Appendix

Detailed protocols for

1. Perfusion fluid
2. ATP quantification
3. Human primary liver cell isolations
4. In vitro assessment of reactive oxygen species production, apoptosis and necrosis in a model of ischemia-reperfusion.
5. Assessment of donor liver viability

1. Perfusion fluid constitution

We used a perfusion fluid developed by our team for resuscitation of discarded livers. This consisted of 3 to 4 units of group-specific Rhesus-negative donor packed RBCs obtained from the local blood bank, or an equivalent volume of Hemopure (Hemoglobin Oxygen Therapeutics LLC, Cambridge, MA). This was supplemented with 1000mL of 5% w/v human albumin solution (Alburex 5, CSL Behring GmbH, Germany), 30mL sodium bicarbonate 8.4% (B. Braun Medical Limited, UK) and 10ml calcium gluconate 10%. The circuit was loaded with 10,000IU heparin (Wockhardt, UK), 500mg vancomycin (Wockhardt, UK) and 60mg gentamicin (Cidomycin, Sanofi, UK) prior to connecting the liver. Epoprostenol (8µg/hour; Flolan, GlaxoSmithKline, UK) was infused once perfusion had started. Further supplementation with 50mL of 10% v/v Aminoplasmal (B.Braun Medical Limited, UK), 0.2mL Cernevit (Baxter Healthcare Ltd., UK) and 0.1mg phytomenadione (Konakion, Roche Products Ltd, UK) was given during the perfusion. Extra buffering capacity was provided by aliquots of 8.4% sodium bicarbonate to maintain a pH of greater than 7.20 as necessary during the perfusion.

2. Tissue ATP quantification

To quantify ATP content, 100mg of frozen liver tissue was taken and immediately homogenised in 1ml SONOP Buffer (0.372g EDTA in 130ml ddH2O (adjusted to pH 10.9 with NaOH) = 370ml of 96% Ethanol) using the GentleMacs system. Particulates were removed by centrifugation at 13,000xg. The protein concentration was determined in the supernatant with the use of a Pierce BCA Protein Assay kit and the concentration adjusted to 300ug/ml protein with the SONOP buffer. Samples were then diluted 10-fold in 100µM Phosphate buffer and ATP concentration determined was using the ATP Bioluminescent Kit (Sigma FL-AA). Concentrations were determined from a calibration curve on the same plate, corrected for amount of protein and expressed as nM/g protein.

2. Protocols for isolation of primary human hepatocytes, sinusoidal endothelial cells and biliary endothelial cells

Human hepatocytes were isolated from liver wedges using a collagenase perfusion technique that we have published previously. Following perfusion, centrifugation was utilized to isolate a highly pure population of human hepatocytes that were plated on rat tail collagen for 72 hours in Williams E media prior to use in experiments.

Human Sinusoidal Endothelial Cells (HSEC) were isolated from liver tissue as previously described. Parenchymal cells were collected after collagenase digestion of liver slices and purified by density gradient centrifugation over Percoll. Endothelial cells were isolated from the resultant heterogeneous cell mixture by positive immunomagnetic selection using antibodies raised against CD31 (Clone JC70A,

Dako, Denmark) according to the manufacturer's protocol. All endothelial cells were maintained in complete media comprising Human Endothelial-Serum Free Media basal growth medium (Invitrogen, UK) containing 104U/mL penicillin and 10µl/mL streptomycin, 10ng/mL epidermal growth factor (R&D Systems, UK), 10µg/mL hydrocortisone (Sigma-Aldrich, UK), and 10% heat-inactivated human serum (TCS Biologicals, UK).

Biliary epithelial cells (BEC) were isolated from liver tissue. The liver (30g) was finely diced and incubated with collagenase type 1A (Sigma, St. Louis, MO, USA). The digest was layered onto a 33% and 77% iso-osmotic Percoll gradient and centrifuged at 500g for 30 minutes. The interface layer was collected, washed three times in phosphate buffered saline and incubated with the BEC-specific mouse antihuman monoclonal antibody to human embryonic antigen 125 (TCS Biologicals Ltd., Botolph Claydon, Bucks, UK). BEC were positively selected by incubating with antimouse IgG1-coated Dynabeads (ThermoFisher Scientific, UK) and by magnetic separation. The cells were cultured in plating media containing: Hams F12, Dulbecco's Eagle medium; heat-inactivated fetal calf serum (10% v/v); penicillin, streptomycin (100ng/mL), glutamine (2mM); epidermal growth factor (10ng/mL); hydrocortisone (2µg/mL); choleratoxin (10ng/mL); tri-iodo-thyronine (2nM); insulin (0.124IU/mL). After 1–2 days in culture, the medium was exchanged for media containing 5% v/v fetal calf serum and 10ng/mL hepatocyte growth factor (R&D Systems Ltd., UK).

4. In vitro model of ischemia-reperfusion injury and assessment of reactive oxygen species production, apoptosis and necrosis

Cells kept at ambient oxygen concentrations were designated as being in normoxia. Those exposed to 0.1% O_2 for 24 hours were classified as being in hypoxia

akin to ischemia. Those cells that were placed into hypoxia for 24 hours and then exposed to ambient oxygen for 24 hours were classified as having undergone hypoxia-reoxygenation (H-R) akin to reperfusion. This experimental model replicates the IRI environment to which the liver cells are exposed during transplantation[33].

Reactive oxygen species (ROS) production, apoptosis and necrosis were determined using a three-color assay. 2,7-dichlorofluorescin reacts with intracellular ROS to produce a signal proportional to the intracellular concentration of ROS. Annexin-V is a specific marker of apoptosis due to its recognition of phosphatidylserine on the outer membrane of the cell membrane when the cell has committed to apoptosis. 7-Aminoactinomycin D (7-AAD) is a vital dye and only binds to DNA once there is disruption of the cell wall and is thus a marker of necrosis. Following exposure to the IRI model all cells were stained with each dye alone and in combination. Using the flow cytometry protocol and design we have detailed previously we set gating strategies that enabled the assessment of the above 3 parameters.

5. Assessment of donor liver viability

For a donor liver to have been considered viable and suitable for transplantation, it needed to meet the following criteria – Metabolism of lactate level to less than or equal to 2.5 within 2 hours or convincing evidence of bile production, in combination with two or more of the following: arterial flow >150ml min and portal flow >500ml/min, maintenance of pH >7.30 and homogenous liver perfusion with soft parenchyma consistency.