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Optimisation of Bile Production during Normothermic Preservation of Porcine Livers

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Machine perfusion of livers may provide a mechanism for extended preservation of marginal donor organs before transplantation, as well as a method for viability assessment. It has proved possible in a series of experimental porcine liver perfusions to maintain liver viability for up to 72 h. However, a reduction in bile production with associated histological evidence of cholestasis was seen after 10 h of perfusion, damaging the biliary canaliculi during the preservation period and leaving these organs in an unacceptable condition for transplantation.
It was proposed that reduction in bile production was the result of a relentless depletion of available bile salts, gut recirculation not being possible and de-novo synthesis being unable to keep up with loss. This was proved by measuring porcine native bile acids within serial perfusate and bile samples using gas chromatography mass spectrophotometry. It was shown that all three native pig bile acids were decreased to 30% of their original value by 20 h of unsupplemented perfusion. An infusion of taurocholate managed to maintain bile production at physiological levels throughout the 20-h period (8 mL/h ± 0.75). It was successfully incorporated by the porcine livers into bile.
We propose to use this circuit as a novel means of preserving donor livers for transplantation in which the organ is maintained at normal body temperature and perfused with blood. This will reduce ischaemia reperfusion injury and may enable prolonged preservation. The modification described ensures optimal bile production over the entire perfusion period, preventing inspissation and subsequent damage to the canaliculus.

Introduction

Extracorporeal liver perfusion has been utilised in an experimental setting for many years (1) to study unique components of liver physiology and more recently as a possible mechanism of liver support (2). One new field of interest involves employing machine perfusion as a possible mechanism for preservation and resuscitation of injured livers before transplantation. For this to provide clinical benefit it would be necessary to maintain functional integrity of the entire organ on an extracorporeal circuit for much longer periods than have previously been investigated.

One important aspect of liver physiology is that of bile production, which is necessary in order for biliary canaliculi to remain in a functional state and excretion of hydrophobic compounds to be maintained (3). This in return relies on a choleretic stimulus from a constant supply of bile acids (1), the majority of which are delivered *in vivo* via the enterohepatic circulation. The isolated circuit clearly precludes the latter because bile is excreted and discarded.

Preliminary data utilising this circuit demonstrated failing bile production after 10 h of perfusion with inspissated bile blocking canaliculi. Hence a method of reproviding bile salts was developed in an attempt to remedy this situation. A porcine model was used in these experiments because of close approximation to human hepatic physiology and anatomy.

Materials and Methods

Experimental plan

Group U (unsupplemented): five normothermic liver perfusions (40 h) were performed as subsequently described, utilising allogeneic blood as the perfusate with no bile acid supplementation.

Group S (supplemented): five liver perfusions were performed as subsequently described with a continuous infusion of sodium taurocholate into the perfusate throughout the 40-h perfusion.

In both groups samples (1 mL) of bile and perfusate (whole blood) were collected every 4 h for bile acid estimation using gas chromatography mass spectrophotometry.

Animals

Twenty large white/landrace pigs were used in these experiments (mean weight 40 kg) in accordance with the Animal Protection Act 1986. Separate pigs were used as blood and liver donors in each perfusion, having been blood matched before surgery.

Premedication

Blood/liver donor pigs were premedicated with 10 mg/kg ketamine and 1 mg/kg midazolam i.m.

Blood donor

Following cannulation of the external jugular vein and instillation of 15 000 U heparin, blood was collected in citrated bags via gravity drainage until circulation ceased.

Liver donors

Animals were premedicated (having had an ear vein cannulated), and anaesthesia was induced with 4 mg/kg of propofol i.v. and maintained with propofol at 10 mg/kg/h throughout the procedure.

A midline laparotomy was performed, the bile duct cut and the hepatic vessels identified and isolated in the standard fashion. The liver was dissected until connected only by its vascular attachments. Twenty thousand units of heparin i.v. was given, and the infrarenal aorta was cannulated with a 20-G cannula. This was connected to a closed system containing Eurocollins solution. The portal vein was then cannulated with a 24-G cannula in a similar fashion, and both were perfused with cold Eurocollins solution as the suprahepatic inferior vena cava was divided in the pericardium.

After 2 L of cold Eurocollins had perfused through the liver, it was removed by excising a cuff of diaphragm around the suprahepatic IVC, dividing the hepatic artery at the coeliac axis, the infrahepatic inferior vena cava (IVC) at the level of the renal veins and the portal vein at the level of the splenic vein. While continuing portal perfusion the liver was placed into a bowl at ice temperature, and the diaphragmatic remnant oversewn with 3.0 prolene to secure haemostasis. The suprahepatic IVC was cannulated with a 28-G cannula with its orifice positioned at the level of the hepatic veins. Pressure monitoring cannula were placed in the inferior vena cava (directly) and portal vein (via a tributary). The hepatic artery was then cannulated with a 10-Fr. cannula. The bile duct was cannulated with a 14-G silastic T-tube with the open end of the T-tube placed into a collection device to monitor bile output and to collect the sample's perfusion circuit.

While backbench preparation of the liver was performed the perfusion apparatus was assembled and primed with 1500 mL of donor pig blood. The circuit consisted of an oxygenator (1500 ECMO Oxygenator Medtronic, Watford, UK), a heat exchanger (Ecmotherm II HE Medtronic) a centrifugal pump (Medtronic BP50 Centrifugal Pump), a reservoir (Venous reservoir bag 800 mL, Medtronic) and tubing (Medtronic, PVC 1/4 and 3/16 inch internal diameter) (see Figure 9). During priming of the circuit, the perfusion circuit was supplemented with 9.2 mmol $CaCl_2$, 20 mmol sodium bicarbonate and 7000 U heparin in all cases. Temperature was maintained at 39 °C. The circuit was supplemented with continuous infusion of 4 mL/h prostacycline, 1% 7 mL/h taurocholic acid (in the supplemented Group S) and 17 mL/h total parenteral nutrition. Total parenteral nutrition (TPN) consisted of amino acids (13.6 g N/2000 mL bag) and glucose (300 g/2000 mL bag), while fats were excluded. Heparin (3000 IU) was added every 4 h. Once the circuit was primed, a sample was obtained for full blood count, electrolytes, urea, creatinine, liver function tests and bile salt concentrations.

Liver perfusion

All livers were perfused at 39 °C with heparinised whole blood at physiological pressures and flows via the portal vein and hepatic artery. During which, pressure and flows within the portal vein (5–8 mmHg, 1.3–1.7 L/min), hepatic artery (70–90 mmHg, 0.3–0.5 L/min) and inferior vena cava (−2 to +2 mmHg, 1.6–2.2 L/min) were kept within normal limits. Blood was returned to the circuit via the vena cava.

As described earlier, in order to replace the enterohepatic circulation of bile salts a 1% solution of sodium, taurocholate (7 mL/h) was instilled into the perfusate in five of the experiments. Perfusate and bile samples were collected for bile acid estimation every 2 h during the 40-h perfusions.

Bile acid estimations

This was performed as described previously by Clayton et al. (4). One mL of plasma/bile was diluted with 3 mL of 0.15 mol/L acetate buffer and incubated with 0.5 mL (90 U) of a cholylglycine hydrolase preparation for 12 h at 37 °C. Fifty μL of 7-ketolithocholic acid in methanol(12.5 nmol) was then added to the deconjugation mixture as an internal standard, and 0.5 g wet-weighed XAD-2 resin was washed with 20 mL sodium hydroxide (0.1 mol/L). The suspension was then mixed on a rotary mixer for 3 h, after which the entire tube contents were poured into a glass column (1 cm internal diameter with a coarse sinter and a P.T.F.E tap). The alkaline solution was drained, and the resin bearing the adsorbed deconjugated bile acids was washed with 20 mL water, followed by two washes with 5 mL of 0.066 mol/L phosphate buffer pH 5.8. Each of the phosphate buffer washes was left in contact with the resin for 20 min.

The resin was then washed with 5 mL hexane to displace excess water, and the bile acids were eluted with 2 mL hexane/chloroform/methanol (1 : 1 : 1, v/v/v) followed by two aliquots of 2 mL methanol. Ten minutes was allowed for each of the three elutions before they were pooled and evaporated to dryness under a stream of nitrogen. Conversion of the free bile acids to bile acid methyl ester trimethylsilyl ethers was achieved as follows: methylation of the free carboxyl groups was performed by reconstituting the residue in 1 mL 5 : 1 diethylether : methanol and by adding 200 μL diazomethane in diethylether (diazomethane was synthesised by refluxing ethanolic KOH with diazald in diethylether at 60 °C). The reaction mixture was then incubated on ice for 20 min and then taken for dryness under vacuum and transferred to a 2-mL glass vial with a Teflon-coated cap. Trimethyl-silyl derivatisation of the hydroxy groups was subsequently performed by the addition of 60 μL Tri-Sil® (Pierce, IL, USA) and 1-h incubation at 60 °C.

Gas chromatography mass spectrophotometry analysis was conducted using a Hewlett-Packard 5890 Series II gas chromatograph coupled to a Hewlett-Packard 5970 mass-selective detector. Using a splitless injection at 250 °C, 2 μL were injected onto an HP-1 cross-linked methyl siloxane capillary column (0.25 μm film thickness, 0.25 mm internal diameter and 30 m length; Hewlett Packard, IA, USA). After a delay of 2 min with the oven temperature at 120 °C, the oven temperature was ramped to 200 °C at 20 °C/min and then to 300 °C at 2 °C/min. The mass spectrometer was scanned between m/z 50–800 with a source temperature of 170 °C and an ionisation energy of 70eV.

Haemolysis (cyanmethaemoglobin colorimetric method)

Free haemoglobin was converted to cyanmethaemoglobin by utilising Drabkin solution. A direct spectrophotometric assay measuring cyanmethaemoglobin was then utilised (diluent 4.0 mL, perfusate sample 20 μL, dilution 1/201) (5).

Histology

At the end of the 40-h perfusion the liver was weighed and then sectioned through its mid-portion. From this slice, five random blocks (5 × 10mm) were immersed in formal saline for paraffin sectioning and haematoxylin and eosin staining.

Results

Mean bile production over a 40-h period of sanguineous liver perfusion (Group U) demonstrated a continuous decrease
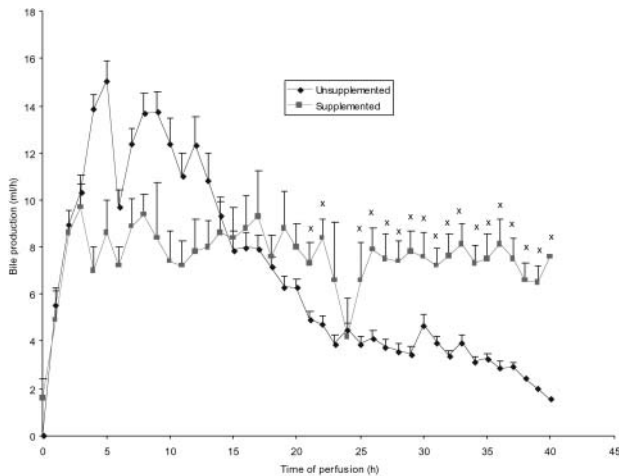
Figure 1: Comparison of hourly bile production during 40-h unsupplemented and supplemented liver perfusion. Bile collected via tubing in common bile duct after cystic duct ligated during five perfusions without bile salt supplementation, and during five perfusions in which a continuous infusion of taurocholate (7 mL/h of 1 g/100 mL concentration) was added. Values expressed as means ±SE.

Figure 3: Changes in three natural porcine bile acids in perfusate during 24-h perfusion. Perfusate (whole blood) was collected during the perfusion and the supernatant was analysed using gas chromatography mass spectrophotometry. The bile acid concentrations are expressed as percentages of their original value at the beginning of the perfusion (means [5] ±SE).
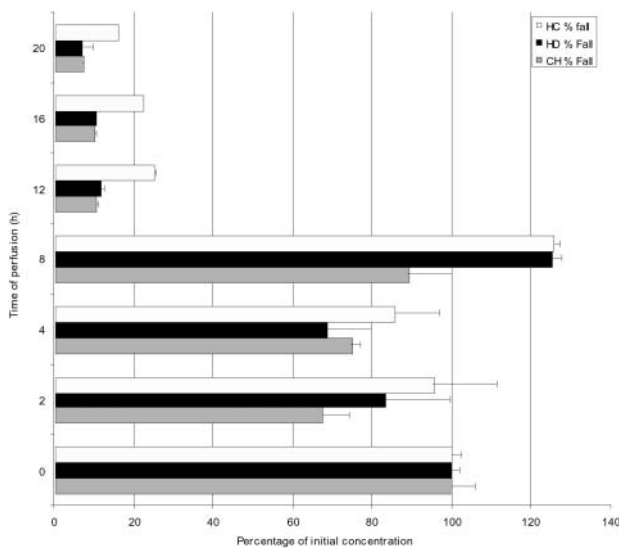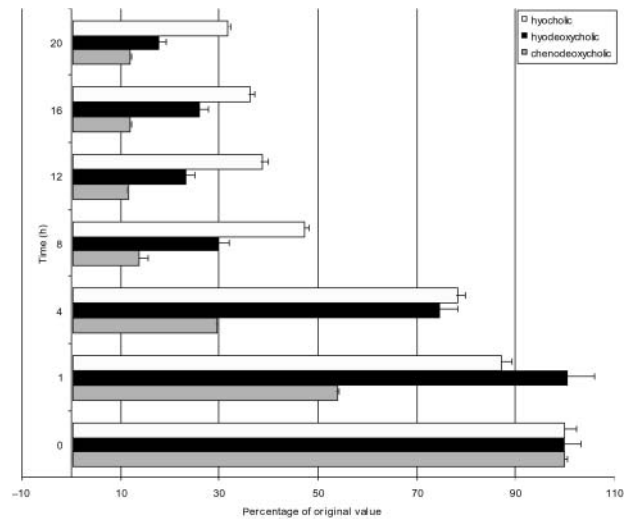

Figure 2: Changes in three natural porcine bile acids in bile during 24-h perfusion. Bile was collected during five perfusions via a silastic tube placed within the common bile duct, the cystic duct having been ligated. The bile acids were measured using gas chromatography mass spectrophotometry on diluted samples, and are expressed as percentages of their original concentration (means [5] ±SE).
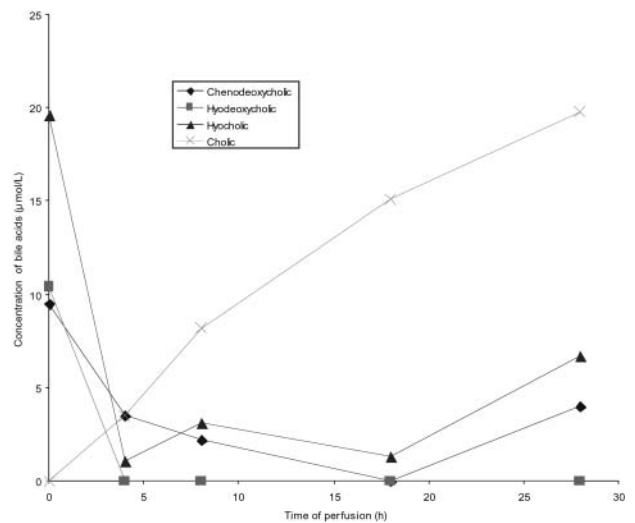

Figure 4: Bile acid concentrations in bile during a supplemented liver perfusion. Bile was collected and analysed by gas chromatography mass spectrophotometry during a 20-h perfusion. Circuit supplemented with continual taurocholate infusion. (1 g/100 mL at 7 mL/h).

from a maximum of 14.8 mL/h (± 0.4) after 6 h (see Figure 1). Towards the end of the perfusion period unsupplemented bile production had dropped to approximately 1.7 mL/h (± 0.03). This was in contrast to the perfusions in which the blood perfusate was supplemented with an infusion of taurocholate (see Figure 1) as a choleretic stimulus to the extracorporeal liver (Group S). Bile production was maintained in this group at approximately 8 mL/h throughout the entire perfusion period.

Analysis of plasma and bile samples of a perfusion from Group U illustrates a fall in all three native porcine bile acids (see Figures 2 and 3). Perfusate levels of chenodeoxycholic, hyodeoxycholic and hyocholic acids dropped to below 50%
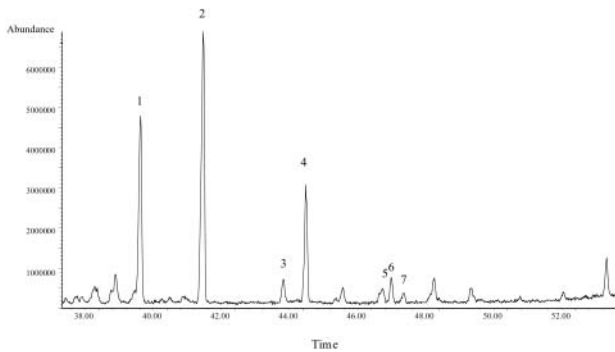
Figure 5: Chromatogram of methyl ester trimethylsilyl ether derivatives of bile acids from perfusate without taurocholate supplementation (Group U). 1 = internal standard, 2 = cholesterol, 3 = chenodeoxycholic acid, 4 = hyodeoxycholic acid, 5 = hyocholic acid, 6 = sitosterol and 7 = iso-fucosterol.
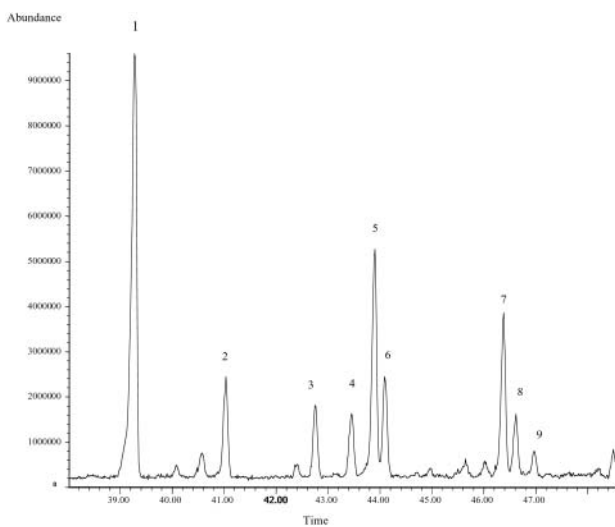

Figure 6: Chromatograph of methyl ester trimethylsilyl ether derivatives of bile acids from bile of a perfusion with taurocholate supplementation (Group S). In contrast to Figure 5, this clearly demonstrates the presence of cholic acid in the bile of a perfused porcine liver. This confirms the capability of a porcine liver to handle foreign bile acids. 1 = internal standard, 2 = cholesterol, 3 = deoxycholic acid, 4 = chenodeoxycholic acid, 5 = cholic acid, 6 = hyodeoxycholic acid, 7 = hyocholic acid, 8 = sitosterol and 9 = iso-fucosterol.

of their original levels by 8-h perfusion, whereas levels within the bile were maintained until 12-h perfusion. Levels in both plasma and bile fell to less than 30% of their original levels by 20 h continuous perfusion. In Group S perfusions, all three native porcine bile acids were seen to decrease in concentration in a similar fashion to that just described (Figure 4). In contrast the concentration of cholic acid rose within the bile in a linear fashion, reaching a peak of 20 μmol/L, confirming that the pig liver was able to absorb a foreign bile acid and excrete it in bile.

Figures 5 and 6 demonstrate chromatograms of trifluoroace-

tyl methyl derivatives of bile acids from perfusate with and without taurocholate supplementation. They allow quantitation of native porcine bile acids, as well as cholic acid in supplemented perfusions, against internal standards (7-ketolithocholic acid in methanol).

No significant increase in red-cell haemolysis occurred in a perfusion supplemented with bile, as compared to an un-supplemented perfusion (p < 0.05).

Discussion

For more than a century extracorporeal liver perfusion has been extensively used to study liver physiology and since the 1960s has been practiced as an experimental and unproven method of clinical liver support in acute liver failure. By modifying and improving the existing circuit design, we have been able to maintain good liver viability during a 48-h sanguineous liver perfusion, and have thus also been able to study this technique as a possible means of organ preservation. We have demonstrated superior preservation utilising a warm perfusion, as compared to the cold University of Wisconsin solution, over a 24-h period in a porcine model (6).

One of the aims of a successful preservation technique is to maintain an organ in a functional state, with no deterioration between procurement and subsequent transplantation. Despite the maintenance of good hepatocyte viability in terms of synthetic, metabolic and excretory liver functions over 40-h sanguineous perfusion, bile production deteriorated inexorably after 10 h from a peak level of approximately 15 mL/h (mean of five perfusions). This was associated with inspissated bile, present both macroscopically and microscopically in all livers (Figure 7).

Several mechanisms account for bile excretion. One requires energy-dependent active biliary secretion by hepatocytes of bile acids, other organic solutes and electrolytes (7). The other is an energy-independent passive diffusion of water and electrolytes (8). The availability of bile salts to the hepatocyte is determined by the enterohepatic circulation, estimated to be 4.4 mmol in the mini-pig (9), and by the synthesis of new bile salts. The latter accounts for only 5% of the total bile salt pool, which compensates for that lost in the faeces.

In this model of isolated perfusion the existing bile acid pool was continually depleted because no such recirculation of the bile acids was incorporated into the extracorporeal circuit. In addition, nutrients were provided to the liver in the form of total parenteral nutrition without fat, and hence very little cholesterol would have been available, necessitating de-novo synthesis. This was borne out by assessing the change in concentration of the three natural bile acids in the pig, chenodeoxycholic, hyodeoxycholic and hyocholic acid within the bile and the plasma of five consecutive liver perfusions.
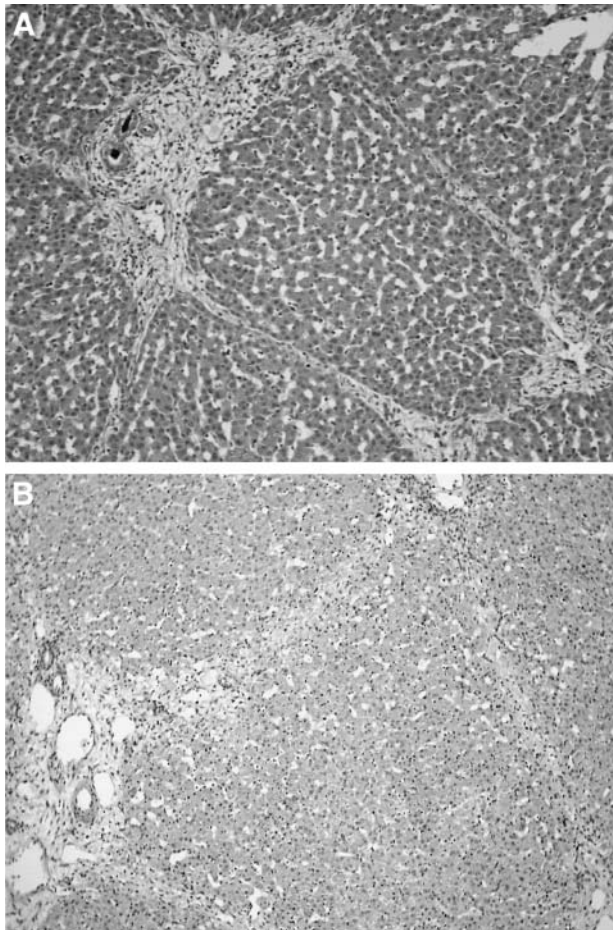
Figure 7: Section (×40) of porcine liver following 40 h unsupplemented (A) and supplemented (B) perfusions stained with H&E. Inspissated bile is clearly visible within the canaliculi of the unsupplemented perfusion in contrast to the perfusion receiving bile salts.
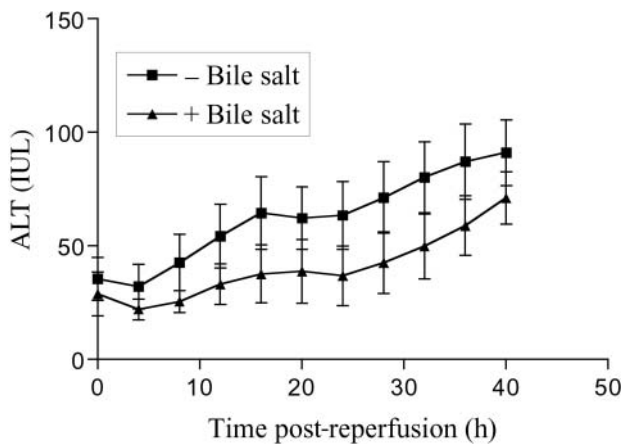

Figure 8: Comparison of alanine aminotransferase levels in livers supplemented with bile salts and those without. No significant difference was discernable between the two groups.

It was clearly demonstrated by the quantification of chenodeoxycholic, hyodeoxycholic and hyocholic acids over time that perfusate depletion preceded bile depletion. It is proposed that this lack of bile salts may be responsible for the reduction of bile production by bile-salt dependent secretion from the canaliculus. It has been shown that the choleretic effects of bile salts in an isolated porcine liver perfusion are similar to those in an intact animal (10), and that in a dual vessel perfusion the secretion of phospholipids and cholesterol (i.e. hydrophobic compounds) into bile is tightly coupled to biliary secretion of bile salts (3).

To counter this it was proposed that we mimic the enterohepatic circulation by infusing taurocholate to maintain a constant bile acid pool and hence continue to stimulate bile production. Porcine bile is known to contain mainly hyocholic, hyodeoxycholic, and chenodeoxycholic acids. In the present study a commercially available taurine conjugate of cholic acid was utilised and as such was not strictly physiological. Results of isolated porcine liver studies, however, have demonstrated that this organ is capable of handling foreign bile salts even during heterologous perfusion (11). Hardison et al. (10) were able to perfuse pig livers *ex vivo* with human blood and demonstrate cholic acid, a bile acid specific to the human, to be a prominent constituent of subsequent bile samples. This was verified in our data (see Figures 4 and 6), where cholic acid was identified in bile samples during a
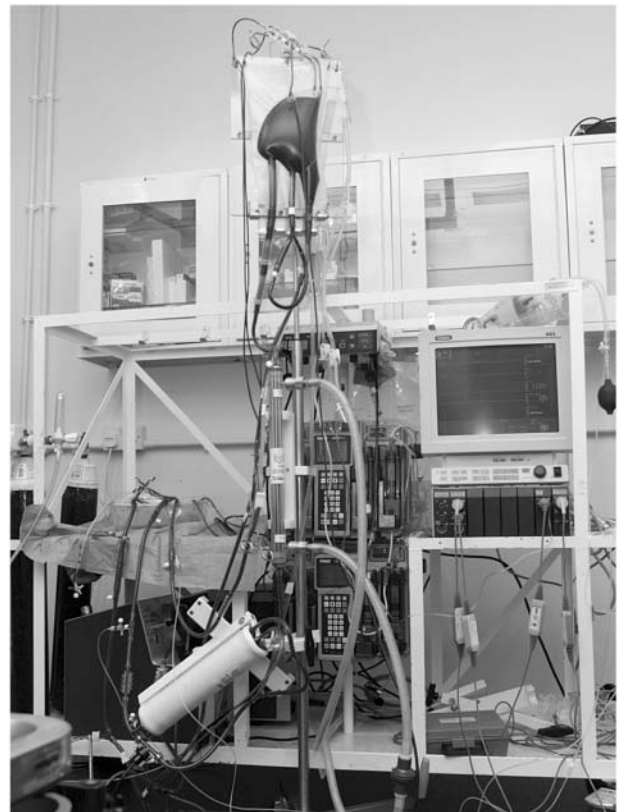

Figure 9: Extracorporeal perfusion circuit.

supplemented perfusion. Previous studies have shown that 5-μmol/min infusion of 1% sodium taurocholate solution can maintain a constant bile acid concentration within the perfusate and hence stimulate bile production to a reasonable level (12): in this series of experiments it was utilised and managed to generate bile production, which was maintained at significantly greater values than unsupplemented perfusions (p <0.05). One further advantageous characteristic of this bile acid is that it is only weakly haemolytic in the presence of erythrocytes (13) and is hence not detrimental in a perfusion that utilises whole blood as the perfusate.

The continued secretion of bile is essential to maintain the functional integrity of the liver. Some endogenous compounds eliminated in bile are toxic to hepatocytes (14) including bile acids, endotoxin, metals and porphyrins. As the continuing presence of these compounds can be harmful, maintenance of bile production during perfusion remains essential. Ursodeoxycholic acid and its taurine conjugate are known to have a hepatoprotective effect in certain cholestatic disorders (15). Foley et al. clearly demonstrated that augmentation of bile production in the isolated *ex-vivo* perfused liver is essential for the optimal excretion of phospholipid, and subsequently in providing an enhanced hydrophobic environment cholesterol (3). His group also established that dual vessel perfusion (via both portal vein and hepatic artery), as utilized in this study, was the optimal method for ensuring hydrophobic toxin secretion.

We were unable to show any significant difference in alanine aminotransferase levels between those livers supplemented with bile salts and those without (see Figure 8). This intracellular enzyme is a marker of global liver injury released following severe and catastrophic hepatocyte injury, and as such is relatively insensitive and was unable to differentiate the two groups studied over a 40-h period.

Several groups have shown that despite several bile acids improving bile production in isolated liver models, they have no demonstrable biochemical or histological attenuation of ischaemia reperfusion injury (16). This is in contrast to Ono et al. (17) who, using higher doses of intravenous tauroursodeoeycholic acid, showed lower levels of myeloperoxidase (representing neutrophil sequestration) activity in liver tissue together with histological evidence of ischaemia-reperfusion attenuation. Although not directly investigated in this study, any mechanism that has been shown to reduce ischaemia-reperfusion injury would seem intuitively to be a worthwhile addition to a circuit designed for improved liver preservation.

We propose to utilise extracorporeal perfusion of the liver as a novel mechanism for normothermic preservation before transplantation. Apart from resetting high-energy phosphate levels and therefore attenuating ischaemia-reperfusion injury, one enormous advantage this may provide is an accurate estimation of organ viability before transplantation and hence a subsequent reduction in levels of primary dysfunction/nonfunction. By optimising bile production and maintaining func-

tional integrity of the biliary apparatus of extracorporeal livers, lengthy normothermic perfusion can be further considered as a possible mechanism for advantageous preservation.

Furthermore, bile flow had been proposed as a reliable index of ischaemic damage and severely impaired bile flow appears to be predictive of poor survival in experimental studies (18, 19). Hence by removing any alternative explanation for poor bile production and by infusing all livers with identical bile salt infusions, any flux in bile production can be directly attributed to liver viability, and thus used as a prediction marker.

Acknowledgments

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