expected to result in significant inflammation as a result of reflux excretion and reperfusion injury, leading to exocrine tissue damage. The GSIS

here assessed islet function, which is not expected to coincide with early acute exocrine damage.<sup>31</sup> This lack of correlation highlights the lack of specificity and poor correlation between the enzymatic biomarkers, which are largely used in clinical transplantation, and endocrine function. This further emphasizes the dissociation between exocrine inflammation and endocrine function, as well as the lack of clinically useful biomarkers.

### Undetermined cfDNA Kinetics in an Ex Vivo Circuit

The significant decrease and low absolute values of cfDNA at the end of NR in the SCS group are challenging to explain, but 1 theory is that it reflects significant irreversible reperfusion injury resulting in perfusion shutdown at the microvascular or lobular level, compounded by the short half-life of this marker. This can be as short as a few minutes, potentially shortened further in an ex vivo circuit at 37 °C with whole blood as perfusate.<sup>28,32,33</sup> This theory is supported by limited evidence available of cfDNA time-dependent changes during ex vivo kidney hypothermic perfusion<sup>34</sup> and liver and lung normothermic perfusion.<sup>28,35–37</sup>

### Histological Assessment

Histologically, all pancreases showed mild damage at the start of hypothermic preservation, specifically minimal loss of lobular architecture. This could be explained by the warm ischemic injury sustained during retrieval, suggesting that, at baseline, this model was more injured than other animal and human models previously used to investigate HMP or HMPO<sub>2</sub>.<sup>12,13,15–17</sup> There was no difference demonstrated at the end of hypothermic preservation across the 3 groups; in contrast, there was notably more severe histological evidence of damage at the end of NR, particularly in the SCS group.

### Functional Assessment Differences Between Groups

Despite the small sample size and technical challenges, a clear difference in insulin secretion could still be demonstrated. The continuous HMPO<sub>2</sub> group exhibited a preserved biphasic insulin secretory response during GSIS, which was not observed in the other 2 groups, suggesting that continuous HMPO<sub>2</sub> preserved islet function more efficiently. The highly variable insulin secretion in the SCS group can be explained by beta-cell injury. Absolute insulin values were within the range reported for large-animal ex vivo pancreas models and should be interpreted relative to baseline within each graft.<sup>14,17</sup>

Previous work using the same abattoir model showed that end-ischemic HMPO<sub>2</sub> for 2 h after 3 h of SCS yielded superior perfusion characteristics, lower lactate levels, and improved insulin secretion during GSIS compared with the SCS group.<sup>14</sup> However, the current study had 5 h of additional CIT, which can explain the difference between our findings.

Furthermore, in a similar study in which porcine pancreases were exposed to 5 h of HMP followed by up to 7 h of SCS, it was shown that 2 pancreases in the HMP group exhibited an insulin response to glucose stimulation, in contrast to none in the SCS-only group (n = 3).<sup>17</sup>

These findings highlight the sensitivity of the porcine pancreas to ischemic injury and how subtle increases in ischemic time can affect beta-cell function. This correlates with the sensitivity of human pancreases to CIT, which is a key factor in

posttransplant outcomes.<sup>38</sup> It also highlights the importance of demonstrating functional difference in preclinical models to support the translation of machine preservation technology into clinical practice.

### Limitations

The primary limitation of this study is the relatively small sample size (n = 18, 6 in each group). This may affect the statistical reliability of the results in particular, markers of damage and histological assessment. Any porcine model has the limitation of subtle differences in pancreatic anatomy and physiology compared with a human model, which therefore affects direct translation. A further limitation is the short duration of the NR model; this may not allow for the full extent of damage to be demonstrated, but our model was sufficient to illustrate a functional assessment, which was the primary aim.

### CONCLUSIONS

Further progress is needed to improve pancreas graft preservation and assessment, increase donor organ utilization, and decrease morbidity associated with pancreas transplantation. Although HMPO<sub>2</sub> is now suitable for evaluating safety and feasibility in a clinical trial, the best mode of application of this technology is still under investigation.

Continuous HMPO<sub>2</sub> was a superior method of preservation of the injured pancreases within the limitations of our model, but this comes with the challenge of initiating perfusion at retrieval and the complexity of pancreas surgical preparation required for perfusion at the donation site, which would pose an immense logistical challenge and increase the risk to the graft during transportation.

The practical implementation of end-ischemic HMPO<sub>2</sub>, immediately before transplant, is clearly more feasible, but its functional benefit might depend on the duration of cold ischemia before the start of perfusion. Technological advances—pancreas-specific devices and consumables, improved perfusate composition, integrated hemofiltration systems, and novel therapeutics—together with further consensus on perfusion protocols will increase feasibility and ease the integration of HMPO<sub>2</sub> into clinical practice.

When HMPO<sub>2</sub> is successfully applied in clinical pancreas preservation, it has the potential to mitigate the effects of IRI, thereby decreasing the intensity and frequency of posttransplant pancreatitis, decreasing the risk of graft vascular thrombosis, and improving pancreas graft survival and long-term function. Safety and feasibility testing will be an appropriate first step in translating this technology for pancreas preservation into clinical practice.

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