Hyperbaric oxygen therapy reduces the severity of ischaemia, preservation and reperfusion injury in a rat model of liver transplantation

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Abstract

Background: Approaches to increase organ availability for orthotopic liver transplantation (OLT) often result in the procurement of marginal livers that are more susceptible to ischaemia, preservation and reperfusion injury (IPRI).

Methods: The effects of post-OLT hyperbaric oxygen (HBO) therapy on IPRI in a syngeneic rat OLT model were examined at various time-points. The effects of IPRI and HBO on hepatocyte necrosis, apoptosis, proliferation, and sinusoidal morphology and ultrastructure were assessed.

Results: Post-OLT HBO therapy significantly reduced the severity of IPRI; both apoptosis [at 12 h: 6.4 ± 0.4% in controls vs. 1.6 ± 0.7% in the HBO treatment group (p < 0.001); at 48 h: 2.4 ± 0.2% in controls vs. 0.4 ± 0.1% in the HBO treatment group (p < 0.001)] and necrosis [at 12 h: 18.7 ± 1.8% in controls vs. 2.4 ± 0.4% in the HBO treatment group (p < 0.001); at 48 h: 8.5 ± 1.3% in controls vs. 3.4 ± 0.9% in the HBO treatment group (p = 0.019)] were decreased. Serum alanine transaminase was reduced [at 12 h: 1068 ± 920 IU/l in controls vs. 370 ± 63 IU/l in the HBO treatment group (p = 0.030); at 48 h: 573 ± 261 IU/l in controls vs. 160 ± 10 IU/l in the HBO treatment group (p = 0.029)]. Treatment with HBO also promoted liver regeneration [proliferation at 12 h: 4.5 ± 0.1% in controls vs. 1.0 ± 0.3% in the HBO treatment group (p < 0.001); at 48 h: 8.6 ± 0.7% in controls vs. 2.9 ± 0.2% in the HBO treatment group (p < 0.01)] and improved sinusoidal diameter and microvascular density index.

Conclusions: Hyperbaric oxygen therapy has persistent positive effects post-OLT that may potentially transfer into clinical practice.

Keywords
ischaemia–reperfusion, transplant, resection, liver, transplant outcomes

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Introduction

Approximately 800 000 people die from end-stage liver disease around the world each year.1 Orthotopic liver transplantation (OLT) is widely accepted as the definitive treatment in end-stage liver disease, selected liver malignancies and acute liver failure. The major limitation of liver transplantation is the availability of suitable donor organs. Increasing demand and rising mortality in patients awaiting transplantation have led to a number of techniques that increase the availability of donor organs.2-5 However, these result in the procurement of organs with marginal functional capacity. Marginal donor organs are more susceptible to the effects of ischaemia, preservation and reperfusion injury (IPRI), which leads to an increased incidence of dysfunction and organ loss following transplantation.6

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Ischaemia, preservation and reperfusion injury is a complex process characterized by intracellular energy depletion leading to activation of the innate immune system, which ultimately affects parenchymal and non-parenchymal cells of the donor organ. Therapies targeting specific aspects of IPRI have been developed and shown to ameliorate such injury in experimental and some clinical settings. Their effects have been demonstrated predominantly when applied prior to the onset of IPRI. Hyperbaric oxygen (HBO) therapy is one such modality that appears to simultaneously impact on multiple aspects of IPRI and to be effective when applied after its onset. The majority of evidence arises from in vitro studies and in vivo studies of warm ischaemia–reperfusion injury. The effects of HBO in a more realistic model of OLT, particularly in the presence of cold preservation as seen in clinical liver transplantation, remain unknown. This study investigates the impact of HBO therapy in a rat model of OLT, when HBO treatment is delivered after the onset of IPRI.

Materials and methods
In this study, irreversible hepatocyte injury was assessed according to measurements of hepatocyte necrosis and apoptosis. Hepatocyte proliferation was measured as a potential surrogate marker of injury. Liver biochemistry was analysed at the relevant timepoints. Changes in sinusoidal architecture and their relation to hepatocyte injury were assessed with scanning electron microscopy (EM) analysis of microvascular resin casts. Effects on endothelial cell and hepatocyte morphology were determined using transmission EM.

Animals and study design
Male Lewis rats (weighing 250–350 g; Laboratory Animal Services, University of Adelaide, Adelaide, SA, Australia) were housed in a temperature- and humidity-controlled room under a constant 12 : 12 h light : dark cycle. Six animals were randomly assigned to each group for this study. Four animals per group were used for scanning EM analysis. Animals had free access to food and water until surgery. All studies were conducted with the approval of the Austin Animal Ethics Committee and in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

The severity of IPRI (hepatocyte necrosis, apoptosis and proliferation) was assessed in rat treatment and control groups. The effects of HBO therapy were also investigated in normal rat liver. In addition, animals underwent immediate OLT without a preservation period and were assessed at 48 h. Outcomes in this group were compared with those in rats that underwent OLT with 24-h preservation to investigate the effects of prolonged preservation on IPRI. The temporal progression of liver injury severity was investigated in animals receiving livers with a preservation time of 24 h and killed at specific endpoints of 6 h, 12 h, 24 h, 48 h and 7 days after OLT. The effects of IPRI and HBO therapy on liver sinusoidal architecture were assessed by scanning and transmission EM at 48 h after OLT. Treatment with HBO began within 3 h of OLT as this regimen has been shown to significantly reduce IPRI in a rat model. Rats in the HBO treatment groups received two (12 h), three (24 h), five (48 h) or 15 (7 days) treatment sessions.

Donor hepatectomy and storage procedure
An orthotopic syngeneic liver transplant model with a 24-h period of preservation in University of Wisconsin (UW) solution was chosen as the model for IPRI in this study as it is highly reproducible and features the same mechanisms and pathophysiology as human liver transplantation. The donor hepatectomy procedure has been described previously. Briefly, male Lewis rats were anaesthetized with 1.5% isoflurane (David Bull Laboratories Pty Ltd, Mulgrave, Vic, Australia) and continuous anaesthesia was maintained using face masks. Laparotomy was undertaken, the liver mobilized and the vascular system flushed with heparin prior to perfusion with cold UW solution. Flushed livers were placed in 30 ml of UW solution at 4 °C and stored for 24 h at 4 °C prior to transplantation.

Liver transplantation
Recipient rats were anaesthetized and subjected to midline laparotomy. Recipient hepatectomy was performed and recipient vessels prepared for transplantation. Donor livers were removed from the preservation solution and OLT performed using an arterialized technique previously described by Howden et al. The anhepatic time during the transplantation procedure was limited to <15 min in order to produce an extremely low mortality rate despite the severity of liver injury.

Administration of HBO therapy
Rats were recovered from anaesthesia and transferred to the HBO chamber within 3 h of liver reperfusion. Treatment with HBO was continued twice daily at 12-h intervals until the selected endpoints. Each treatment session (153 kPa with 100% oxygen) was delivered for 90 min, which is the standard treatment time adopted for rodent experiments by this study group.

Collection of liver samples
The study endpoints were defined as 12 h, 24 h and 48 h after reperfusion. Rats were anaesthetized with an intraperitoneal injection of 400 μl ketamine (100 mg/kg) (Pfizer Australia, West Ryde, NSW, Australia) and xylazine (10 mg/kg) [Troy Laboratories (Australia) Pty Ltd, Glenendenning, NSW, Australia]. The liver was removed from the anaesthetized rat, weighed and fixed in 10% buffered formalin (Sigma-Aldrich Corp., St Louis, MO, USA). Because of the hepatic anatomy and the variable sizes of the...
rat liver lobes, which may influence the degree of IPRI, each liver was divided into three parts for assessment: the median, left and right lobes.

**Assessment of hepatic necrosis**

After 48 h of fixation, livers were preserved in 50% ethanol and each lobe was sliced into 1.5-mm-thick sections using a multiblade fractionator. Two random slices from each liver lobe (right, median and left) were processed for paraffin blocks and stained with haematoxylin and eosin. Images from each section of liver were captured using a randomized pattern to ensure that the areas captured were representative of the entire tissue slice. Each image was then analysed to determine the area of necrosis using an image analysis program (Image Pro Plus; CyberMedia, Perth, WA, Australia), in which the areas of necrosis and subsequently the total liver areas were traced electronically. The areas were used to calculate the percentage of necrosis as a function of the total liver area and were determined for each lobe for all livers. The mean percentage of necrosis in each liver lobe was expressed as the mean ± standard error of the mean (SEM) and was used to indicate the extent of IPRI in the control and treatment groups. This method has been previously validated by this study group and the levels of injury shown in the individual lobes are considered to be representative of an entire rat liver in this model.

**Immunohistochemistry**

Paraffin-embedded liver tissue sections (4 μm) were stained with monoclonal antibodies using an indirect polymer immunoperoxidase technique. The tissues were stained for proliferating cells (Ki67) and apoptotic cells (active caspase-3; Sapphire Bioscience Pty Ltd, Waterloo, NSW, Australia). One random slice from each of the three liver lobes (right, median and left) in each rat was stained. Specific binding of primary antibodies was detected using a polymer detection system for either rabbit or mouse primary antibodies. (DAKO Envision Plus; Dako Australia Pty Ltd, Botany, NSW, Australia) using standard procedures. The reaction was developed using 3,3-diaminobenzidine tetrachloride (Sigma-Aldrich Australia Pty Ltd, Castle Hill, NSW, Australia) and hydrogen peroxide (H2O2), counterstained with Mayer’s haematoxylin (Sigma-Aldrich Australia Pty Ltd), dehydrated, and mounted in DPX mountant (Merck Pty Ltd, Kilsyth, Vic, Australia).

**Quantitative assessment of immunohistochemical staining**

Staining of proliferating hepatocytes and apoptotic cells was analysed using Image Pro Plus®. In these analyses, the observer was blinded to the treatment group. Twelve random, non-overlapping images from each liver lobe were captured at ×80 magnification for each slice. Quantitation of stained cells was performed by identifying and counting positively stained cells. Non-stained cells were also counted. Counts were expressed as percentages of the total number of hepatocytes determined for each lobe for all livers. The percentage of proliferating or apoptotic cells in each liver lobe was expressed as the mean ± SEM and used to compare findings in control and treatment groups.

**Liver biochemistry**

Blood was collected at the respective endpoints by cardiac puncture to produce serum samples. Minimum quantities of 160 μl of serum from each sample were placed in separate microcentrifuge tubes and stored at −20 °C. The samples were analysed for liver biochemistry [bilirubin, alanine transaminase (ALT), alkaline phosphatase (ALP), γ-glutamyl transferase (GGT)] using a timed endpoint Diazo method, an enzymatic rate method and a kinetic rate method to measure the levels of the respective factors (Department of Anatomical Pathology, Austin Health, Melbourne, Vic, Australia).

**Microvascular resin casting and scanning EM analysis**

Microvascular resin casting was undertaken as previously described. Briefly, left thoracotomy was performed in anaesthetized rats and the left lung mobilized to the right to expose the thoracic aorta. A catheter was inserted caudally into the thoracic aorta to flush the circulation with a mixture of warm (37 °C) saline solution (0.9% NaCl), 60 mg/l polyvinyl-pyrolidone, heparin (10 units/ml) and papaverine (0.25 ml/l) (at a constant pressure of 120 mmHg) and vented through an incision in the heart. When the effluent was clear (approximately 100 ml of saline infusion), the pressure was released. A resin mixture was then infused (at a pressure of 160 mmHg). The resin was allowed to polymerize in situ overnight and the liver carefully removed. Organic tissue was dissolved using several changes of 20% potassium hydroxide (KOH). The resulting resin casts were rinsed to remove debris, and frozen in milliQ water to enable the casts to be cut into smaller pieces using a hand electric rotary saw (Dremel Moto Tool Co., Racine, WI, USA). The casts were then prepared by standard methods for scanning EM. Each cast was examined under scanning EM and images of randomly selected areas were captured in order to obtain measurements of sinusoidal diameters and microvascular index.

**Measuring sinusoidal diameters**

Analysis of the microvascular images captured using scanning EM was conducted using an image analysis program (Image Pro Plus®). Images were calibrated for assessment using the magnification bar on each image. A grid mask was then placed over the image and the largest vessel in each grid was measured using a drag line. Analysis was performed by measuring 20 sinusoidal diameters from images at ×1000 magnification. Sixteen images from each liver were analysed and data expressed as the mean ± standard deviation (SD) for each group. Sinusoidal diameters were measured only in regions without large empty spaces corre-
sponding to areas of coagulative necrosis to represent the microvascular changes occurring in the surviving viable regions of the liver.

**Assessing changes to the microvascular density index**
The microvascular density index (MDI) was measured using scanning EM images (×1000 magnification) of resin casts from rats in the control and treatment groups at 48 h after transplantation. Images were captured in locations where no physical damage was present and the lobular architecture was maintained. Image Pro Plus® was used to apply a colour mask to the area occupied by all the vessels in the foreground of each image. A measuring tool was used to determine the area occupied by vascular structures and the MDI was calculated as the percentage of the area of vascular structures divided by the area of image sampled. Individual MDIs were used to determine the mean MDI in each treatment group.9

**Transmission EM**
Tissue samples from the left liver lobe were collected from the control and HBO treatment groups at 48 h after OLT and immediately cut into 1-mm³ pieces and fixed in 2.5% gluteraldehyde in phosphate buffer overnight prior to being placed in phosphate buffered saline. Transmission EM was performed on these tissues after the routine preparation of sections using standard techniques to obtain a descriptive analysis of the cellular morphological changes seen in liver transplantation and HBO treatment.

**Statistical analysis**
All data (including graphical representations) are expressed as the mean ± SEM unless otherwise stated. Statistical analysis was conducted in spss Version 13.0 (SPSS, Inc., Chicago, IL, USA) using both parametric and non-parametric tests as appropriate in normality testing. Animal numbers were based on previous studies conducted by this group and were calculated to achieve 95% confidence with a power of 0.8. Data were tested for normality using descriptive statistics such as skewness and kurtosis of the data, and the Kolgomorov–Smirnov test. Animal numbers were based on previous studies conducted by this group and were calculated to achieve 95% confidence with a power of 0.8. Data were tested for normality using descriptive statistics such as skewness and kurtosis of the data, and the Kolgomorov–Smirnov test. An analysis of variance (ANOVA) was performed for normally distributed data and post hoc analysis was performed if significance was determined [least significant difference (LSD) or Scheffe’s procedure]. Kruskal–Wallis followed by Mann–Whitney rank sum tests were used to analyse data that were not normally distributed. A P-value of ≤0.05 was deemed to indicate statistical significance in all analyses.

**Results**

**Effects of HBO therapy on normal liver**
Livers from normal rats were compared with livers from animals exposed to two doses of HBO therapy and differences in hepatocyte necrosis, apoptosis and proliferation were analysed. Neither normal livers nor HBO-treated livers demonstrated any measurable evidence of coagulative necrosis. The baseline rate of hepatocyte apoptosis in normal livers was low in all lobes (left, median and right lobes) and was not altered by HBO therapy (0.1 ± 0.0% vs. 0.1 ± 0.0%; p > 0.05) (Fig. S1, online). Baseline hepatocyte proliferation in all three normal liver lobes assessed was unchanged by HBO therapy (0.9 ± 0.2% vs. 1.2 ± 0.2%; p > 0.05) (Fig. S2, online).

**Effects of prolonged preservation on IPRI severity**
Outcomes in rats that underwent immediate OLT with minimal cold preservation were compared with those in rats that underwent OLT using donor organs preserved for 24 h (Fig. 1a). Rats in both groups were killed 48 h after OLT and the severity of IPRI assessed by comparing hepatocyte necrosis, apoptosis and cell proliferation. Prolonged cold preservation resulted in significantly greater hepatocyte necrosis, apoptosis and proliferation compared with minimal cold preservation (p < 0.001) (Fig. 1a). These differences were similar across individual lobes (data not shown).

**Effects of IPRI on development of hepatocyte necrosis, apoptosis and proliferation**
Prolonged organ preservation and subsequent OLT produced severe IPRI (Fig. 1b, Fig. S3). Coagulative hepatocyte necrosis was apparent at 6 h after reperfusion, peaked at 12 h and slowly declined to 24 h and 48 h (Fig. 1b). Necrosis was no longer evident by day 7. Hepatocyte apoptosis followed a similar pattern, increasing rapidly to 6 h, peaking at 12 h and declining to baseline levels by day 7. Hepatocyte proliferation in response to IPRI increased slowly, peaked at 48 h and remained elevated until day 7.

**Effects of HBO therapy on hepatocyte necrosis in the left, median and right liver lobes**
Coagulative necrosis in the individual lobes of control livers showed that the left lobe developed a significantly greater degree of necrosis at 12 h compared with the median (p < 0.001) and right (p < 0.001) lobes (Fig. S4). Similar patterns were seen at both 24 h and 48 h. Therapy with HBO significantly decreased the development of hepatocyte necrosis at all time-points (Fig. 2). The difference was most marked at 12 h (p < 0.001), but remained significant at 24 h and 48 h (p < 0.001 and p < 0.05, respectively). The effects were statistically significant in each individual lobe at these time-points except in the right lobe at 24 h and the left lobe at 48 h, when they approached but did not reach significance (Fig. S4). At 7 days after OLT, necrosis was no longer evident in either the control or treatment groups.

**Effects of HBO therapy on hepatocyte apoptosis in the left, median and right liver lobes**
The percentage of hepatocytes undergoing apoptosis demonstrated a 60-fold increase from a baseline normal value of 0.10 ± 0.0% by 12 h (p < 0.001) (Fig. 2), which progressively reduced at the 24-h and 48-h time-points. The left lobe developed a higher rate of apoptosis than the median and right lobes (Fig. S4). The
rate of apoptosis in HBO-treated animals increased slightly but significantly at 12 h compared with normal liver \((p < 0.001)\) and was found to progressively reduce at 24 h and 48 h after OLT \((p < 0.001 \text{ and } p < 0.001, \text{ respectively})\) (Fig. 2). This difference was also reflected in the individual lobes at each time-point (Fig. S4). At day 7 after OLT, the rate of apoptosis had significantly reduced to similar levels in both the control and treatment groups.

**Effects of HBO therapy on hepatocyte proliferation in the left, median and right liver lobes**

The rate of hepatocyte proliferation following OLT remained unchanged at 6 h and 12 h compared with normal liver \((P = 0.7)\). However, by 24 h, a progressive but significant increase became apparent and remained at 48 h \((p < 0.05)\) (Fig. 2). Individual lobes
demonstrated similar patterns of proliferation at each time-point (Fig. S4). Therapy with HBO resulted in a rapid increase in hepatocyte proliferation. This was significantly increased as early as 12 h and continued to increase rapidly at 24 h and 48 h ($p < 0.001$). At day 7, hepatocyte proliferation remained significantly elevated in control rats but had returned to normal levels in HBO-treated rats ($p < 0.001$).

### Changes in liver biochemistry with IPRI and HBO therapy

Alterations in serum ALT occurred early after OLT in IPRI control rats. Serum ALT increased significantly at 6 h and peaked at 12 h (1068 ± 920 IU/l) after OLT, declining to normal levels at day 7 (Fig. 3). Therapy with HBO significantly attenuated the ALT rise at the 12-h, 24-h and 48-h time-points ($P = 0.03$). By contrast, serum bilirubin, ALP and GGT were minimally elevated at the 24-h time-point, but rose rapidly to 48 h in the IPRI controls. In the HBO treatment group, serum bilirubin, ALP and GGT levels were found to be slightly higher than in controls in the first 24 h, but reduced significantly at 48 h for ALP ($P = 0.03$) and GGT ($P = 0.03$). Serum bilirubin reduced from 45.0 ± 19.0 μmol/l in controls to 8.0 ± 2.6 μmol/l with HBO treatment over this period; however, sample variation did not allow statistical significance to emerge ($P = 0.1$).

![Figure 3](image)

**Figure 3** Effects of hyperbaric oxygen (HBO) therapy on liver biochemistry. Serum alanine transaminase (ALT) levels peaked at 12 h and progressively reduced. Therapy with HBO significantly reduced the rise in ALT at all time-points. Serum bilirubin, alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) followed a common trend, demonstrating an increase only at 48 h. Therapy with HBO significantly reduced this rise at the 48-h time-point. All levels had normalized by day 7 (data not shown). Error bars represent the standard error of the mean. *$p = 0.03$

### Scanning EM features of liver sinusoids after IPRI and HBO treatment

Scanning EM images of resin casts from livers of normal rats demonstrated the complete filling of sinusoids organized in a typical hepatic lobular arrangement. At low magnification (×200), cross-sections of vascular casts revealed hepatic sinusoids radiating from the portal venous branches within portal triads and converging towards the central venules (Fig. 4a). At higher magnification (×1000), sinusoids were densely arranged, complete and had multiple branches (Fig. 4d).
Figure 4 Effects of hyperbaric oxygen (HBO) therapy on liver microvasculature. (a–f) Scanning electron microscopy images of microvascular casts from normal rat liver at (a, d) baseline, (b, e) 48 h after orthotopic liver transplantation (OLT) and (c, f) 48 h after OLT with HBO therapy. The architecture in the normal liver is seen to be significantly disrupted in the OLT control group (b, e). Reductions in diameter, blind-ended sinusoids and extravasation of casting material indicate microvascular disruption. Therapy with HBO maintained normal structure and prevented disruptive changes. S, liver sinusoids; CV, central vein; P, portal triad; white arrows, absence of filling showing blockage; B, blind-ended vessels; E, extravasated casting material. [Original magnification: (a–c) $\times 150$, (d–f) $\times 1000$.] (g, h) Effects of HBO on sinusoidal diameter and microvascular density index. The OLT control livers demonstrated a significant reduction in mean sinusoidal diameter ($8.9 \pm 0.9 \mu m$ vs. $10.8 \pm 0.2 \mu m; p < 0.01$) and density index (percentage of vascularity) ($68.6 \pm 1.0\%$ vs. $59.9 \pm 1.4\%; p < 0.001$) at 48 h compared with normal livers. Therapy with HBO prevented these changes in mean sinusoidal diameter ($10.3 \pm 0.1 \mu m$ vs. $10.8 \pm 0.2 \mu m; p > 0.05$) and density index ($68.6 \pm 1.0\%$ vs. $68.8 \pm 0.9\%; p > 0.05$) compared with normal liver. All groups: $n = 4$; 16 images at each time-point for each liver. Error bars represent the standard error of the mean.
Control rat livers at 48 h demonstrated areas of incomplete filling, particularly in perivenular regions of the hepatic lobule (Fig. 4b). Sinusoids were seen to end blindly, suggesting microvascular occlusion. An obvious and typical feature was a reduction in vessel density represented by clusters of discontinuous and blind-ended sinusoids (Fig. 4e). Analysis of multiple scanning EM fields at low magnification ($\times 125$) revealed areas lacking in microvascular filling, indicative of necrosis (Fig. 4b).

Exposure to HBO therapy appeared to prevent these changes. Although HBO reduced sinusoidal density, sinusoids remained patent and continuous. On low-power imaging, it was not possible to differentiate between images from normal and HBO-treated animals (Fig. 4a, c). Rat livers treated with HBO demonstrated intact microvascular architecture (Fig. 4c, f). Cross-sections of resin casts showed normal hepatic architecture with no areas of unfilled vessels or extravasation of casting material, and vessels were seen to radiate towards the portal vein, as in control livers.

**Effects of IPRI and HBO therapy on microvascular diameter**

The mean diameter of liver sinusoids in normal rat liver was $10.8 \pm 0.2 \mu m$. This decreased significantly at 24 h after OLT and even further by 48 h ($p < 0.01$). The effect of HBO therapy in IPRI on sinusoidal diameter assessed at the 48-h time-point showed that HBO therapy prevented the decrease in microvascular diameter so that no significant difference in this parameter emerged in a comparison with normal rat liver ($p > 0.05$). At the 48-h time-point, sinusoidal vessels in HBO-treated livers were significantly greater in diameter than those in controls ($p < 0.05$) (Fig. 4g).

**Effects of IPRI and HBO therapy on MDI**

The mean MDI of normal rat liver was determined to be $68.6 \pm 1.0\%$. At 48 h post-OLT, mean MDI was markedly reduced ($p < 0.001$) (Fig. 4h). Treatment with HBO reversed the reduction in MDI after OLT so that mean MDI in HBO-treated rat liver was found to mirror that in normal liver.

**Transmission EM of liver sinusoids and effects of IPRI and HBO treatment**

In normal rat livers, hepatocytes with distinct intracellular organelles were seen. Sinusoidal endothelial cells were seen to have clear margins and thin cytoplasm with normal nuclear morphology (Fig. 5a). At high magnification, endothelial fenestrae were present and were visualized as discontinuous pores. Mitochondria and rough endoplasmic reticulum were seen. Red blood cells were seen traversing the sinusoidal spaces.

At 48 h post-transplantation, significant ultrastructural changes were visible. The general architecture of the hepatic lobules was somewhat retained, although a range of degenerative changes were apparent. The hepatocyte microvilli were reduced in number and completely absent in some areas. Extensive vacuolization within hepatocytes was also widely seen. Lysosomes were more abundant than in normal hepatocytes. Mitochondria were often swollen, rounded and surrounded by fragmented rough endoplasmic reticulum (Fig. 5b). Changes in endothelial cells were heterogeneous. Many endothelial cells appeared swollen and detached, and were located within the sinusoidal lumen. Widening of the endothelial fenestrae was also more prominent (Fig. 5b).

Liver tissues from HBO-treated OLT rats demonstrated evidence of improvement in cellular architecture and ultrastructure compared with control livers. The lobular arrangement of hepatocytes was well preserved and showed little evidence of vacuoles. However, hepatocytes contained an increased number of lysosomes. The mitochondria displayed reduced swelling and the rough endoplasmic reticulum was more organized than that seen...
in controls. Normal morphological nuclear structure was also observed. Microvilli extending from the hepatocytes into the space of Disse were present, although absent from some areas. The majority of endothelial cells retained normal ultrastructure, although some remained swollen. By contrast with the control group, no endothelial cells were seen to be detached or lying within the sinusoidal lumen and their long elongated nuclei were seen to spread along the length of the sinusoid (Fig. 5c). Some discontinuity along the sinusoidal lining and widening of fenestrae were apparent, but the severity of these changes appeared to be significantly less than in the control group.

Discussion

Orthotopic liver transplantation is the only potentially curative therapy in end-stage liver disease, selected liver malignancies and fulminant hepatic failure. Rising demand for OLT has resulted in an increase in the use of donor organs in which functional capacity is reduced for a variety of reasons. These marginal donor organs appear to be more susceptible to post-OLT dysfunction.20 The major contributor to post-OLT organ dysfunction is IPRI. The implementation of viable therapeutic strategies to minimize IPRI would not only improve outcomes of OLT, but might potentially increase the safety of using marginal donor organs.

Ischaemia, preservation and reperfusion injury is a complex process involving interactions among a wide variety of donor and recipient cell types, including hepatic parenchymal and non-parenchymal cells and constituents of the innate and adaptive immune systems. A number of targeted therapies have demonstrated amelioration of IPRI in mostly animal studies and some clinical studies. The majority of in vivo animal studies evaluating these therapies used a warm ischaemia–reperfusion model. These are supplemented by in vitro studies mimicking similar conditions by providing an environment of hypoxia and hypoglycaemia.21 However, these therapies generally target only one component of this complex process and usually require application prior to the onset of IPRI. Inhibition of Kupffer cells by glycine or gadolinium reduces severe IPRI with sequential warm ischaemia, cold preservation and warm reperfusion and is representative of conditions in human OLT. These experiments demonstrate the efficacy of HBO therapy in reducing IPRI in an animal model that closely reproduces the pathophysiological changes that occur in clinical transplantation.

Despite the importance of evaluating the degree of liver injury after OLT, the assessment of severity of IPRI remains an inexact science. The most common techniques used to quantify IPRI are liver biochemistry, bile production, cytokine release from tissue injury, histopathological scoring systems and overall survival. This study used hepatocyte cell death, indicated by necrosis and apoptosis, as a marker of the severity of IPRI. A quantitative stereological technique was applied to areas of necrosis to allow objective measurements in preference to the commonly used subjective histopathological scoring systems. This technique has been used extensively in the present study group’s department to determine the percentage volume of liver metastases.31 Given previous findings that HBO may divert the cell death pathway from necrosis to apoptosis, the quantification of both of these processes was deemed essential. Serum bilirubin, ALT, ALP and GGT were also measured as markers of liver injury at all endpoints in keeping with standard practice. In this OLT model, hepatocyte injury peaked at 12 h after reperfusion, as evidenced by maximum necrosis, apoptosis and serum ALT levels, which then progressively reduced. Serum bilirubin, ALP and GGT remained slightly elevated in the first 24 h and demonstrated a rapid rise by 48 h. All serum biochemistry had normalized by day 7. An interesting phenomenon was the consistently higher severity of injury in the left lobe of the liver. This effect may be associated with the fact that, in the rat, the left lobe represents half of the total liver volume. Alternatively, it may reflect unique hepatic blood supply or flow characteristics. This finding reinforces the importance of assessing several different areas within each liver when investigating IPRI in the rat model, or consistently assessing the left lobe in order to determine the maximum severity.

The application of HBO delivered in the post-OLT setting significantly reduced the severity of IPRI. This was seen across all liver lobes and was demonstrated by a major reduction in both apoptosis and necrosis, along with improvements in serum biochemistry. It is clear that HBO therapy acts by limiting the process of IPRI, not simply by altering the cell death pathway, as both necrosis and apoptosis were significantly reduced; these findings support recent research focusing on HBO therapy as a molecular switch that acts at the cellular level.32 In vitro studies recently showed the ability of HBO to inhibit cell surface membrane-bound guanidyl cyclase on neutrophils.33 Activation of this enzyme is an essential prerequisite for neutrophil expression of β-integrins, which play an integral and early role in the process of
IPRI. It appears that HBO treatment leads to an increase in reactive oxygen and reactive nitrogen species, which promotes the excessive S-nitrosylation of β-actin. The high concentration of myeloperoxidase in polymorphonucleocytes (PMN) is probably the reason why this phenomenon is seen specifically in neutrophils and not in other leukocytes. This study group has preliminary data that suggest HBO therapy acts on PMNs in this model by reducing the recruitment of PMNs within liver tissue. The impact and mechanisms of action of HBO therapy on other non-parenchymal cells remain to be investigated.

Emerging evidence also strongly suggests that HBO therapy promotes liver regeneration in the presence of liver injury. Therapy with HBO has been shown to stimulate hepatocyte proliferation after liver resection in animal and, more recently, human studies. The present study group has demonstrated that hepatocyte proliferation may be used as a surrogate marker of liver injury. Organs transplanted immediately demonstrated significantly less necrosis and apoptosis and were associated with a lower proliferation index. Organs transplanted after 24 h of preservation clearly showed more severe injury, as well as a resultant increase in proliferation. The present study therefore aimed to assess the cumulative effects of HBO therapy in minimizing IPRI and thus reducing liver injury, and its capacity to stimulate proliferation. Despite the significant reduction in liver injury produced by HBO therapy, hepatocyte proliferation was markedly increased in HBO-treated liver at 48 h. However, by day 7, proliferation had returned to normal in HBO-treated rat livers but remained elevated in non-treated livers. This suggests that, as well as reducing hepatocyte injury, HBO therapy may independently promote liver regeneration; this latter finding may have important clinical implications.

Although acute hepatocyte injury peaked at 12 h after OLT in this model, ongoing liver injury is clear. This is evidenced by the later rises in bilirubin, ALP and GGT levels. The ultrastructural studies of the hepatic microvasculature performed at the single time-point of 48 h after OLT further support this. Mean sinusoidal diameter and sinusoidal density were both significantly reduced compared with those in normal liver. Strong experimental evidence suggests the hepatic microcirculation is regulated by endothelin-1 (ET-1) and NO. During ischaemia, an imbalance occurs in these vasoactive substances as the focus of regulation shifts towards ET-1, resulting in marked vasoconstriction of the hepatic microcirculation. Therapy with HBO has been shown to improve the microcirculation by a number of mechanisms. These include the reduction of endothelial swelling and the inhibition of PMN adhesion, both of which improve vascular diameter. This study assessed changes in the microcirculatory architecture at 48 h after OLT to determine persisting changes following initial injury and found that sinusoidal diameter and density remained significantly reduced in OLT liver compared with normal liver. Treatment with HBO reversed this reduction to normal levels and, similarly, transmission EM demonstrated improvement in the cellular architecture of both hepatocytes and liver sinusoidal endothelial cells after HBO therapy. Therefore, HBO therapy appears to have persistent positive effects on both parenchymal and non-parenchymal cells up to 48 h following OLT.

This study clearly demonstrates that HBO therapy delivered in the post-OLT period causes a significant reduction in IPRI severity. The present authors believe this to be the first study to demonstrate such an effect using a true liver transplantation model that includes sequential warm ischaemia, cold preservation and warm reperfusion phases and thus closely resembles human OLT. Treatment with HBO resulted in significant reductions in hepatocyte necrosis and apoptosis and concurrently stimulated hepatocyte proliferation and maintained a positive effect on the microvascular and cellular architecture. These changes were demonstrated despite the fact that HBO was delivered after the onset of IPRI in the post-OLT period. This has significant clinical implications as this represents the most practical time-point to deliver HBO.

The technical and logistic impediments to delivering HBO therapy to critically ill patients have been largely overcome by the introduction of large multi-chamber HBO units with easy access and abundant internal facilities. Although multi-occupancy chambers are now commonplace, treatment must be administered by high-quality, fully trained teams of staff who are familiar with the care and management of critically ill patients. The availability of such facilities within specialty transplant centres could conceivably be readily achieved. Dose–response studies and investigations into the outcomes of pre-OLT treatment of donor organs and recipients, as well as into the mechanisms and specific effects of HBO therapy on individual cell types in the liver, are required to further characterize this therapeutic option. Experimental evidence also supports the potential application of HBO in livers that are small for size, live donor transplantation (donors and recipients), hepatic artery thrombosis, marginal donor organs and acute liver failure. Results thus far are promising and may lead to the consideration of HBO therapy for translation into routine clinical practice in transplantation.

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Conflicts of interest

None declared.

References


Supporting information
Additional supporting information may be found in the online version of this article.

**Figure S1.** Effects of hyperbaric oxygen therapy on normal liver. Normal liver showed no evidence of necrosis.

**Figure S2.** Light microscopy of liver tissue sections from tissues collected at 48 h post-transplantation from normal liver, normal liver with hyperbaric oxygen treatment, liver without preservation (immediate transplantation) with ischaemia, preservation and reperfusion injury (IPRI), and liver with 24-h preservation with IPRI.

**Figure S3.** Light microscopy of liver tissue sections in ischaemia, preservation and reperfusion injury with and without hyperbaric oxygen treatment investigated at 6 h, 12 h, 24 h and 48 h.

**Figure S4.** Comparison of the severity of injury in three liver lobes, according to hepatocyte necrosis, apoptosis and proliferation, in hyperbaric oxygen-treated and control livers at 12 h, 24 h and 48 h after transplantation.

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