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Extended preservation of non-heart-beating donor livers with normothermic machine perfusion

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Background Non-heart-beating donor (NHBD) livers represent an important organ pool, but are seldom utilized clinically and require rapid retrieval and implantation. Experimental work with oxygenated perfusion during preservation has shown promising results by recovering function in these livers. This study compared sanguinous perfusion with cold storage for extended preservation of the NHBD liver in a porcine model.

Methods: Porcine livers were subjected to 60 min of *in vivo* total warm ischaemia before flushing, after which they were preserved by one of two methods: group 1 ($n = 4$), University of Wisconsin (UW) solution by standard cold storage for 24 h; group 2 ($n = 4$), oxygenated autologous blood perfusion on an extracorporeal circuit for 24 h. All livers were subsequently tested on the circuit during a 24-h reperfusion phase.

Results: Livers in group 1 showed no evidence of viability during the reperfusion phase with no bile production or glucose utilization; they also displayed massive necrosis. Livers in group 2 demonstrated recovery of function by synthetic function, substrate utilization and perfusion haemodynamics; these livers displayed less cellular injury by hepatocellular enzymes. All differences in parameters between the two groups were statistically significant ($P < 0.05$). These findings were supported by histological examination.

Conclusion: Warm ischaemia for 1 h and simple cold storage (UW solution) for 24 h renders the liver non-viable. Oxygenated, sanguinous perfusion as a method of preservation recovers liver function to a viable level after 24 h of preservation.

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Introduction

Non-heart-beating donor (NHBD) livers represent a potential pool of organs that could significantly reduce the large discrepancy between supply and demand for liver transplantation¹. The current method of preserving livers by simple cold storage subjects the organ to a further period of ischaemia, increasing the level of injury, and usually leaving the organ unfit for transplantation². The cold-stored NHBD organ undergoes injury at four consecutive stages: (1) prior to retrieval (warm ischaemia), (2) during cold storage ischaemia, (3) during ischaemic rewarming (warm ischaemia) and (4) at reperfusion. Consequently, clinical experience with the NHBD liver has been limited^{3–5}. When cardiac arrest occurs outside the

operating room, there is a high rate of primary non-function⁴. It is for these reasons that the NHBD liver is rarely used; however, in view of the excess of demand over supply in liver transplantation, and the lack of any alternative means of providing long-term support to patients with liver failure, it is important to develop methods that make use of NHBD livers.

The use of NHBD donor livers that have experienced substantial injury from warm ischaemia during the period of arrest would require the capability of ameliorating the ischaemic cellular changes either before or during the preservation period. It was hypothesized that perfusing the liver with an oxygenated perfusate supplemented with nutritional support at body temperature during the preservation period might be a way of accomplishing this

goal. By these means, depleted cellular substrates could be restored during preservation, allowing the organ to tolerate the warm ischaemic period of surgical anastomosis and reperfusion.

This study investigated the use of oxygenated, normothermic perfusion of NHBD livers during the preservation period as an alternative to cold storage. Preservation by normothermic blood perfusion was compared directly with simple cold storage in the porcine model after 60 min of warm ischaemia.

Methods

Operation

White Landrace pigs (40 kg) were premedicated intramuscularly with ketamine (Ketaset®; IDIS, Surbiton, UK) 10 mg/kg and midazolam (Hypnovel®; Roche, Welwyn Garden City, UK) 1 mg/kg. General anaesthesia was induced with propofol (Diprivan®; AstraZeneca, King’s Langley, UK) 0.5 mg/kg given intravenously, and maintained by an intravenous infusion of propofol (1 mg per kg per h) together with halothane inhalation via an extended endotracheal tube.

Upon laparotomy the porta hepatis was dissected to delineate the anatomy. The common bile duct was ligated and divided. The infrahepatic vena cava, portal vein and arterial anatomy were each identified. After 20 000 units of standard human heparin (Multiparin®; CP Pharmaceuticals, Wrexham, UK) had been injected directly

into the aorta and allowed to circulate, a 10-Fr cannula (Medtronic, Watford, UK) was placed into the abdominal aorta and 4 units blood were taken. Cardiac arrest was then induced by intracardiac injection with 10 ml potassium chloride. A period of circulatory arrest of exactly 60 min was observed in every case before flushing the liver with 3 litres cold Eurocollins solution (Soltran®; Baxter, Compton, UK). The livers were then assigned to a preservation period of either simple cold storage in University of Wisconsin (UW) solution (Viaspan®; Du Pont, Stevenage, UK) or machine perfusion according to the experimental design outlined below.

Experimental design (Fig. 1)

The livers were preserved for 24 h in one of two groups: group 1 (n = 4) – flushed and preserved in cold storage for 24 h in UW solution; group 2 (n = 4) – placed on an isolated perfusion circuit with whole blood at 38°C.

Reperfusion period

All livers were tested for function after the preservation period by using an extracorporeal circuit to perfuse the livers with whole blood at body temperature as a surrogate for transplantation. To replicate surgical anastomosis, both groups were flushed after preservation with 2 litres non-oxygenated cold Eurocollins solution and left for 45 min before being flushed with a warm plasma substitute (Gelofusine®; B. Braun, Sheffield, UK) and reperfused on

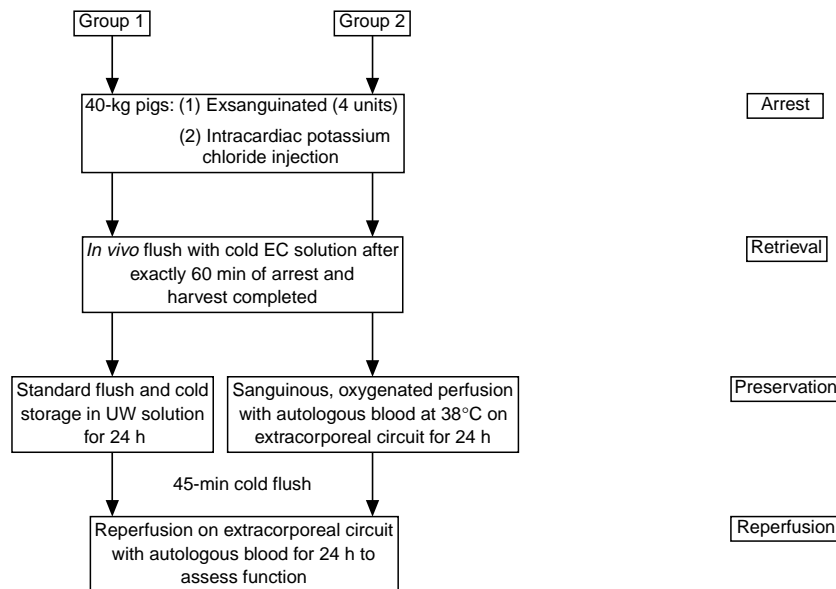


Fig. 1 Experimental design. EC, Eurocollins; UW, University of Wisconsin

the extracorporeal circuit. The blood used to perfuse livers in group 2 was changed between the preservation and reperfusion phases so that a fair comparison of physiological parameters could be made.

Assessment of function

Arterial blood gases, venous blood gases, blood glucose, haemodynamics and bile production were also documented every hour of the reperfusion phase. Perfusate was sampled every hour for the first 8 h of the reperfusion period and every 4 h thereafter for conventional liver function testing and estimation of urea and electrolyte levels.

Full blood count and factor V samples were taken every 2 h for the first 8 h, and then every 4 h. Galactose clearance

was measured at 12 and 24 h of reperfusion. At the end of each perfusion, the liver was sectioned and multiple random samples were assessed histologically for evaluation of reperfusion injury.

Perfusion circuit

The circuit consisted of an oxygenator (1500 ECMO Oxygenator; Medtronic), a heat exchanger (Ecmotherm II HE; Medtronic), a centrifugal pump (BP50 Centrifugal Pump; Medtronic), an 800-ml venous reservoir bag (Medtronic) and polyvinylchloride tubing (Medtronic) with an internal diameter of 1/4 and 3/16 inches (Fig. 2). During priming, the perfusion circuit was supplemented with 9.2 mmol calcium chloride, 20 mmol sodium bicar-

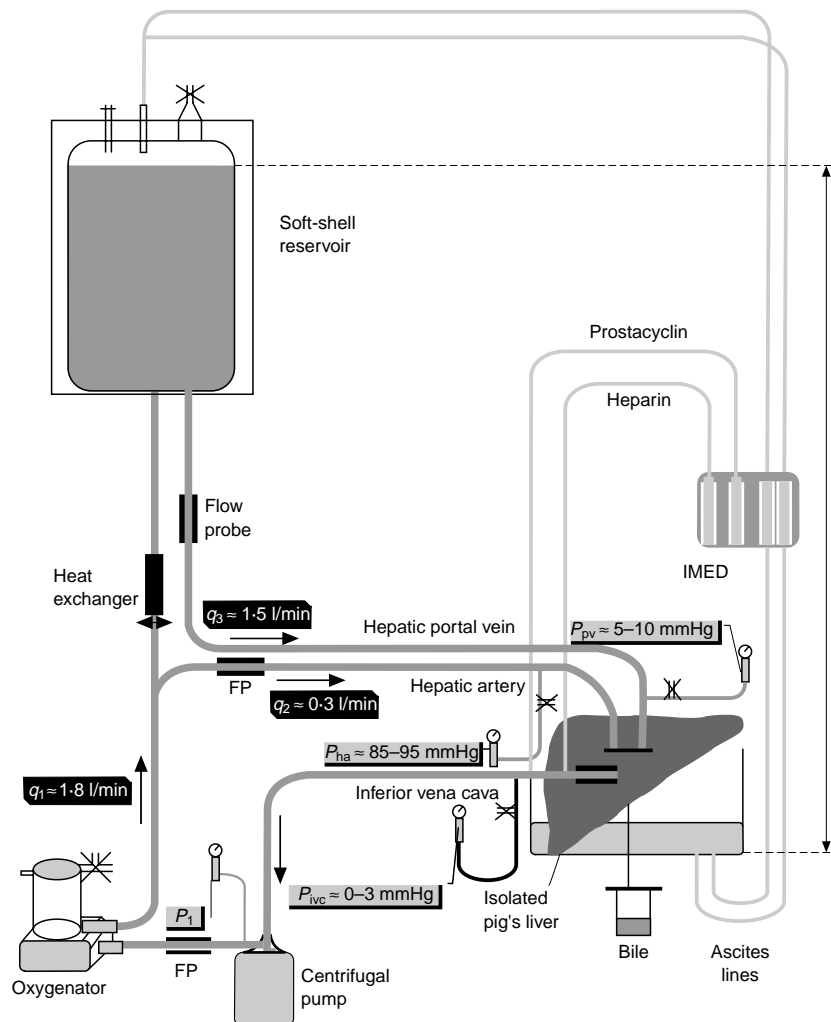


Fig. 2 Extracorporeal perfusion circuit. FP, flow probe; IMED, infusion pump; P, pressure, q, flow; ivc, inferior vena cava; ha, hepatic artery; pv, portal vein

bonate and 7000 units heparin in all cases. Temperature was maintained at 38°C. The circuit was supplemented with a continuous infusion of prostacyclin (Flolan®; Glaxo Wellcome, Uxbridge, UK) at 4 ml/h, 2 per cent taurocholic acid (Sigma, Watford, UK) at 7 ml/h and nutrition (Nutriflex®; B. Braun) at 17 ml/h. Nutrition consisted of 96 g amino acids and 300 g anhydrous glucose in 2 litres of solution, while fats were excluded. Regular human insulin (Humulin®; CP Pharmaceuticals), 100 units, was added to each bag. Heparin, 3000 units, was added every 4 h.

While attempting to keeping the extracorporeal circuit user-friendly, it is imperative that it simulates the *in vivo* physiological environment as closely as possible. Thus it was made up of two circuits in parallel, the arterial arm being fed directly from the centrifugal pump (high pressure) and the portal arm being fed passively from a venous reservoir by gravity (low pressure). Efferent was retrieved via the suprahepatic caval cannula and blood returned to the pump, after which the blood was oxygenated and warmed. During preservation, physiological flows and pressures were maintained in the hepatic artery, portal vein and vena cava by controlling pump head speed and adjustment of gate clamp resistance. At the initiation of the reperfusion phase, the pump was maintained at a set rate (2600 cycles per minute) so that the flows and pressures within the vessels could be used as an indication of viability.

Statistical analysis

Statistical analysis was performed using Welch's *t* test (assumption of unequal variances), with each timepoint reflecting the mean of four perfusions. $P \leq 0.05$ was considered significant. The comparisons were made between the two groups for each individual parameter at each equal timepoint in perfusion.

Results

Upon reperfusion, none of the cold-stored livers of group 1 demonstrated any sign of viability throughout the reperfusion phase. The machine-perfused livers in group 2 functioned well throughout the reperfusion phase. The comparative data are outlined below.

Hepatocellular damage

Mean levels of alanine aminotransferase (ALAT) rose sharply upon reperfusion in group 1 livers (Fig. 3) compared with levels seen in group 2. This was statistically significant by 1 h of reperfusion ($P = 0.016$) and at all timepoints thereafter. The rise in ALAT concentration continued throughout reperfusion of the livers in group 1,

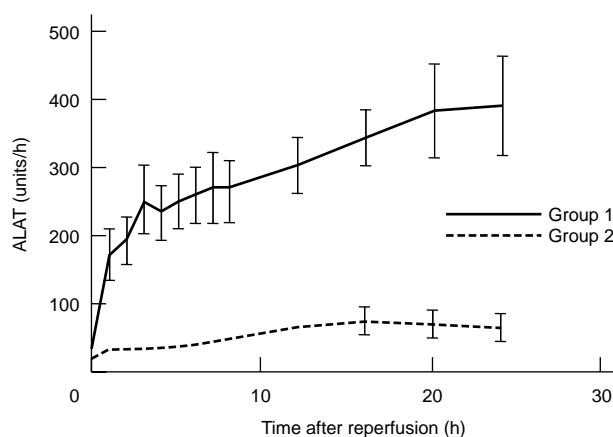


Fig. 3 Demonstration of massive hepatocellular lysis in group 1 livers with a progressive rise in alanine aminotransferase (ALAT) levels. Values are the mean (s.e.m.) of four experiments. $P \leq 0.05$ for all timepoints (Welch's *t* test)

with relatively little increase seen in group 2 during the same time period. Mimicking the ALAT curves, aspartate aminotransferase (ASAT) levels in group 1 livers rose rapidly to 3810 units/l after 1 h of reperfusion, whereas the mean level of ASAT in group 2 was only 259 units/l after 1 h of reperfusion ($P = 0.036$). The differences between the two groups were significant at all timepoints. The mean level of lactate dehydrogenase rose to 2985 units/l in group 1 livers by 1 h of reperfusion, compared with 1158 units/l in group 2. The differences between the two groups continued to grow throughout reperfusion and were statistically significant from 2 h onwards.

Synthetic function

The cold-stored livers of group 1 produced no bile after reperfusion in any of the four perfusions. In addition, these livers displayed haemorrhagic output from the biliary system. The perfused livers of group 2 recovered bile function of up to 10 ml/h. The differences were statistically significant at all timepoints (Fig. 4).

Factor V levels fell in group 1 to 12 units/dl at 24 h, while livers in group 2 maintained synthetic function throughout reperfusion, with a level of 263 units/dl at 24 h. The differences were statistically significant from 4 h onwards.

Metabolic function

Livers in group 1 showed no evidence of glucose consumption. Perfusate glucose levels remained above the upper limit of detection throughout reperfusion. In contrast,

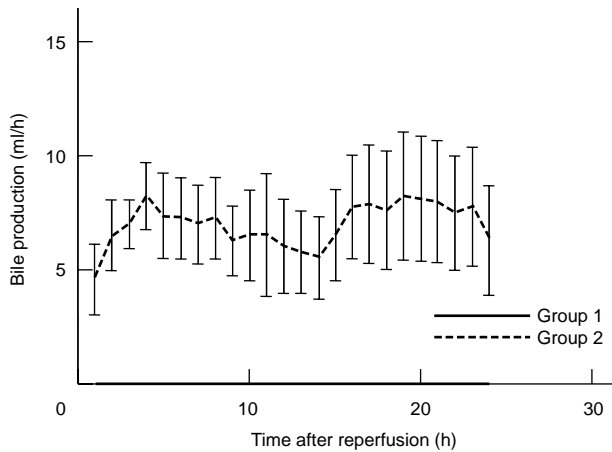


Fig. 4 Group 2 livers produced bile throughout the reperfusion phase, whereas group 1 livers made no bile. Values are the mean(s.e.m.) of four experiments. $P \leq 0.05$ for all timepoints (Welch's *t* test)

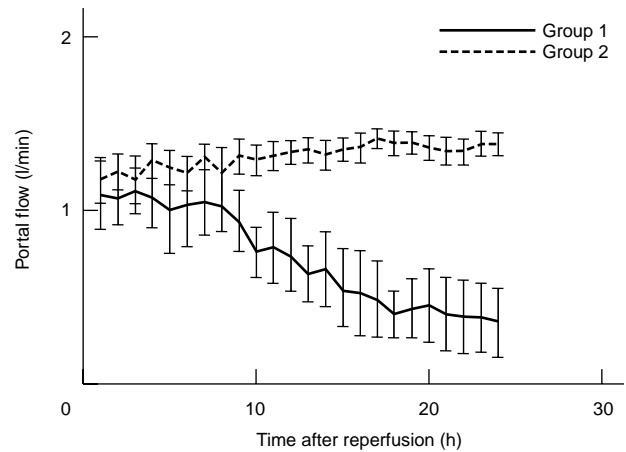


Fig. 6 Portal flow deteriorates throughout reperfusion in group 1 livers, presumably due to progressive destruction of sinusoidal and tissue architecture. Values are the mean(s.e.m.) of four experiments. $P \leq 0.05$ for all timepoints after 10 h (Welch's *t* test)

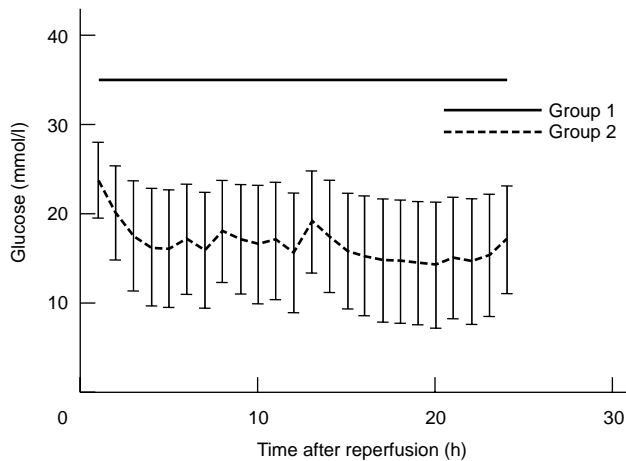


Fig. 5 Metabolic activity is still present after 24 h of machine preservation as demonstrated by the glucose utilization curve for group 2 livers. Values are the mean(s.e.m.) of four experiments. $P \leq 0.05$ for all timepoints (Welch's *t* test)

group 2 livers demonstrated glucose utilization throughout reperfusion (Fig. 5).

Haemodynamics

The mean total hepatic blood flow in group 1 decreased throughout the reperfusion phase to 0.44 l/min at 24 h, compared with 1.40 l/min at 24 h in group 2 ($P = 0.005$).

Both portal and arterial flow followed similar curves, with diminishing values seen in group 1. The portal flow, similar to total flow, was significantly greater in group 2 livers, with a widening difference throughout reperfusion (Fig. 6).

Haemolysis

Despite both groups having no significant difference in haemoglobin concentration at the commencement of reperfusion (mean(s.d.) 7.6(1.3) g/dl in group 1 versus 6.8(2.2) g/dl in group 2), the perfusate in group 1 demonstrated massive haemolysis immediately, such that the blood gas machine could no longer detect the sample shortly after reperfusion. A measurable level of haemoglobin was present in the 2-h sample in only one of the experiments from this group (mean 0.98 g/dl at 2 h). By contrast, livers in group 2 maintained measurable haemoglobin levels throughout reperfusion. The mean haemoglobin at 2 h was 5.55 g/dl ($P = 0.02$).

Histological findings

The group 2 livers showed diffuse, mild vacuolization and sinusoidal dilatation with minimal to no necrosis and preserved tissue structure. Livers in group 1 displayed diffuse hepatocellular necrosis (more than 66 per cent per high-power field), sinusoidal haemorrhage and architectural destruction (Fig. 7).

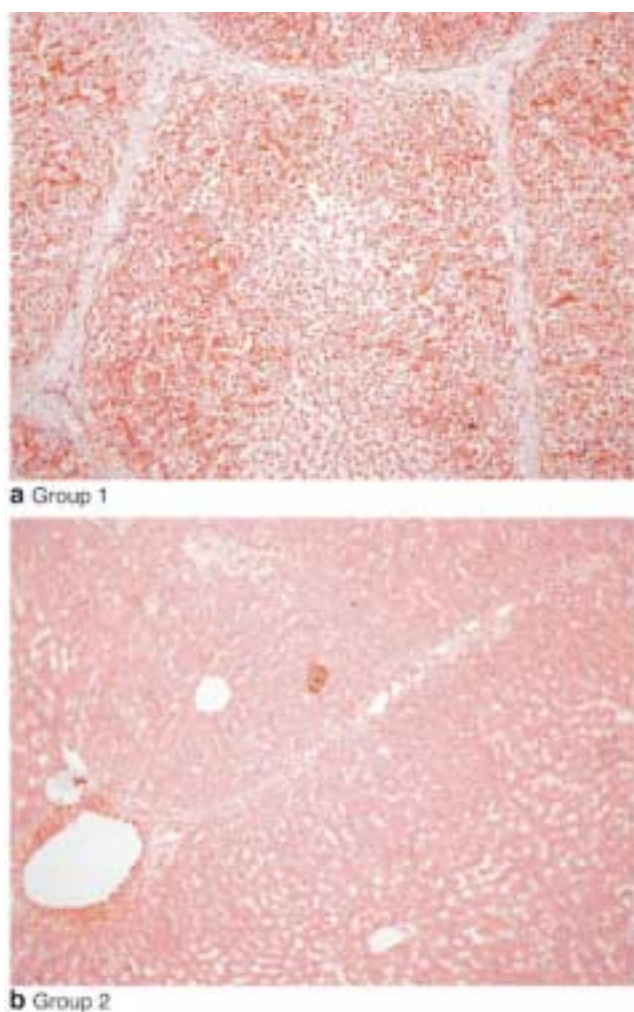


Fig. 7 **a** Gross hepatocellular necrosis, sinusoidal haemorrhage and architectural destruction diffusely present. **b** Mild vacuolization and sinusoidal dilatation with viable hepatocytes and preserved tissue structure

Discussion

This experiment was based on data that oxygenated perfusion of NHBD livers will promote cellular recovery from warm ischaemic injury. Without perfusion, cold storage exacerbates the injury by subjecting the organ to additional (cold) ischaemia; then, during surgical anastomosis in the recipient, another period of warm ischaemia. These consecutive ischaemic periods facilitate the progression of cellular injury to a state that is not compatible with recovery upon reperfusion. It is therefore essential to restore cellular energy levels between retrieval and reimplantation for NHBD livers.

Previous studies have shown that perfusion prior to harvesting of the NHBD liver can restore cellular energy levels before cold storage. Total body reperfusion with cardiopulmonary bypass (CPB) using autologous blood after 30 min without ventilation was shown to revive total adenosine 5'-triphosphate (ATP) content in porcine livers and kidneys⁶. Conditioning the liver with 30 min of normothermic isolated perfusion *in vivo*, using whole blood, has been shown to increase mitochondrial ATP content after 10 min of circulatory arrest in the porcine model⁷. CPB for only 10 min after 30 min of arrest, at 37°C, provided functional recovery of the heart, liver and kidney in the canine model⁸. In the porcine transplant model, CPB for systemic perfusion with cold, oxygenated Eurocollins solution restored tissue energy charge after 10 min of arrest⁹. These livers were then cold-stored for 5 h and transplanted with a survival rate of 100 per cent in the perfused group, while there were no recipient survivors in the control group (no perfusion). The largest study utilizing preharvest perfusion to salvage NHBD livers in the transplant model comes from Barcelona¹⁰. In this study, pigs were assigned progressive circulatory arrest time, followed by CPB to perfuse the livers *in vivo* for 30 min prior to cold storage and transplantation. The control group did not undergo bypass before cold storage. The transplant survival rate was related significantly to warm ischaemia time. However, even after 40 min of arrest, 50 per cent survived in the perfused group, whereas there were no survivors in the control group after only 20 min of arrest. This work showed that bypass did regenerate tissue energy charge, and that maintenance of these energy stores after arrest correlated with post-transplant survival^{11,12}.

Cellular substrates can also be restored after the NHBD liver has been harvested. Sanguinous perfusion of isolated porcine livers at body temperature for 3 h has been shown to resuscitate basic liver function on a perfusion circuit after 75 min of arrest¹³. In this study, perfusion was initiated immediately after harvest, representing the final phase of the experiment. This shows that sanguinous perfusion upon retrieval does restore liver integrity after a period of warm ischaemia. The present study confirmed these findings with the excellent function observed in group 2 livers during the preservation period.

Delivery of oxygen during the preservation period has previously been shown to be successful in the NHBD model by various methods. Persufflation of oxygen during cold storage in UW solution resulted in 100 per cent recipient survival in an experimental group of pigs after 45 min of arrest, while all the controls without persufflation died¹⁴. In a separate study, machine perfusion of the pig liver through the hepatic artery with acellular perfusate (UW-gluconate) resulted in a transplant survival rate of 80 per cent after

90 min of hypotension and a brief period of arrest, while the control group (cold storage) displayed a 25 per cent survival rate¹⁵.

Schon *et al.*¹⁶ subjected porcine livers to 1 h of warm ischaemia before harvesting, but by preserving them for 4 h with normothermic extracorporeal perfusion using blood as the perfusate they were able to display excellent post-transplant function in a group of six pigs. Also in this study, a group of six pigs with the same warm ischaemia time showed no survivors after preservation in cold UW solution for 4 h. This work clearly delineates the benefit of sanguinous perfusion during preservation, but it should be noted that the preservation period was only 4 h, which is of marginal clinical applicability. Also, their perfusion circuit included diaphragmatic replication and dialysis, based on previous work^{17–19}. In the present study excellent results were achieved after 24 h of perfusion without these additions. Schon's group did not provide nutrients, bile salts or prostacyclin for their perfused livers.

In the present study normothermic perfusion was used for an extended preservation period of 24 h. To simulate the transplant model as closely as possible, the livers in group 1 were taken off the circuit after 24 h of machine preservation, and a 45-min period of ischaemia was initiated by flushing with cold Eurocollins solution followed by warm colloid. This subjected the livers to ischaemic periods of cooling and rewarming before reperfusion.

The results of this experiment show, as expected, that 1 h of warm ischaemia followed by 24 h of cold storage in UW solution results in a completely non-viable organ. However, 24 h of normothermic perfusion preservation produces a functioning liver, despite the same ischaemic injury. The perfused livers in group 2 made bile, utilized glucose, maintained stable haemodynamics and displayed histological features of viability, confirming the hypothesis that perfusion during the preservation period restricts the level of ischaemic injury in the NHBD liver. These results provide a powerful argument for the value of oxygenated perfusion during preservation as the length of both the arrest time and the preservation time were longer than those in other studies. A preservation period of 24 h is much longer than the pig liver will normally tolerate, yet the livers in group 2 demonstrated good preservation even when this was compounded by 60 min of arrest. Furthermore, perfusing NHBD livers throughout the preservation period not only replenished cellular substrate, ameliorating the ischaemic injury, but provided a clear assessment of liver function. This technique, in combination with the use of a variety of viability markers, could permit the use of severely injured organs with reassurance of function.

It is concluded that sanguinous perfusion as a method of preservation is potentially a technically feasible means of

providing viable organs after significant warm ischaemic injury and an extended period of preservation. The next step in validating this technique of perfusion preservation to salvage NHBD organs will require demonstration of function and recovery after transplantation.

Acknowledgements

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