Transplant International

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Erythropoietin reduces ischemia-reperfusion injury after liver transplantation in rats

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Keywords
epo, erythropoietin, ischemia-reperfusion, liver transplantation.

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Received: 12 December 2008
Revision requested: 9 January 2009
Accepted: 14 February 2009

doi:10.1111/j.1432-2277.2009.00861.x

Abstract

Human recombinant Erythropoietin (rHuEpo) has recently been shown to be a potent protector of ischemia- reperfusion injury in warm-liver ischemia. Significant enhancement of hepatic regeneration and survival after large volume partial hepatic resection has also been demonstrated. It was the aim of this study to evaluate the capacities of rHuEpo in the setting of rat liver transplantation. One-hundred-and-twenty Wistar rats were used: 60 recipients received liver transplantation following donor organ treatment (60 donors) with either 1000 IU rHuEpo or saline injection (controls) into portal veins (cold ischemia 18 h, University of Wisconsin (UW) solution). Recipients were allocated to two groups, which either received 1000 IU rHuEpo at reperfusion or an equal amount of saline (control). Animals were sacrificed at defined time-points (2, 4.5, 24, 48 h and 7 days postoperatively) for analysis of liver enzymes, histology [hematoxylin-eosin (HE) staining, periodic acid Schiff staining (PAS)], immunostaining [terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), Hypoxyprobe] and real-time polymerase chain reaction (RT-PCR) of cytokine mRNA (IL-1, IL-6). Lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) values were significantly reduced among the epo-treated animals 24 and 48 h after liver transplantation (LT). The TUNEL and Hypoxyprobe analyses as well as necrotic index evaluation displayed significant reduction of apoptosis and necrosis in rHuEpo-treated graft livers. Erythropoietin reduces ischemia-reperfusion injury after orthotopic liver transplantation in rats.

Introduction

In transplantation surgery, ischemic organ damage and reperfusion injury after cold storage remain as major risk factors for impaired graft function and potential failure of the transplanted organ.

Substantial logistical efforts are undertaken in order to limit cold ischemic time, yet, as the demand for available transplant organs increases, periods of cold ischemia are often extended. This often leads to reduced organ quality at the time of transplantation.

Various studies have been conducted to define substances carrying the potential to minimize ischemia-reperfusion injury [1–10]. However, clinical improvements concerning this problem have been limited in recent years.

Erythropoietin (rHuEpo) has been shown to protect various tissue types from ischemic damage improving post-traumatic organ function [11–15]. Our group as well as others [16–18] have detected protective capacities for rHuEpo in warm hepatic ischemia in a rat model. Additionally, we were able to display enhanced regeneration and survival for rHuEpo-treated rats after large-volume hepatectomy [19].

Erythropoietin is an endogenous hormone, which has been routinely applied to patients for many years.

Current indications include tumor-related anemia and substitution for patients on dialysis. Large doses of rHuEpo can significantly increase the risk of thrombosis; however, the overall spectrum of side-effects is rather limited [20,21].

Various studies have elucidated the mechanisms of rHuEpo-mediated cell protection and reduction of apoptosis [22,23]. The tissue-protective capacities of erythropoietin are mediated via the Jak-2 pathway and an Akt-dependent intracellular cascade down-regulating the expression of pro-apoptotic mediators and molecules [24,25]. The synthesis of cell-protective substances and signaling proteins such as signal-transducing activator 3 (STAT-3) is increased facilitating intracellular homeostasis by blocking apoptotic mechanisms. The exact molecular cascade by which rHuEpo eventually mediates cellular injury remains to be elaborated completely at this point.

The aim of this study was to evaluate the potential effect of erythropoietin preconditioning and treatment on post-transplant graft function in a liver transplantation rat model.

Materials and methods

Animals

Male Wistar rats (body weight 250–300 g; Charles River Laboratories, Sulzfeld, Germany) were used for the experiments. Animals were housed in standard animal laboratories with a 12-h light-dark cycle and had free access to water and standard laboratory chow *ad libitum*. The experimental design was reviewed and approved by the local government (Senator fuer Gesundheit und Soziales, Berlin), and carried out according to the European Union regulations for animal experiments and the 'Guide for the Care and Use of Laboratory Animals' [DHEW Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

One-hundred-and-twenty rats were used for the experiments
Sixty rats served as liver donors. Of these donors, 30 received a dose of 4,000 IU rHuEpo/kg body weight (0.1 ml NeoRecormon [Epoetin beta; Roche Pharma, Welwyn Garden City, UK] diluted in saline solution) into the portal vein 30 min before harvest. The other 30 donor rats were treated with an intraportal venous saline injection of identical volume 30 min before organ removal.

Sixty rats served as recipients and were allocated to two groups
Group 1 ($n = 30$) received a bolus injection of 4000 IU rHuEpo/kg body weight into the portal vein immediately at reperfusion. Donor livers for these recipients had been pretreated with an equal dose 30 min before harvesting. Epo dosages were chosen in line with previously published protocols [14,17,19].

Group 2 ($n = 30$) received 0.1 ml saline as a bolus injection (0.1 ml) into the portal vein at the time of reperfusion. Liver graft for these recipients had not been pretreated with rHuEpo but with saline injection.

Animals were sacrificed 2, 4.5, 24, 48 h and 7 days after transplantation ($n = 6$ each) for collection of serum and liver tissue.

Surgical procedure

Donor organ harvest
Anesthesia was performed using isoflurane/air inhalation with 40% oxygen. The abdomen was opened by a midline incision and the liver was mobilized from all ligamentous attachments, the hepatic artery and the portal vein were isolated. The distal aorta was cannulated with a 14 G catheter; the supra truncal aorta and both renal arteries were ligated. Aortic perfusion was performed by gravity with a pressure of 120 cm H_2O, portal perfusion was executed with a pressure of 20 cm H_2O. Taking into account the gradual rewarming after cold storage perfusion in the clinical setting of multiorgan procurement, all livers remained within the body for an additional 30 min before removal and back-table perfusion.

Allotransplantation
Donor livers underwent 18 h of cold ischemic storage in UW solution. To resemble the warm ischemia time during human liver transplantation procedures the livers were kept at 21 °C (room temperature) for 15 min before surgical implantation started. All livers were re-flushed with 10 ml saline solution via the portal vein and weighed. Orthotopic rat liver transplantation was performed with hepatic artery revascularization and bile duct reconstruction by stent implantation. Except for hepatic artery and bile duct, all anastomoses were conducted by hand-sewn sutures. Portal clamping time in all transplantation procedures included in this study was less than 18 min.

After reperfusion and control of potential bleeding sources the abdominal cavity was closed and animals were placed in their cages for postoperative monitoring. All animals received analgesic treatment with 10 mg/kg of tramadol by intraperitoneal injection immediately before closure of the abdomen.

Animal sacrifice and organ harvest

All animals received complete anesthesia at 2, 4.5, 24, 48 h or 7 days after transplantation respectively. The abdomen was opened again and a clamp was placed on

the infrarenal aorta. Blood was drawn from the aorta until circulation completely stopped. The heart was incised to secure animal's death; liver tissue was harvested and forwarded to histological and PCR evaluation.

Hepatocellular damage

The extent of hepatocellular damage was assessed at 4.5, 24 and 48 h after ischemia by spectrophotometric determination of aspartate aminotransferase (AST), ALT, LDH and glutamate dehydrogenase (GLDH), using a commercially available reaction kit (Roche Diagnostics, Mannheim, Germany). (Evaluation of liver enzymes 2 h after LT was not performed because of preliminary experiments showing a transaminase peak at approximately 6 h postoperatively. We therefore abstained from evaluation of 2-h enzyme values).

Histology

Remnant liver tissue was fixed in 4% phosphate buffered formalin for 2–3 days and then embedded in paraffin. From the paraffin-embedded tissue blocks, 5 μm sections were cut and stained with HE and PAS. For each animal and time-point, nine fields were analysed and necrotic, apoptotic and mitotic cells counted.

To evaluate hepatocyte replication, mitotic figures were counted in 1000 hepatocytes (200-fold magnification) and analysed as mitotic index (number of mitotic figures per 1000 hepatocytes). All slides were judged by the same investigator who had been blinded to the corresponding study group.

TUNEL/apoptosis detection

To identify apoptosis in hepatocytes the TACS *TdT in situ* apoptosis detection kit was used in sequential order according to the procedures of the manufacturer (R&D Systems, Wiesbaden, Germany). Briefly, after cryopreservation and cutting of tissue slices the cryosection was first fixed in cold acetone and permeabilized in Cytonin solution for 60 min. Thereafter, chymase was stained by adding the primary anti-chymase mAb (1 mg/ml) for 60 min at 37 °C, the alkaline phosphatase-conjugated secondary Ab (1:100) (Vector) for 60 min at room temperature, and finally Red Label Substrate (R&D Systems) for 30 min. Next, endogenous peroxidase activity was quenched in 3% H_2O_2 for 5 min followed by incubation in the TdT labeling buffer for 5 min. The labeling reaction was initiated by adding 50 ml per slide of the TdT labeling buffer containing TdT-dNTPs, Mn_2, and TdT enzyme.

After 60 min reaction period at 37 °C, the labeling was stopped in TdT Stop buffer for 5 min. The slides were washed twice in H_2O, incubated in streptavidin-HRP for 10 min, washed again twice in H_2O, and finally incubated in TACS Blue Label for 2–5 min. DNase-free deionised water was used throughout the procedure. The slides were processed through increasing concentrations of ethanol, then xylene and finally covered with Depex.

TACS-nuclease-treated sample was used as a control for labeling reaction and a slide stained without TdT enzyme was used as a negative control. Positive tissue control slides for apoptosis were provided by R&D Systems. The hepatocyte was judged to be apoptotic when the red color for cytoplasmic chymase and bluish color for nuclear apoptosis were seen in close contact and the morphologic criteria for apoptosis were fulfilled. The cells were counted as explained above and the apoptosis index (%) was defined as the number of apoptotic hepatocytes in relation to all chymase-positive cells. Examination and grading of all tissue samples were performed by the same investigator who had been blinded to the corresponding study group.

Hypoxyprobe

We used the HypoxyprobeTM-1 Kit for detection of tissue hypoxia (Chemicon, Temecula, CA, USA).

The marker Pimonidazole possesses a heterocyclic nitro-group (nitroimidazole). This is a substance which is reduced to a nitro-radical anion by intracellular nitroreductases. This anion binds to intracellular macro-molecules, usually RNA. The reduced anion is stabilized for this binding procedure only in the absence of oxygen, therefore stable binding can only occur in hypoxic or anoxic cells.

Animals were treated with 60 mg/kg body weight of hypoxyprobe 30 min before they were sacrificed. Slices of paraffinized liver tissue of 5-μm size were used for staining according to the manufacturer's recommendations in the Dako Cytomation Autostainer (Dako Cytomation, Hamburg, Germany).

IL-1β and IL-6 mRNA expressions were analysed using non competitive semi-quantitative RT-PCR

Expression of mRNA in liver and tissue samples was analysed by reverse transcriptase-polymerase chain reaction (RT-PCR). For RNA isolation by phenol extraction, we used a TRIZOL© reaction kit. Every sample was incubated with Dexoxypolymerase I (DNase) for 15 min to exclude DNA contamination thereafter. After photometric determination of RNA purity and concentration, it was stored in aliquots of 10 μl at −80 °C. RNA was amplified using a RT-PCR kit (Boehringer, Mannheim, Germany) that combines reverse transcriptase and taq polymerase

activity in a final reaction volume of 50 µl. Controls using no RNA or only taq polymerase without reverse transcriptase activity were performed. Amplification products were analysed for size and quantity by gel electrophoresis (agarosis 2%). In addition to cytokines, β-actin was also determined for each sample to assess differences in RNA concentration. Primers for β-actin were designed to anneal in two different exons to further control possible DNA contamination that would result in a bigger size PCR product including the intron in-between. Finally, the amplification products of all cytokines were analysed for correct sequence by restriction analysis. Sequences for primers used were as follows (all commercially available from Interactive Corp., Ulm, Germany):

β-actin: Antisense: 5′ ACC CAC ACT GTG CCC ATC TA 3′; Sense: 5′ CGG AAC CGC TCA TTG CC 3′.

Il-1β: Antisense: 5′AAG AAG GTG CTT GGG TCC TCA TCG 3′; Sense: 5′ CTT CCT TGT GCA AGT GTC TGA AGC 3′.

Il-6 Antisense: 5′ GAG CAT TGG AAG TTG GGG TA 3′; Sense: 5′ TGT GCA ATG GCA ATT CTG AT 3′.

Statistical analysis

Results of liver enzyme measurement were expressed as mean values with standard deviations. After proving the assumption of normality and equal variance across respective groups, differences between groups were assessed using analysis of variance (overall differences) followed by Fisher's exact test. In all instances, *P*-values <0.05 were considered to be statistically significant. Longitudinal lab data were analysed using a linear regression model.

SPSS software was used for statistical analysis (SPSS 16.0; SPSS Inc., Chicago, IL, USA), graphical design was performed using Sigma-Plot (Sigma-Plot 8.0; SPSS Inc.).

Results

Liver enzymes

All liver enzymes measured in this experimental setting increased extensively after LT, which can be identified in Fig. 1.

Mean total enzyme values were lower for epo-treated animals at all time-points and for all enzyme types measured. Standard deviations were large and statistically significant reduction was only present for LDH 24 h after LT and ALT 48 h after LT ($n = 6$ for each time-point).

Histology

Hematoxylin-eosin (HE) staining and PAS staining revealed the amount of necrotic cell damage after LT and the associated ischemia-reperfusion injury: While in the epo-treated group there were hardly any necroses detectable 2 h after reperfusion, the control group animals displayed up to 30% necrotic areas. After 4.5 h these differences were even more pronounced with very few necroses among the epo-treated rats and up to 40% in the control collective (see Figs 2 and 3).

As early as 2 and 4.5 h after reperfusion mitoses could hardly be detected in both study groups. At later time-points, epo-treated livers displayed slightly more mitotic cells (data not shown).

Immunohistologic staining: TUNEL/Hypoxyprobe

Two hours after reperfusion, TUNEL-staining displayed reduced apoptosis rates among epo-treated animals as compared with controls (2.9% vs. 4.3%). At 4.5 h after reperfusion, we detected a mean of 2.6% apoptotic cells in the epo-group as compared with 4.1% in controls. At later time-points, very few apoptotic cells were identified in TUNEL-staining.

Two hours after LT hypoxyprobe-1 antibody-stained tissue revealed a markedly reduced number of hypoxic cells for epo-treated animals as compared with controls.

All animals in the control group showed approximately 30% hypoxic cells per analysed section whereas only two of the epo-treated rats reached this extent. Most of these animals displayed only 1–5% hypoxic tissue (see Figs 4 and 5).

IL-1β mRNA expression

Reverse transcriptase PCR-based evaluation of IL-1β mRNA expression revealed highest levels at 24 h after LT for both groups with a more pronounced increase among the control animals.

At 48 h after LT, these values dropped and mean values of both groups merged. Standard deviations were large; none of the differences between the study groups did reach statistical significance (see Fig. 6).

IL-6 mRNA expression

Interleukin (IL)-6 mRNA showed the strongest increase in values between those at 2 and 4.5 h after LT. No statistically significant difference in expression could be detected between the two groups (see Fig. 6).

Discussion

The presented data demonstrate that rHuEpo application to donor livers and to recipients at reperfusion attenuates ischemia-reperfusion injury after rat liver transplantation.

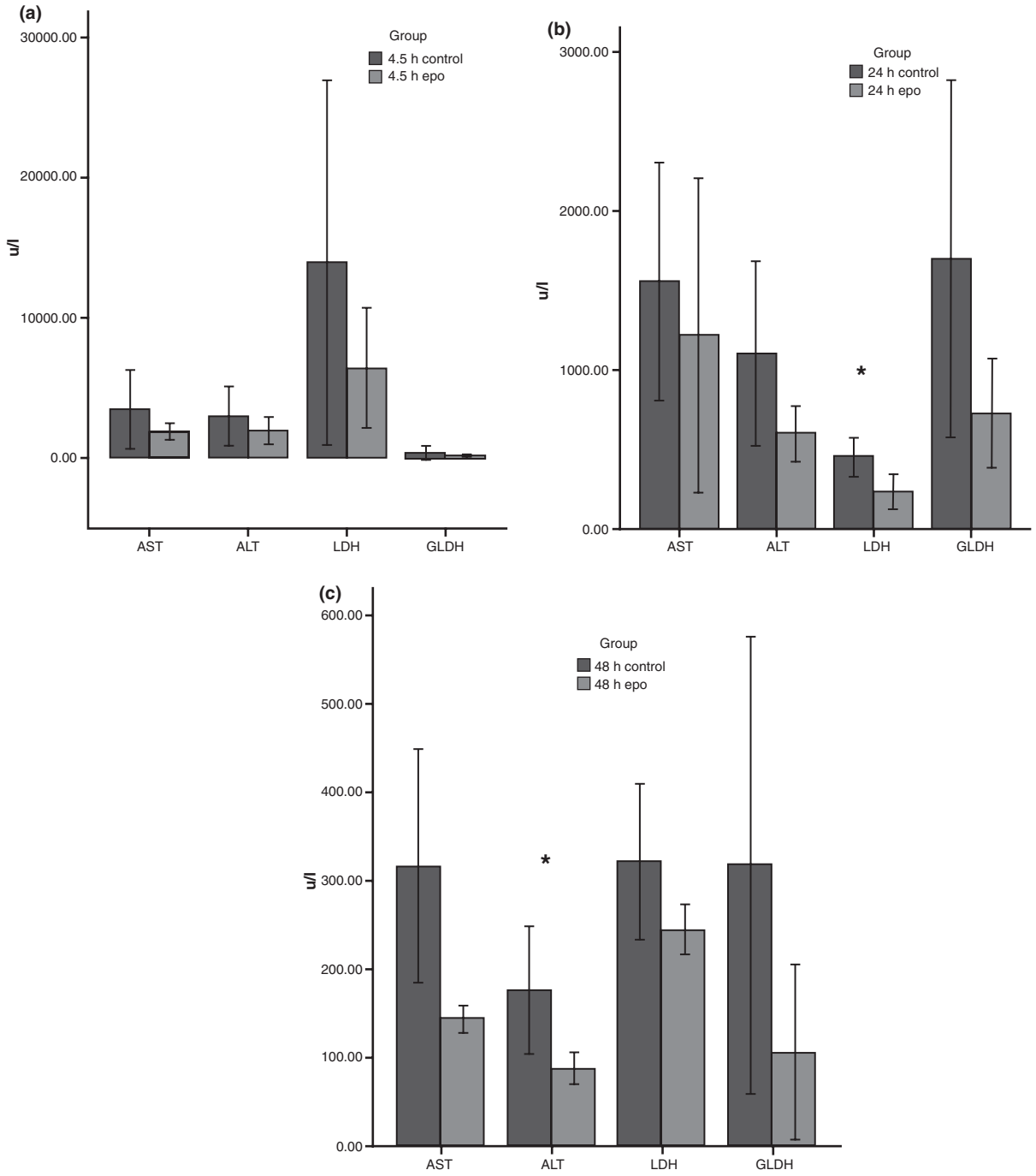

Figure 1 (a) Liver enzyme values 4.5 h after reperfusion. Enzyme values were reduced in epo-treated animals; however, differences were not significant. (b) Liver enzyme values 24 h after reperfusion. Enzyme values were reduced among epo-treated animals with statistical significance for LDH values (*$P < 0.05$). (c) Liver enzyme values 48 h after reperfusion. Enzyme values were reduced in epo-treated animals with statistical significance for ALT values (*$P < 0.05$).

This is reflected by a reduction in liver enzyme levels (ALT, LDH) and decreased apoptosis rates. Reduced transaminase levels were recorded at all time-points for rHuEpo-treated animals as compared with controls; however, significance was only reached 24 h post-transplantation for LDH and 48 h after LT for ALT. This fact might be attributed to the relatively small sample size and rather wide range of standard deviations.

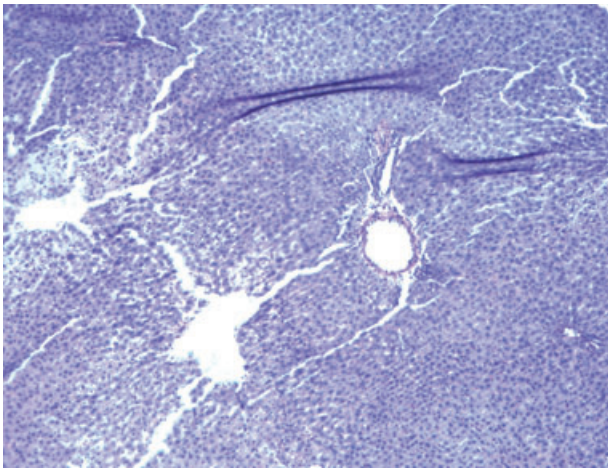

Figure 2 Control group: PAS staining 2 h after reperfusion demonstrates onset of necrosis (original magnification ×200).

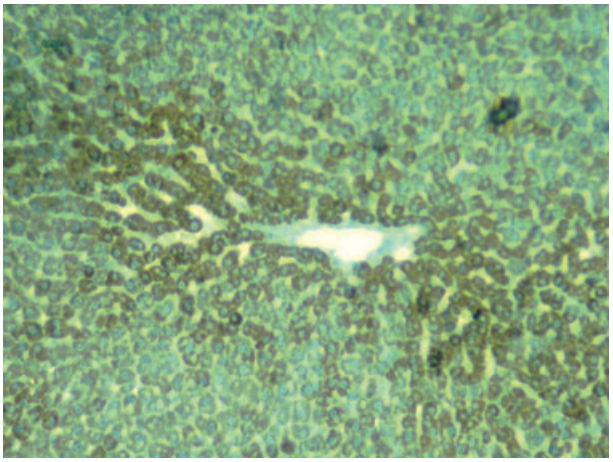

Figure 4 Control group: Hypoxyprobe staining 2 h after reperfusion. Brownish color marks hypoxic cells (original magnification ×400).

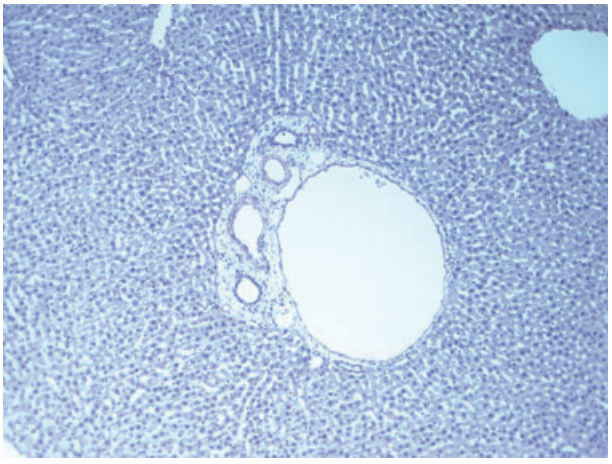

Figure 3 Epo-treated group: PAS staining 4.5 h after reperfusion displays minimal parenchymal alteration without significant necrosis (original magnification ×200).

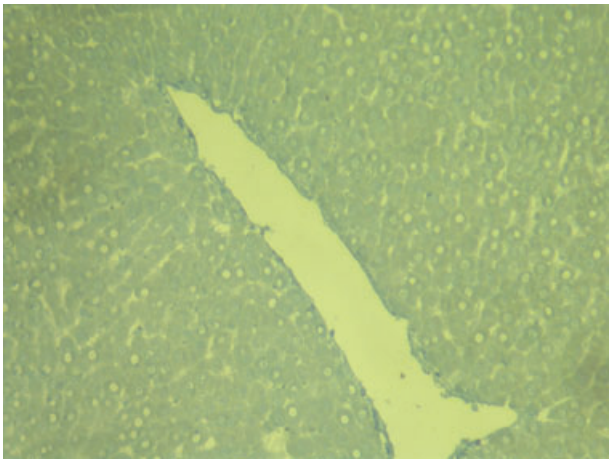

Figure 5 Epo-treated group: Hypoxyprobe staining 2 h after reperfusion. Brownish color marks hypoxic cells (original magnification ×400).

Hypoxyprobe-immunostaining displayed less hypoxic alteration in epo-treated graft hepatocytes resulting in reduced extent of liver tissue necrosis as compared with untreated controls.

While saline-treated rats had immunohistologically visualized hypoxia in up to 40% of transplant livers, rHuEpo-recipients displayed hypoxic damage only in about 10% of graft tissue. TUNEL-staining as well as evaluation of HE- and PAS-stained tissue revealed increased apoptosis and necrosis rates in control animal livers as compared with epo-treated rats as early as 2 h after ischemia-reperfusion injury. Malhi *et al.* [26] published a thorough analysis on the importance of both apoptosis and necrosis in liver cell death after I/R injury, underlining the relevance of our data.

While interleukin-1β mRNA expression was elevated among control animals as compared with epo-treated rats, no difference could be detected for interleukin-6 mRNA intensity. The reason for this finding remains unclear, however the role of IL-6 in I/R injury has been discussed controversially with IL-6 overexpression leading to detrimental effects in some studies [27,28].

Our study design was based on previous work from our group [17,19] and the results of multiple studies in neuronal and cardiac tissue demonstrating reduced cellular injury after rHuEpo pretreatment [11–15]. We have previously shown that rHuEpo treatment reduces ischemia-reperfusion injury after 45 min of warm-liver ischemia in rats [17].

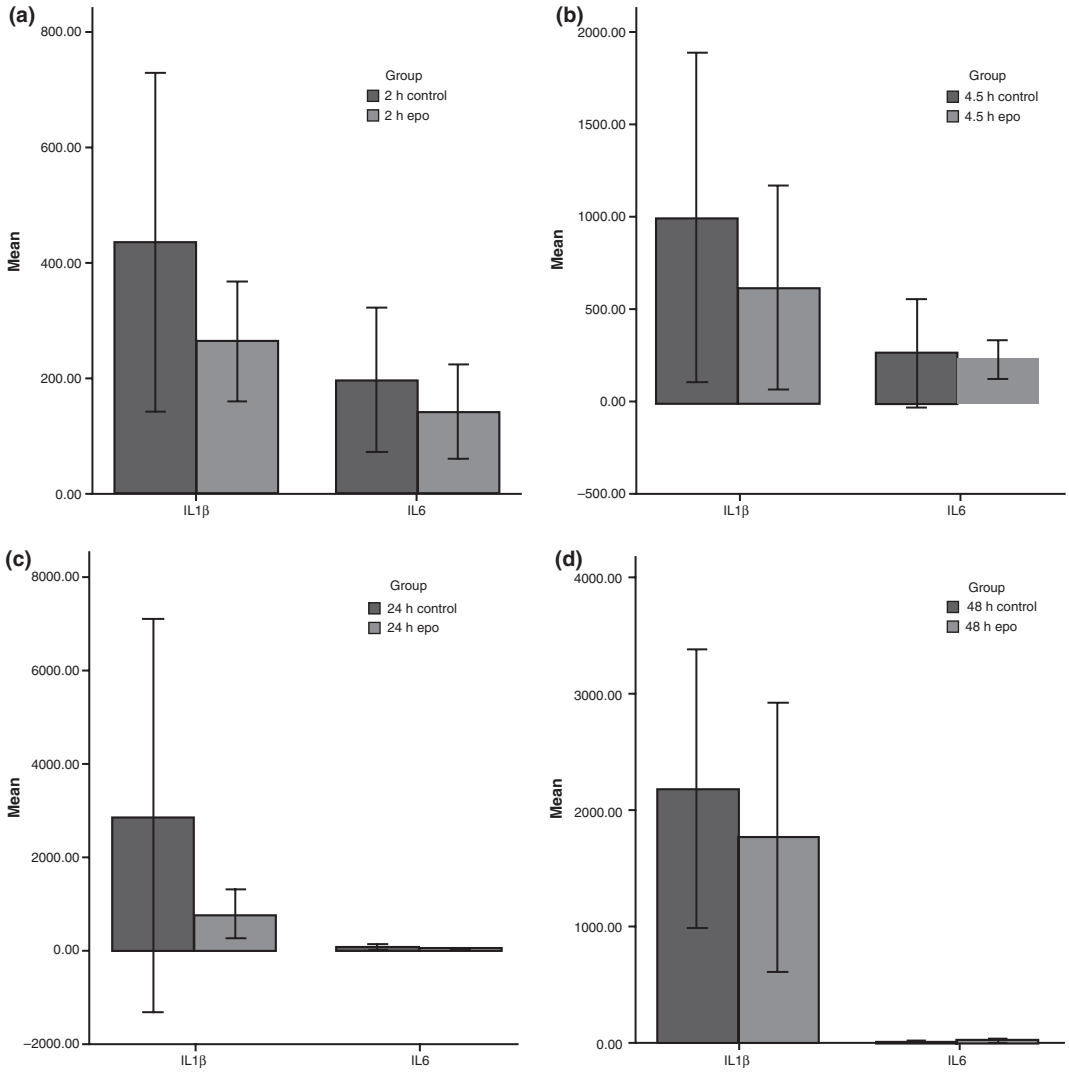

Figure 6 Interleukin-1β and Interleukin-6 mRNA values (RT-PCR) 2 (a), 4.5 (b), 24 (c) and 48 h (d) after reperfusion. Although values were elevated for control animals as compared with epo-treated rats no statistical significance was reached.

Recent studies have demonstrated the effectiveness of erythropoietin treatment concerning the reduction of ischemic tissue damage in the clinical setting [29]. However, the exact mechanisms of epo-mediated cell protection remain to be clarified. It has been demonstrated that both PI3-kinase/Akt-triggered procedures and the JAK/STAT pathway play a decisive role in the intracellular signaling cascade between extracellular epo-receptor binding and gene transcription [30–32]. Pro-apoptotic gene expression is down-regulated by epo while Akt activation induces HIF-gene transcription and consecutive up-regulation of epo-gene transcription [33,34]. A recent study from Sweden demonstrated additional epo-mediated effects in astrocytes, which are not dependent on gene-activation but depend on direct interference with astroglial water channels [35].

Tissue protection from ischemia-reperfusion injury has been a major field of research for many years [1,2,7,36] however, few treatment protocols have been incorporated into clinical routines:

Steroids, for instance, are accepted as organ-protective, limiting the extent of I/R injury when applied prior to ischemia [37]. Side-effects such as destabilization of the glucose-metabolism remain to be a problem, though.

Sileri *et al.* [38] even found enhanced liver injury after corticosteroid application.

Ischemic or hyperosmolar preconditioning also have been shown to positively influence the postoperative organ function [39–42]. However, this procedure is

restricted to surgical resection and not applicable in the transplant setting.

Lately, matrix-metalloproteinases have been found to be a potential target in the prevention of ischemia-reperfusion injury [43,44].

Rentsch *et al.* [45] demonstrated reduced I/R injury in rat livers after LT when animals were pretreated with adenoviral bcl-2 gene-transfer, a promising approach which applies further downstream in the same signaling cascade as epo does.

In order to limit I/R injury, many different substances have been studied in recent years, partially with great success, in small or even large animal models. Most of the examined drugs, though, are not approved for medical use in humans, which makes their clinical application even more complicated. rHuEpo has been used in clinical routines for many years and can be regarded as a fairly safe substance with few side-effects [3,7,44, 46–49].

The combination of several different approaches to minimize ischemia-reperfusion injury may enable us to optimize organ function even for donor grafts, which currently would be regarded as 'marginal'. This may offer a valuable option to expand the continuously limited donor organ pool.

Conclusion

In conclusion, rHuEpo attenuates ischemia-reperfusion injury after orthotopic liver transplantation in rats. rHuEpo is a clinically well known drug and may therefore be more readily applicable for use in humans than many other experimental substances. Further studies are needed to verify the presented findings.

Authorship

MS: designed the study, analysed data, wrote the paper. GH: Performed research/experiments, collected data. VA, SR and SL: performed research/experiments. SBK: collected data, analysed data. PN: contributed important reagents and material. UPN: designed the study, analysed data.

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