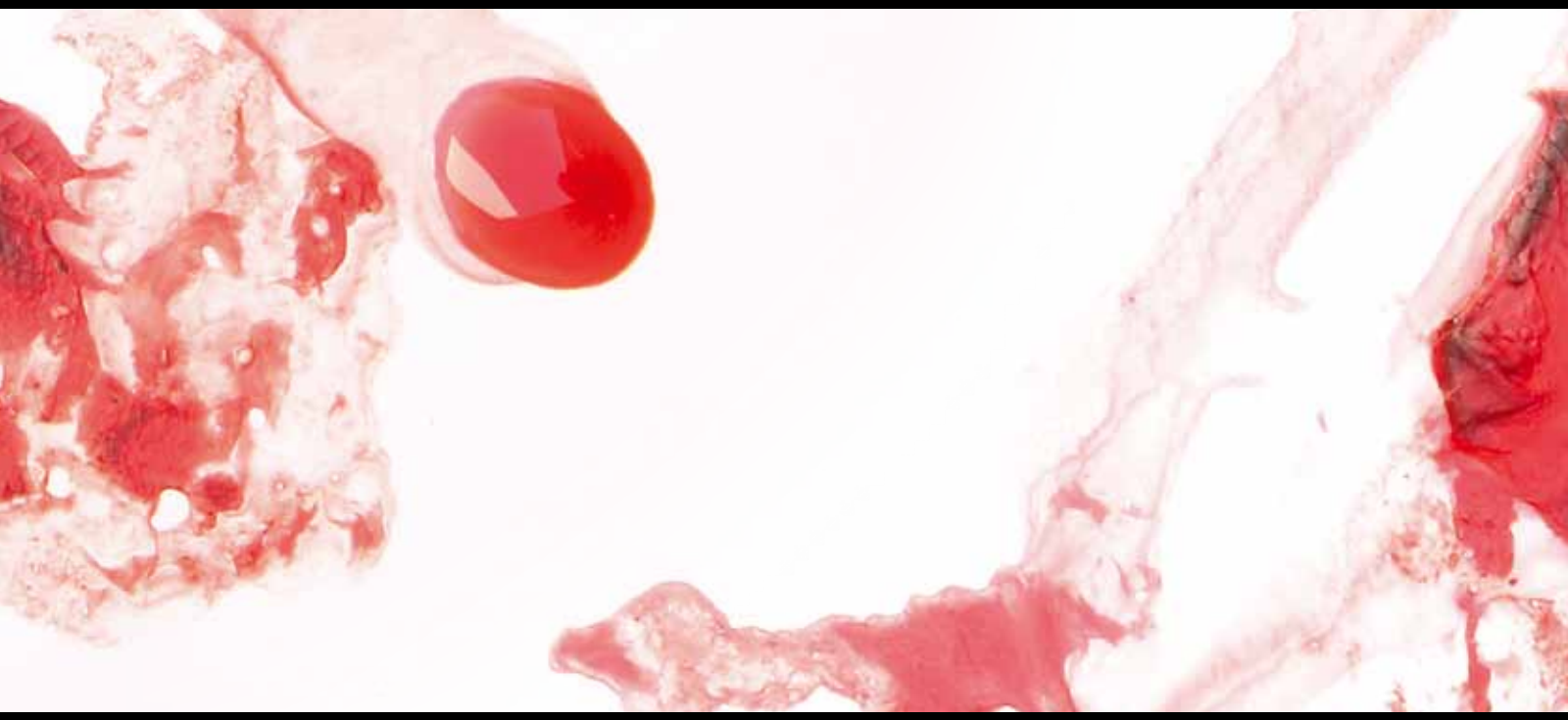
HPB Surgery

Ischemia/Reperfusion Injury in Liver Surgery and Transplantation

Guest Editors: Peter Schemmer, John J. Lemasters, and Pierre-Alain Clavien



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Editorial

Ischemia/Reperfusion Injury in Liver Surgery and Transplantation

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Despite medical advances, there is unfortunately still no guarantee for adequate liver function after extended resection and transplantation. Impaired liver function is associated with high morbidity and mortality. While the underlying mechanisms are only partly understood, they seem to have similar pathophysiological pathways.

Ischemia/reperfusion injury (IRI) is one of the main contributors to decreased liver function after liver surgery. Posthepatectomy failure is reported in up to 60–90% of cases, despite the fact that the liver remnant's volume in itself should be sufficient to maintain adequate function. After liver transplantation (LT), IRI cannot be avoided and is one of the ultimate factors that limits liver function after LT. Taking into account the various definitions of primary dysfunction and primary nonfunction as well as the number of grafts with high risk of failure and other risk factors, poor graft function is reported in up to 88% of patients after LT. While techniques that do not require hepatic vascular occlusion for liver resection (LR) have evolved, inevitable surgical manipulation itself creates hypoxia to liver tissue during LR, donor hepatectomy, and LT.

Organ specific parameters, such as preexisting damage (i.e., steatosis/steatohepatitis), and additional liver injury, such as surgical trauma, perfusion/preservation solutions, cold/warm ischemia time, and reperfusion, have been identified as contributing towards IRI.

Although some risk factors for mortality and morbidity after hepatic surgery including LT are defined, little is known about the mechanisms of injury. To date, no valid clinical concepts to preserve hepatic integrity and guarantee adequate regeneration in the context of both LT and LR have evolved. Indeed, many protective strategies have been proposed with the aim of preemptively inducing tolerance against IRI or interfering with the pathways of injury and regeneration—either by inhibiting deleterious molecules or enhancing protective pathways. Thus, the focus of this special issue of HPB surgery is on donor preconditioning, warm ischemia, nonheart-beating donors, hemorrhage and resuscitation-related liver injury, antioxidants, liver resection and transplantation, hepatic microperfusion, anesthetic considerations, and small-for-size phenomena.

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Research Article

Ischemic Preconditioning of Rat Livers from Non-Heart-Beating Donors Decreases Parenchymal Cell Killing and Increases Graft Survival after Transplantation

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A critical shortage of donors exists for liver transplantation, which non-heart-beating cadaver donors could help ease. This study evaluated ischemic preconditioning to improve graft viability after non-heart-beating liver donation in rats. Ischemic preconditioning was performed by clamping the portal vein and hepatic artery for 10 min followed by unclamping for 5 min. Subsequently, the aorta was cross-clamped for up to 120 min. After 2 h of storage, livers were either transplanted or perfused with warm buffer containing trypan blue. Aortic clamping for 60 and 120 min prior to liver harvest markedly decreased 30-day graft survival from 100% without aortic clamping to 50% and 0%, respectively, which ischemic preconditioning restored to 100 and 50%. After 60 min of aortic clamping, loss of viability of parenchymal and nonparenchymal cells was 22.6 and 5.6%, respectively, which preconditioning decreased to 3.0 and 1.5%. Cold storage after aortic clamping further increased parenchymal and non-parenchymal cell killing to 40.4 and 10.1%, respectively, which ischemic preconditioning decreased to 12.4 and 1.8%. In conclusion, ischemic preconditioning markedly decreased cell killing after subsequent sustained warm ischemia. Most importantly, ischemic preconditioning restored 100% graft survival of livers harvested from non-heart-beating donors after 60 min of aortic clamping.

1. Introduction

Liver transplantation surgery is a viable alternative for patients with end-stage liver disease but the number of heart-beating cadavers suitable for liver donation remains a key limitation. In human kidney transplantation, organ donation from non-heart-beating cadavers is now employed successfully at many centers [1]. Organ donors are typically terminally ill patients who do not meet the criteria of brain death and whose life support is withdrawn at the request of the family. After cardiac arrest occurs and death is pronounced several minutes later, the organs are harvested.

The use of livers from non-heart-beating donors is also emerging as an important stratagem to expand the liver donor pool [2]. Organs from non-heart-beating cadaver donors typically experience several minutes of warm ischemia prior to cold preservation. Warm ischemic injury that occurs to livers after cardiac arrest can severely compromise graft viability. Early clinical results with livers from non-heart-beating donors were poor, and two-month graft survival was only 50% even for donors that were extubated in an operating room setting [3]. With more rapid organ harvesting, clinical outcomes have improved, but rates of primary nonfunction, initial poor function, and ischemic-type biliary strictures remain greater than with donor livers from heat-beating cadaver donors [2]. Consequently, new and different strategies are needed to block warm ischemic injury in this context and to improve the outcome of non-heart-beating cadaver donation in liver transplantation.

Ischemic conditioning is the application of brief episodes of nonlethal ischemia and reperfusion to confer protection against sustained ischemia, which is showing therapeutic

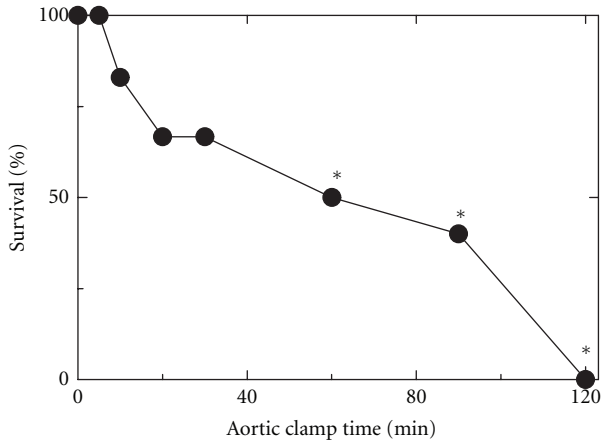

Figure 1: Loss of graft survival after aortic clamping. Aortas were clamped for 0 to 120 min. After clamping, livers were flushed with ice-cold UW solution. After 2 h of cold storage, the livers were transplanted into recipient rats, as described in section 2. Data are from 5 to 12 transplantations per time point. $^*P < 0.05$ compared to 0 min clamping by Fisher's exact test.

potential in various clinical settings [4, 5]. In rodent studies, ischemic preconditioning of the liver protects against injury after subsequent prolonged warm ischemia, particularly in fatty livers [6–8]. Decreases of transaminase release and sinusoidal endothelial cell killing also occur after cold preservation, which improve graft survival after orthotopic transplantation [9, 10]. In human liver surgery, ischemic preconditioning decreases postoperative transaminases and hepatic apoptosis, particularly in patients with mild-to-moderate steatosis, but in liver transplantation the benefit of ischemic preconditioning remains to be conclusively established [11–14]. The effect of ischemic preconditioning on graft injury and survival after transplantation of livers from non-heart-beating donors is not well studied. Here, we show that preconditioning with 10 min of warm hepatic ischemia markedly decreases hepatocellular and endothelial cell killing after subsequent sustained warm ischemia and after sustained warm ischemia followed by cold storage. Most importantly, ischemic preconditioning restores graft survival of livers harvested from non-heart-beating donors.

2. Methods

2.1. Orthotopic Rat Liver Transplantation. All animal protocols conformed to criteria of the Institutional Animal Care and Use Committee. Orthotopic rat liver transplantation was performed in male Lewis rats (220–280 g) under ether anesthesia using an arterialized two-cuff method by slight modification of the procedure of Steffen et al. [15]. For the donor operation, the liver was freed from its peritoneal attachments, and the common bile duct was cannulated with a polyethylene tube and divided. Cold University of Wisconsin (UW) solution (Viaspan, Dupont Pharma, Wilmington, DE) was infused through the portal vein. The suprahepatic inferior cava, subhepatic inferior cava, portal vein, and celiac artery were divided at the level of the diaphragm, left renal vein, splenic vein, and splenic artery, respectively. The liver was excised and placed in a bath of ice-chilled UW solution. Cuffs were then placed on the portal vein and subhepatic inferior cava before storage at 0–1°C in an ice water bath.

In recipient rats, the proper hepatic and gastroduodenal arteries were divided at their origin, leaving a stump of the common hepatic artery. The stump was clamped at the base of the dissected segment. The bifurcation of the proper hepatic and gastroduodenal arteries was cut, leaving a funnel-shaped opening to which a cuff was attached. After dividing the bile duct at the hilum, the suprahepatic inferior cava, portal vein, and subhepatic inferior cava were clamped and divided, and the recipient liver was removed. The donor liver was then rinsed with 10 mL of Ringer's solution at 37°C. Subsequently, the suprahepatic inferior cava was anastomosed with a running suture, and the portal vein, subhepatic inferior cava, and hepatic artery were connected in sequence by insertion of cuffs. The bile duct was anastomosed over an intraluminal polyethylene splint.

2.2. Ischemic Preconditioning and Aortic Clamping. To induce ischemic preconditioning, the abdomen was opened under ether anesthesia, and the hepatic artery and portal vein were occluded with a mini-bulldog vascular clamp for 10 min. The clamp was then released, and the liver was reperfused for 5 min prior to harvesting. To simulate non-heart-beating organ donation, the thorax was opened under ether anesthesia, and the ascending aorta clamped for 5 to 120 min, followed immediately by harvest of the liver.

2.3. Cell Killing. To assess cell viability after storage, livers were reperfused for 15 min with Krebs-Henseleit bicarbonate buffer (KHB) containing 500 μM trypan blue at an initial flow rate of 5 mL/min increasing to 30 mL/min over the first 5 min. In some experiments, stored livers were reperfused for 5 min with ice-cold UW solution containing 500 μM trypan blue at an initial flow rate of 5 mL/min increasing to 15 mL/min after 5 min [16, 17]. After trypan blue infusion, livers were fixed by perfusion for 2 min with 2% paraformaldehyde in 0.1 M NaPi buffer, pH 7.4. The temperature of the fixation was the same as the preceding perfusion. After this initial fixation, the left lateral lobe was cut into 1 cm slices and placed in ice-cold fixative. The tissue was then embedded in paraffin, sectioned, and stained with eosin or hematoxylin/eosin. For each liver, nuclear trypan blue uptake in parenchymal and nonparenchymal cells was determined in 5 random periportal and pericentral regions in eosin-stained sections using a 60X objective and expressed as the percentage of total nuclei counted in hematoxylin/eosin stained sections.

2.4. Statistics. Differences in survival were analyzed using Fisher's exact test, and differences between means were analyzed by analysis of variance (ANOVA). One-tailed tests were used to test unidirectional hypotheses, for example, that ischemic preconditioning decreases storage/reperfusion

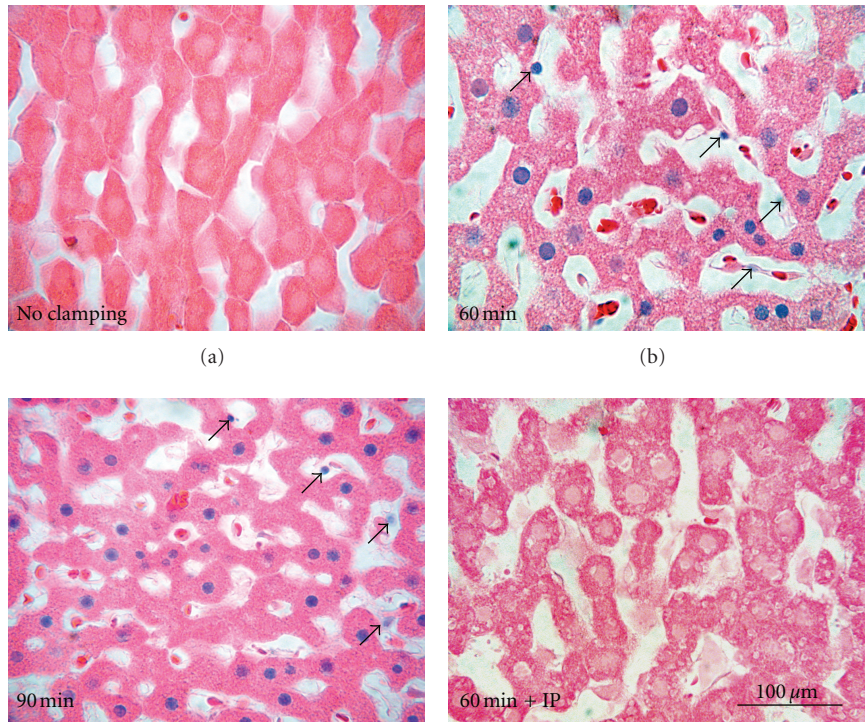

FIGURE 2: Light microscopy of livers after aortic clamping. In (a, b, c), the aortas of anesthetized rats were clamped for 0, 60, and 90 min, respectively, and their livers were then infused with cold UW solution containing trypan blue to label nonviable cells, as described in section 2. In d, a liver was first subjected to 10 min of ischemia followed by 5 min of reperfusion before 60 min aortic clamping. Trypan blue was then infused, as described for (a–c). Blue nuclei in eosin-counterstained sections represent mostly nonviable parenchymal cells. Arrows identify examples of nonviable nonparenchymal cells.

injury and improves graft survival. Group sizes are given in the legends to the figures. Errors represent the standard error of the mean. *P* values of less than 0.05 were considered to be significant.

3. Results

3.1. Decreased Graft Survival after Warm Ischemia Prior to Liver Harvest. Harvesting livers from non-heart-beating cadaver donors represents a potential means to expand the donor pool for human clinical liver transplantation. Accordingly, we harvested rat livers after various periods of aortic clamping and transplanted them into syngeneic recipient Lewis rats. When the aorta was not cross-clamped prior to liver harvest, 30-day graft survival was 100% after 2 h of cold storage (Figure 1). Graft survival was also 100% after 5 min of aortic clamping. After 10 min and longer, graft survival decreased progressively from 83% after 10 min to 50% after 60 min and 0% after 120 min. Thus, the livers tolerated only a very short period of warm ischemia before graft survival after transplantation began to decline. Necropsies revealed that none of the deaths was due to an obvious surgical error, such as a leaky arterial or venous anastomosis.

3.2. Loss of Parenchymal and Nonparenchymal Cell Viability after Warm Ischemia and Warm Ischemia Followed by Cold Storage. In another set of experiments, we assessed what cell types were losing viability as a consequence of various periods of warm ischemia. Aortas were cross-clamped, and after various periods of time, cold UW solution containing trypan blue was infused for 5 min followed by perfusion fixation with cold phosphate-buffered paraformaldehyde. Viable cells exclude trypan blue, whereas nonviable cells take up trypan blue into their nuclei. Thus, by counting trypan blue positive nuclei in eosin-counterstained histological sections, loss of viability of both parenchymal and nonparenchymal cells was determined.

Without aortic cross-clamping, trypan blue-stained nuclei were extremely rare in histological sections (Figure 2(a)). By contrast, with aortic cross-clamping, trypan blue labeling progressively increased (Figures 2(b) and 2(c) and Figure 3). Most trypan blue labeling occurred in hepatic parenchymal cells (hepatocytes), but some nonparenchymal cells also began to label after longer periods of clamping (Figures 2(b) and 2(c), arrowheads). The percentage of trypan blue-labeled parenchymal and nonparenchymal nuclei was averaged for several livers subjected to various times of aortic cross-clamping. After 0 and 5 min of aortic cross-clamping, trypan blue labeling of parenchymal or nonparenchymal cells was less than 0.5% (Figure 3). After longer times of cross-clamping, parenchymal cell killing (loss of viability) steadily increased from 12% after 30 min to 40% after 90 min. Nonparenchymal cell killing increased to a lesser extent to a maximum of 6% after 60 min. Nonparenchymal

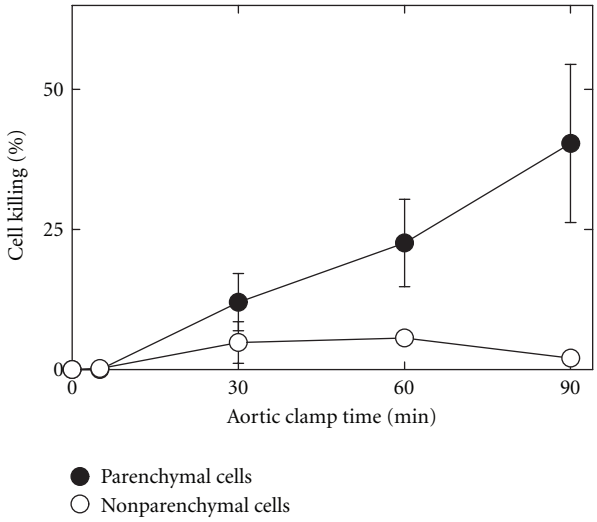

Figure 3: Increased parenchymal and nonparenchymal cell killing after aortic clamping. Aortas were clamped for 0 to 90 min. After clamping, livers were immediately infused with cold UW solution containing trypan blue and fixed, as described in Figure 2. Data represent means ± S.E.M. from 3 to 5 rats per group per time point.

killing appeared to decrease after 90 min, but this finding may be artifactual due to masking of nonparenchymal labeling by parenchymal nuclear labeling or to washout of nuclei of nonviable nonparenchymal cells as the livers were infused with trypan blue-containing UW solution.

To assess the additional effect of cold storage and reperfusion, aortas were cross-clamped for 0 to 90 min, and the livers were then infused with cold UW solution and stored for 2 h. After storage, livers were reperfused at 37°C for 15 min with KHB containing trypan blue and fixed for histology. Cold storage and reperfusion in the absence of aortic clamping led to virtually no trypan blue staining of either parenchymal or nonparenchymal cells (Figure 4(a)). With increasing times of aortic clamping followed by cold storage and reperfusion, parenchymal cell killing increased markedly (Figures 4(b), 4(c), and 5). After 30 and 60 min of cross-clamping and cold storage, parenchymal cell killing was 3.8 and 1.8 times that observed after clamping but no cold storage (Figure 5 compared to Figure 3, $P < 0.05$). After 90 min of cross-clamping, cold storage caused 28% more parenchymal cell killing than in the absence of cold storage, but this difference was not statistically significant (Figure 4(d) and Figure 5). None of the differences between nonparenchymal cell killing after clamping alone and nonparenchymal cell killing after clamping plus storage were statistically significant.

3.3. Improved Survival after Ischemic Preconditioning of Liver Grafts Subjected to Warm Ischemia and Cold Storage. In an effort to make donor livers resistant to the deleterious effects of aortic clamping prior to organ harvest and cold storage, we performed an ischemic preconditioning protocol whereby the hepatic artery and portal vein were occluded for 10 min. Subsequently, the vascular clamp was removed to allow the reflow of blood to the liver. After 5 more min, the aorta was cross-clamped for 60 to 120 min, and the livers were harvested, stored 2 h in UW solution, and transplanted. In comparison to non-preconditioned liver grafts, 30-day survival of liver grafts subjected to ischemic preconditioning increased from 50 to 100% after 60 min of aortic clamping ($P < 0.05$), 40 to 67% after 90 min of clamping ($P = 0.39$), and 0 to 50% after 120 minute of clamping ($P < 0.05$) (Figure 6). When it occurred, graft failure developed relatively rapidly. After clamp times of 60, 90 and 120 min, survival of animals that did not live to 30 days averaged 2.4, 2.5, and 4.8 days, respectively, without preconditioning. With preconditioning, average time to death of animals not surviving 30 days was 3.0 and 4.8 days after 90, and 120 min of aortic clamping.

3.4. Decreased Parenchymal and Nonparenchymal Cell Killing by Ischemic Preconditioning in Livers Subjected to Warm Ischemia. Since ischemic preconditioning improved survival of liver grafts subjected to warm ischemia before storage, we investigated how ischemic preconditioning influenced cell killing during these treatments. Livers were subjected to ischemic preconditioning or a sham operation. Subsequently, aortic clamping was imposed for 60 min, and the livers were infused with cold trypan blue-containing UW solution followed by fixation for histology. Our goal was to determine how ischemic preconditioning affected parenchymal and nonparenchymal cell killing prior to cold storage. As shown in Figure 7, ischemic preconditioning (IP) decreased parenchymal (a) and nonparenchymal (b) cell killing determined by trypan blue labeling to 3.0 to 1.5%, respectively, from 22.5% and 5.6 % after sham treatment (control) ($P < 0.05$).

Similarly, we subjected livers to ischemic preconditioning (IP) or sham treatment followed by 1 h of aortic clamping and 2 h of cold storage in UW solution. At the end of storage, the livers were either flushed with cold trypan blue-containing UW solution to assess cell killing at the end of storage (unreperfused) or reperfused with warm trypan blue-containing KHB for 15 min to assess cell killing after reperfusion (reperfused). As shown in Figure 8, parenchymal (left panel) and nonparenchymal (right panel) cell killing without preconditioning was about the same in stored livers that were not reperfused as in those that were reperfused. By contrast, ischemic preconditioning prior to aortic clamping and cold storage caused large and statistically significant decreases of both parenchymal and nonparenchymal cell killing measured at the end of cold storage with and without reperfusion.

4. Discussion

The success of liver transplantation surgery for patients with end-stage liver disease has caused growth worldwide of waiting lists for liver transplantation surgery, which greatly outnumber the available donor livers. The use of livers from non-heart-beating donors is one potential source for increasing the organ donation pool. Non-heart-beating donors have become an important source of kidney

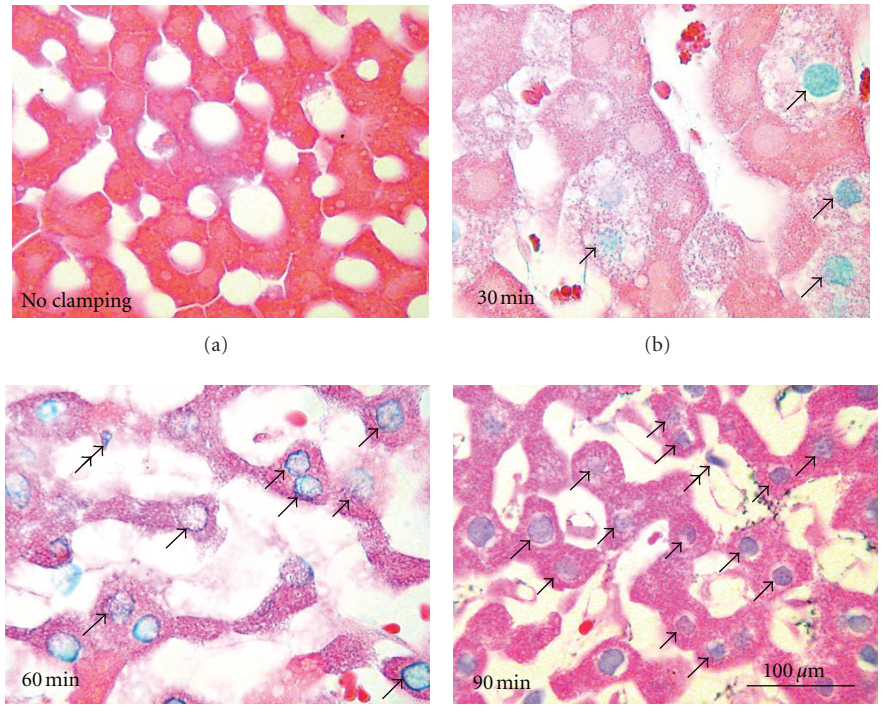

Figure 4: Light microscopy of livers after aortic clamping and cold storage/reperfusion. Aortas were clamped for 0 to 90 min. After clamping, livers were stored for 2 h in cold UW solution, followed by 15 min of warm reperfusion with KHB containing trypan blue to label nonviable cells, as described in section 2. Blue nuclei in eosin-counterstained sections represent nonviable parenchymal and nonparenchymal cells, as illustrated by arrows and double arrows respectively.

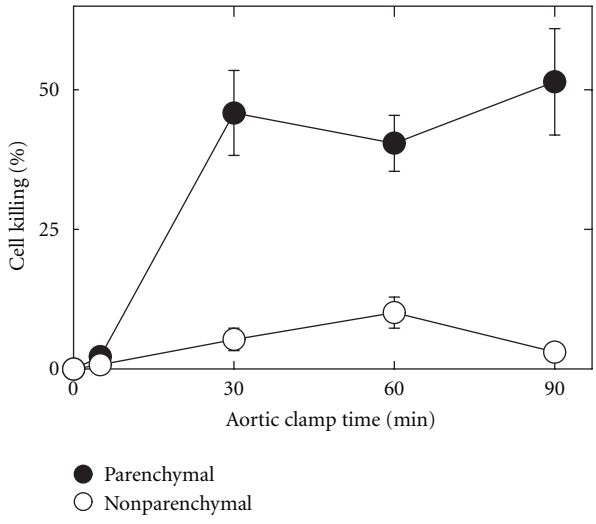

Figure 5: Parenchymal and nonparenchymal cell killing after aortic clamping and cold storage/reperfusion. Aortas were clamped for 0 to 90 min, and livers were cold stored and reperfused, as described in Figure 4. Data represent means ± S.E.M. from 5 rats per group per time point.

donations and are becoming an increasing source for liver donation [1, 2]. However unlike kidney grafts, liver grafts must recover function much more quickly and appear to be more susceptible to harvest, preservation, and reperfusion injury.

In our non-heart-beating model, the aortas of Lewis rats were cross-clamped for a specified period of time, stored for 2 h in UW solution, and transplanted. After as little as 10 min of aortic clamping, graft survival decreased after orthotopic rat liver transplantation (Figure 1). With increasing aortic clamp time, there was a continued decline in graft survival to 50% after 60 min of aortic clamping and 0% after 120 min of warm ischemia.

To better understand the injury caused by aortic clamping, we infused trypan blue at the end of aortic clamping to label nonviable cells. To minimize the effects of reperfusion we infused cold UW solution containing trypan blue for 5 min. Trypan blue labeling indicated that cellular killing was predominately to parenchymal cells (Figure 2). Trypan blue nuclear labeling signifies onset of necrotic cell death. Although not analyzed in detail, apoptosis was largely absent as shown by the lack of nuclear condensation, lobulation, and chromatin aggregation, which is consistent with earlier studies of both warm and cold ischemia (reviewed in [18]). Parenchymal cell killing started after as little as 10 min of aortic clamping and steadily increased over time (Figure 3). Parenchymal cell killing increased further after subsequent cold storage in UW solution and reperfusion, but no significant differences in nonparenchymal cell killing were

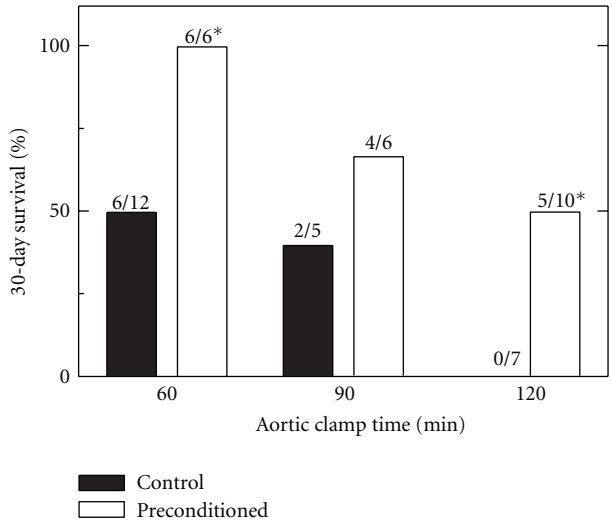

Figure 6: Improved graft survival by ischemic preconditioning before aortic clamping. Anesthetized rats were given a sham operation (control) or ischemic preconditioning by clamping of the hepatic artery and portal vein for 10 min followed by 5 min of blood reperfusion (preconditioned). The aortas of the rats were then clamped for 60, 90, or 120 min. After clamping, livers were infused with cold UW solution, and stored for 2 h. After storage, livers were transplanted into recipient rats, and recipient survival after 30 days was determined. Numbers above the bars indicate survivors/total transplants. $^*P < 0.05$ versus control by Fisher's exact test.

observed between aortic clamping and aortic clamping plus cold storage and reperfusion (Figures 4 and 5). Unlike cold ischemia [19, 20], warm ischemia involved principally parenchymal cells. Injury to parenchymal cells began to increase after 10 min of aortic clamping and continued to increase over time, which correlated with transplantation studies where graft failure also began to occur and progressively increased after 10 min or more of aortic clamping.

In heart and other organs including liver, preconditioning by short periods of ischemia followed by reperfusion protects against longer periods of ischemia [4]. Our model of ischemic preconditioning entailed clamping the hepatic artery and portal vein for 10 min, followed by 5 min of blood reperfusion. Such ischemic preconditioning markedly increased graft survival after 60 and 120 min of aortic cross-clamping (Figure 6). Substantial survival after as much as 120 min of aortic cross-clamping illustrates the robustness of protection by ischemic preconditioning. Likewise, ischemic preconditioning decreased parenchymal and nonparenchymal cell killing after aortic clamping and after aortic clamping followed by cold storage plus reperfusion (Figures 7 and 8). In some models, repetitive (up to 3) intervals of short ischemia/reperfusion further improve protection by ischemic preconditioning [4, 5]. Future studies will be needed to optimize preconditioning in terms of ischemia time and number of repetitions for non-heart-beating liver donation, although use of repetitions of ischemia/reperfusion may be impractical in a clinical donor situation. Ischemic-type biliary strictures can develop 1 to 4 months after liver transplantation whose incidence increases with non-heart-beating donation [2, 21, 22]. Future studies will also be needed to determine if ischemic preconditioning can also decrease the incidence of such strictures.

The mechanisms of action for ischemic preconditioning of the liver involve a variety of factors and pathways, including adenosine, nitric oxide, and activation of protein kinases (e.g., phosphatidylinositol 3-kinase, protein kinase C, p38 MAP kinase) and transcription factors (e.g., signal transducer and activator of transcription 3, nuclear factor-kB and hypoxia-inducible factor 1) [5]. In the context of cold storage/reperfusion injury to liver, we and others showed in a rat model of orthotopic liver transplantation that ischemic preconditioning improves survival of liver grafts harvested from heart-beating rat donors [10, 23, 24]. Improved graft survival is associated with decreased sinusoidal endothelial cell killing and Kupffer activation mediated at least in part by an adenosine A_2 receptor pathway coupled to increased cAMP [9]. Ischemic preconditioning also decreases hepatic injury, attenuates mitochondrial dysfunction, reduces free radical formation, and improves regeneration of small-for-size liver grafts, possibly by increasing mitochondrial superoxide dismutase expression [25]. Increasingly, ischemic preconditioning is being employed clinically to decrease hepatic injury after liver transplantation and other surgeries [12].

By contrast to the predominantly nonparenchymal cell injury that occurs after cold storage/reperfusion, the findings of the present study show that warm ischemia induced by aortic clamping caused loss of viability predominantly to parenchymal cells, namely, the hepatocytes. This lethal parenchymal cell injury then led to graft failure after orthotopic rat liver transplantation. Reperfusion was not a key factor contributing to cell killing after aortic clamping, since cell death could be shown in the absence of warm reperfusion by direct infusion of cold UW solution containing trypan blue. Nonetheless, subsequent brief cold storage and warm reperfusion did increase cell killing moderately. The change in cell type and role of reperfusion in hepatic cell killing after warm versus cold ischemia suggests different mechanisms of injury. Since parenchymal cell injury after aortic clamping occurred before cold storage, assessment of the suitability of donor livers from non-heart-beating cadavers might be possible before cold storage by examining trypan blue labeling in biopsies.

In conclusion, ischemic preconditioning protected strongly against parenchymal cell killing after aortic clamping and markedly improved survival of grafts from non-heart-beating donors. In a clinical setting, uncontrolled episodes of hypoxia and hypoperfusion may contribute to protective preconditioning, but organ manipulations such as ischemic preconditioning are currently prohibited prior to declaration of donor death. However, future changes in living wills and accepted ethical practices may permit use of ischemic preconditioning in terminally ill donors just prior to withdrawal of life support. Moreover, better understanding of the mechanisms underlying protection by ischemic preconditioning in the specific context of non-heart-beating liver donation may permit

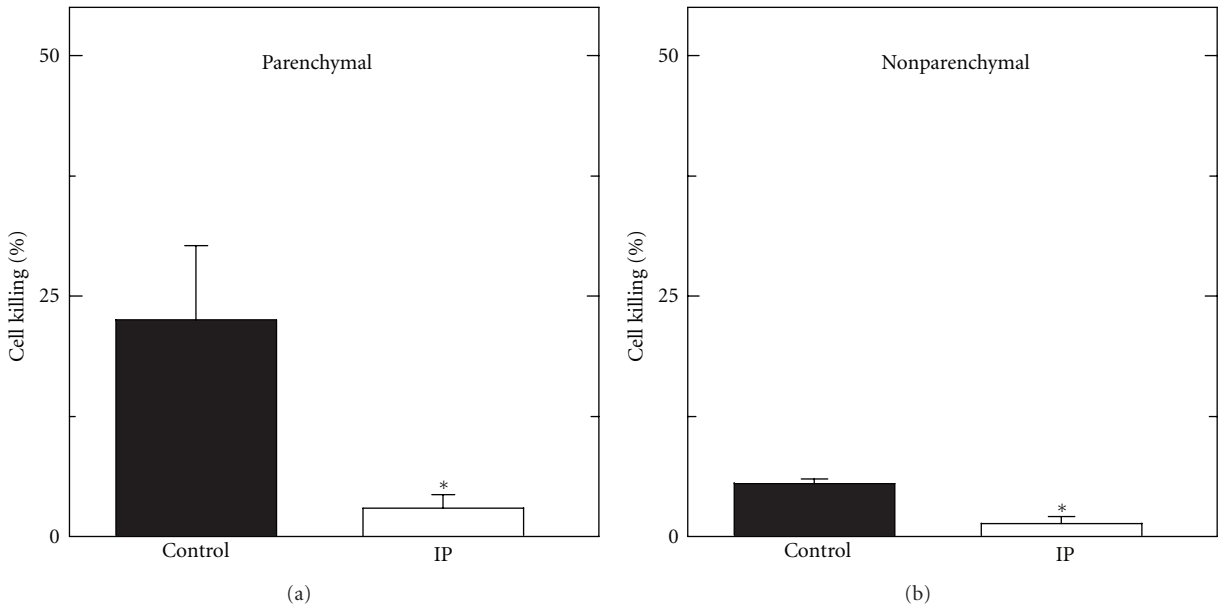

Figure 7: Decreased parenchymal and nonparenchymal cell killing by ischemic preconditioning after aortic clamping. Anesthesized rats were given a sham operation (control) or ischemic preconditioning by clamping of the hepatic artery and portal vein for 10 min followed by 5 min of blood reperfusion (IP). The aortas of the rats were then clamped for 60 min. After clamping, the livers were infused with cold UW solution containing trypan blue to label nonviable parenchymal (a) and nonparenchymal (b) cells. Data represent means ± S.E.M. from 5 rats per group per time point. $^*P < 0.05$ versus control by ANOVA.

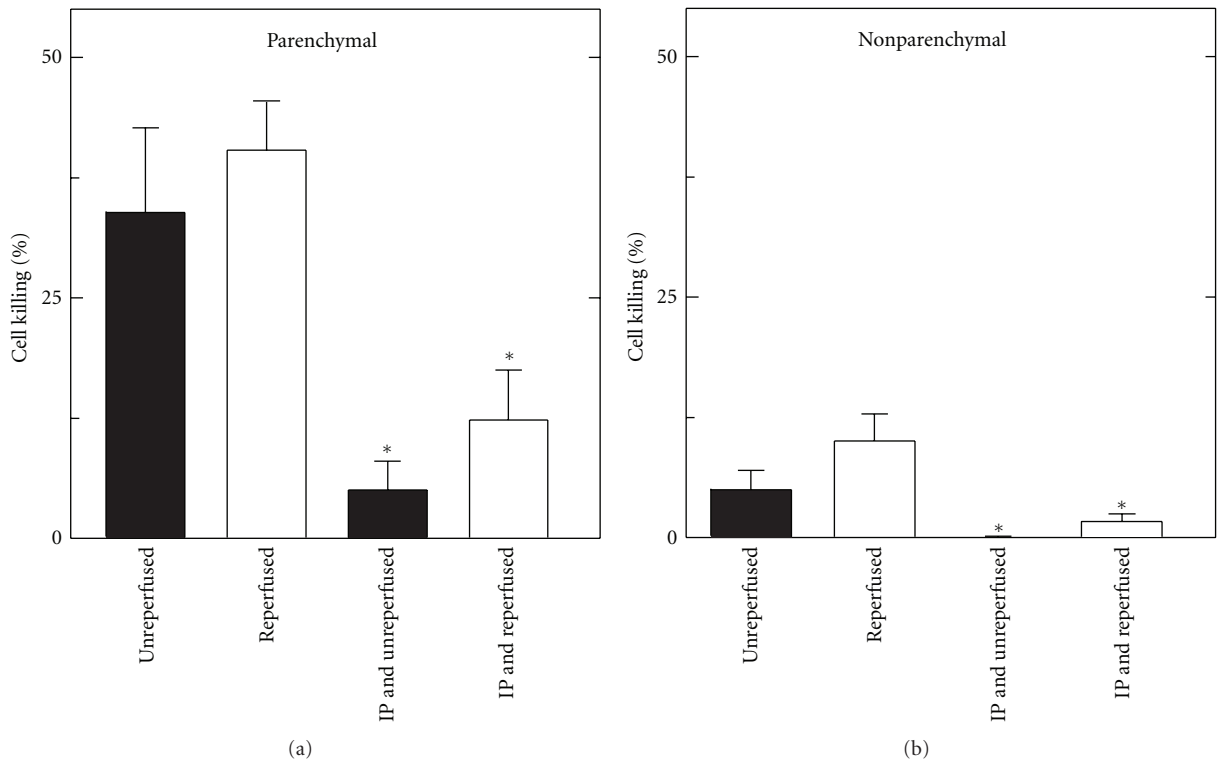

Figure 8: Decreased parenchymal and nonparenchymal cell killing by ischemic preconditioning after aortic clamping and cold storage with and without reperfusion. Anesthesized rats were given a sham operation or ischemic preconditioning (IP) by clamping of the hepatic artery and portal vein for 10 min followed by 5 min of blood reperfusion. The aortas of the rats were then clamped for 60 min. After clamping, the livers were stored for 2 h in cold UW solution. Subsequently, the livers were infused for 5 min with cold UW solution containing trypan blue to label nonviable cells in the absence of warm reperfusion (unreperfused) or infused for 15 min with warm KHB containing trypan blue to label nonviable cells after warm reperfusion (reperfused), as described in section 2. Nonviable parenchymal cells (a) and nonparenchymal cells (b) were counted from 5 livers per group. $^*P < 0.05$ compared to the corresponding non-IP group by ANOVA.

use of pharmacological strategies to simulate ischemic preconditioning. In this way, ischemic preconditioning or its pharmacological surrogate might one day be applied clinically in advance of liver donation after planned removal of life-sustaining treatment such as mechanical ventilation.

Acknowledgments

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Research Article

C-Jun N-Terminal Kinase 2 Promotes Liver Injury via the Mitochondrial Permeability Transition after Hemorrhage and Resuscitation

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Hemorrhagic shock leads to hepatic hypoperfusion and activation of mitogen-activated stress kinases (MAPK) like c-Jun N-terminal kinase (JNK) 1 and 2. Our aim was to determine whether mitochondrial dysfunction leading to hepatic necrosis and apoptosis after hemorrhage/resuscitation (H/R) was dependent on JNK2. Under pentobarbital anesthesia, wildtype (WT) and JNK2 deficient (KO) mice were hemorrhaged to 30 mm Hg for 3 h and then resuscitated with shed blood plus half the volume of lactated Ringer's solution. Serum alanine aminotransferase (ALT), necrosis, apoptosis and oxidative stress were assessed 6 h after resuscitation. Mitochondrial polarization was assessed by intravital microscopy. After H/R, ALT in WT-mice increased from 130 U/L to 4800 U/L. In KO-mice, ALT after H/R was blunted to 1800 U/l ($P < 0.05$). Necrosis, caspase-3 activity and ROS were all substantially decreased in KO compared to WT mice after H/R. After sham operation, intravital microscopy revealed punctate mitochondrial staining by rhodamine 123 (Rh123), indicating normal mitochondrial polarization. At 4 h after H/R, Rh123 staining became dim and diffuse in 58% of hepatocytes, indicating depolarization and onset of the mitochondrial permeability transition (MPT). By contrast, KO mice displayed less depolarization after H/R (23%, $P < 0.05$). In conclusion, JNK2 contributes to MPT-mediated liver injury after H/R.

1. Introduction

Multiple trauma is the principal cause of hemorrhagic shock and is typically the consequence of traffic accidents, falls, and, in time of war, casualties of combat [1, 2]. After hemorrhagic shock, resuscitation can lead to multiple organ dysfunction syndrome (MODS), which remains the most significant contributor to late mortality and intensive care unit resource utilization in critical care medicine [3, 4]. The liver is quite vulnerable to injury after ischemia and reperfusion (I/R). After I/R, hepatic necrosis is the predominant mode of cell death, whereas apoptosis is of less importance [5–7]. However, apoptosis and necrosis share common pathways, particularly the mitochondrial permeability transition (MPT) [8].

The MPT is caused by opening of high conductance MPT pores in the mitochondrial inner membrane, which leads to mitochondrial depolarization, uncoupling of oxidative phosphorylation, and large amplitude mitochondrial swelling [9]. The MPT plays a prominent role in the pathogenesis of cell death after I/R injury and a variety of other stresses [9–12]. After onset of the MPT, necrotic cell killing (oncosis) can occur as a consequence of ATP depletion, whereas swelling of mitochondria after the MPT leads to rupture of the outer membrane and release of proapoptotic proteins like cytochrome c. The extent of ATP depletion is crucial to

whether necrosis or apoptosis occurs, since caspase-dependent apoptosis requires ATP, and necrosis does not occur until ATP is depleted by more than 85%.

c-Jun N-terminal kinase (JNK) is a stress-activated protein kinase that becomes activated after stresses like ultraviolet (UV) radiation, I/R and inflammation [13–16]. JNK-dependent phosphorylation of the transcription factor c-Jun/AP-1 promotes gene expression for an enhanced immune response [17]. JNK can also induce apoptosis via JNK-mediated phosphorylation of proapoptotic Bcl2 family proteins, such as Bim and Bmf, leading to mitochondrial outer membrane permeabilization, release of cytochrome c, and caspase activation [18, 19]. Moreover, translocation of activated JNK to mitochondria promotes the MPT [20, 21]. JNK becomes activated after experimental liver transplantation, warm hepatic I/R and hemorrhage/resuscitation (H/R), and pharmacological inhibition of JNK decreases liver injury, improves liver function, and increases survival in these settings [14, 15, 22–25]. Liver expresses two isoforms of JNK—JNK1 and JNK2 [26]. In models of acetaminophen hepatotoxicity, TNFα-dependent hepatic injury, warm I/R to liver and liver transplantation, JNK2 deficient mice are relatively protected against injury compared to wildtype mice [27–30].

Another organ vulnerable to injury during H/R is the gut. H/R compromises the barrier function of the gut, causing toxins and bacterial products like lipopolysaccharide (LPS) to enter the liver via the portal vein [31]. LPS and other gut-derived toxins entering the liver after H/R stimulate free radical generation and proinflammatory cytokine release by Kupffer cells to contribute to hepatic injury and increased cytokines in the blood stream [32–35]. Since JNK2 is also associated with the loss of barrier function of the gut [36, 37], we hypothesized that JNK2 is important for promotion of liver injury after H/R. Here, we test this hypothesis and show that liver injury decreases and hepatic function improves after H/R to JNK2 deficient mice in comparison to wildtype mice. These improvements are associated with improved mitochondrial function.

2. Materials and Methods

2.1. Chemicals and Reagents. Rhodamine 123 (Rh123) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals. Experiments were performed using protocols approved by the Institutional Animal Care and Use Committee. C57BL/6 (wildtype) and JNK2-deficient (B6.129S2-Mapk9tm1Flv/J on a C57BL background) mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice used were males of 8 to 10 weeks of age and weighing 21–25 g.

2.3. Hemorrhagic Shock and Resuscitation. After an overnight fast, mice were anesthetized with sodium pentobarbital (80 mg/kg body weight). Under spontaneous breathing, the left and right femoral arteries were exposed and cannulated with polyethylene-10 catheters (SIMS Portex), as described [15]. Before insertion, the catheters were flushed with normal saline containing heparin (100 IU/l). One catheter was connected via a transducer to a pressure analyzer (Micro-Med; Louisville, KY, USA), and blood was withdrawn over 5 min via the second catheter into a heparinized syringe (10 units) to a mean arterial pressure of 30 mm Hg. This pressure was maintained for 3 h by the reinfusion or withdrawal of shed blood. An animal temperature controller was used to maintain rectal temperature between 36.6 and 37.3°C. After 3 h, mice were resuscitated with the shed blood followed by lactated Ringer's solution corresponding to 50% of the shed blood volume infused with a syringe pump over 30 min. Adequacy of resuscitation was determined by the restoration of blood pressure to ~80 mm Hg. After resuscitation, the catheters were removed, the vessels were ligated, and the groin incisions were closed. Sham-operated animals underwent the same surgical procedures without hemorrhage. In sham-operated mice, pentobarbital anesthesia lasted up to 120 min before the animals began to awaken, and a second injection was required to continue the anesthesia. In mice undergoing H/R, a second injection of pentobarbital was not necessary to maintain anesthesia, most likely due to decreased pentobarbital metabolism by the hypoperfused liver. Over the course of the experiments, no mortality in any group occurred. For the determination of H/R-dependent liver damage, mice were anesthetized, and the two right dorsal liver lobes were snap frozen in liquid nitrogen. The remaining liver was flushed with saline through the portal vein, fixed by infusion of 4% buffered paraformaldehyde, and embedded in paraffin.

2.4. Alanine Aminotransferase (ALT). Blood samples to measure ALT were collected from the inferior vena cava 6 h after H/R for analysis using a kit (Sigma Chemical, St. Louis, MO, USA).

2.5. Histology. Necrosis was evaluated 6 h after H/R in 4-μm paraffin sections stained with hematoxylin and eosin (H&E). Necrosis was identified by standard morphologic criteria (e.g., loss of architecture, karyolysis, vacuolization, increased eosinophilia). Areas of necrosis were outlined in 10 random fields for each liver. Images were captured (Olympus BH-2 Microscope; Micropublisher 5.0 RTV, Center Valley, PA, USA), and the area percentage of necrosis was quantified using a computer program (BioQuant BQ Nova Prime 6.7, R&M Biometrics, Nashville, TN, USA).

2.6. Caspase-3. Liver tissue (~100 mg) was homogenized (Polytron PT-MR2100, Kinematica, Luzern, Switzerland) in 1 mL of lysis buffer containing 0.1% 3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid, 5 mM DTT, 2 mM EDTA, 1 mM pefabloc, 10 ng/mL pepstatin A, 10 ng/mL aprotinin, 20 μg/mL leupeptin and 10 mM HEPES buffer, pH 7.4. After centrifugation at 15,000 rpm for 30 min, activity of caspase-3 in the supernatant was determined using a Caspase-3 Colorimetric Assay Kit (R&D Systems,

Minneapolis, MN) according to the manufacturer's instructions. Activity was normalized to protein concentration and expressed as fold increase compared to sham.

2.7. 4-Hydroxynonenal. Paraffin sections were deparaffinized, rehydrated, and incubated with polyclonal antibodies against 4-hydroxynonenal (4-HNE, Alpha Diagnostics; San Antonio, TX, USA) in PBS (pH 7.4) containing 1% Tween 20 and 1% bovine serum albumin. Peroxidase-linked secondary antibody and diaminobenzidine (Peroxidase Envision Kit, DAKO) were used to detect specific binding.

2.8. Intravital Microscopy. At 4 h after H/R, mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and connected to a small animal ventilator via a tracheostomy and respiratory tube (22-gauge catheter), as described [29]. Laparotomy was performed, and a polyethylene-10 catheter was inserted into the distal right colic vein. Using a syringe pump, a membrane potential indicating fluorophore, Rh123 (1 μmol/mouse), was infused via the catheter over 10 min. After prone positioning of the mouse, the liver was gently withdrawn from the abdominal cavity and placed over a glass coverslip on the stage of an inverted microscope. Rh123 fluorescence was excited with 820 nm light from a Chameleon Ultra Ti-Sapphire pulsed laser (Coherent, Santa Clara, CA, USA) and imaged with a Zeiss LSM 510 NLO laser scanning confocal microscope using a 63 × 1.3 NA water-immersion objective lens. Green Rh123 fluorescence was collected through a 525 ± 25 nm band pass filter. During image acquisition, the respirator was turned off for ~5 sec to eliminate breathing movement artifacts. In 20 fields per liver, hepatocytes were scored for bright punctate Rh123 fluorescence signifying polarized mitochondria or a dimmer diffuse cytosolic fluorescence denoting depolarized mitochondria. Image analysis was performed in a blinded manner.

2.9. Statistical Analysis. Data are presented as means ± S.E., unless noted otherwise. Statistical analysis was performed by ANOVA with Student-Newman-Keuls test, as appropriate, using $P < 0.05$ as the criterion of significance.

3. Results

3.1. Decreased ALT Release and Liver Necrosis after Hemorrhage and Resuscitation of JNK2-Deficient Mice. After sham operation, serum ALT averaged 112 ± 15 U/L in wildtype and JNK2 deficient mice (Figure 1). After H/R, ALT increased to 4860 ± 538 U/L 6 h after resuscitation in wildtype mice compared to 1806 ± 126 U/L in JNK2-deficient mice ($P < 0.001$, Figure 1).

In sham-operated wildtype and JNK2-deficient mice, liver histology was normal and indistinguishable from untreated mice (Figure 2(a) and data not shown). At 6 h after H/R to wildtype mice, large areas of hepatic necrosis developed with a predominantly pericentral and midzonal distribution (Figure 2(b)). In JNK2-deficient mice, hepatic necrosis after H/R decreased from 24.5 ± 1.5% in wildtype mice to 6.6 ± 1.5% ($P < 0.05$, Figures 2(c) and 2(d)).

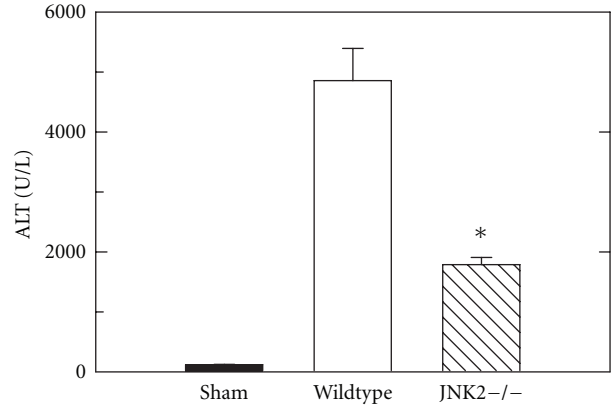

Figure 1: Decreased alanine aminotransferase (ALT) release after hemorrhage/resuscitation in JNK2-deficient mice. Wildtype and JNK2-deficient (JNK2−/−) mice were subjected to sham operation or bled to a mean arterial pressure of 30 mm Hg and resuscitated after 3 h, as described in Section 2. Blood was collected at 6 h after resuscitation for ALT measurement. Group sizes were 5-6 mice/group. $^*P < 0.05$ versus wildtype. Average ALT values of wildtype and JNK2 deficient mice after sham operation were not statistically significantly different and are pooled.

Thus, hepatic necrosis in JNK2-deficient mice after H/R was decreased by more than two-thirds in comparison to wildtype mice (Figure 2(d)).

3.2. Decreased Apoptosis after Hemorrhage and Resuscitation of JNK2-Deficient Mice. Caspase 3 activity was measured in liver extracts at 6 h after H/R of wildtype- and JNK2-deficient mice in comparison to sham-operated mice. After sham operation, caspase 3 activity in the liver was nearly undetectable (Figure 3). After H/R of wildtype mice, caspase 3 activity increased significantly by 7.6-fold. By contrast after H/R of JNK2-deficient mice, hepatic caspase 3 activity increased only 2.6-fold ($P < 0.05$ versus wildtype, Figure 3).

3.3. Improved Mitochondrial Function In Vivo after Hemorrhage and Resuscitation of JNK2-Deficient Livers. Intravital multiphoton microscopy revealed bright fluorescence of Rh123 in hepatocytes at 4 h after sham operation. The punctate pattern denoted polarization of individual mitochondria. No differences in Rh123 fluorescence were observed between livers of wildtype- and JNK2-deficient mice (Figure 4(a) and data not shown). We then imaged Rh123 fluorescence at 4 h after H/R. This time point was selected because previous studies of liver transplantation after cold ischemic storage showed that 4 h after reperfusion was a time point where mitochondrial dysfunction could be detected prior to onset of cell death [38]. At 4 h after H/R in wildtype mice, Rh123 staining became diffuse and dim in many hepatocytes indicative of depolarized mitochondria (Figure 4(b)). By contrast, after H/R of JNK2-deficient mice, mitochondria depolarized in fewer hepatocytes than in wildtype mice (Figure 4(c)). Rather, most hepatocytes

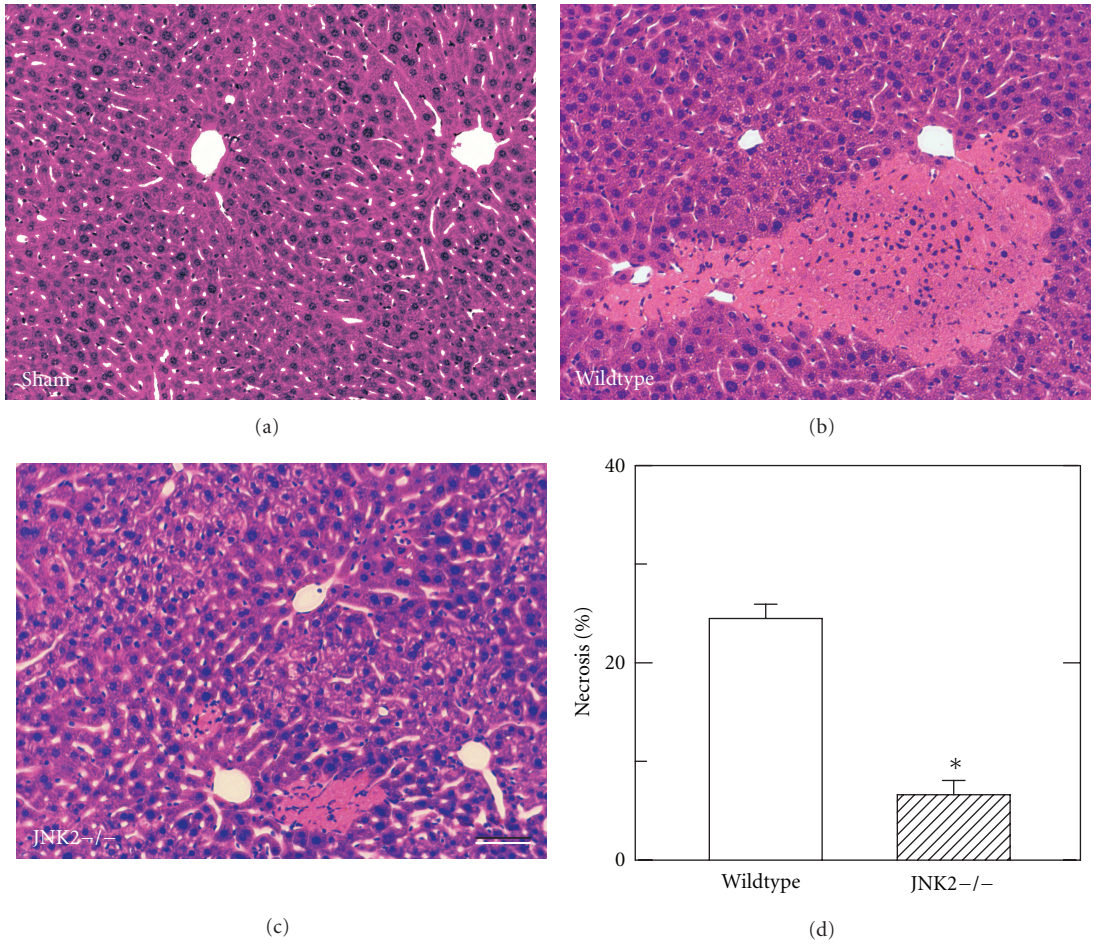

Figure 2: Decreased necrosis after hemorrhage and resuscitation in JNK2 deficient mice. At 6 h after resuscitation, necrosis was assessed by H&E in livers from sham-operated wildtype mice (a) and from wildtype- (b) and JNK2-deficient (c) mice after H/R. Bar is 50 μm. In (d), the percent area of necrosis is averaged from 5 livers per group. Necrosis was not present after sham operation of either wildtype- or JNK2 deficient mice and is not plotted. $^*P < 0.05$.

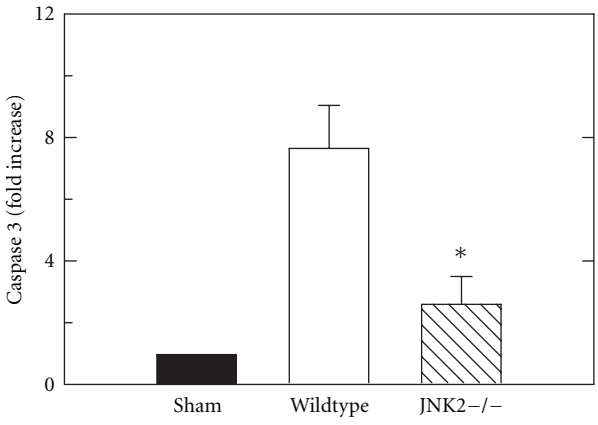

Figure 3: Decreased caspase 3 activation after hemorrhage and resuscitation of JNK2-deficient mice. At 6 h postoperatively, caspase 3 activity was assessed after sham operation and after H/R of wildtype and JNK2-deficient (JNK2−/−)mice, as described in Section 2. $P < 0.05$ versus wildtype, $n = 5$ per group.

exhibited bright, punctate staining by Rh123 in JNK2-deficient mice. In these experiments, hepatocytes were scored for Rh123 staining. In sham-operated mice, virtually no hepatocytes contained depolarized mitochondria. At 4 h after H/R of wildtype mice, 58% of hepatocytes contained depolarized mitochondria (Figure 4(d)). By contrast, at 4 h after H/R of JNK2-deficient mice, hepatocytes with depolarized mitochondria became 23%, less than half of that in wildtype mice ($P < 0.05$ versus wildtype, Figure 4(d)).

3.4. Decreased Oxidative Stress after Hemorrhage and Resuscitation of JNK2-Deficient Mice. We used 4-HNE immunohistochemistry to evaluate oxidative stress in mouse livers 6 h after H/R. 4-HNE is a product of lipid peroxidation that forms protein adducts that are recognized by anti-4-HNE antibodies. After sham operation, the brown reaction product of 4-HNE immunohistochemistry was virtually undetectable (Figure 5(a)). By contrast at 6 h after H/R of wildtype mice, wide confluent areas of HNE

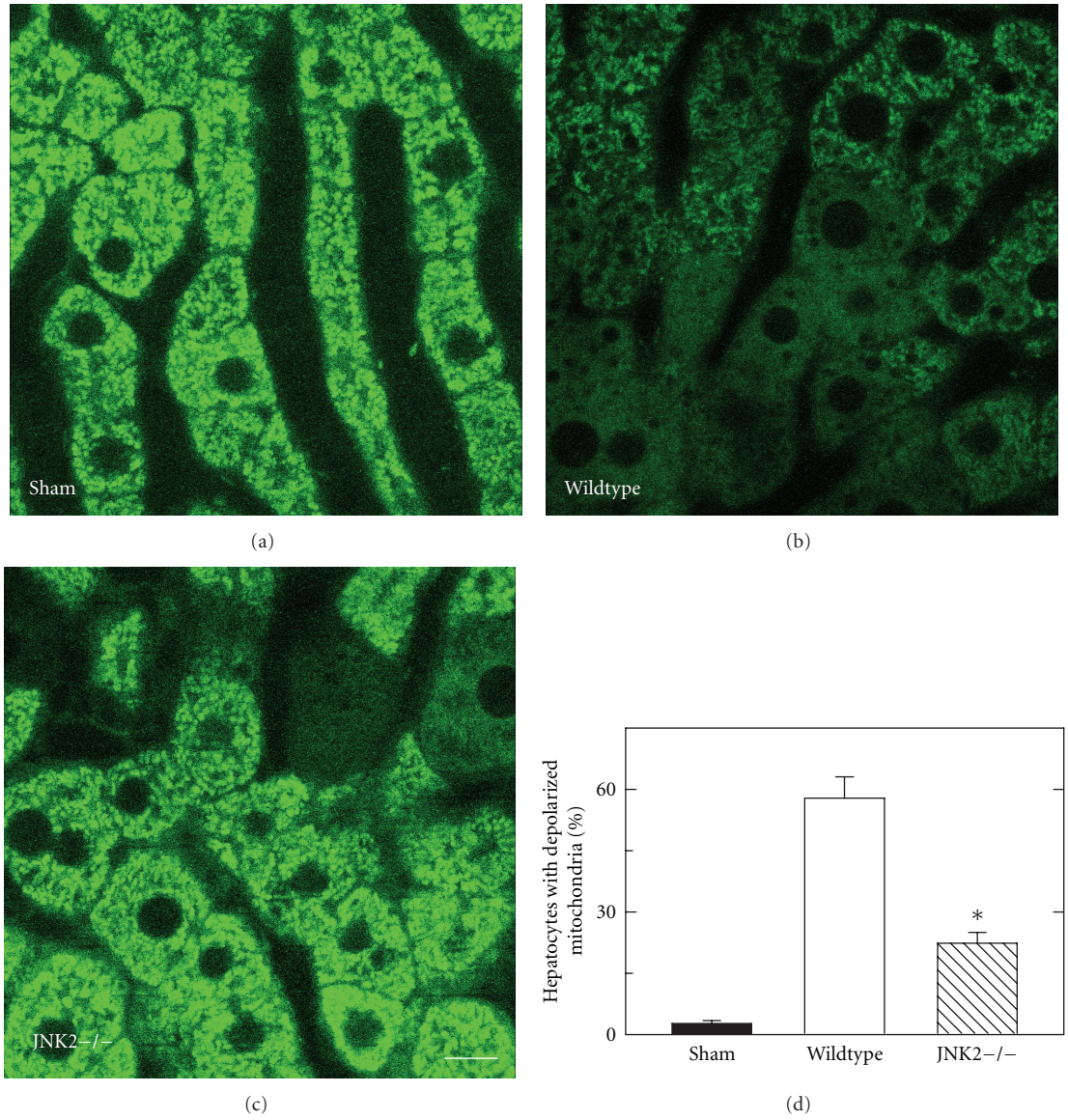

Figure 4: Decreased mitochondrial depolarization after hemorrhage and resuscitation of JNK2-deficient mice. Multiphoton imaging of hepatic Rh123 fluorescence was performed at 4 h after sham operation to wildtype mice (a) and H/R of wildtype- (b) and JNK2-deficient (c) mice, as described in Section 2. The percentage of hepatocytes per HPF with depolarized mitochondria is plotted in (d). Bar is 10 μm. $P < 0.05$ versus other groups; $n = 3$ per group.

immunoreactivity developed in pericentral and midzonal areas with relative sparing the periportal regions (Figure 5(b)). After H/R of JNK2-deficient mice, HNE immunoreactivity was substantially decreased and confined mostly to pericentral regions (Figure 5(c)).

4. Discussion

4.1. Decreased Liver Injury after Hemorrhagic Shock and Resuscitation of JNK2-Deficient Mice. Systemic inflammatory response syndrome (SIRS) and MODS following H/R are major problems after multiple trauma [3, 4]. H/R also causes hepatic necrosis and apoptosis [15, 23, 39]. The goal of this study was to evaluate the impact of JNK2 on hepatic injury and mitochondrial dysfunction after H/R. Our findings show a specific role for JNK2 in liver injury after H/R, since JNK2-deficient mice had decreased hepatic injury and mitochondrial dysfunction after H/R in comparison to wildtype mice (Figures 1–4).

4.2. Reperfusion Injury after Hemorrhagic Shock and Resuscitation Induces Necrosis and Apoptosis through JNK2 Signaling. JNK becomes activated in various models of liver injury, and pharmacological inhibition of JNK decreases liver injury [14, 15, 22–24, 40–42]. In particular, JNK inhibition with the peptide inhibitor, DJNKI-1, decreases hepatic damage and inflammation after H/R [23]. However, JNK inhibitors are nonspecific with regards to the two isoforms of JNK, JNK1

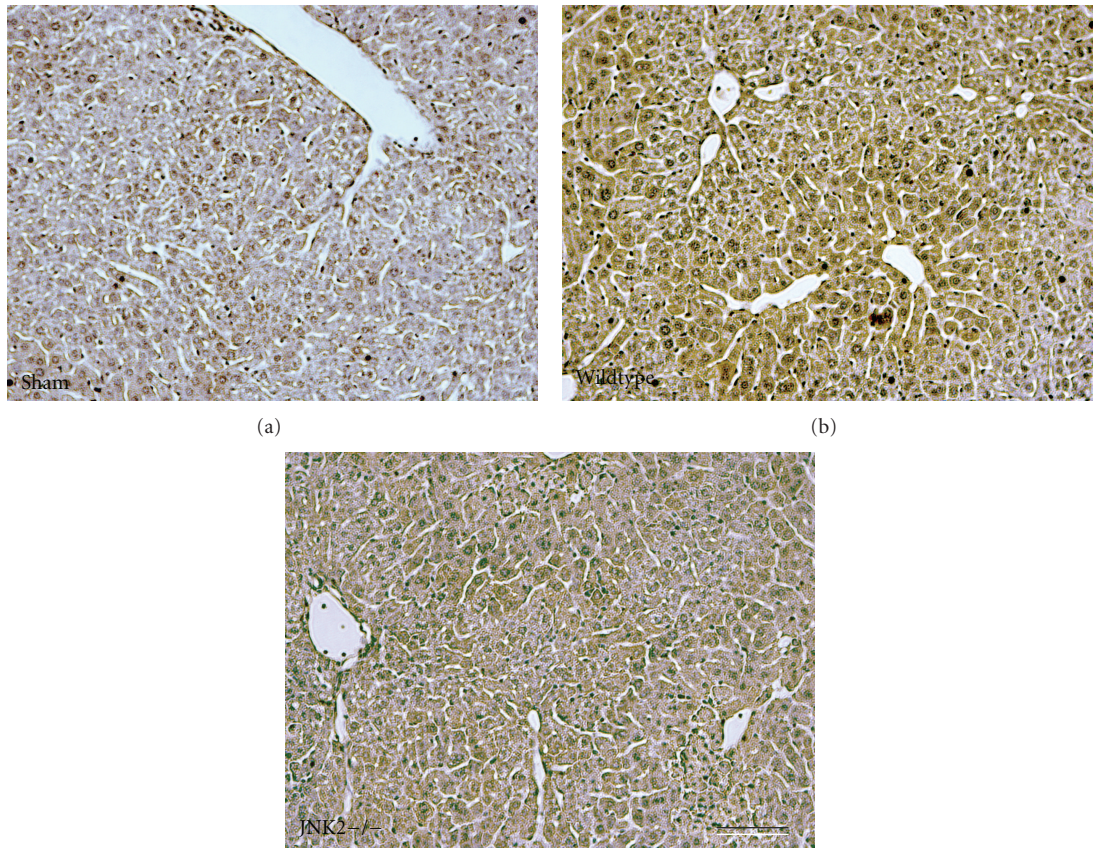

Figure 5: Decreased 4-hydroxynonenal immunostaining after hemorrhage and resuscitation of JNK2-deficient mice. ROS generation was assessed by 4-hydroxynonenal immunocytochemistry livers at 6 h after sham operation of wildtype mice (a) and after H/R to wildtype- (b) and JNK2-deficient (c) mice, as described in Section 2. Bar is 50 μm. $n = 5$ per group.

and JNK2, that are expressed in liver. Previous studies show that injury after orthotopic mouse liver transplantation and warm hepatic I/R decreases in JNK2-deficient livers compared to wildtype [29, 30]. In H/R, the specific roles of JNK isoforms are unknown. Therefore, we investigated the role of JNK2 by comparing JNK2-deficient mice and wildtype mice.

JNK2 deficiency decreased both necrosis and apoptosis in liver after H/R. Necrosis assessed by ALT and histology and apoptosis assessed by caspase 3 activity were decreased by 60% or more in JNK2-deficient mice compared to wildtype (Figures 1 and 2). Nonetheless, necrosis was the predominant mode of cell death after H/R. These results are in agreement with earlier results after liver transplantation and warm I/R [29, 30].

4.3. JNK2 Deficiency Attenuates Formation of Reactive Oxygen Species after Hemorrhage and Resuscitation. Reactive oxygen species (ROS) mediate, at least in part, liver injury after H/R, warm I/R, and storage/reperfusion injury occurring in liver transplantation. A consequence of ROS formation is peroxidation of polyunsaturated fatty acids, such as linoleic and arachidonic acids, which leads to 4-HNE generation and formation of 4-HNE-protein adducts. In the present study, hepatic 4-HNE immunostaining was marked after H/R to wildtype mice but substantially diminished in JNK2-deficient mice (Figure 5). This indicates that JNK2 signaling has a role in promoting ROS generation after H/R. Such ROS can directly damage proteins, lipids, and DNA, as well as to help induce the MPT.

4.4. JNK2 Signaling after H/R Induces Mitochondrial Depolarization and Promotes Liver Injury. To test the hypothesis that the JNK2 isoform specifically promotes mitochondrial dysfunction after H/R, we used intravital multiphoton microscopy of Rh123 to assess mitochondrial polarization. This technique allows direct assessment of mitochondrial polarization in livers of living animals. Four hours after H/R of wildtype livers, mitochondrial depolarization occurred in more than 50% of hepatocytes. Mitochondrial depolarization occurred prior to cell death, since after 4 h few cells labeled with propidium iodide, a marker of nonviable cells (data not shown), as described previously [29]. After H/R of JNK2-deficient mice, mitochondrial depolarization was markedly decreased in comparison to wildtype mice (Figure 4). Minocycline and N-methyl-4-isoleucine cyclosporin are specific inhibitors of the MPT that prevent mitochondrial depolarization after I/R and orthotopic rat liver transplantation with no direct effect

on mitochondrial respiration and oxidative phosphorylation [29]. Thus, mitochondrial depolarization visualized by intravital multiphoton microscopy, which was attenuated in JNK2-deficient mice, most likely represents onset of the MPT. Several studies indicate involvement of the MPT in acetaminophen hepatotoxicity [12, 20]. In acetaminophen hepatotoxicity, activated JNK translocates to mitochondria to induce MPT onset, which can be prevented by JNK inhibitors [20]. Thus, protection against mitochondrial depolarization in JNK2-deficient livers after H/R implies that JNK2 is directly involved in promoting the MPT in wildtype livers after H/R stress.

4.5. Other Mechanisms Promoting JNK2-Dependent Toxicity. H/R is also associated with a proinflammatory milieu in the gut lumen that promotes loss of barrier function [31]. Moreover, JNK2 mediates osmotic stress-induced tight junction disruption in the intestinal epithelium [36], although JNK1 is reported to mediate apical junction disassembly triggered by calcium depletion [37]. Impaired intestinal barrier function promoted by JNK during H/R may therefore also lead to portal vein endotoxemia, activation of TLR4 with phosphorylation of MAPKs, and increased production of inflammatory cytokines and ROS by hepatic Kupffer cells [34, 35, 43, 44]. Future studies will be needed to characterize how JNK2-dependent actions inside and outside hepatocytes contribute causally to liver injury, mitochondrial dysfunction, and development of MODS/SIRS after H/R.

4.6. Therapeutic Implications. An important implication of the present findings is that JNK2 represents a unique therapeutic target for treatment and prevention of hepatic injury and possibly SIRS and MODS after H/R. D-JNKI-1 and other existing JNK inhibitors are nonspecific and inhibit all JNK isoforms: JNK1, JNK2, and JNK3 [45]. JNK2 in our model of H/R plays a detrimental role, but JNK1 and/or JNK3 may have beneficial effects in liver and other tissues, especially since JNK1/JNK2 double knockout mice are not viable [46]. Thus, a specific JNK2 inhibitor might provide greater and more specific benefit after H/R and decrease the potential of toxicity by JNK1 and/or JNK3 inhibition, but such an inhibitor still awaits development.

List of Abbreviations

4-HNE: 4-hydroxynonenal
ALT: Alanine aminotransferase
H/R: Hemorrhage and resuscitation
H&E: Hematoxylin and eosin
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPF: High-power field
MODS: Multiple organ dysfunction syndrome
MPT: Mitochondrial permeability transition
Rh123: Rhodamine 123
ROS: Reactive oxygen species
SIRS: Systemic inflammatory response syndrome.

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Research Article

Effects of a Preconditioning Oral Nutritional Supplement on Pig Livers after Warm Ischemia

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Background. Several approaches have been proposed to pharmacologically ameliorate hepatic ischemia/reperfusion injury (IRI). This study was designed to evaluate the effects of a preconditioning oral nutritional supplement (pONS) containing glutamine, antioxidants, and green tea extract on hepatic warm IRI in pigs. *Methods*. pONS (70 g per serving, Fresenius Kabi, Germany) was dissolved in 250 mL tap water and given to pigs 24, 12, and 2 hrs before warm ischemia of the liver. A fourth dose was given 3 hrs after reperfusion. Controls were given the same amount of cellulose with the same volume of water. Two hours after the third dose of pONS, both the portal vein and the hepatic artery were clamped for 40 min. 0.5, 3, 6, and 8 hrs after reperfusion, heart rate (HR), mean arterial pressure (MAP), central venous pressure (CVP), portal venous flow (PVF), hepatic arterial flow (HAF), bile flow, and transaminases were measured. Liver tissue was taken 8 hrs after reperfusion for histology and immunohistochemistry. *Results*. HR, MAP, CVP, HAF, and PVF were comparable between the two groups. pONS significantly increased bile flow 8 hrs after reperfusion. ALT and AST were significantly lower after pONS. Histology showed significantly more severe necrosis and neutrophil infiltration in controls. pONS significantly decreased the index of immunohistochemical expression for TNF-α, MPO, and cleaved caspase-3 ($P < 0.001$). *Conclusion*. Administration of pONS before and after tissue damage protects the liver from warm IRI via mechanisms including decreasing oxidative stress, lipid peroxidation, apoptosis, and necrosis.

1. Introduction

During liver surgery, the inflow occlusion maneuver to prevent blood loss as well as the liver manipulation itself have been shown to induce a cascade of molecular events, referred to as ischemia-reperfusion injury (IRI). IRI leads to the activation of Kupffer cells (KCs), the release of reactive oxygen species (ROS) and proinflammatory cytokines, microcirculatory disturbances, and eventually liver dysfunction and failure [1–10]. Different strategies have been proposed to prevent or ameliorate IRI. Among others, pharmacological preconditioning has been shown to be effective via mechanisms including, but not limited to, the direct neutralization of ROS, upregulation of anti-inflammatory, and downregulation of proinflammatory signaling pathways [11–27].

During IRI, intestinal endotoxins (LPS) leak through the altered gut membrane into the portal circulation and enhance the phagocytosis in hepatic KCs [28–35]. This interrelation between intestinal LPS and hepatic KCs makes the gastrointestinal tract an attractive target for the pharmacological preconditioning strategies against hepatic IRI. We hypothesized that an oral pharmacological preconditioning supplement, tailored not only to exert direct ROS-scavenging activity but also to stabilize the gut epithelium during IRI, would tackle the warm hepatic IRI in a porcine model. To

the best of our knowledge, this work is the first report of an oral pharmacological preconditioning against hepatic IRI in a larger animal model.

2. Materials and Methods

2.1. Animal Care. German landrace pigs (32.3 ± 0.9 kg) were given access to standard laboratory chow (ssniff R/M-H, ssniff Spezialdiäten, Soest, Germany) and tap water *ad libitum* before experiments. All experimental procedures were reviewed and approved by the responsible authority (Regierungspräsidium Karlsruhe, Baden-Württemberg, Germany) according to the animal welfare legislation (§ 8 Abs. 1 Tierschutzgesetz (TierSchG) dated 18 May, 2006 (BGBI. I S. 1206)) and were performed according to institutional guidelines at the Ruprecht-Karls University of Heidelberg.

2.2. Experimental Procedure. Pigs underwent general anesthesia. After premedication with Azaperone (Stresnil, Janssen-Cilag Pharma, Wien, Austria, 1-2 mg/kg, i.m.) and midazolamhydrochloride (Dormicum 15 mg/3 mL, Roche, Grenzach-Wyhlen, Germany, 0.5–0.7 mg/kg, i.m.), anesthesia was induced with Esketaminhydrochloride (KETANEST S 25 mg/mL, Parke-Davis, Berlin, Germany, 10 mg/kg i.v.) and midazolam hydrochloride (1–1.4 mg/kg i.v.). After endotracheal intubation, animals were ventilated with a mixture of 1.5–2.0 L/min oxygen, 0.5–1.0 L/min air, and 0.75%–1.5% isoflurane (Isofluran-Baxter, Baxter, Unterschleißheim, Germany, semiopen ventilation). For analgesia, Piritramide (Dipidolor, Janssen-Cilag, Neuss, Germany, 3.75 mg/h intravenously) was administered. Body temperature was maintained using warming blankets (WarmTouch, Maleinckrodt Medical GmbH, Hennet/Sieg, Germany) and monitored by continuous rectal temperature probes. Systemic hemodynamic parameters, including mean arterial pressure (MAP) and central venous pressure (CVP), were measured continuously (Stetham Transducer, Hellige Monitoring, Freiburg, Germany) by indwelling polypropylene catheters (Braun, Melsungen, Germany) in the common carotid artery and internal jugular vein, respectively. Heart rate (HR) was monitored by body surface electrocardiogram recordings. Experimental groups were given a preconditioning oral nutritional supplement (pONS, 70 g per serving, Fresenius Kabi, Germany) containing glutamine, green tea extract (the resource, method of extraction, and composition of green tea extract has been published elsewhere [36]), vitamin C, vitamin E, beta carotene, selenium, zinc, and carbohydrates (1 sachet = 70 g) (Table 1) dissolved in 250 mL tap water 24 hrs (p.o.) and 12 hrs (p.o.) before the operation. The animals were then fasted overnight. On the day of operation and after performing a midline laparotomy, a third dose of pONS was applied via a jejunostomy tube. The portal vein and common hepatic artery were then mobilized and encircled by elastic bands. Two hrs after the administration of the third dose of pONS, the portal vein and the common hepatic artery were closed with Yasargil clamps (Aesculap, Tübingen, Germany) for 40 min to induce warm ischemia. Common bile duct was cannulated to collect bile continuously. After 40 min, the liver was reperfused by removing the clamps. A fourth dose of pONS was given 3 hrs after reperfusion. Controls were given the same amount of cellulose with the same volume of water. Serial blood samples were drawn and spun at 0.5, 3, 6, and 8 hrs after reperfusion and serum samples were kept at −20°C for the analysis of transaminases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) serum concentrations with standard enzymatic methods [37]. The changes in bile production during each time interval were documented and the amount of the newly produced bile was plotted at the end of each time interval to assess the bile flow rate over time. Liver tissue was taken 8 hrs after reperfusion for histology (hematoxylin and eosin (H&E) staining) and immunohistochemistry (TNF-α, myeloperoxidase, cleaved caspase-3). Hemodynamic parameters (HR, MAP, CVP, PVF, HAF) were continuously monitored throughout the experiments; ultrasonic probes (Transsonic System Inc, New York, NY, USA) were used for the measurement of portal venous flow (PVF) and hepatic arterial flow (HAF). The experimental design is outlined in Figure 1. After the completion of experimental procedures 8 hrs after reperfusion, animals were sacrificed in deep anesthesia through the intravenous application of a high dose of potassium chloride.

Table 1: Composition of the pONS.

Component	Dry weight per sachet (g)
Glutamine	15
Antioxidants	
Green tea extract	1
Vitamin C	0.75
Vitamin E	0.25
ß-carotene	0.005
Selenium	0.00015
Zinc	0.01
Carbohydrates	50

1 sachet of pONS was dissolved in 250 mL tap water before use. Conditions as mentioned in Materials and Methods. pONS: preconditioning oral nutritional supplement.

2.3. Histology. Liver samples were fixed by perfusion with 5% paraformaldehyde in Krebs-Henseleit bicarbonate buffer (118 mmol/L NaCl, 25 mmol/L NaHCO3, 1.2 mmol/L KH2PO4, 1.2 mmol/L MgSO4, 4.7 mmol/L KCl, and 1.3 mmol/L CaCl2) at pH 7.6, embedded in paraffin, and processed for light microscopy (H&E) 8 hrs after warm ischemia. In order to assess the histomorphological changes, 40 areas of 0.15 mm^2 were evaluated per slide using a point-counting method as described previously [38]: grade 0, minimal or no evidence of injury; grade 1, mild injury, including cytoplasmic vacuolation and focal nuclear pyknosis; grade 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders; grade 3, severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration. To describe leukocyte infiltration

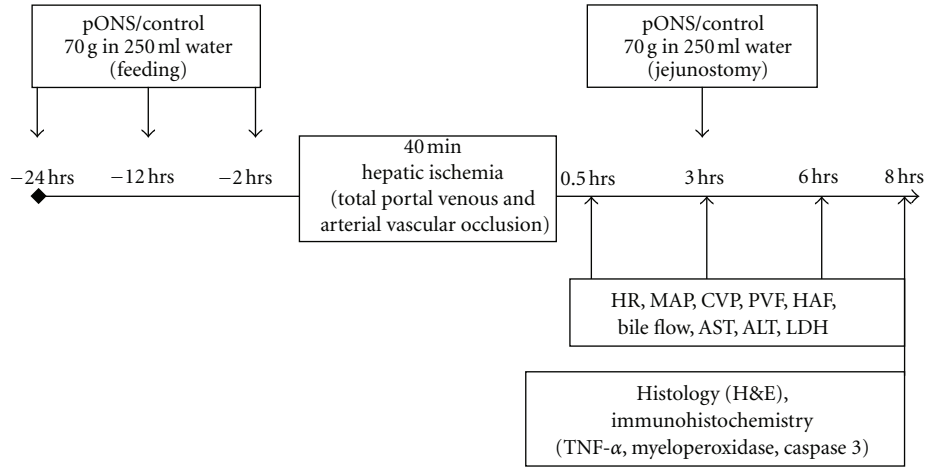

Figure 1: Experimental design. pONS (70 g in 250 mL tap water) was given to overnight-fasted German Landrace pigs 24, 12, and 2 hrs before warm ischemia of the liver. A fourth dose was given 3 hrs after reperfusion. Controls were given the same amount of cellulose. Two hrs after administration of the third dose, both the portal vein and the hepatic artery were clamped for 40 min. After reperfusion, hemodynamic parameters, bile production, and transaminases were measured serially. Liver tissue was taken 8 hrs after reperfusion for histology (H&E) and immunohistochemistry (TNF-a, myeloperoxidasby the Mann-Whitney rank sume, cleaved caspase-3) as described in Materials and Methods. pONS: preconditioning oral nutritional supplement; hrs: hours; min: minutes; PR: pulse rate; MAP: mean arterial pressure; CVP: central venous pressure; PVF: portal venous flow; HAF: hepatic artery flow; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

into the hepatic tissue, a scale from 1 to 4 was used: grade 1, <10 leukocytes/field (focal infiltration); grade 2, 10–20 (mild infiltration); grade 3, 21–50; grade 4, >50 leukocytes/field.

2.4. Immunohistochemistry. Paraffin sections from liver tissue obtained 8 hrs after reperfusion were deparaffinized in xylene and rehydrated with graded ethanol. Antigen retrieval was performed via microwave pretreatment in EDTA buffer (pH 9.0) three times for 5 min. The specimens were then cooled and treated with 30% hydrogen peroxidase (H_2O_2) in phosphate-buffered saline (PBS)—final H_2O_2 concentration: 1%—to block endogenous peroxidases. Nonspecific antibody binding was blocked by normal rabbit serum. Sections were incubated with rabbit polyclonal anti-mouse tumor necrosis factor-alpha (TNF-α) antibody (Biosource Europe, Nivelles, Belgium) at the dilution of 1 : 500, and rabbit polyclonal anti-cleaved caspase-3 antibody (DCS, Hamburg, Germany) at a 1 : 200 dilution. After incubation, secondary biotinylated polyclonal rabbit anti-mouse immunoglobulin (Dako, Hamburg, Germany) was applied at a dilution of 1 : 200 for 1 hr followed by streptavidin-biotin complex. For myeloperoxidase (MPO) immunohistochemistry analysis, the sections were pretreated with proteinase K (1 : 40 dilution) and then blocked with bovine serum albumin. They were then incubated with the primary antibody, a polyclonal rabbit anti-MPO antibody (Dako, Carpinteria, CA, USA) at a dilution of 1 : 200, for 60 min at room temperature. A biotinylated swine anti-rabbit antibody (diluted 1 : 300) was used as the secondary antibody.

Positive cells for immunohistochemistry were counted in 10 microscopic fields per slide and slides were evaluated with a semiquantitative technique, relating the score of 0 to 4 points to the fraction of stained cells: scale 0, 0% cells; 1, <5% cells; 2, 5%–20% cells; 3, >20%–40% cells; 4, >40% positive cells as described elsewhere [12].

2.5. Statistics. Mean values ± SEM were compared using one-way ANOVA with the Students-Newman-Keuls post hoc test for the analysis of differences in hemodynamic values, vascular flow measurements, bile production, and transaminases. Differences in histological grading of injury as well as in immunohistochemical staining were tested by the Mann-Whitney rank sum test. $P < 0.05$ was selected prior to the investigation as the criterion for significance of differences between groups.

3. Results

3.1. General and Hemodynamic Data. Hematocrit, body weight, and temperature were not different between control and pONS groups ($n = 6$ in each group) (Table 2). Continuous postperfusion monitoring of the hemodynamic parameters (HR, MAP, CVP, PVF, HAF) also showed no significant differences between the two groups (Table 2).

3.2. Liver Injury and Bile Production. While serum ALT increased in controls after warm ischemia/reperfusion to the liver, pONS prevented this effect; the difference between the two groups started to be significant 6 hours after reperfusion (49 ± 3 U/L in controls versus 35 ± 3 U/L in pONS; $P = 0.01$). This difference continued to exist until the end of experiments, 8 hrs after perfusion (50 ± 3 U/L in controls versus 33 ± 4 U/L in pONS; $P = 0.02$). pONS had the same effect on serum AST levels after reperfusion. The difference between the groups was significant 8 hrs after reperfusion (140 ± 52 U/L in controls versus 46 ± 7 U/L in pONS;

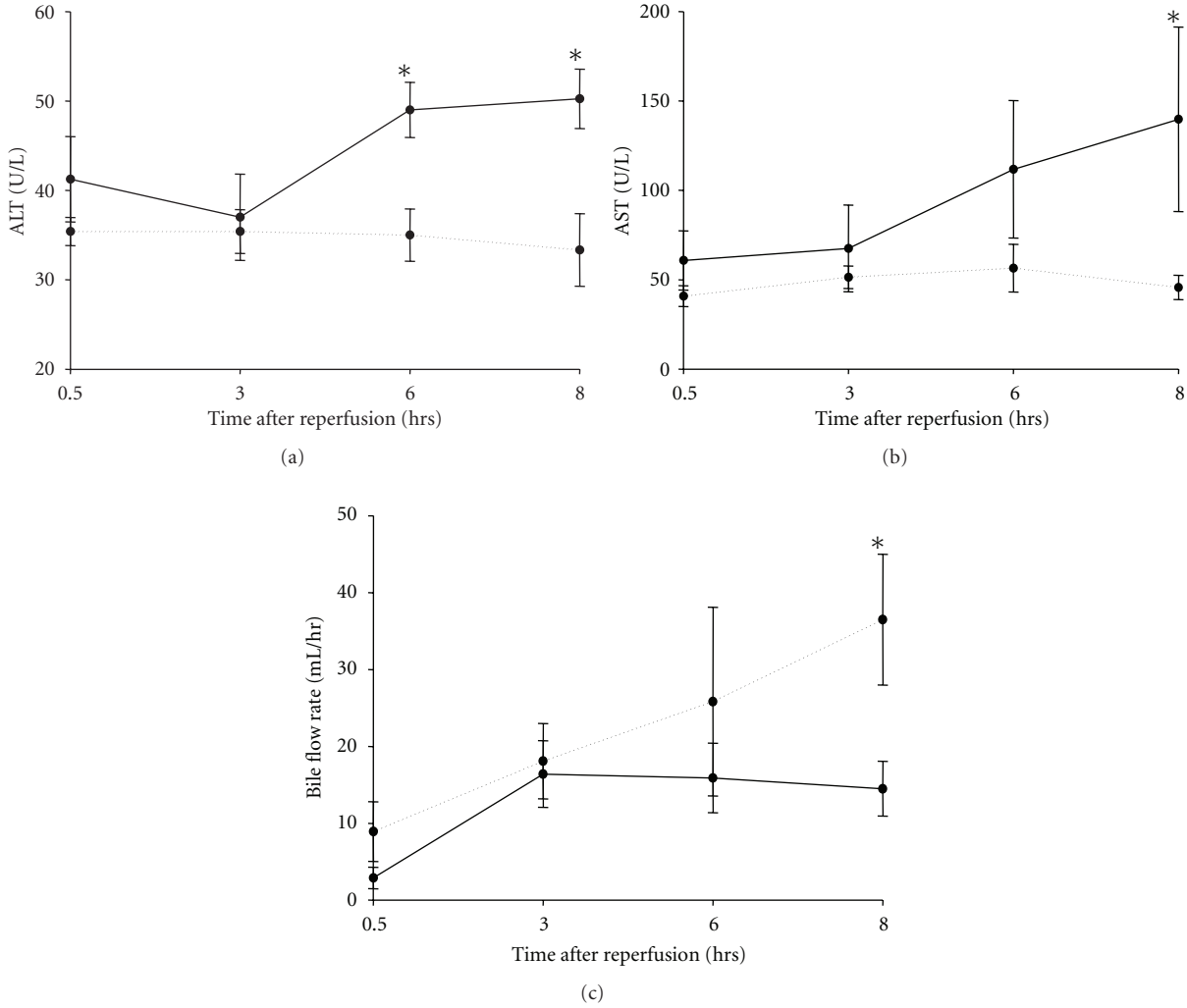

Figure 2: Effects of pONS on serum transaminases and bile production after reperfusion. ((a), (b)) Serial measurement of transaminases was performed after reperfusion as described in Materials and Methods. (c) Bile flow rate (mL/hr) has been depicted over time after reperfusion. Values are mean ± SEM ($P < 0.05$ by one-way ANOVA with Students-Newman-Keuls post hoc test, $n = 6$ per group); $^*P < 0.05$ for comparison to controls; AST: aspartate aminotransferase; ALT: alanine aminotransferase; pONS: preconditioning oral nutritional supplement.

Table 2: Basic parameters.

	Control ($n = 6$)	pONS ($n = 6$)	P
Body weight (kg)	33.1 ± 1.7	31.4 ± 0.8	0.4
Temperature (°C)	36.9 ± 0.2	36.8 ± 0.2	0.7
Respiratory rate (/min)	12 ± 1	12 ± 1	0.9
Hct (%)	30 ± 0.9	33 ± 1.7	0.1
HR (/min)	177 ± 10	185 ± 9	0.6
MAP (mmHg)	90 ± 4.1	91 ± 3.7	0.9
CVP (mmHg)	15 ± 1.7	14.8 ± 0.5	0.9
PVF (L/min)	1.4 ± 0.6	1.9 ± 0.2	0.3
HAF (dL/min)	114 ± 22	117 ± 12	0.9

Table shows basic parameters (body weight, temperature, and respiratory rate) as well as postperfusion data for hematocrit (Hct), heart rate (HR), mean arterial pressure (MAP), central venous pressure (CVP), portal venous flow (PVF), and hepatic arterial flow (HAF). Values are mean ± SEM.

$P = 0.01$) (Figure 2). There was significantly more severe necrosis with disintegration of hepatic cords, hemorrhage, and neutrophil infiltration (the median grade for necrosis and leukocyte infiltration were 3 and 4, resp.) in control tissue taken 8 hrs after reperfusion. pONS decreased the severity of the above-mentioned histomorphological changes in the liver (the median grade of necrosis and leukocyte infiltration of 1; $P < 0.001$) (Figure 3). Bile flow rate (mL/hr) was significantly higher in the pONS group 8 hrs after reperfusion (Figure 2).

3.3. Immunohistochemistry. The immunohistochemical analysis of sections obtained 8 hrs after reperfusion indicated positive staining for TNF-α, MPO, and cleaved Caspase-3. pONS reduced the number of positively stained hepatocytes against all of three above enzymes (Figure 4).

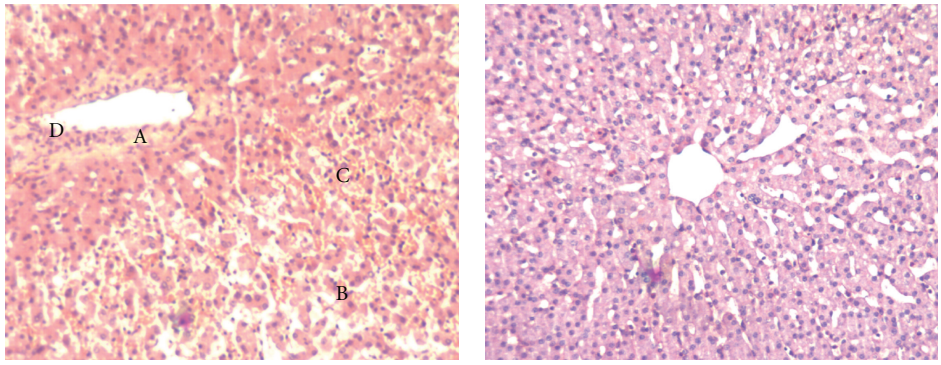

Figure 3: Liver injury eight hours after reperfusion. Liver tissue was taken 8 hrs after reperfusion and processed for light microscopy by H&E staining. (a), control; (b), pONS; controls displayed severe focal necrosis (A) with disintegration of hepatic cords (B), hemorrhage (C), and neutrophil infiltration (D) 8 hrs after reperfusion; this effect was significantly blunted by pONS. Pictures depict typical pattern of pathology; pONS: preconditioning oral nutritional supplement.

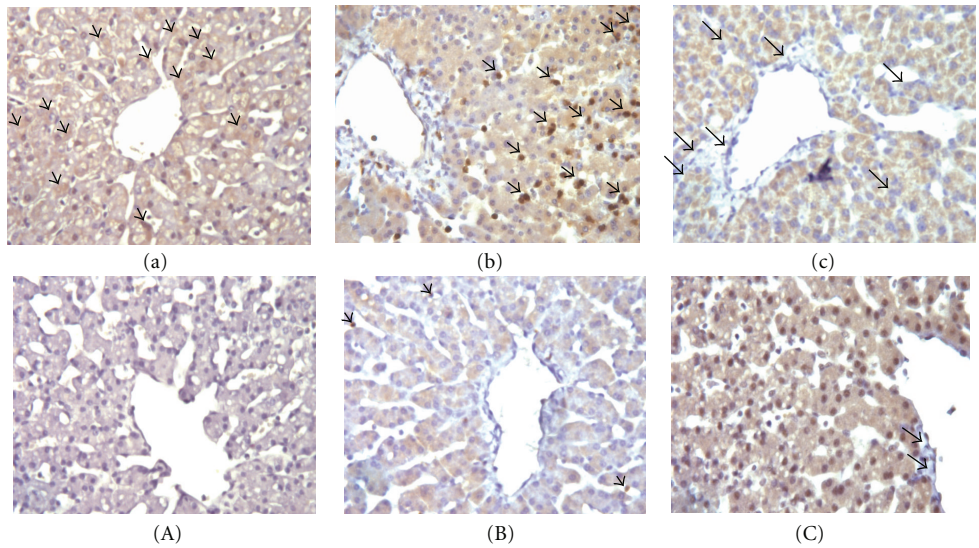

Figure 4: Immunohistochemistry for TNF-α ((a), (A)), MPO ((b), (B)), and cleaved Caspase-3 ((c), (C)). Conditions as described in Materials and Methods. Eight hrs after reperfusion, tissue was collected and processed for immunohistochemical analysis with light microscopy. The intensity of TNF-α expression (brown staining, black arrows), MPO expression (brown staining, black arrows), and cleaved Caspase-3 (blue halo, black arrows) was significantly higher in controls ((a), (b), (c), resp.) compared to their pONS counterparts ((A), (B), (C), resp.). Pictures depict typical pattern of staining (original magnification: $\times 200$). TNF-α: tumor necrosis factor-alpha; MPO: myeloperoxidase; pONS: preconditioning oral nutritional supplement.

The quantitative assessment of immunohistochemical findings is presented in Table 3.

4. Discussion

The manipulation of the liver during hepatic surgery activates Kupffer cells, which leads to the release of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interlukin-1 (IL-1) as well as free radicals, thus initiating an inflammatory cascade [2–7]. This phenomenon is usually further complicated through the application of the Pringle maneuver (hepatic inflow occlusion) in an attempt to prevent blood loss during the hepatic transection [39]. The cumulative effect induces warm IRI to the liver, which results, depending on the duration of ischemia, in microcirculatory disturbances, liver cell damage, and—in severe cases—liver failure [40]. Several approaches have been proposed to pharmacologically tackle hepatic IRI; none, however, has found its way into clinical routine yet.

To prevent IRI, it is important to neutralize reactive oxygen and nitrogen species, either by administering radical scavengers or by enhancing the capacity of endogenous redox defense systems [41]. A vast variety of dietary constituents can exert radical scavenging effects in vivo. Among all, hydrophilic ascorbic acid (vitamin C) and lipophilic α-tocopherol (vitamin E) are the important components of

Table 3: Quantitative assessment of immunohistochemical findings.

Expression	Control ($n = 6$)				pONS ($n = 6$)				P
	n	median	25%	75%	n	median	25%	75%	
TNF-α	84	2	2	3	102	1	1	1	<0.001
MPO	79	4	3	4	99	2	2	3	<0.001
Caspase-3	81	4	3	4	100	2	1	2	<0.001

Conditions as described in Materials and Methods; $n = 6$ in each group; median values of indices for immunohistochemical expression of TNF-α, MPO, and cleaved caspase-3 with interquartile range 8 hrs after warm ischemia/reperfusion have been compared with Mann-Whitney rank sum. TNF-α: tumor necrosis factor-alpha; MPO: myeloperoxidase; pONS: preconditioning oral nutritional supplement.

the human antioxidant system [42]. Carotenoids, the principal dietary source of vitamin A in humans [43], polyphenolic compounds in green tea extract [36, 44], selenium [45], and zinc [46] all exert antioxidant action; a synergism among the different antioxidants as part of an antioxidant network has been shown [47–49].

Large amounts of Gram-negative bacteria and endotoxins (LPS) are normally present in the intestines. A reduction in splanchnic blood flow and ischemia damages the intestinal wall and changes the permeability of the gut membrane, leading to excessive leakage of LPS and bacterial translocation into the portal circulation. It has been shown that LPS can activate KCs directly [50]. Scavenger receptors, including the scavenger receptor cystein-rich (SRCR) superfamily members that are expressed on KCs, are involved in the bactericidal action by binding and endocytosis of endotoxin [51, 52]. The CD11/CD18 receptor of KCs, the pattern recognition receptors (PRRs) CD14, and the Toll-like receptor 4 (TLR4) in combination with the adaptor protein MD2 are reported to be essentially involved in the LPS-associated KC activation [50, 53]. This may reflect an evolutionary adaptation by KCs to their local hepatic environment and strategic anatomic position in the portal circuit, which is optimal for the removal of endotoxin and, thus, for the protection of the host [54]. Glutamine has been shown to have a positive impact on the intestinal barrier by reducing permeability and bacterial translocation and preserving mucosal integrity [55, 56]. It can, therefore, prevent Kupffer cell activation and results in a more favorable outcome [57].

However, we did not measure blood LPS levels or histology of intestine, which might have further documented the protective effects of pONS on intestine.

In many models of liver injury, TNF-α levels are elevated and correlate with injury; the inhibition of TNF-α activity can attenuate liver injury, protect hepatic morphology, and decrease mortality [54]. MPO has been shown to be largely responsible for the neutrophil-induced parenchymal cell killing [58]. Released from the neutrophil's azurophilic granules, MPO can generate hypochlorous acid, a diffusible oxidant and chlorinating agent that gives rise to other toxic species, such as chloramines [59]. Apoptotic cell death can trigger neutrophil transmigration, severely aggravating apoptotic cell injury; caspase inhibitors can have a significant overall protective effect on hepatic IRI [60]. In the present study, we have shown that the oral administration of consecutive doses of a preconditioning supplement in experimental pigs significantly reduced the transaminases compared to controls after hepatic warm IRI. Furthermore, pONS resulted in significantly milder histological changes as well as a significant increase in bile production. The milder histological changes as well as improved sinusoidal bile production after pONS represents reduced postreperfusion injury. This has been further proved by the immunohistochemical analysis of the tissues obtained 8 hrs postreperfusion; pONS reduced the expression of TNF-α, MPO, and cleaved Caspase-3. pONS most likely exerts these protective effects via different mechanisms including direct antioxidative effects of its various antioxidant constituents including vitamin C, vitamin E, β-carotene, polyphenolic compounds in green tea extract, selenium and zinc. Furthermore, pONS most likely exerts an inhibitory effect on LPS-associated KC activation through glutamine.

To the best of our knowledge, this work is the first report of an oral pharmacological preconditioning against hepatic IRI in a larger animal model. The application of an oral nutritional substance in pigs is safe, reproducible, and well-deals with the current obstacles faced within the context of hepatic surgery and warm IRI. Tailoring such clinically-oriented experiments may finally help improve bench-to-bedside preconditioning protocols.

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Research Article

Desferrioxamine Attenuates Pancreatic Injury after Major Hepatectomy under Vascular Control of the Liver: Experimental Study in Pigs

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Introduction. Pancreatic injury can manifest after major hepatectomy under vascular control. The main mechanism involved seems to be remote oxidative injury due to "spillage" of reactive oxygen species and cytokines from the liver. The aim of this study is to evaluate the role of desferrioxamine in the prevention of pancreatic injury following major hepatectomy. *Methods*. Twelve Landrace pigs were subjected to a combination of major hepatectomy (70–75%), using the Pringle maneuver for 150 minutes, after constructing a porta-caval side-to-side anastomosis. The duration of reperfusion was 24 hours. Animals were randomly divided into a control group ($n = 6$) and a desferrioxamine group (DFX, $n = 6$). DFX animals were treated with continuous IV infusion of desferrioxamine 100 mg/kg. Pancreatic tissue injury, c-peptide and amylase concentrations, and pancreatic tissue oxidative markers were evaluated. *Results*. Desferrioxamine-treated animals showed decreased c-peptide levels, decreased acinar cell necrosis, and decreased tissue malondialdehyde levels 24 hours after reperfusion compared with the control group. There was no difference in portal pressure or serum amylase levels between the groups. *Conclusions*. Desferrioxamine seems to attenuate pancreatic injury after major hepatectomy under vascular control possibly by preventing and reversing production and circulation of oxidative products.

1. Introduction

Ischemia and reperfusion injury takes place during major hepatectomies due to the need for the use of vascular control techniques, as well as in liver transplantation and liver trauma. Although such maneuvers are invaluable in preventing excessive blood loss, they result in the production of cytokines and reactive oxygen species (ROS), which are responsible for induction of oxidative stress to the liver as well as to distant organs [1, 2]. Spillage of cytokines and inflammatory mediators has been shown to promote remote injury [3].

Hyperamylasemia and pancreatic injury has been well documented following major liver resections in patients with or without chronic liver disease [4, 5], as well as in experimental models [6]. Portal congestion, liver failure, and remote oxidative stress have been proposed as pathophysiologic mechanisms [3, 7]. Similar finding have been reported after liver transplantation [8, 9]. Although most often pancreatic injury can be subclinical, it can manifest as severe pancreatitis resulting in multiple organ failure, increasing morbidity and mortality following liver surgery [9, 10].

Desferrioxamine has been used in the past as an antioxidant in liver ischemia and reperfusion, as well as for the protection of remote organ injury after hepatectomy [11–13].

The aim of this study was to investigate the role of desferrioxamine as an antioxidant agent in the prevention of pancreatic injury that follows major hepatectomy under vascular control.

2. Methods

This protocol was approved by the Animal Research Committee of the University of Athens and the Committee of Bioethics of Aretaieion Hospital. Care and handling of the animals was in accordance with European guidelines for ethical animal research. Twelve female Landrace pigs weighing 30–35 kg were used. The animals were randomly divided in two groups: a desferrioxamine treatment group (DFX, $n = 6$) and a control group ($n = 6$). DFX animals received a constant intravenous infusion of desferrioxamine beginning at the time of initiation of hepatic ischemia, until the end of the experiment.

2.1. Surgical Procedure. All animals were subjected to major hepatectomy by removal of the left and median lobes, combined with ischemia of 150 minutes and a 24-hour reperfusion period, as described in the past [6]. A side-to-side portacaval anastomosis was performed using continuous 5-0 prolene sutures, in order to prevent splanchnic congestion. During the creation of the anastomosis, care was taken not to interrupt blood flow through the vessels. After the anastomosis, the left hepatic artery was ligated, and the hepatoduodenal ligament was clamped (Pringle maneuver). Afterwards, 70% hepatectomy was performed by resection of the median and left liver lobes. The liver remnant was kept ischemic for 150 minutes, and then the portacaval anastomosis was clamped, and portal blood flow was redirected back to the liver remnant by unclamping the hepatoduodenal ligament. A 20G catheter was then inserted in the portal vein through a side branch for portal pressure monitoring and portal blood sampling. The abdomen was closed, and the liver was reperfused for a 24-hour period during which the animals were kept under mechanical ventilation and monitored. Mean arterial pressure (MAP) and portal pressure (PP) were recorded, and blood samples were taken at the beginning of the reperfusion period and at 0, 6, 12, and 24 hours of reperfusion. At the end of the experiment, all animals were euthanized with intravenous infusion of thiopental 5 mg/kg and 2 g KCl, and pancreatic tissue was sampled for histological studies and measurement of malondialdehyde (MDA) and protein carbonyls content.

2.2. Desferrioxamine Administration Protocol. Desferrioxamine 100 mg/kg was administered continuously IV starting at the time of occlusion of the hepatoduodenal ligament (start of ischemia), until the end of the experiment. The total dose was divided in 66 mg/kg that was administered during the ischemic period until the 6th hour of reperfusion and 34 mg/kg that was administered after the 6th hour, until the end of the experiment. Animals in the control group received an equal volume of normal saline 0.9%.

2.3. Tissue Oxidative Markers Content Assay. Blood samples were separated by centrifugation at 4000 rpm at 4°C for 20 minutes and stored at −80°C until analysis. Pancreatic tissue was placed to liquid nitrogen immediately after collection and then stored at −80°C until analysis. MDA content was determined in the membrane fraction of the tissue according to the protocol we have already described [6]. The total protein of the membrane fraction was determined by the Bradford method [14], and the MDA content was determined according to the method of Jentzsch et al. [15] using 100 μg of membrane protein. Protein *carbonyls* were measured using the colorimetric assay kit from *Cayman Chemical* (Ann Arbor, MI). Results are expressed as nmoL per mg of tissue homogenate protein.

2.4. Histological Evaluation. Pancreatic tissues sampled were fixed in 4% formaldehyde, embedded in paraffin, and then cut into 3–5 μm sections and stained with haematoxylin-eosin (HE) staining.

An expert pathologist then studied five sights of each sample in a blind manner. A modification of Schmidt et al. [16] grading was used in which the pancreatic injury was evaluated regarding congestion, pancreatic cell necrosis, inflammation, and hemorrhage and static necrosis according to Table 1.

2.5. Biochemical Analysis. After collection, blood samples were separated by centrifugation at 4000 rpm for 20 minutes, and amylase levels were determined in systemic circulation using the Dimension RXL system (Dade Behring, Dupond). c-peptide concentration was determined in portal circulation as a marker of endocrine pancreatic cell injury, using a porcine c-peptide RIA kit (Linco Research, Missouri, USA).

2.6. Statistical Analysis. Analysis of variance was used in order to determine statistical significance when the distribution was normal. When the distribution was not normal, when the data was ordinal, and when standard deviations differed significantly, the nonparametric Man-Whitney test was used. Normality was tested using the Kolmogorov-Smirnov technique. All calculations were carried out using SPSS 15.0 for Windows. The level of statistical significance was set to $P < 0.05$. Data are expressed as mean ± SD.

Table 1: Pancreatitis histological grading.

Edema	
No edema present	0
Interlobular edema	1
Interacinar edema	2
Intercellular edema	3
Acinar cell necrosis	
No necrotic cells	0
1–4 necrotic cells/field	1
5–10 necrotic cells/field	2
>10 necrotic cells/field	3
Inflammation	
No leukocytes	0
1–4 leukocytes/field	1
5–10 leukocytes/field	2
>10 leukocytes/field	3
Hemorrhage and fat necrosis	
Absent	0
1–3 foci	1
3–5 foci	2
>5 foci	3

3. Results

3.1. Portal Pressure. There were no statistically significant differences in portal pressure between the two groups during the reperfusion period. In addition, there were no significant changes in portal pressure within groups during the reperfusion period.

3.1.1. Pancreatic Histology. Pancreatic microscopy revealed pancreatitis in both groups. Necrosis, edema, hemorrhage, and leukocyte accumulation were present in both groups. Necrosis was significantly lower in the DFX group compared with control animals (2.7 ± 0.5 versus 1.5 ± 0.5, $P < 0.05$). No differences were noted in inflammatory infiltration, edema, and hemorrhagic foci. Although total pancreatitis score was lower in the DFX group, the difference failed to achieve statistical significance ($P = 0.052$), as shown in Table 2.

3.1.2. Serological Markers of Pancreatic Injury. C-peptide levels followed an increasing pattern in both the groups during the first hours of reperfusion. In the control group, c-peptide was significantly higher compared to values immediately after reperfusion throughout the experiment ($P < 0.05$). In animals treated with desferrioxamine, c-peptide increased significantly after reperfusion until the 12-hour time point ($P < 0.05$). After 12 hours of reperfusion, c-peptide had a decreasing trend in the DFX group and did not have significant differences compared to values immediately after reperfusion. C-peptide values were significantly lower in the DFX group 24 hours after reperfusion compared with the control group ($P = 0.037$), as shown in Table 3.

Serum amylase levels increased during reperfusion in both groups until 12 hours after reperfusion, but the increase was not statistically significant. At 24 hours, amylase levels continued to increase in control group, still with no statistical significance. No significant differences between groups were observed in any time point, as shown in Table 4.

3.1.3. Pancreatic Tissue Protein Carbonyls and MDA Content. Pancreatic MDA content was decreased in the DFX group compared with the control group 24 hours after reperfusion ($P = 0.005$). In the contrary, there was no difference in pancreatic protein carbonyls content as shown in Table 5.

4. Discussion

Ischemia and reperfusion injury that occurs during liver transplantation, liver resections under vascular control, and liver trauma surgery has an impact on the liver as well as remote organs [6, 8, 11, 13, 17, 18]. The spillage of cytokines and reactive oxygen species have been implicated in the pathogenesis of remote organ injury [3, 6, 11, 13, 17, 19–21]. Pancreatic injury has been reported following liver ischemia reperfusion in liver transplantation and in major liver resections [18, 20, 21].

Antioxidants have been widely used to prevent or reverse reperfusion injury of the liver [22]. Desferrioxamine has been commonly used in ischemia and reperfusion injury [23–25] and has been shown to attenuate ischemic and oxidative injuries to the liver and other tissues [12, 26–33], as well as remote injury to the intestinal mucosa, the lung, and the myocardium [11, 13, 17]. Desferrioxamine chelates iron, preventing the production of oxygen free radicals though the Fenton equation, and induces the expression of hypoxia-inducible factor 1-alpha (HIF-1alpha) giving protection against hypoxic states [34, 35]. Desferrioxamine blocks an alternate pathway (Fenton reaction) in the production of reactive oxygen species compared to other antioxidants. Desferrioxamine has also been shown to have antioxidative properties by means of scavenging free radicals [36]. Recently, desferrioxamine was used for the prevention of pancreatitis induced by liver transplantation in rats [18].

We have already shown that the pathophysiology of pancreatitis following major hepatectomy under vascular control is multifactorial [6]. It involves portal hypertension during the Pringle maneuver leading to direct congestion of the pancreas [7], postoperative portal hypertension due to the decreased intrahepatic portal vasculature following massive resections [37–40], spillage of cytokines, oxidative substances, and reactive oxygen species which produce tissue injury [3, 20].

We have used an already described experimental model of major hepatectomy under vascular control without intraoperative portal hypertension during the Pringle maneuver [41]. Our data show that treatment with desferrioxamine attenuates pancreatic tissue necrosis compared with the control group 24 hours after reperfusion, while there is no difference in tissue edema, hemorrhage, and inflammatory infiltration. In both groups, interlobular and interacinar edema was noticed, while tissue was disorganized, showing necrosis of acinar cells, infiltration of a few leukocytes, and interstitial hemorrhage. Li et al. have reported similar

Table 2: Pancreatic tissue injury scoring.

	Group	Mean	Std. deviation	P value compared to control group
Total score	Control	9.5	1.4	0.052
	DFX	7.7	1.5	
Edema	Control	2.2	0.4	0.34
	DFX	2.0	0	
Necrosis	Control	2.7	0.5	**0.004**
	DFX	1.5	0.5	
Inflammation	Control	2.5	0.5	0.11
	DFX	1.8	0.8	
Hemorrhage	Control	2.2	0.8	0.66
	DFX	2.3	0.5	

Table 3: C-peptide levels (ng/mL) in portal circulation during the reperfusion period.

Timepoint	Group	Mean c-peptide levels in portal circulation (ng/mL)	Std. deviation	P value compared to the same time point of the control group
Baseline	Control	0.42	0.17	0.98
	DFX	0.42	0.22	
0 hours of reperfusion	Control	0.71	0.29	0.70
	DFX	0.78	0.34	
6 hours of reperfusion	Control	$0.97^{\dagger}$	0.30	0.25
	DFX	$1.20^{\ddagger}$	0.34	
12 hours of reperfusion	Control	$2.02^{\dagger}$	1.34	0.15
	DFX	$1.27^{\ddagger}$	0.35	
18 hours of reperfusion	Control	$1.56^{\dagger}$	0.67	0.08
	DFX	0.95	0.39	
24 hours of reperfusion	Control	$1.62^{\dagger}$	0.78	**0.037**
	DFX	0.90	0.26	

$^{\dagger}P < 0.05$ compared to c-peptide values on the control group immediately after reperfusion.
$^{\ddagger\dagger}P < 0.05$ compared to c-peptide values on the DFX group immediately after reperfusion.

Table 4: Serum amylase levels (U/mL) in systemic circulation during the reperfusion period.

Timepoint	Group	Mean serum amylase concentration (U/mL)	Std. deviation	P value compared to the same time point of the control group
0 hours after reperfusion	Control	1201	244	0.65
	DFX	1300	465	
6 hours after reperfusion	Control	1555	584	0.91
	DFX	1599	859	
12 hours after reperfusion	Control	1732	474	0.88
	DFX	1686	576	
24 hours after reperfusion	Control	1828	508	0.055
	DFX	1252	138	

findings after liver transplantation in rats. They reported attenuation of acinar cell necrosis and decreased edema 6 hours after liver transplantation in animals treated with desferrioxamine. This effect was even more remarkable 24 hours after transplantation [18].

According to the histological findings, serum markers of pancreatic cell injury were increased during the reperfusion period. C-peptide has been used in the past as a marker of endocrine cell damage, as it has been shown to correlate with morphological changes to the pancreas during injury [6, 42]. C-peptide was significantly lower in animals treated with desferrioxamine at the end of the experiment, while differences in amylase levels failed to achieve statistical significance in the same group. We have already shown that pancreatic

Table 5: Pancreatic tissue protein carbonyls and malondialdehyde (MDA) levels (nmoL/mg protein) 24 hours after reperfusion.

	Group	Mean value	Std. deviation	*P* value compared to the same time point of the control group
Tissue protein carbonyls (nmoL/mg protein)	Control	3.2	1.8	0.15
	DFX	4.8	3.9	
Tissue MDA (nmoL/mg protein)	Control	2.4	0.8	**0.005**
	DFX	1.1	0.1	

injury takes place early in the postoperative period, and that portal MDA content increases during the first hours of the reperfusion period after hepatectomy under vascular control. This effect peaks at 12 hours postoperatively and afterwards starts to disappear, as the percentage increase of portal MDA content in our previous study was higher in the 12-hour reperfusion time point compared to 24 hours [43]. This pattern is in accordance with the early phase of the ischemia reperfusion injury that has been documented in the literature [44, 45]. We attributed the sudden increase of c-peptide at 12 hours in both groups to oxidative injury to the pancreas that peaked at 12 hours postoperatively as demonstrated in our previous work.

In our study, we evaluated pancreatic tissue protein carbonyls and malondialdehyde (MDA) as markers of lipid peroxidation and oxidative injury. Pancreatic MDA was significantly lower following desferrioxamine treatment 24 hours after reperfusion. However, there was no difference in the levels of pancreatic tissue protein carbonyls. This could be explained by the fact that these biochemical processes are modulated by different mechanisms in this organ. Alexandris et al. have reported that lipid and protein oxidation can have different kinetics, resulting in different recovery times, thus influencing tissue levels [46].

In conclusion, our study supplies evidence that desferrioxamine attenuates pancreatic injury after major hepatectomy under vascular control. Desferrioxamine can decrease the production and systemic "spillage" of inflammatory and toxic mediators (oxidative products), that are produced during liver oxidative injury. The protective mechanism of desferrioxamine seems to be a combination of (1) chelation of redox active iron and prevention of oxygen free radicals production in the liver during reperfusion, thus preventing the production of inflammatory mediators and their circulation; (2) scavenging of reactive oxygen species produced in the liver and in other organs; (3) binding of redox active iron and prevention of its release from the liver during reperfusion.

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Research Article

ICAM-1 Upregulation in Ethanol-Induced Fatty Murine Livers Promotes Injury and Sinusoidal Leukocyte Adherence after Transplantation

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Background. Transplantation of ethanol-induced steatotic livers causes increased graft injury. We hypothesized that upregulation of hepatic ICAM-1 after ethanol produces increased leukocyte adherence, resulting in increased generation of reactive oxygen species (ROS) and injury after liver transplantation (LT). *Methods*. C57BL/6 wildtype (WT) and ICAM-1 knockout (KO) mice were gavaged with ethanol (6 g/kg) or water. LT was then performed into WT recipients. Necrosis and apoptosis, 4-hydroxynonenal (4-HNE) immunostaining, and sinusoidal leukocyte movement by intravital microscopy were assessed. *Results*. Ethanol gavage of WT mice increased hepatic triglycerides 10-fold compared to water treatment ($P < 0.05$). ICAM-1 also increased, but ALT was normal. At 8 h after LT of WT grafts, ALT increased 2-fold more with ethanol than water treatment ($P < 0.05$). Compared to ethanol-treated WT grafts, ALT from ethanol-treated KO grafts was 78% less ($P < 0.05$). Apoptosis also decreased by 75% ($P < 0.05$), and 4-HNE staining after LT was also decreased in ethanol-treated KO grafts compared to WT. Intravital microscopy demonstrated a 2-fold decrease in leukocyte adhesion in KO grafts compared to WT grafts. Conclusions. Increased ICAM-1 expression in ethanol-treated fatty livers predisposes to leukocyte adherence after LT, which leads to a disturbed microcirculation, oxidative stress and graft injury.

1. Introduction

After cold ischemic liver storage for transplantation, reperfusion injury may lead to poor initial graft function and even graft failure. This injury is more severe and causes increased morbidity and mortality when steatotic donor livers are used [1, 2]. Because of the increasing incidence of nonalcoholic steatohepatitis in the general population and the association of vehicular accidents with steatosis-causing alcohol use and abuse, an important fraction of potential human donor livers is steatotic. Such marginal steatotic livers are increasingly used as liver grafts because of the liver donor shortage and the expanding waiting list for liver transplantation.

Sinusoidal endothelial cells and hepatocytes are particularly susceptible to ischemia/reperfusion (I/R) injury and consequent apoptotic and necrotic cell death, as shown by both *in vitro* and *in vivo* studies [3–6]. After liver I/R, recruitment of neutrophils and other inflammatory cells aggravates injury [7, 8]. Neutrophil recruitment also contributes to liver injury after endotoxin, sepsis, and chronic ethanol treatment [9–12]. Hepatic infiltration with neutrophils results in production of reactive oxygen species

(ROS) and oxidative stress, resulting in neutrophil-mediated liver cell killing.

Intercellular adhesion molecule-1 (ICAM-1) is an endothelial- and leukocyte-associated transmembrane protein important in adherence of neutrophils to liver cells, including hepatocytes [13], and promotion of adherence-dependent oxidant stress, a major factor in neutrophil-mediated hepatocyte killing [10, 14]. However in a previous study, antibody blockade of ICAM-1, although decreasing white blood cell adherence, did not protect against I/R injury in a rat model of lean liver transplantation [15]. The importance of ICAM-1 in fatty liver transplantation has not been assessed. Accordingly, we compared liver injury in a murine model of fatty liver transplantation using wild-type and ICAM-deficient liver grafts. Our results show that ethanol treatment increases hepatic ICAM-1. Such ICAM-1 upregulation predisposes to leukocyte adherence, microcirculatory disturbances, oxidative stress, and increased graft injury after liver transplantation.

2. Materials and Methods

2.1. Ethanol Treatment and Donor Operation. All experiments were conducted using protocols approved by the Institutional Animal Care and Use Committee. Male C57BL/6 (wild-type) and ICAM-1-deficient mice, B6.129S4-*ICAM1*^{tm1Jcgr}/*J* on a C57BL/6 background, were gavaged with 6 g/kg ethanol or water alone. Twelve hours after gavage, livers were harvested under ether anesthesia and stored in ice cold University of Wisconsin (UW), as previously described [16]. Time for the donor operation averaged 22 min.

2.2. Recipient Operation. Livers from wild-type and ICAM-1-deficient mice were transplanted without rearterialization into wild-type mouse recipients under ether anesthesia, as previously described [16]. Both donor and recipient mice weighed 19–24 g. The recipient operation averaged 45 min, and portal vein clamp time averaged 15 min. For sham operations under ether anesthesia, wild-type and ICAM-1-deficient mice were laparotomized. After 45 min, the abdomen was closed.

2.3. Tissue Triglyceride Content. Liver tissues (50 mg) of both wild type and ICAM-1 deficient mice were homogenized in water, and lipids were extracted into $CHCl_3$ [17], dried in a vacuum centrifugal evaporator (Jouan RC 10.10, Thermo Scientific Inc., Atlanta, GA), and resuspended in 1 mL of $CHCl_3$. An aliquot (50 μL) was dried and resuspended in 100 μL of isopropyl alcohol, 1% Triton X-100. Triacylglycerol (TAG) content was then determined using an enzymatic colorimetric method (Triglyceride Test Kit, Stanbio Laboratory, Boerne, TX).

2.4. Alanine Aminotransferase (ALT). Blood samples to measure ALT were collected from the inferior vena cava 8 h after transplantation for analysis by standard methods.

2.5. Histology. Histology was evaluated 8 h after liver transplantation. Liver tissues were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin (H&E). Ten random fields were assessed for necrosis by standard morphologic criteria (e.g., loss of architecture, vacuolization, karyolysis, increased eosinophilia). Images were captured on a microscope (Zeiss Axiovert 100 microscope, Thornwood, NY), and the area percentage of necrosis was quantified using a computer program (AxioQuant, BD Bioimaging Systems, San Jose, CA).

2.6. Cell Death Immunohistochemistry. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed on paraffin sections using an *in situ* cell death detection kit (Roche Diagnostics, Penzberg, Germany). TUNEL-positive parenchymal and nonparenchymal cells were counted by light microscopy in 10 random high-power fields (HPFs).

2.7. Lipid Peroxidation Immunohistochemistry. Lipid peroxidation was assessed immunocytochemically by detecting 4-hydroxy-2-nonenal (HNE) adducts with a rabbit 4-HNE antibody (Alpha Diagnostic International, San Antonio, TX) with visualization by anti-rabbit IgG horse radish peroxidase (HRP) and diaminobenzidine (DAB) chromogen according to the manufacturer's instructions (DAKO corporation, Carpinteria, CA). The slides were then counterstained with hematoxylin.

2.8. Intravital Imaging of White Blood Cell Adherence. At 4 h after transplantation, recipients were anesthetized with pentobarbital (50 mg/kg) and connected to a small animal ventilator via a tracheostomy and respiratory tube (20-gauge catheter), as previously described [16]. Briefly, a catheter (0.4 mm inner diameter, Zeus, Inc., Orangeburg, SC) was inserted into the right carotid artery. Using a syringe pump, rhodamine 6G (1 μmol/mouse) was infused via the catheter over 20 min. During this time a laparotomy was performed using the previous incision line. After prone positioning of the mouse, the liver was gently withdrawn from the abdominal cavity and placed over a glass coverslip on the stage of a Zeiss Axiovert 100 microscope (Thornwood, NY). Images of rhodamine 6G fluorescence were collected with a 40X 1.2 NA water-immersion objective lens through a spinning disk confocal imaging attachment (Attofluor CARV Optical Module, BD Bioimaging Systems, San Jose, CA) to a 12-bit cooled CCD camera (Hamamatsu, Bridgewater, NJ). In 10 sec movies of 5 random fields per liver, white blood cells were scored for sticking (permanent adherence) and rolling (margination and slowing of white blood cell flow). Image analysis was performed in a blinded fashion using MetaFluor v.5.0 (Universal Imaging Corp., Downingtown, PA).

2.9. Statistical Analysis. Data are presented as means ± S.E., unless otherwise noted. Statistical analysis was performed by

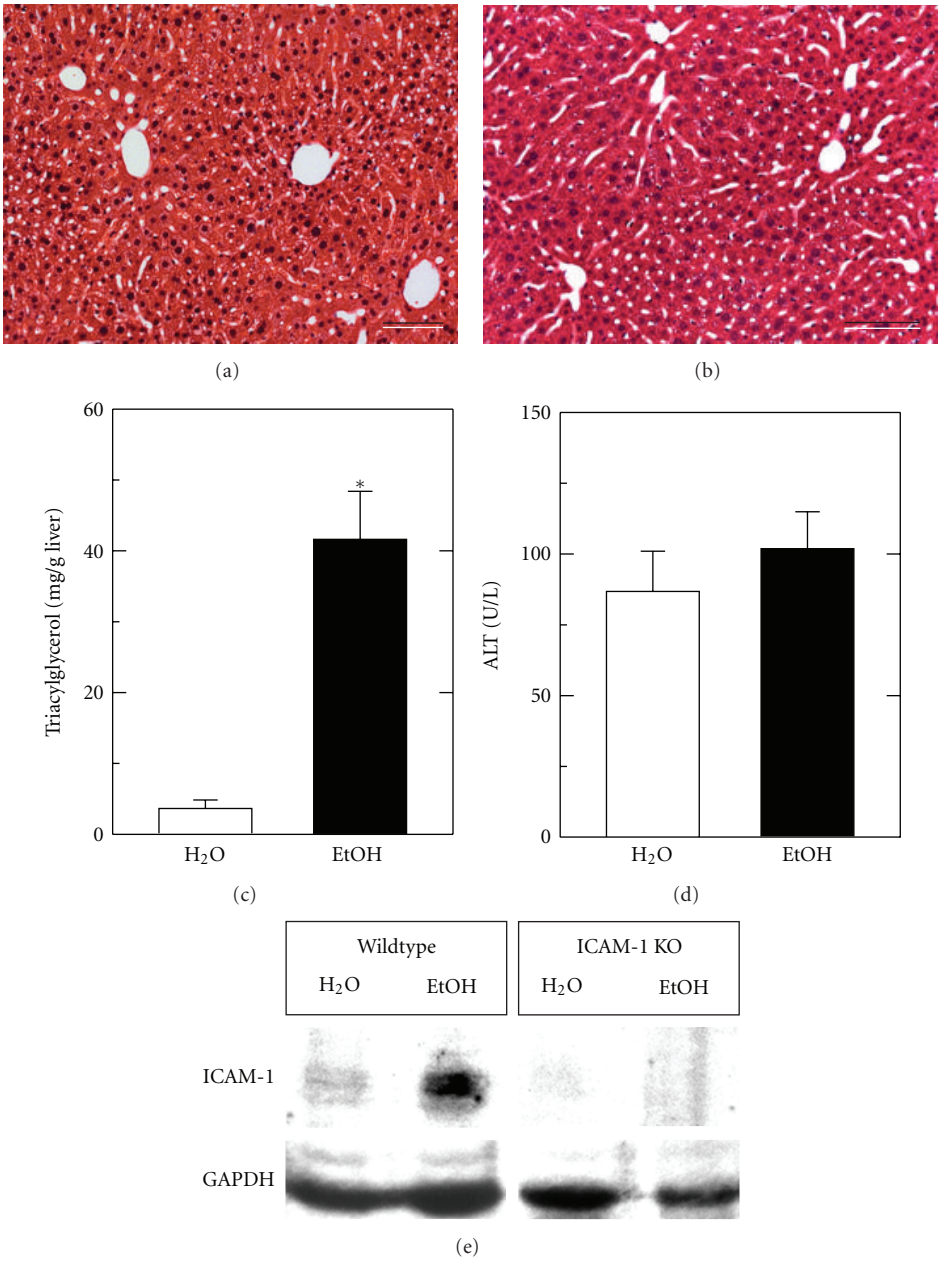

Figure 1: After ethanol gavage, mouse livers have increased fat content and ICAM-1 protein expression. Representative images of hepatic histology after ethanol gavage in wild type (a) and ICAM-1 deficient mice (b) are shown. Hepatic triacylglycerol (c), serum ALT (d), and hepatic ICAM-1 protein expression by Western blot (e) were assessed 12 h after water (H_2O) and ethanol (EtOH) gavage, as described in Section 2. (e) shows upregulation of ICAM-1 after ethanol treatment and the absence of ICAM-1 in ICAM-1 deficient (KO) mice. Size of individual groups was 3-4. Bar is 50 μm. $^*P < 0.05$ versus H_2O.

Student's t-test or ANOVA plus Student-Newman-Keuls test as appropriate, using $P < 0.05$ as the criterion of significance.

3. Results

3.1. ICAM-1 Upregulation in Ethanol-Induced Fatty Livers. Wild type and ICAM-1 deficient mice were gavaged with ethanol or water, as described in Section 2. At 12 h after ethanol gavage, marked steatosis occurred to an equal extent in the livers of wild type and ICAM-1 deficient mice, which were indistinguishable histologically (compare Figures 1(a) and 1(b)). Overall, no differences in histology in livers of wild type and ICAM-1 deficient mice were observed either before or after ethanol treatment. After 12 h, serum ALT levels were normal and comparable in both wild type and ICAM-1 deficient mice (Figure 1(c)). However, triacylglycerol levels increased 10-fold after ethanol treatment compared to water

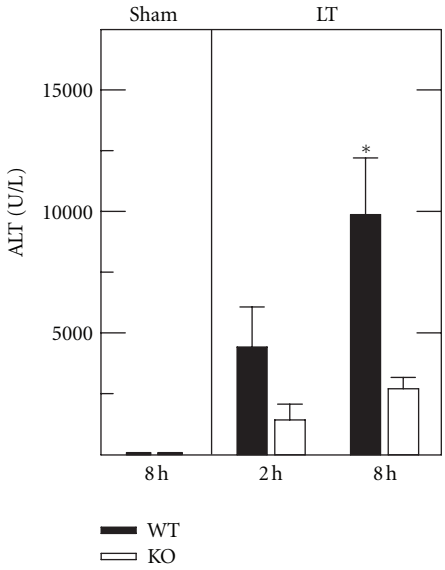

Figure 2: ALT release is decreased after transplantation of ICAM-1 deficient fatty livers. Serum ALT was assessed 8 h after sham operation (Sham) and 2 and 8 h after mouse liver transplantation (LT), as described in Section 2. Transplants were performed into WT recipients after ethanol treatment of wild type (WT) or ICAM-1 deficient (KO) liver donors. Sham groups had normal and comparable values to unoperated animals, and only sham is shown. Group sizes were sham WT and KO, 3; LT WT, 5; LT KO, 6. $^*P < 0.05$ versus KO grafts.

treatment ($P < 0.01$, Figure 1(d)). Western blotting of homogenized liver tissue revealed increased ICAM-1 expression at 12 h after ethanol treatment and, as expected, absent ICAM-1 expression in ICAM-1 deficient livers (Figure 1(e)). Overall, although ICAM-1 upregulation did not occur in ICAM-1 deficient mice, hepatic histology, steatosis, and ALT release were comparable in wild type and ICAM-1 deficient mice after acute ethanol treatment.

3.2. Decreased ALT Release and Graft Necrosis after Transplantation of ICAM-1 Deficient Fatty Livers. At 8 h after sham operation in ethanol-treated animals, wild type and ICAM-1 deficient mice had normal and comparable serum ALT averaging 118 ± 13 U/L (Figure 2). In contrast, ALT increased markedly in recipient mice after ethanol treatment, 12 h storage, and transplantation of livers. At 2 h after transplantation, ALT increased to 4,431 ± 1,636 U/L and 1,423 ± 656 U/L ($P = 0.1$), respectively, in recipients of wild type and ICAM-1 deficient livers. At 8 h after transplantation, ALT increased to 9,870 ± 2,344 U/L and 2709 ± 458 U/L, respectively ($P < 0.05$) (Figure 2). Thus, at 8 h following transplantation, ALT was 72% less in ICAM knockout than wild type liver recipients.

Graft injury was also assessed histologically. Liver histology was normal and indistinguishable in wild type and ICAM-1 deficient mice with and without sham operation (Figure 3(a) and data not shown). At 8 h after transplantation of wild type livers, large areas of necrosis were present with a predominantly pericentral and midzonal distribution (Figure 3(b)). By comparison, necrosis was decreased after transplantation of ICAM-1 deficient livers (Figure 3(c)). Morphometry revealed a decrease of hepatic necrosis from 25 ± 5.3% after wild type liver transplantation to 6.5 ± 3.1% after transplantation of ICAM-1 deficient livers ($P < 0.05$) (Figure 3(d)). Thus, transplantation of ICAM-1 deficient fatty livers decreased hepatic necrosis by three quarters.

3.3. Decreased Graft Apoptosis after Transplantation of ICAM-1 Deficient Fatty Livers. TUNEL was performed on tissue sections to assess double-stranded DNA breaks that are characteristic of apoptosis. TUNEL-positive cells were rare in wild type and ICAM-1 livers with and without sham operation, averaging less than 1 cell/HPF (Figures 4(a) and 4(d), and data not shown). At 8 h after transplantation with wild type livers, TUNEL in nonnecrotic areas increased to 12.2 ± 4.8 cells/HPF without apparent zonal localization (Figures 4(b) and 4(d)). After transplantation of ICAM-1 deficient livers, TUNEL decreased by about two-thirds to 3.5 ± 1.1 cells/HPF ($P < 0.05$, Figures 4(c) and 4(d)). As a percentage of all cells, TUNEL was 2.6 ± 1.3% after transplantation of wild type livers versus 0.7 ± 0.3% after transplantation of ICAM-1 deficient livers ($P < 0.05$).

3.4. Decreased White Blood Cell Adhesion in Fatty ICAM-1 Deficient Liver Grafts. At 4 h after sham operation, intravital confocal microscopy revealed bright fluorescence of rhodamine 6G-labeled white blood cells moving through hepatic sinusoids. No differences were seen in wild type or ICAM-1 deficient mice after sham operation and only occasional margination (rolling) and sticking of rhodamine 6G-labeled cells were noted in sinusoids (Figures 5(a) and 5(b), and Video A of supplemental data in Supplementary Material available online at doi:10.11/2012/480893). By contrast, at 4 h after liver transplantation of fatty wild type livers, marginating (rolling) and adherent (stickers) rhodamine 6G-stained cells increased markedly (Figure 5, Video B of supplemental data).

After transplantation of ICAM-1 deficient livers, fewer nonmobile rhodamine 6G-stained cells (stickers) were present in hepatic sinusoids (Video C of supplemental data). Rhodamine 6G-stained rollers and stickers were scored and counted for each liver. In ethanol-treated sham-operated livers, 1.6 ± 0.36 stickers/100 μm^2 were identified. After transplantation of fatty WT livers, stickers increased to 15.0 ± 4.04/100 μm^2, a more than 8-fold increase. Similarly, rollers increased by 2.5-fold after wild type transplantation compared to sham (Figures 5(a) and 5(b)). After transplantation of ICAM-1 deficient livers, stickers decreased 54% to 6.9 ± 1.04 per 100 μm^2 ($P < 0.05$ versus wild type) (Figure 5(b)). By contrast, rollers did not decrease in ICAM-1 compared to wild type liver grafts (Figure 5(a)). Thus, sinusoidal adherence (sticking) but not initial margination (rolling) of white blood cells was decreased in ICAM-1 deficient compared to wild type grafts after liver transplantation.

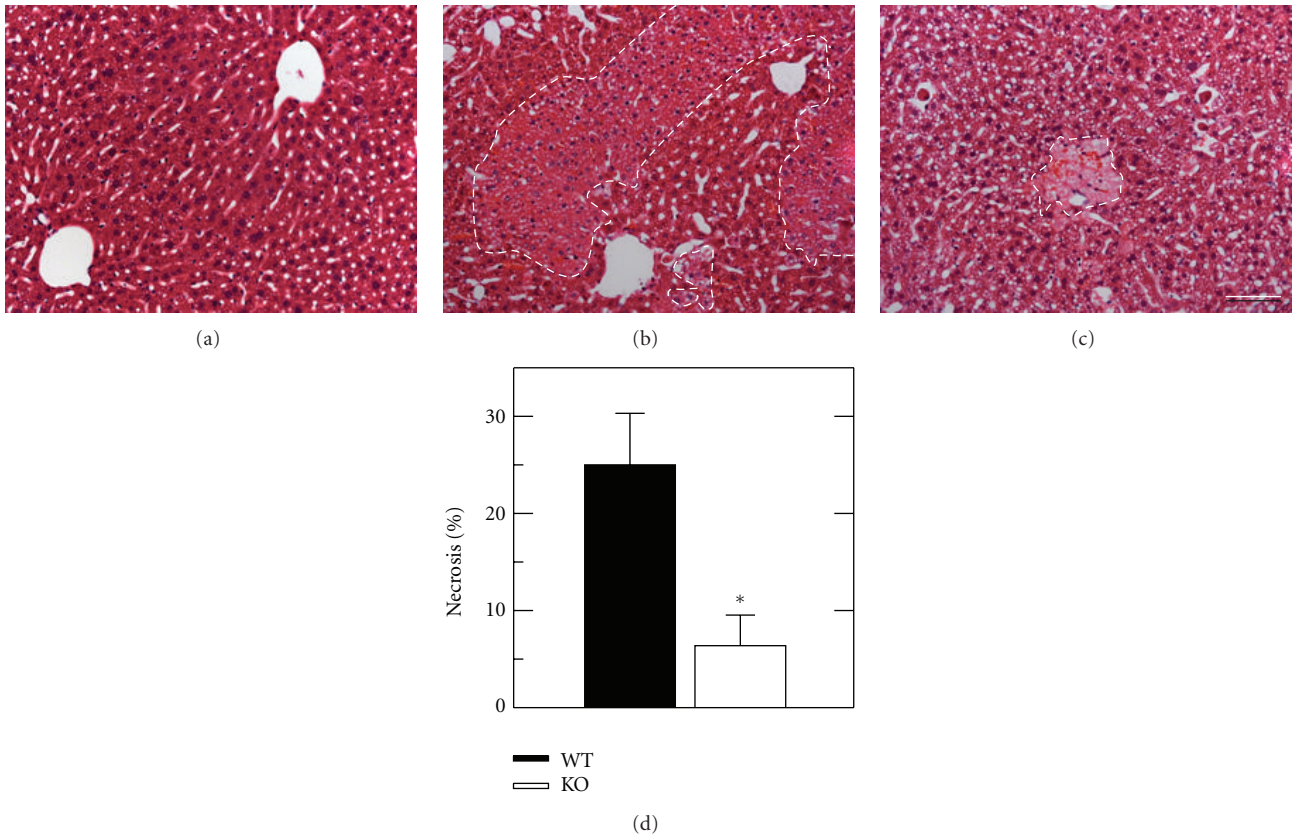

Figure 3: Necrosis is decreased after transplantation of ICAM-1 deficient fatty livers. Mouse livers were transplanted, as described in Section 2. At 8 h postoperatively, necrosis was assessed by H&E histology after sham operation (a), transplantation of wild type (WT) livers (b), and transplantation of ICAM-1 deficient (KO) livers (c). (d) shows necrosis as percent area in liver sections averaged from 5 livers per group. Necrosis in sham-operated WT and KO livers was absent. Bar is 50 μm. $^*P < 0.05$ versus WT grafts.

3.5. Decreased Oxidative Stress after Transplantation of Fatty ICAM-1 Deficient Livers. We used 4-HNE immunocytochemistry as a marker of oxidative stress in liver grafts at 2 h and 8 h after reperfusion. HNE adduct formation is a consequence of lipid peroxidation resulting from ROS generation. In livers of ethanol-treated wild type and ICAM-1 deficient mice at 8 h after sham operation, HNE brown staining was nearly undetectable (Figures 6(a) and 6(b)). By contrast, at 2 h after transplantation of fatty wild type livers, HNE staining in the cytoplasm and nuclei of hepatocytes was present in a mosaic pattern throughout the tissue sections (Figure 6(c)). After 8 h, HNE staining became confluent in midzonal and pericentral areas with sparing of periportal areas (Figure 6(e)).

Compared to wild type, far fewer hepatocytes of fatty ICAM-1 deficient liver grafts stained for HNE both at 2 h and 8 h after reperfusion (Figures 6(d) and 6(f)). Thus, oxidative stress in ICAM-1 deficient fatty liver grafts was decreased compared to wild type fatty grafts.

4. Discussion

The limiting factor in clinical liver transplantation is donor shortage, which leads to death of patients on the waiting list. Expanding the donor pool by including marginal steatotic donor livers would help shorten wait times and increase the availability of donor livers for transplantation. However, such increased use requires overcoming the increased susceptibility of fatty liver grafts to poor initial function and failure. Targeting specific pathways to decrease reperfusion injury of cold stored steatotic livers might thus be a beneficial approach to improve the function and survival of fatty liver grafts.

The aim of the present study was to evaluate the importance of ICAM-1 in graft injury after transplantation of ethanol-induced steatotic mouse livers. ICAM-1 has previously been shown to contribute to hepatic injury after various nonsurgical liver stresses [18, 19] and ICAM-1 blockade demonstrated less white blood cell adherence after lean rat liver transplantation [15]. We tested the hypothesis that ICAM-1 upregulation in ethanol-induced fatty livers leads to necrosis and apoptosis after transplantation through sinusoidal leukocyte adherence and subsequent ROS generation. We evaluated graft injury by enzyme release, necrosis and apoptosis, white blood cell adherence by intravital microscopy, and oxidative stress by HNE immunocytochemistry after transplantation. Our findings demonstrate that ethanol treatment upregulates ICAM-1 expression and that

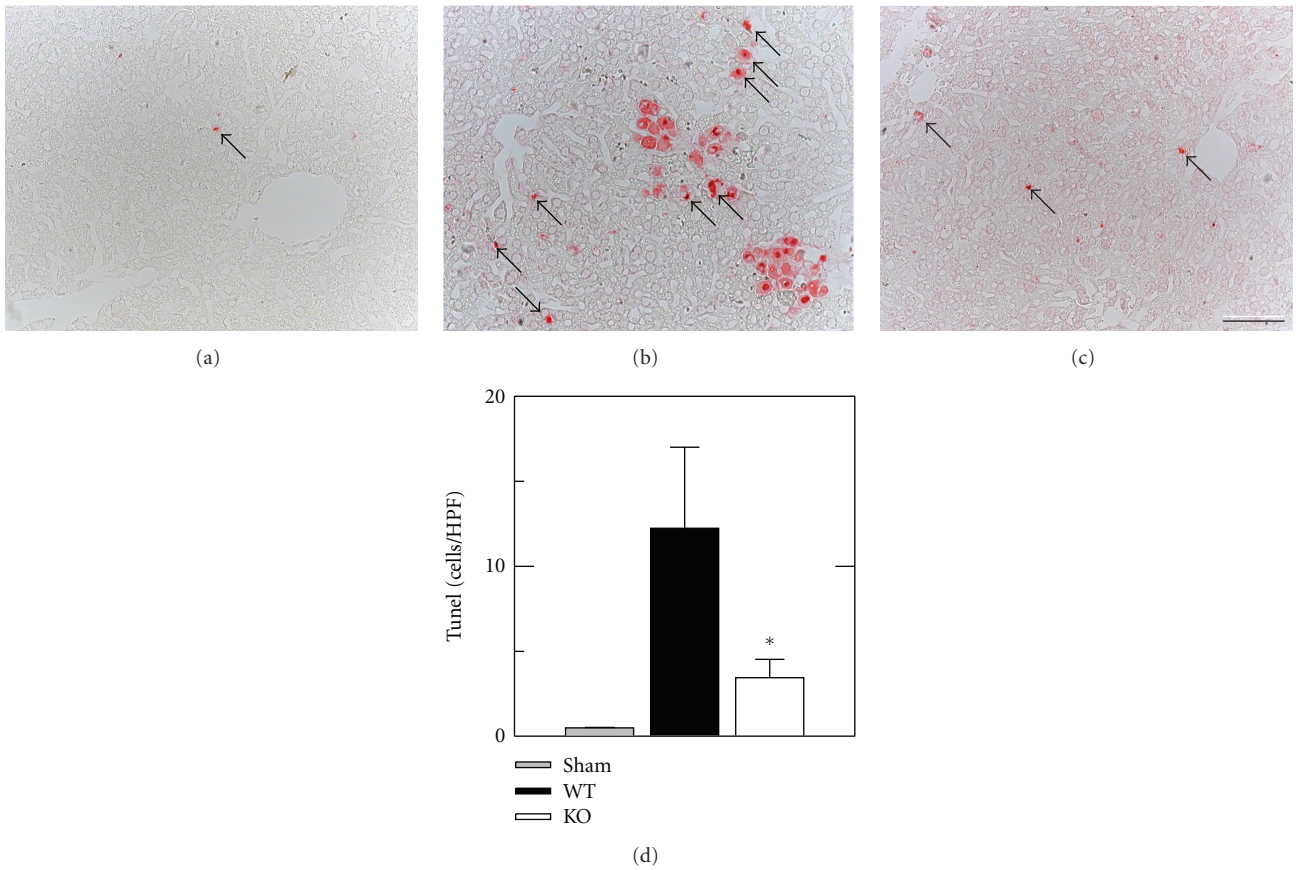

Figure 4: Apoptosis is decreased after transplantation of ICAM-1 deficient fatty livers. Mouse livers were transplanted, as described in Section 2. At 8 h postoperatively, TUNEL was assessed in tissue sections after sham operation (a), transplantation of wild type (WT) livers (b), and transplantation of ICAM-1 deficient (KO) livers (c). (d) quantifies the TUNEL-positive cells per high power field (HPF). TUNEL for WT sham was virtually zero and comparable to KO sham, and only KO sham is plotted. Bar is 50 μm. $^*P < 0.05$ versus WT grafts.

ICAM-1 deficiency decreases injury, leukocyte adherence and oxidative stress in ethanol-induced steatotic liver grafts. Our results are consistent with the conclusion that protection in ICAM-1 deficient grafts is the consequence of decreased sinusoidal adherence of white blood cells and decreased ROS formation.

Previous studies utilizing rodents demonstrate that doses of 5-6 g/kg ethanol increase hepatic triglyceride and decrease graft survival after transplantation substantially [20, 21]. Such a dose produces a peak blood ethanol concentration of about 370 mg/dL after 2 h in rats, which declines to undetectable levels in 8 to 10 h. No respiratory suppression is observed in these ethanol-treated animals receiving this treatment. In humans, fatty liver occurs to a similar extent after an acute ethanol binge [22]. Accordingly, rodent models have been used for decades to investigate the extent of ethanol-induced liver steatosis in a variety of contexts. In our experimental setting in mice, a single high dose of ethanol (6 g/kg) administered by gavage produced prominent hepatic steatosis 12 h later and a 10-fold increase of hepatic triacylglycerol content (Figure 1). Steatosis was associated with increased hepatic ICAM-1 expression (Figure 1(e)). Although blood alcohol peaked at about 580 mg/dL at 40 min after this treatment (data not shown), increases of necrosis, apoptosis, and serum ALT were negligible, and mortality did not occur. Interestingly, ethanol-induced upregulation of ICAM-1 alone did not increase white blood cell adherence before cold storage and reperfusion (Figure 5 and Video A of supplemental data). However, after transplantation of ethanol-induced fatty livers, white blood cell adherence increased markedly, an effect attenuated by more than half in ICAM-1 deficient liver grafts (Figure 5 and Videos B and C of supplemental data). Injury was also decreased in ICAM-1 deficient grafts (Figures 2, 3, and 4).

Previously in a study of warm liver I/R, antibody against ICAM-1 did not improve outcome when used alone but only when combined with antibodies against lymphocyte-function-associated antigen-1 (LFA-1) and beta 2 integrin, (CD-18), which are binding partners for ICAM-1 present on leukocyte membrane surfaces [23]. Similarly, after lean liver transplantation, ICAM-1 antibodies failed to prevent graft injury, although leukocyte adherence was decreased [15]. Thus, ICAM-1-dependent leukocyte adherence may itself be insufficient to cause liver injury and instead may act synergistically with other alterations to cause tissue damage. In the setting of transplantation of ethanol-induced

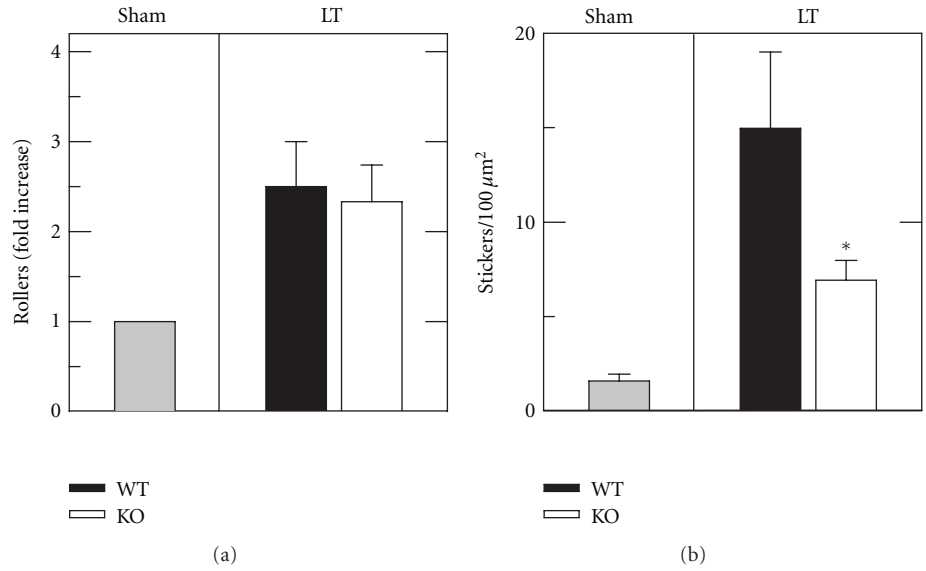

Figure 5: ICAM-1 deficient grafts show decreased leukocyte adherence but unchanged rolling margination after ethanol-induced fatty mouse liver transplantation. Livers were transplanted (LT) or subjected to sham operation (Sham) and visualized by intravital confocal microscopy of rhodamine 6G fluorescence after 4 h, as described in Section 2. Rhodamine 6G-fluorescing leukocytes were scored for slow flow rolling margination (Rollers, (a)) and no flow adherence to sinusoidal walls (Stickers, (b)) as either fold increase versus sham (a) or as absolute number per μm^2 (b). WT and KO sham groups were comparable, and only the KO sham group is plotted. Individual group size was 4. $^*P < 0.05$ versus WT grafts.

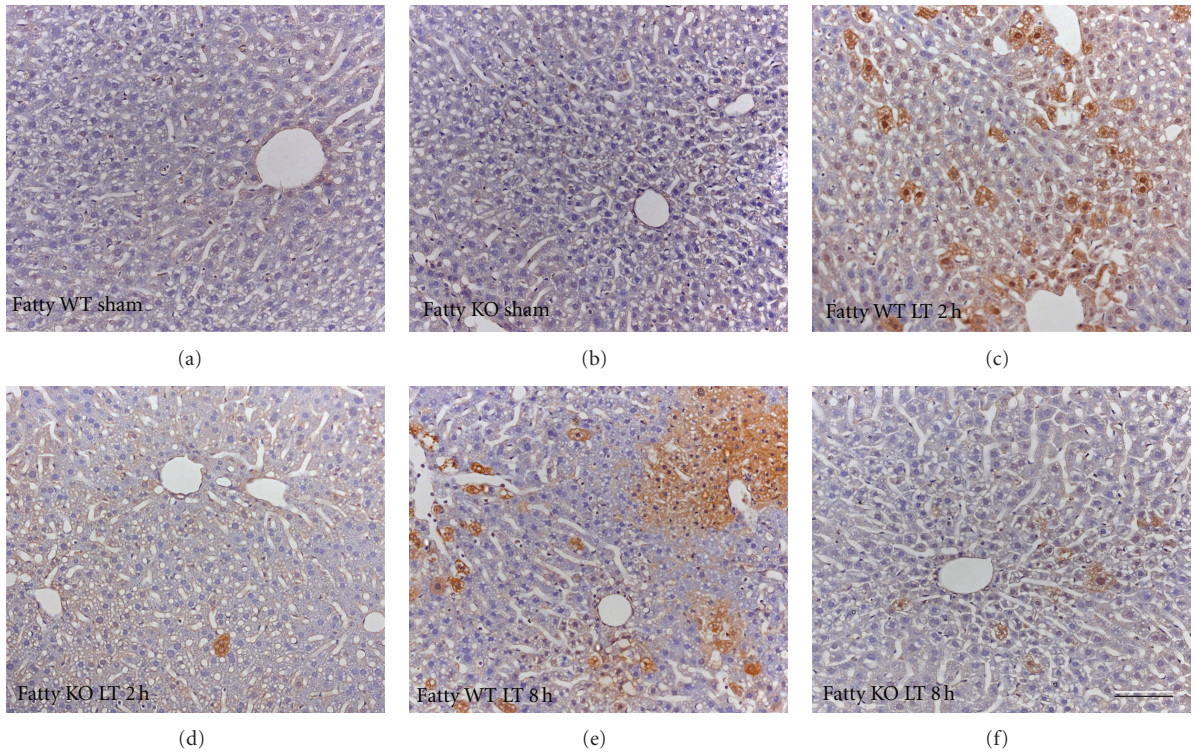

Figure 6: Decreased lipid peroxidation occurs in ICAM-1 deficient liver grafts. At 2 and 8 h after transplantation or sham operation, immunohistochemical staining was performed for 4-hydroxynonenal-modified proteins (4-HNE, *brown*), as described in Section 2. Panels are sham-operated wild type (WT) livers (a), sham-operated ICAM-1 deficient (KO) livers (b), WT liver grafts at 2 h after reperfusion (c), KO liver grafts at 2 h after reperfusion (d), WT liver grafts at 8 h after reperfusion (e), and KO liver grafts at 8 h after reperfusion (f). Brown HNE immunoreactivity was present in WT grafts after 2 and 8 h ((c) and (e)), which was markedly decreased in KO livers ((d) and (f)). Size of groups was 4. Bar is 50 μm.

fatty livers, our studies would suggest that ethanol-induced sinusoidal ICAM-1 upregulation is a first hit that alone is insufficient to cause hepatic injury. After transplantation, however, activation of leukocytes may represent a second hit, which when combined with the first hit causes hepatic necrosis, apoptosis, and enzyme release. ICAM-1 in our setting of fatty liver transplantation had more impact on injury than in previous models of warm hepatic I/R and lean liver transplantation. Ethanol-induced upregulation of ICAM-1 prior to I/R or transplantation stress may be particularly important in predisposing livers to injury, and ICAM-1 upregulation could potentially represent a biomarker to susceptibility of fatty livers to storage/reperfusion injury in human clinical transplantation.

Although leukocyte adherence (sticking) was decreased in ICAM-1 deficient liver grafts, rolling margination of leukocytes was unchanged (Figure 5, Videos B and C in supplemental data). These observations are consistent with earlier studies showing that endothelial selectins mediate rolling margination of leukocytes in response to chemokines and other proinflammatory signals, whereas ICAM-1 mediates adherence and infiltration into tissue [24].

ROS production by ischemic tissue after reperfusion is widely held as a major factor contributing to I/R injury. ROS generation also contributes to cold storage/reperfusion injury [21, 25–32]. ROS induces tissue damage by activating mitochondrial pathways leading to necrosis and apoptosis and through direct attack on proteins, lipids, and DNA. HNE is a product of ROS-dependent peroxidation of ω-6 polyunsaturated fatty acids, such as linoleic and arachidonic acid [33]. HNE reacts with protein sulfhydryls to form covalent adducts that can be detected by immunocytochemistry. In the present study, HNE adducts developed after wild type liver transplantation that were markedly decreased in ICAM-1 deficient liver grafts (Figure 6). Even at 2 hours after transplantation before onset of necrosis, HNE adducts were increased in wild type grafts but markedly decreased in ICAM-1 deficient grafts (Figure 6). These findings indicate that ROS production is temporally upstream of necrosis, which does not occur maximally until 4 or more hours after transplantation (Figures 2 and 3 and data not shown, see also [25, 26, 34–36]). Thus, ICAM-1-dependent leukocyte margination likely contributes to ROS generation and graft injury after wild type liver transplantation.

In summary, our results demonstrate involvement of ICAM-1 in storage/reperfusion injury to ethanol-induced fatty liver grafts. Prior to transplantation, ethanol treatment causes steatosis and upregulation of ICAM-1 expression in donor wild type livers. Such ICAM-1 upregulation was associated with increased leukocyte adherence, ROS generation, and injury to liver grafts. Thus, ICAM-1 upregulation and signaling in fatty liver grafts could represent a biomarker and target to identify and decrease susceptibility to storage/reperfusion injury of fatty liver grafts. However, the effects of chronic alcohol exposure or other means of fatty liver induction on graft injury, oxidative stress, and leukocyte recruitment may be different from those observed after acute ethanol administration. Thus, future studies will be needed to determine what benefit, if any, ICAM-1 targeting might have on fatty liver grafts in human clinical liver transplantation.

Abbreviations

ALT: Alanine aminotransferase
ATP: Adenosine triphosphate
CD-18: Beta 2 integrin
DAB: 3,3′-diaminobenzidine
H&E: Hematoxylin and eosin
HNE: 4-hydroxynonenal
HPF: High power field
HRP: Horse radish peroxidase
ICAM-1: Intercellular adhesion molecule-1
I/R: Ischemia/reperfusion
LFA-1: lymphocyte function-associated antigen-1
LT: Liver transplantation
ROS: Reactive oxygen species
TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
UW: University of Wisconsin cold storage solution.

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Research Article

Minocycline Decreases Liver Injury after Hemorrhagic Shock and Resuscitation in Mice

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Patients that survive hemorrhage and resuscitation (H/R) may develop a systemic inflammatory response syndrome (SIRS) that leads to dysfunction of vital organs (multiple organ dysfunction syndrome, MODS). SIRS and MODS may involve mitochondrial dysfunction. Under pentobarbital anesthesia, C57BL6 mice were hemorrhaged to 30 mm Hg for 3 h and then resuscitated with shed blood plus half the volume of lactated Ringer's solution containing minocycline, tetracycline (both 10 mg/kg body weight) or vehicle. Serum alanine aminotransferase (ALT), necrosis, apoptosis and oxidative stress were assessed 6 h after resuscitation. Mitochondrial polarization was assessed by intravital microscopy. After H/R with vehicle or tetracycline, ALT increased to 4538 U/L and 3999 U/L, respectively, which minocycline decreased to 1763 U/L ($P < 0.01$). Necrosis and TUNEL also decreased from 24.5% and 17.7 cells/field, respectively, after vehicle to 8.3% and 8.7 cells/field after minocycline. Tetracycline failed to decrease necrosis (23.3%) but decreased apoptosis to 9 cells/field ($P < 0.05$). Minocycline and tetracycline also decreased caspase-3 activity in liver homogenates. Minocycline but not tetracycline decreased lipid peroxidation after resuscitation by 70% ($P < 0.05$). Intravital microscopy showed that minocycline preserved mitochondrial polarization after H/R ($P < 0.05$). In conclusion, minocycline decreases liver injury and oxidative stress after H/R by preventing mitochondrial dysfunction.

1. Introduction

Trauma and surgical procedures, including gastrointestinal and hepatobiliary surgery, can lead to severe hemorrhage and hypovolemic shock. Fluid resuscitation after less than one hour of severe hemorrhagic shock restores hemodynamics and typically leads to full recovery. By contrast although restoring hemodynamics, resuscitation after greater than an hour may lead instead to multiple organ dysfunction syndrome (MODS), which is associated with mortality of 30% [1]. Effective strategies to extend this golden hour for resuscitation are therefore needed to improve the treatment of hemorrhagic shock and decrease the incidence of MODS and its lethal consequence. Hemorrhage/resuscitation (H/R) is an example of ischemia/reperfusion (I/R) and hypoxia/reoxygenation injuries, for which mitochondrial dysfunction plays a major pathophysiological role [2–4]. Moreover, the liver with its crucial involvement in metabolism and homeostasis is among the most frequently affected organs after hemorrhage-induced hypotension in humans [5].

I/R injury leads to both necrotic cell death and apoptosis. A common pathway for hepatic apoptosis and necrosis after I/R is the mitochondrial permeability transition (MPT) [6]. Opening of permeability transition (PT) pores in the mitochondrial inner membrane causes the MPT with

consequent mitochondrial depolarization and uncoupling of oxidative phosphorylation. ATP depletion after uncoupling produces necrotic cell killing, the main pathway of cell death after I/R, whereas cytochrome *c* release due to MPT-driven mitochondrial swelling induces caspase-dependent apoptosis. Previously, experimental strategies to inhibit the MPT after liver transplantation in rats improved survival and decreased mitochondrial dysfunction [7].

Minocycline is a semisynthetic tetracycline antibiotic, which is protective against neurodegenerative disease, trauma, and hypoxia/ischemia [8–12]. Mechanisms by which minocycline exerts neuroprotection include inhibition of apoptotic pathways, decreased mitochondrial release of proapoptotic factors like cytochrome *c*, and upregulation of antiapoptotic Bcl-2 and inhibitor of apoptosis proteins (IAPs) [13, 14]. In orthotopic rat liver transplantation, minocycline cytoprotection against storage/reperfusion injury is mediated by suppression of the MPT through inhibition of the mitochondrial calcium uniporter [7]. Here, we investigated whether resuscitation with minocycline also decreases liver injury after H/R.

2. Materials and Methods

2.1. Chemicals and Reagents. Minocycline, tetracycline, rhodamine 123, and other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animals. Male C57BL/6J mice (8–10 wk of age, 23–27 g) were obtained from Jackson Laboratory (Bar Harbor, ME). Animal protocols were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina.

2.3. Hemorrhagic Shock and Resuscitation. After an overnight fast, mice were anesthetized with pentobarbital sodium (80 mg/kg body weight). Under spontaneous breathing, both femoral arteries were exposed and cannulated with polyethylene-10 catheters (SIMS Portex). The catheters were flushed with normal saline containing heparin (100 IU/l) before insertion. One catheter was connected via a transducer to a pressure analyzer (Micro-Med; Louisville, KY), and blood was withdrawn via the second catheter into a heparinized syringe (10 units) over 5 min to a mean arterial pressure of 30 mm Hg. This pressure was maintained for 3 h by withdrawal or reinfusion of shed blood [15]. Body temperature was monitored and maintained at 37°C. After 3 h, mice were resuscitated with a syringe pump over 30 min with shed blood followed by a volume of lactated Ringer's solution corresponding to 50% of the shed blood volume [16, 17]. As indicated, the resuscitating Ringer's solution contained minocycline (10 mg/kg body weight), tetracycline (10 mg/kg), or vehicle. Doses of minocycline and tetracycline were based on a prior study [7]. Adequacy of resuscitation was determined by the restoration of blood pressure. Catheters were then removed, the vessels were ligated, and the groin incisions were closed. Sham-operated animals underwent the same surgical procedures, but hemorrhage was not carried out. No mortality in any group occurred over the course of the experiments.

For the determination of hemorrhage-/resuscitation-dependent liver damage, mice were anesthetized and killed by exsanguination 6 h after the end of resuscitation. For each mouse, the two right dorsal liver lobes were snap-frozen in liquid nitrogen. The remaining liver was flushed with normal saline, infused and fixed with 4% buffered paraformaldehyde through the portal vein, and embedded in paraffin sections.

2.4. Alanine Aminotransferase (ALT). Blood samples to measure ALT were collected from the inferior vena cava 6 h after H/R for analysis by standard methods.

2.5. Histology. Sections (4 μm) were stained with hematoxylin and eosin (H&E). Ten random fields were assessed for necrosis by standard morphologic criteria (e.g., loss of architecture, vacuolization, karyolysis, increased eosinophilia). Images were captured by an image analysis system (Olympus BH-2 Microscope; Micropublisher 5.0 RTV, Center Valley, PA), and the area percentage of necrosis was quantified using a computer program (BioQuant BQ Nova Prime 6.7, R&M Biometrics, Nashville, TN).

2.6. TUNEL. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed on paraffin sections using an *in situ* cell death detection kit (Roche Diagnostics, Penzberg, Germany). TUNEL-positive cells were counted by light microscopy in 10 random high-power fields (HPF).

2.7. Caspase-3. Liver tissue (~100 mg) was homogenized (Polytron PT-MR2100, Kinematica, Luzern, Switzerland) in 1 mL of lysis buffer containing 0.1% 3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid, 2 mM EDTA, 5 mM dithiothreitol, 1 mM Pefabloc, 10 ng/mL pepstatin A, 10 ng/mL aprotinin, 20 μg/mL leupeptin, and 10 mM HEPES buffer, pH 7.4. The lysate was centrifuged at 15,000 rpm for 30 min. Activity of caspase-3 in the supernatant was determined using a Caspase-3 Colorimetric Assay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Activity was normalized to protein concentration of each sample and expressed as fold increase compared to sham.

2.8. 4-Hydroxynonenal. Paraffin-embedded sections were deparaffinized, rehydrated, and incubated with polyclonal antibodies against 4-hydroxynonenal (4-HNE, Alpha Diagnostics; San Antonio, TX) in PBS (pH 7.4) containing 1% Tween 20 and 1% bovine serum albumin. Peroxidase-linked secondary antibody and diaminobenzidine (Peroxidase Envision Kit, DAKO) were used to detect specific binding. A Universal Imaging Metamorph image acquisition and analysis system (Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss; Thornwood, NY) was used to capture and analyze the immunostained tissue sections at 40x magnification. The extent of labeling was determined in randomly selected fields as the percentage of area within

a preset color range determined by the software. Data from each tissue section (10 fields/section) were pooled to determine means, as described previously [18].

2.9. Intravital Multiphoton Microscopy. At 4 h after H/R, mice were anesthetized with pentobarbital (50 mg/kg) and connected to a small animal ventilator via a tracheostomy and respiratory tube (22-gauge catheter), as described previously [19]. Laparotomy was performed, and a polyethylene-10 catheter was inserted into the distal part of the right colic vein. Using a syringe pump, rhodamine 123 (1 μmol/mouse), a membrane potential-indicating fluorophore, was infused via the catheter over 10 min. After prone positioning of the mouse, the liver was gently withdrawn from the abdominal cavity and placed over a glass coverslip on the microscope stage. Rhodamine 123 fluorescence was excited with 820 nm light from a Chameleon Ultra Ti-Sapphire pulsed laser (Coherent, Santa Clara, CA) and imaged with a Zeiss LSM 510 NLO inverted laser scanning confocal microscope using a 63x 1.3 NA water-immersion objective lens. Green rhodamine 123 fluorescence was collected through a 525 ± 25 nm band pass filter. During image acquisition, the respirator was turned off for ~5 sec to eliminate movement artifacts from breathing. In 20 fields per liver, parenchymal cells were scored for bright punctate rhodamine 123 fluorescence representing hepatocytes with polarized mitochondria or dimmer diffuse cytosolic fluorescence representing hepatocytes with depolarized mitochondria. Image analysis was performed in a blinded fashion.

2.10. Statistical Analysis. Data are presented as means ± S.E., unless otherwise noted. Statistical analysis was performed by ANOVA plus Student-Newman-Keuls test, as appropriate, using $P < 0.05$ as the criterion of significance.

3. Results

3.1. Decreased ALT Release and Liver Necrosis after Resuscitation with Minocycline. C57BL6 mice were hemorrhaged for 3 h and resuscitated with shed blood followed by half the volume of lactated Ringer solution, containing minocycline (10 mg/kg), tetracycline (10 mg/kg), or vehicle. As described previously [20], resuscitation restored mean arterial pressure to ~80 mm Hg, which was nearly identical to blood pressure before hemorrhage (data not shown). At 6 h postoperatively, sham-operated mice had serum ALT of 105 ± 15 U/L (Figure 1). After H/R, ALT after vehicle treatment increased to 4538 U/L ± 557 U/L, which decreased to 1763 ± 213 U/L after resuscitation with minocycline ($P < 0.01$). Identical treatment with tetracycline did not cause a statistically significant change of serum ALT (3999 ± 491 U/L) compared to vehicle (Figure 1).

Liver injury was also assessed histologically at 6 h postoperatively. In sham-operated mice, liver histology was normal and indistinguishable from untreated mice (Figure 2(a) and data not shown). After H/R with vehicle and tetracycline treatments, large areas of necrosis developed 6 h

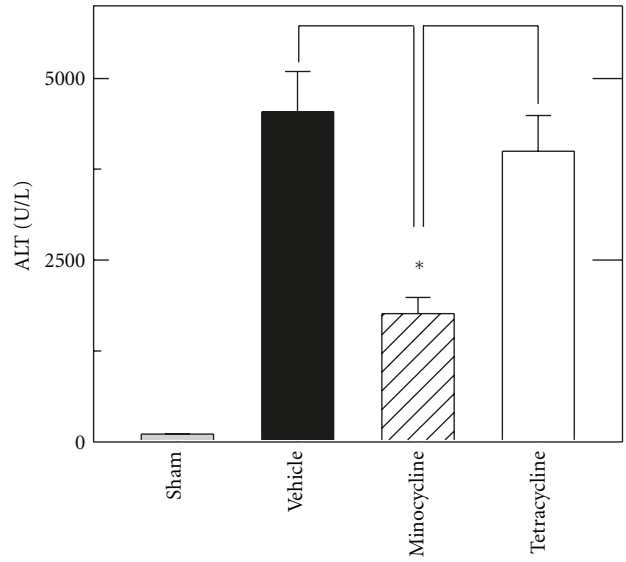

Figure 1: Minocycline decreases ALT release after hemorrhage and resuscitation. Mice were resuscitated with shed blood and then half the volume of lactated Ringer's solution containing tetracycline or minocycline (10 mg/kg body weight) or vehicle, as described in materials and methods. Serum ALT was assessed 6 h after resuscitation. Group sizes were sham, 4; vehicle, 7; minocycline, 7; tetracycline, 7. $^*P < 0.01$ versus vehicle and tetracycline.

postoperatively with a predominately pericentral and midzonal distribution, which was decreased after resuscitation with minocycline (Figures 2(b)–2(d)). Resuscitation with minocycline decreased hepatic necrosis from 24.5 ± 1.5% after vehicle to 8.3 ± 1.4% ($P < 0.05$) (Figure 2(e)). By contrast, resuscitation with tetracycline did not decrease liver necrosis after H/R (23.3 ± 1.5%) in comparison to vehicle treatment. Overall, minocycline treatment decreased hepatic necrosis by nearly two-thirds.

3.2. Decreased Liver Apoptosis after Resuscitation with Minocycline and Tetracycline. TUNEL was performed on tissue sections to assess double-stranded DNA breaks that are characteristic of apoptosis. TUNEL-positive parenchymal cells were rare after sham operation, averaging less than one cell per high power field (HPF). At 6 h after H/R with vehicle, TUNEL of parenchymal cells in nonnecrotic areas increased to 17.7 ± 3.2 cells/HPF (Figure 3). Treatment with minocycline decreased TUNEL by half to 8.7 ± 1.7 cells/HPF ($P < 0.05$ compared to vehicle, Figure 3). After resuscitation with tetracycline, TUNEL-positive cells in nonnecrotic areas decreased to 9 ± 2.2 cells/HPF ($P < 0.05$ compared to vehicle, Figure 3) as well.

To further investigate the extent of apoptosis after minocycline and tetracycline treatment, caspase-3 activity was measured in liver extracts at 6 h after resuscitation with vehicle, minocycline and tetracycline in comparison to sham operation (Figure 4). After sham operation, caspase-3 activity was very low. After H/R with vehicle, caspase-3 activity increased 8.6-fold, which decreased to 2.8-fold

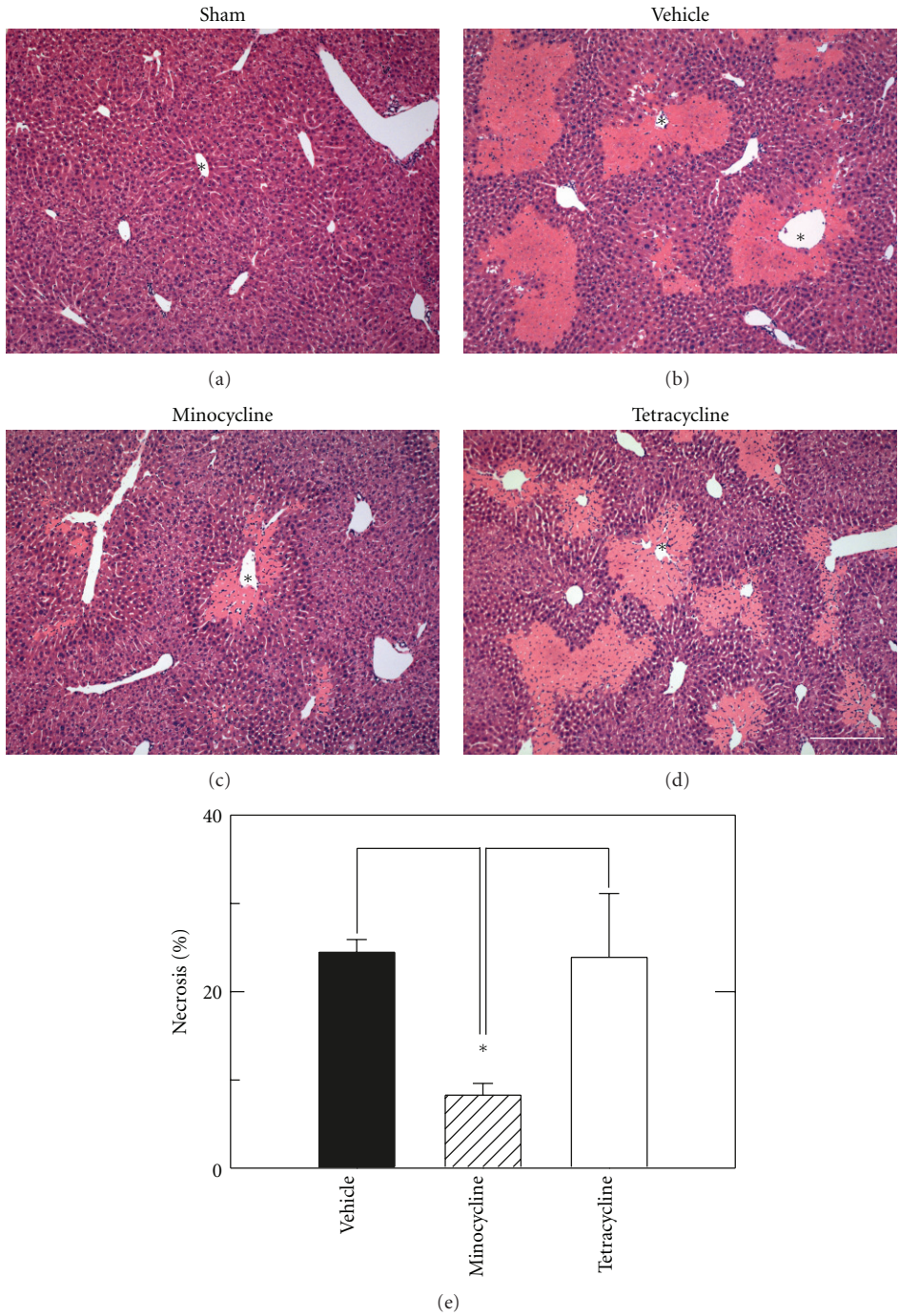

FIGURE 2: Minocycline decreases necrosis after hemorrhage and resuscitation. H/R was performed, as described in Figure 1. Necrosis was assessed by H&E histology at 6 h after sham operation (a) or resuscitation with vehicle, minocycline, or tetracycline (b–d). In (e), necrosis as percent area in liver sections was averaged from 5 livers per treatment group. Necrosis in sham-operated mice was absent and not plotted. *, central vein. Bar is 100 μm. $^*P < 0.05$ versus vehicle and tetracycline.

after minocycline and to 2-fold after tetracycline ($P < 0.05$ compared to vehicle, Figure 4).

3.3. Decreased Oxidative Stress after Resuscitation with Minocycline. We used 4-HNE immunohistochemistry to evaluate oxidative stress in livers 6 h after hemorrhage and resuscitation. HNE is an aldehyde product of lipid peroxidation that forms covalent adducts with proteins that are recognized by anti-HNE antibodies. After sham operation, the brown reaction product of HNE immunohistochemistry was virtually undetectable (Figure 5(a)). By contrast at 6 h after resuscitation with vehicle or tetracycline, wide confluent areas of HNE immunoreactivity developed in pericentral

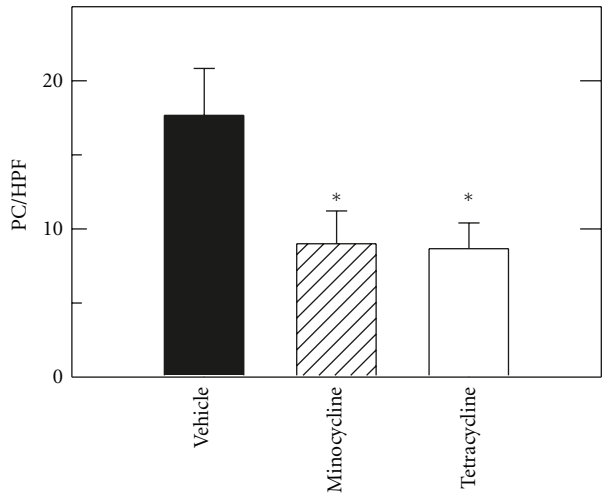

Figure 3: Minocycline and tetracycline decrease apoptosis after hemorrhage and resuscitation. H/R was performed, as described in Figure 1. Apoptosis of parenchymal cells was assessed by TUNEL in nonnecrotic areas at 6 h after sham operation or resuscitation with vehicle, minocycline, or tetracycline. The average number of TUNEL positive cells is plotted for each treatment group. TUNEL for sham was virtually zero and is not plotted. Bar is 50 μm. $^*P < 0.05$ versus vehicle.

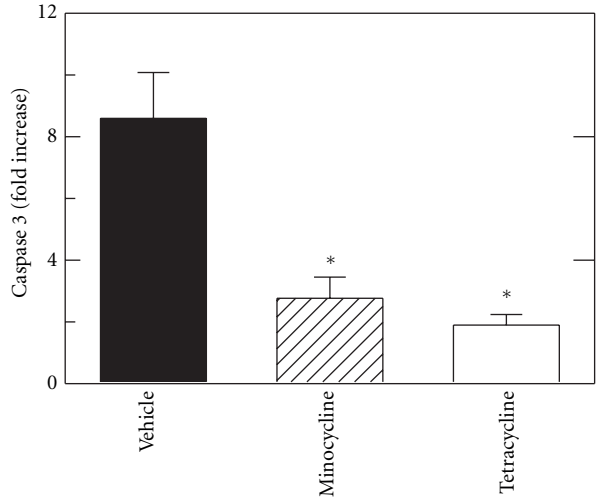

Figure 4: Minocycline and tetracycline decrease caspase 3 activation after hemorrhage and resuscitation. H/R was performed, as described in Figure 1, and caspase 3 activity was assessed in liver homogenates after sham operation or resuscitation with vehicle, minocycline, or tetracycline. Activity is expressed as fold increase over sham-operated mice. $^*P < 0.05$ versus vehicle.

and midzonal areas with relative sparing the periportal regions (Figures 5(b) and 5(d)). However, after H/R with minocycline, HNE immunoreactivity was decreased about 70% compared to vehicle and tetracycline treatments. HNE staining with minocycline was confined mostly to pericentral regions. ($P < 0.05$ compared to vehicle and tetracycline, Figures 5(c) and 5(e)).

3.4. Mitochondrial Dysfunction In Vivo after Hemorrhage and Resuscitation: Protection by Minocycline. At 4 h after sham operation, intravital multiphoton microscopy revealed bright fluorescence of rhodamine 123 in virtually all hepatocytes whose punctate pattern signified polarization of individual mitochondria and normal mitochondrial function (Figure 6(a)). Cytosolic and nuclear areas had little fluorescence. By contrast at 4 h after H/R with vehicle treatment, rhodamine 123 staining became diffuse and dim in many hepatocytes (Figure 6(b)), which indicated mitochondrial depolarization and dysfunction. Similar to the necrosis and HNE immunoreactivity that became present at 6 h after H/R (see Figures 2 and 5), mitochondrial depolarization after 4 h had a predominantly pericentral and midzonal distribution (data not shown). After H/R with minocycline, fewer hepatocytes contained depolarized mitochondria (Figure 6(c)), whereas mitochondrial depolarization after tetracycline treatment was indistinguishable from vehicle-treated liver after H/R (Figure 6(d)). At 4 h postoperatively, livers were scored and counted for rhodamine 123 staining (Figure 6(e)). In sham-operated mice, 0.05 ± 0.002 hepatocytes/HPF contained depolarized mitochondria. After H/R with vehicle treatment, 12.2 ± 0.9 hepatocytes/HPF contained depolarized mitochondria, which corresponded to depolarization of 57.8 ± 5.2% of hepatocytes. After H/R with minocycline treatment, hepatocytes with depolarized mitochondria decreased to 5.4 ± 0.7 hepatocytes/HPF ($P < 0.05$ versus vehicle and tetracycline). By contrast, after H/R with tetracycline, 12.7 ± 0.9 hepatocytes/HPF contained depolarized mitochondria, which was not different from vehicle treatment (Figure 6(e)).

4. Discussion

Hemorrhage is a risk of trauma and major surgery, particularly gastrointestinal and hepatobiliary surgery, and tissue damage after hemorrhage and resuscitation is a variant of ischemia/reperfusion injury. Despite advances in medical and surgical treatment, the golden hour for resuscitation remains a time limit and barrier to effective treatment of hemorrhagic shock. Moreover, the liver is among the most frequently affected organs after hemorrhage-induced hypotension in humans [5]. Here in a mouse model of H/R, we show that minocycline substantially decreases hepatic injury after resuscitation following 3 h of profound hemorrhagic hypotension. Specifically after 3 h of hemorrhage followed by resuscitation with shed blood and then lactated Ringers solution, hepatic necrosis, apoptosis, and enzyme release decreased by 50% or more after minocycline treatment (Figures 1–4). Minocycline also improved mitochondrial function as assessed by intravital multiphoton imaging of the fluorescence of the mitochondrial membrane potential-indicating fluorophore, rhodamine 123 (Figure 6). Notably, minocycline protected even when used after blood resuscitation as a component of Ringer's solution.

Previous studies show cytoprotection by minocycline in a variety of settings, including rat liver transplantation, ischemic renal injury, and various injuries to the central

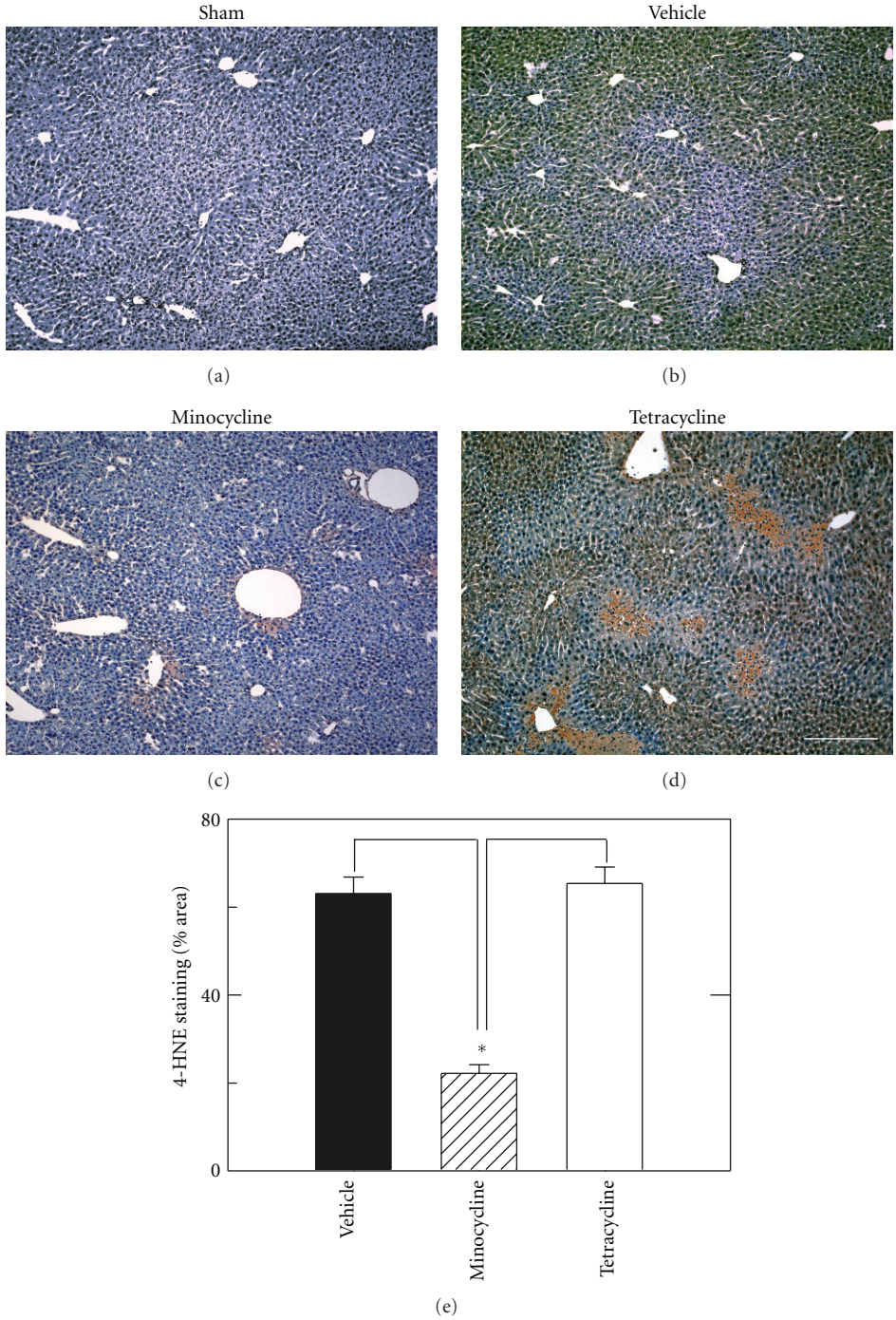

FIGURE 5: Minocycline decreases oxidative stress after hemorrhage and resuscitation. H/R was performed, as described in Figure 1, and immunohistochemical staining was performed for 4-HNE adducts at 6 h after sham operation (a) or resuscitation with vehicle, minocycline, or tetracycline (b–d). In (e), HNE staining as percent area in liver sections was averaged from 5 livers per group. HNE in sham-operated livers was virtually zero and not plotted. Individual group size was 5. Bar is 50 μm. $^*P < 0.05$ versus vehicle.

nervous system [7–12]. In our model of mouse H/R, minocycline protected even when used late during resuscitation after the initial blood resuscitation. Hepatic necrosis assessed by ALT and histology decreased by half at 6 h after H/R with minocycline treatment, and apoptosis assessed by TUNEL and caspase-3 activity also decreased by more than half (Figures 1–4). Necrosis represents the predominant mode of cell death in the setting of hepatic I/R with apoptosis contributing to a lesser extent [21, 22]. However, both modes of cell death, namely, apoptosis progressing to necrosis, can occur through a common mitochondrial pathway involving the MPT, a phenomenon of necrapoptosis [23–25].

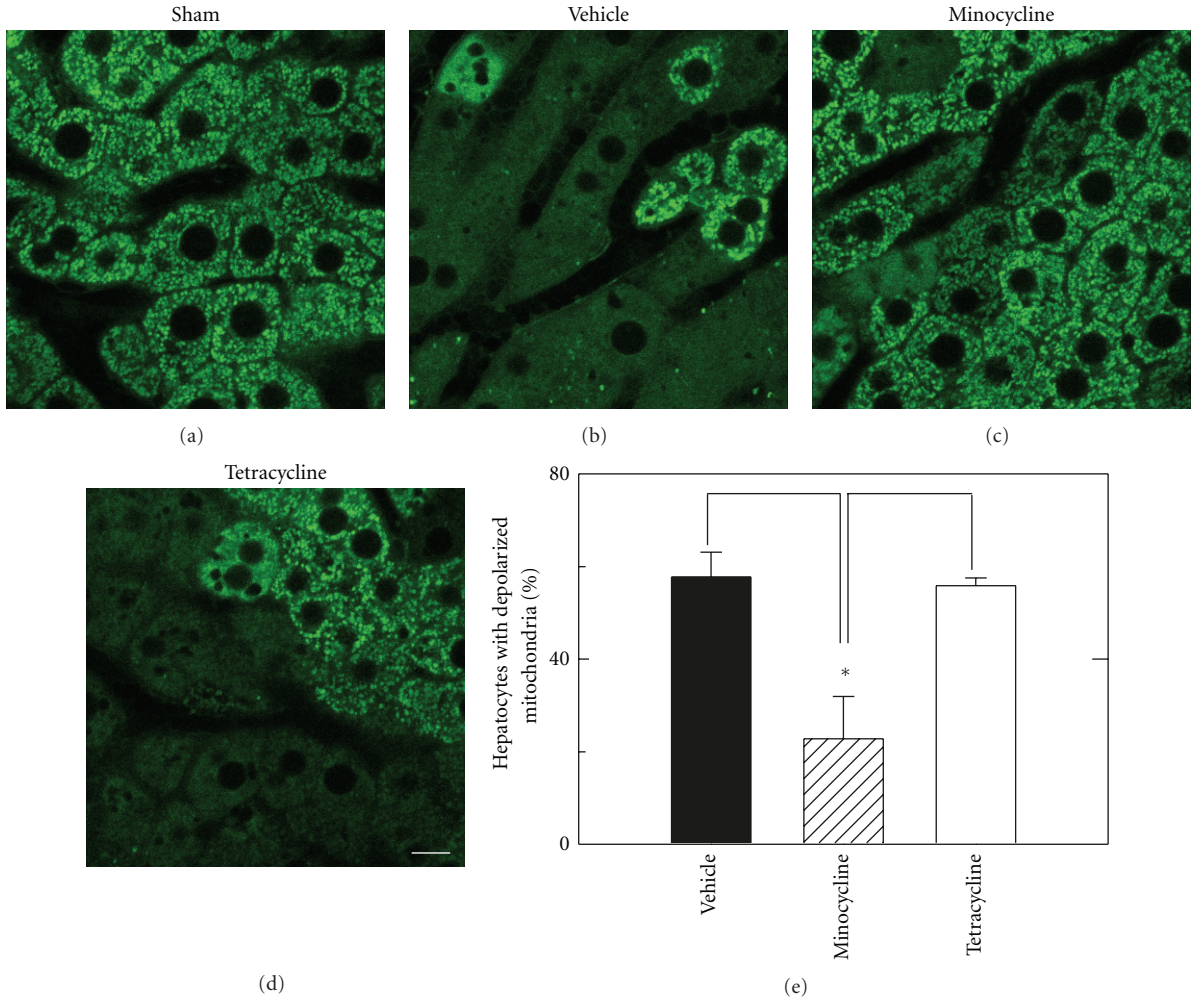

Figure 6: Minocycline decreases mitochondrial depolarization after hemorrhage and resuscitation. H/R was performed, as described in Figure 1, and intravital multiphoton microscopy of rhodamine 123 fluorescence was performed 4 h after sham operation (a) or resuscitation with vehicle, minocycline, or tetracycline (b–d), as described in materials and methods. Punctate staining of rhodamine 123 denoted polarization of individual mitochondria, whereas dim diffuse cellular staining indicated mitochondrial depolarization. In (e), the average percentage of hepatocytes with depolarized mitochondria is plotted for each H/R treatment group. Mitochondrial depolarization in sham-operated livers was virtually zero and not plotted. Size of individual groups was 5. Bar is 30 μm. $^*P < 0.05$ versus vehicle.

After orthotopic rat liver transplantation, minocycline cytoprotection against hepatic necrosis, apoptosis, and enzyme release is virtually identical to the cytoprotection of *N*-methyl-4-isoleucine cyclosporin (NIM811), a specific inhibitor of the MPT [7]. Minocycline also inhibits calcium-induced MPT onset in isolated mitochondria. Unlike NIM811 which inhibits the MPT pore component, cyclophilin D, minocycline prevents MPT onset by blocking electrogenic calcium uptake by the mitochondrial calcium uniporter. Since H/R caused mitochondrial depolarization that was virtually identical to mitochondrial depolarization after liver transplantation, and since minocycline protected against this depolarization (Figure 6), it is likely the minocycline protects against hepatic injury after H/R by blocking MPT onset. Importantly, minocycline-sensitive mitochondrial depolarization signifying the MPT preceded necrotic cell death and thus was not a consequence of cell death, since after 4 h few cells labeled with propidium iodide, a marker of nonviable cells, as described previously [7]. Tetracycline, which did not decrease hepatic necrosis and ALT release after H/R, did not prevent mitochondrial depolarization after H/R (Figure 6). Because minocycline protected against mitochondrial depolarization, necrosis, and apoptosis, liver damage after H/R would appear to be largely a necroapoptotic phenomenon [26].

In storage/reperfusion injury during liver transplantation and in isolated mitochondria, tetracycline does not protect against hepatic damage, mitochondrial depolarization, and onset of the MPT [7]. Similarly in the present work, tetracycline did not protect against hepatic necrosis, enzyme release, and mitochondrial depolarization after H/R (Figures 1, 2 and 6). By contrast, tetracycline protected similarly to minocycline against apoptosis, as assessed by TUNEL and caspase 3 (Figures 3 and 4). This finding suggests

different protective actions—one unique to minocycline and another shared by both tetracycline and minocycline. One shared action is that minocycline and tetracycline are both calcium chelators [27, 28], although only minocycline blocks mitochondrial calcium uptake [7]. Thus, suppression of apoptosis by tetracycline and minocycline might be due to calcium chelation. Alternatively in necrapoptosis, apoptosis progresses to necrosis with increasing severity of an inducing stress. Consequently, protective strategies may revert necrosis to apoptosis, such that protected necrotic areas begin toshow apoptosis. Accordingly, protection against apoptosis by an agent like minocycline may be offset in part by increased apoptosis in areas that otherwise would have become necrotic. Tetracycline, by contrast, did not decrease necrosis, and tetracycline may simply represent a much weaker protective agent than minocycline that protects partially against apoptosis but not at all against necrosis. Future studies will be needed to distinguish between these possibilities.

In I/R, oxidative stress after reperfusion promotes the MPT, and antioxidants are protective. After H/R in our mouse model, 4-HNE immunostaining increased substantially as an indicator of lipid peroxidation and oxidative stress (Figure 5). Minocycline decreased this 4-HNE staining after H/R. Since minocycline is not an antioxidant, decreased HNE staining by minocycline suggests that oxidative stress is occurring as a consequence of the MPT and cell death. However, much HNE staining occurred in regions that had not yet become necrotic, and this oxidative stress might nonetheless be contributing to the progression of injury.

Endotoxin acting through lipopolysaccharide-binding protein contributes to H/R injury to liver [15]. As an antibiotic, minocycline might alter intestinal flora and hence endotoxemia after H/R. However, tetracycline is also a broad spectrum antibiotic, and tetracycline did not protect after H/R. The danger of bacterial infection necessitates prophylactic use of antibiotics, such as broad spectrum cephalosporins, after multiple trauma and in advance of major surgery [29–31]. Since minocycline is a broad spectrum antibiotic with an excellent safety record, one-time treatment of hemorrhagic shock patients with minocycline would be consistent with current clinical practice and has the additional benefit of decreasing injury from H/R and the subsequent development of MODS. Future studies will be needed to determine what benefit, if any, minocycline might have in a clinical setting of hemorrhagic shock and resuscitation.

List of Abbreviations

4HNE: 4-Hydroxynonenal
ALT: Alanine aminotransferase
ATP: Adenosine triphosphate
H/R: Hemorrhage and resuscitation
H&E: Hematoxylin and eosin
HEPES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPF: High power field
MODS: Multiple organ dysfunction syndrome
MPT: Mitochondrial permeability transition
NIM811: *N*-Methyl-4-isoleucine cyclosporin
PT: Permeability transition
SIRS: Systemic inflammatory response syndrome
TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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Review Article

Ischemia/Reperfusion Injury in Liver Surgery and Transplantation: Pathophysiology

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Liver ischemia/reperfusion (IR) injury is caused by a heavily toothed network of interactions of cells of the immune system, cytokine production, and reduced microcirculatory blood flow in the liver. These complex networks are further elaborated by multiple intracellular pathways activated by cytokines, chemokines, and danger-associated molecular patterns. Furthermore, intracellular ionic disturbances and especially mitochondrial disorders play an important role leading to apoptosis and necrosis of hepatocytes in IR injury. Overall, enhanced production of reactive oxygen species, found very early in IR injury, plays an important role in liver tissue damage at several points within these complex networks. Many contributors to IR injury are only incompletely understood so far. This paper tempts to give an overview of the different mechanisms involved in the formation of IR injury. Only by further elucidation of these complex mechanisms IR injury can be understood and possible therapeutic strategies can be improved or be developed.

1. Introduction

Ischemia/reperfusion (IR) injury of the liver results from a loss of blood supply reducing oxygen supply to the organ. Upon revascularisation the liver undergoes reperfusion injury. Together these factors lead to affection of oxygen-dependent cells within the liver causing impairment of organ function. Affected are all cells requiring mitochondrial oxidative phosphorylation for their metabolism [1]. Warm IR injury can be separated from cold IR injury. Warm IR injury occurs during prolonged surgical liver resection using clamping of the perfusion [2]. Other aetiologies are reduced liver perfusion due to shock, heart failure, respiratory failure, hemorrhage, trauma, and sepsis [3–5]. In contrast, cold IR injury follows liver transplantation with the necessity of cold preservation of the donor organ, followed by reperfusion after implantation [6, 7]. Furthermore, it has been demonstrated that tissue damage occurs in two phases, an early and a late phase [8, 9]. The early phase which occurs within the first 6 hours following reperfusion is thought to be the consequence of the fast change in the redox state of the liver tissue [9, 10]. Most likely, this change is caused by hepatocytes, Kupffer cells (KCs), and sinusoidal endothelial cells (SECs) [8, 9, 11]. In contrast, the late phase of IR injury is caused by the production of cytokines and chemokines followed by the infiltration of leukocytes into the liver tissue [8, 9, 12].

Of clinical relevance is that liver IR injury results in elevated liver enzymes, biliary strictures, clinical dysfunction, or even liver failure [13]. Furthermore, other organs can develop dysfunction secondary to the liver damage. Possible affected organs are lungs, heart, kidneys, and blood vessels [14–17]. Risk factors for IR injury include age of the liver, sex and others [18–21].

A complex network and cross talk of multiple molecular mechanisms and cellular interactions lead to liver IR injury [22, 23]. The result of these processes is cell death by apoptosis and necrosis via different pathways. Redox status,

cellular ionic disturbances, cytokines, chemokines, other mediators and molecular mechanisms as well as many different cells like KC, SEC, dendritic cells, leukocytes, and lymphocytes are involved in this process and are closely interlocked. Therefore, there are still many open questions regarding this inflammatory response. This paper tempts to give a systematic overview of the different components and signalling pathways leading to IR injury.

2. Altered Redox Status and Reduced Microcirculatory Blood Flow

IR injury starts with reduced blood flow and a lack of oxygen supply [24, 25]. This ischemia leads to a lack of adenosine triphosphate (ATP) production in hepatocytes, KC, and SEC [26]. As a result the function of the ATP-dependent sodium/potassium plasma membrane pump (Na^+/K^+ ATPase) is impaired. This results in an increase of intracellular Na^+, which is followed by a swelling of the hepatocytes, KC and SEC. Narrowing of the sinusoidals is the consequence. Within minutes after reperfusion, enhanced levels of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^\bullet$), can be measured [8, 9, 11, 27]. Cellular sources for these ROS are mitochondrial metabolism, hepatocyte-derived xanthine oxidase, and KC- and SEC-associated NADPH oxidase [8, 9, 11, 28]. This increase of ROS is accompanied by reduced nitric oxide (NO), most likely due to a decreased function of the NO synthase (NOS) in SEC [29, 30]. Since NO is a vasodilator [31], reduced NO aggravates the sinusoidal narrowing. However, these mechanisms are still controversially discussed [32, 33]. Especially the role of the different NOS isoforms is still unclear [34]. The effects of NO are well known. It increases the sinusoidal diameter and increases intrahepatic ATP levels via better oxygen supply. Thereby mitochondrial damage and leukocyte infiltration are reduced. Since NO is synthesized by NOS, NOS should be protective in IR injury. While this is well observed for the endothelial NOS (eNOS), the role of the inducible NOS (iNOS) is less clear [34]. In the late phase of IR injury it probably is also protective, while in the early phase, there may be a harmful role of iNOS.

Nevertheless, the changes occurring in IR injury lead to a more oxidative environment with the ROS leading to both, apoptotic and necrotic cell death of hepatocytes and SEC [35, 36]. ROS causes damage to membrane lipids resulting in cell swelling and death [35]. The damage is not limited to the plasma membrane but includes cell organelles and extracellular matrix. Besides swelling of cells and reduced NO levels, the described damage leads to an increase of vasoconstrictors like endothelin and thromboxane A2 [37]. In addition, adhesion and aggregation of platelets and leucocytes is increased (see below). This leads to further narrowing of the sinusoidals with significant reduction of microcirculatory blood flow including areas with complete absence of blood flow [38], enhancing the lack of oxygen supply. Increased ROS and decreased NO levels play further roles which will be discussed below.

3. Ionic and Mitochondrial Disturbances

In IR injury significant changes of intracellular Ca^{2+} concentration in the hepatocytes can be found [39]. Ca^{2+} is mainly found in three cellular compartments, in the cytosol, the mitochondria, and the endoplasmic reticulum (ER). The homeostatic concentration is regulated by different Ca^{2+} channels. In IR injury cytosolic Ca^{2+} concentration is increased as a result of increased entry across the plasma membrane and release from the ER. Reason for this cytoplasmic Ca^{2+} overload is the activation of the ryanodine receptor in the ER membrane and the so-called transient receptor potential (TRP) channels in the plasma membrane. There is evidence that ROS can activate these channels [40, 41]. Secondary to decreased Ca^{2+} concentration in the endoplasmic reticulum so-called store operated calcium (SOC) channels in the plasma membrane further increase Ca^{2+} influx [42, 43]. In addition, in IR injury the Ca^{2+} ATPase in ER and plasma membrane is inhibited, potentially because of ATP depletion. Normally this Ca^{2+} ATPase discharges cytosolic Ca^{2+} into extracellular space and into the ER counteracting the above mentioned Ca^{2+} channels [44].

Increased cytosolic Ca^{2+} leads to stimulation of the Ca^{2+} uniporter in the mitochondrial membrane [45]. As a result the mitochondrial Ca^{2+} concentration increases as well. The mechanism how the mitochondrial Ca^{2+} uniporter is activated has not been fully resolved, so far. It is believed that the mitochondrial P2Y-like receptor 1 ($mP2Y_1$) is activated by adenosine diphosphate (ADP) and adenosine monophosphate (AMP). The $mP2Y_1$ stimulates the PLC-dependent mP2Y-like receptor resulting in activation of the Ca^{2+} uniporter [46, 47]. In contrast, $mP2Y_2$ activated by ATP leads to inhibition of the uniporter. During IR injury ATP is depleted, as discussed before, possibly leading to activation of the uniporter. As a consequence of this increased mitochondrial Ca^{2+} concentration the mitochondrial transmembrane potential is reduced. To maintain the mitochondrial membrane the mitochondrial ATP-synthase reverses its activity and hydrolyzes ATP to provide energy for different ionic pumps in the mitochondrial membrane [39]. However, this further increases Ca^{2+} influx resulting in ATP consumption instead of production in the mitochondria. This is enhanced by the fact that ROS causes oxidative damage to the enzymes of the respiratory chain in the mitochondria leading to failure of ATP production [48]. Cytosolic and mitochondrial Ca^{2+} and other ionic disturbance lead to damage of plasma and mitochondrial membranes including the formation and opening of mitochondrial permeability transition (MPT) pores [49]. MPT pores are formed from integral not fully identified mitochondrial membrane proteins [50, 51]. Hepatic mitochondria afflicted by MPT pores are permanently damaged due to depolarization of the mitochondria [37]. When only a few mitochondria are afflicted, they are removed from the hepatocyte by lysosomal mitophagy [52]. Such damaged mitochondria are a source for further ROS

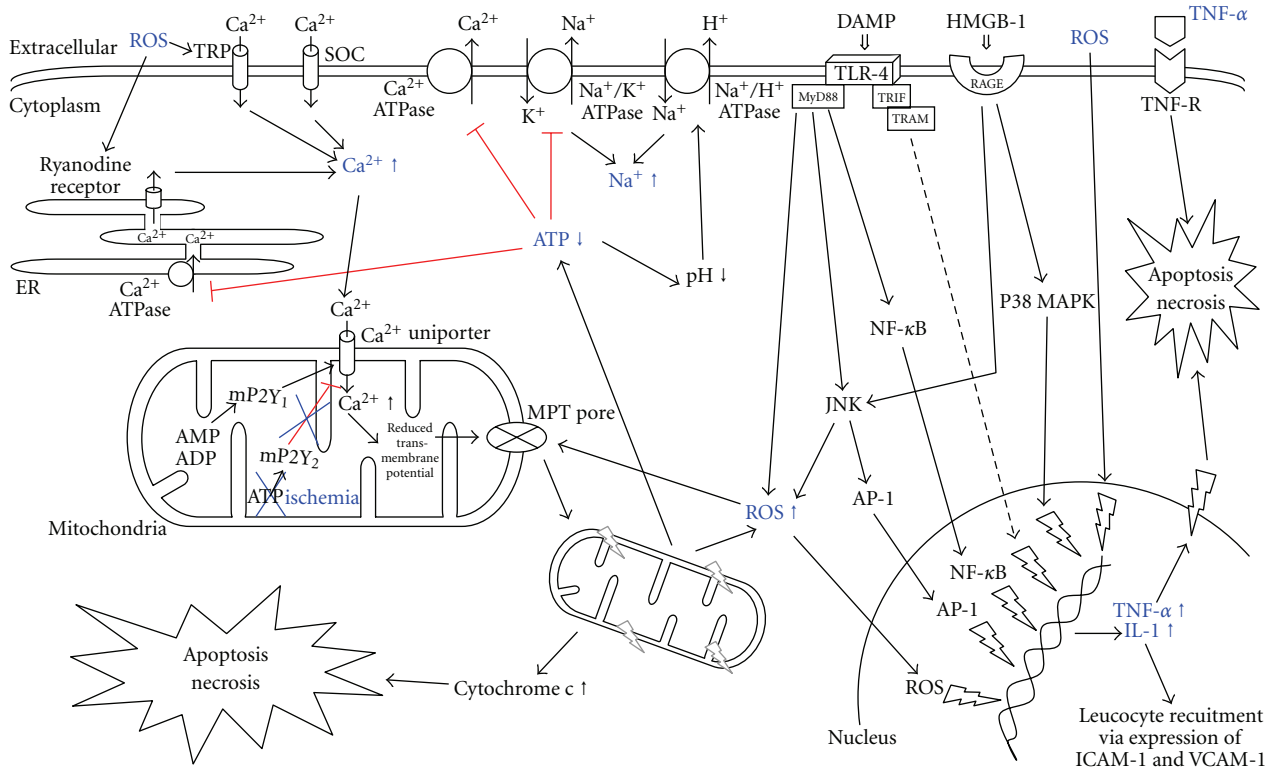

Figure 1: Intracellular signalling pathways and ionic disturbances engaged during IR injury, resulting in cellular swelling, apoptosis, and necrosis. ADP: adenosine diphosphate; AMP: adenosine monophosphate; AP-1: activator protein-1; ATP: adenosine triphosphate; DAMP: danger-associated molecular pattern; HMGB-1: high mobility group box-1; ICAM-1: intercellular adhesion molecule-1; IL-1: interleukin-1; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; MPT pore: mitochondrial permeability transition pore; MyD88: myeloid differentiation factor 88; NF-κB: nuclear factor kappa B; RAGE: receptor for advanced gylcation end product; ROS: reactive oxygen species; SOC: store operated calcium channel; TLR: toll-like receptor; TNF: tumor necrosis factor; TRAM: TRIF-related adaptor molecule; TRIF: TIR domain-containing adaptor inducing interferon; TRP: transient receptor protein; VCAM-1: vascular adhesion molecule-1.

production and ATP consumption [53]. However, ROS themselves induce MPT pore opening. With the number of damaged mitochondria increasing, cytochrome C is released from the mitochondria into the cytosol triggering apoptosis [48]. When the majority of the mitochondria within the hepatocyte are afflicted by MPT pores ATP levels drop too fast resulting in cell death by necrosis [49, 54].

Other important ionic disturbances in IR injury include intracellular Na^+ and hydrogen (H^+) concentrations. Lack of oxygen supply leads to anaerobic respiration of the hepatocytes resulting in intracellular acidosis [55]. To stabilize intracellular pH within normal range the Na^+/H^+ exchanger is activated by the hepatocytes, resulting in reduced cytosolic H^+ and further increased Na^+ levels. In addition, the Na^+/K^+ exchanger is ATP dependent, so ATP depletion, as in IR injury, subsequently blocks this exchange leading to further increase of intracellular Na^+ concentration resulting in cell death [56].

Furthermore, this counteracts the protective effect of an acidic pH during reperfusion [57], for example, the maintenance of an acidic pH prevents the formation of MPT pores [49]. However, these regulations are still based on experimental observations and need to be studied further to understand the relevance in IR injury (Figure 1).

4. Cellular Cascade in IR Injury

Many different cell types are involved in the process of hepatic damage and cell death in IR injury. The key cells initiating IR injury are the KCs [58–60]. Besides their direct damage by ROS, as discussed above, they are also activated by ROS leading to production of more ROS and thereby entering a cycle of self-activation and -destruction. In addition, KC are activated by the systemic complement system [61] which may also be liberated by damaged hepatocytes. In addition, complement leads to further liver damage by formation of a membrane attack complex in the plasma membrane, lysing liver cells [62].

Activated KCs also produce proinflammatory cytokines including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [63]. These cytokines lead to activation and migration of neutrophils and CD4+ T lymphocytes into the liver [64]. Furthermore, these cytokines stimulate SEC and hepatocytes to produce ROS and to express adhesion molecules on the cell surface [65]. As described above, this leads to adhesion and aggregation of leucocytes and platelets [66], influencing the microcirculatory blood flow in the liver.

The recruitment of neutrophils and CD4+ T lymphocytes is further enhanced by the matrix metalloproteinase

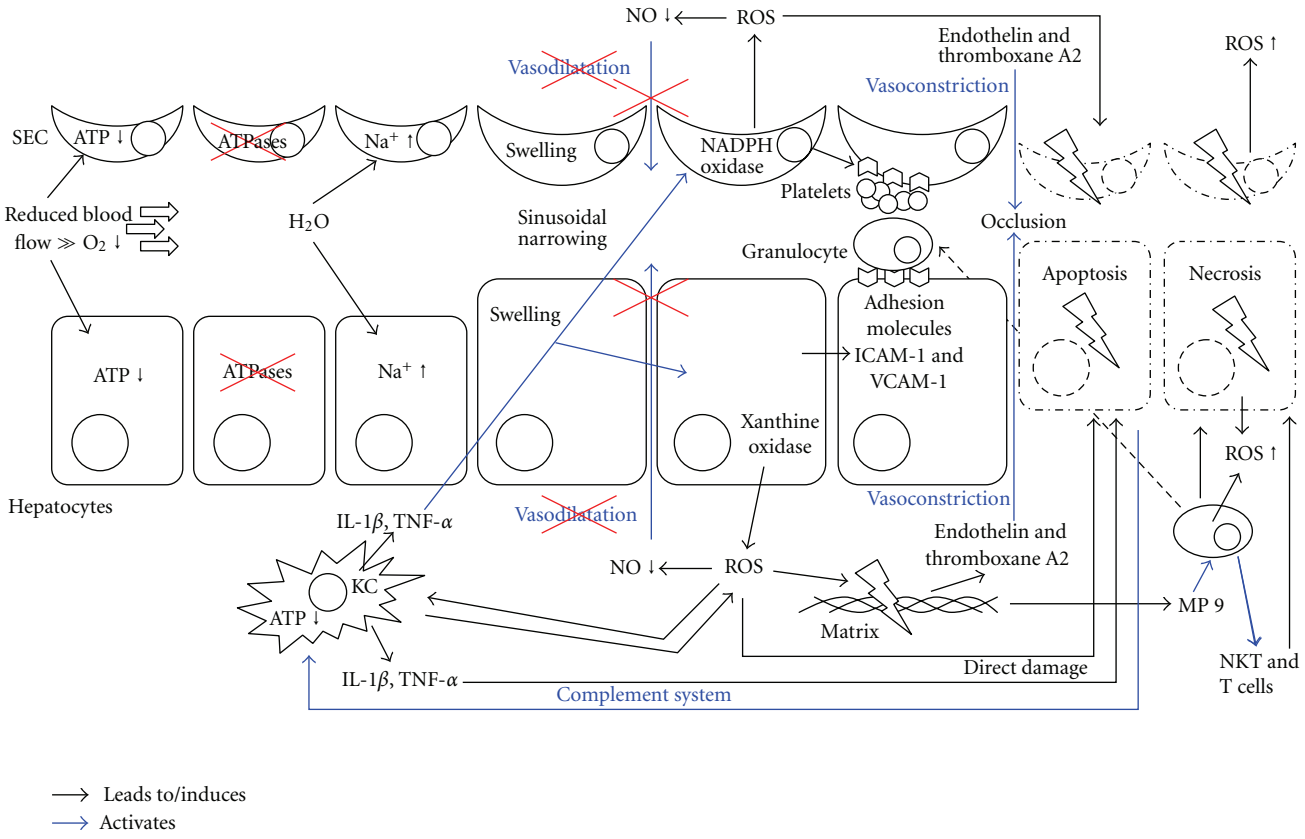

Figure 2: Cellular interaction involved in IR injury, resulting in cellular swelling, apoptosis, and necrosis. ATP: adenosine triphosphate; ICAM-1: intercellular adhesion molecule-1; KC: Kupffer cell; IL-1, interleukin-1; NKT: natural killer T cell; NO: nitric oxide; ROS: reactive oxygen species; SEC: sinusoidal endothelial cells; T cell: CD4+ T lymphocyte; TNF: tumor necrosis factor; VCAM-1: vascular adhesion molecule-1.

9 [67] after ischemic damage of the liver. Via production of interferon-γ (IFN-γ) and IL-17 by activated CD4+ T lymphocytes additional activation of KC and hepatocytes is achieved [68, 69]. Thus CD4+ T lymphocytes and KC reciprocally activate each other [64]. These chemokines furthermore activate natural killer T (NKT) cells. Activated NKT cells directly damage liver tissue and also produce IFN-γ with further activation of KC and hepatocytes [69, 70]. The net result of this circular activation and stimulation of different cell sub types is destruction of hepatocytes and SEC [71, 72].

The expressed cell-surface adhesion molecules on hepatocytes and SEC include intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) [28, 73]. Neutrophils bind to ICAM-1 and VCAM-1 and by doing so migrate across the endothelium into the liver parenchyma enhancing ROS production and degranulation of cytoplasmic vesicles containing enzymes capable to degrade extracellular matrix and hepatocytes [28] (Figure 2).

5. Death Signalling Pathways

Besides direct damage of hepatocytes by neutrophils, NKT cells, the complement system and ROS, the main destruction of cells is mediated by endogeneous pathways leading to apoptosis or necrosis of hepatic cells during IR injury.

This paper is not capable to focus on all cytokine cascades with pro- and antiinflammatory effects [74] as well as their effect during IR injury, but will concentrate on some important signalling pathways. The most important component in IR injury seems to be TNF-α [75, 76]. The pathways leading to up regulation of TNF-α have been described above. TNF-α binds to specific TNF-receptors, as for example TNF-R1 and TNF-R2, on the hepatocyte surface which leads to increased production of cytokines and ROS. In addition, activation of CD95 leads to apoptosis [77–79]. Furthermore, CD95 also binds NKT cells leading to direct destruction of hepatocytes [80].

Furthermore, downstream of the receptor the nuclear factor kappa B (NF-κB), the mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) are activated [81–83]. The various cytokines and the mentioned molecules lead to alteration of various factors further downstream like transcription factors, activator protein-1 (AP-1), heat shock factor, signal transducer and activators of transcription (STATs), antioxidants, inflammation-stimulated inducible enzymes (COX-2), intracellular signalling molecules, antiapoptotic proteins (Bcl-2, Bcl-x), and

many more [37]. The damage in IR injury therefore spreads throughout the entire cell. NF-κB furthermore upregulates the expression of cytokines, like TNF-α [84], and of ICAM-1 and VCAM-1 [75, 82], enhancing the recruitment of neutrophils. AP-1 promotes apoptosis of liver cells by activation of caspase-3 and release of cytochrome C [82].

ROS furthermore inherit a direct oxidative damage of DNA within the nucleus resulting in further failure of protein transcription and translation. In addition, ROS cause post-translational protein modification [85]. These alterations and pathways lead to apoptosis of the damaged cells.

The intracellular damage and alterations as well as the damage of extracellular matrix are followed by the release of danger-associated molecular patterns (DAMPs). Examples of DAMPs released during IR injury are the nuclear transcription factor high mobility group box-1 (HMGB-1), the cytoplasmic Ca^{2+} regulator S100, ATP, DNA, and hyaluronic acid [86–90]. DAMPs bind to a group of so-called pattern recognition receptors (PRRs) on the cell surface as well as in the cytoplasm [37]. In IR injury mainly two PRRs are involved, the toll-like receptors (TLRs), specifically TLR-4, and the receptor for advanced glycation end products (RAGE). To present knowledge TLR-4 provides an important link between liver damage and activation of the immune system. Activation of TLR-4 triggers intracellular signalling cascades in IR injury [91]. The Toll-IL-1 receptor domain (TIR) of TLR-4 interacts with intracellular adaptors. One may be the myeloid differentiation factor 88 (MyD88), others are TIR domain-containing adaptor inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM) [92]. Via production of proinflammatory cytokines the inflammatory response is mediated leading to liver IR injury [91–93]. Included in the downstream process of these activation are further transcription factors like NF-κB, AP-1, STAT, the MAP kinase JNK, and ROS [86, 94–96].

The best characterized DAMP is HMGB-1 which is expressed by all nucleated cells within the liver and is released upon necrosis and apoptosis [86, 97]. HMGB-1 binding to RAGE in IR injury leads to a signalling cascade involving activation of JNK and other kinases, increasing expression and activation of the inducible transcription factor early growth response-1. As a consequence the upregulation of several gene families is found, recruiting immune cells into the post ischemic liver [98]. RAGE is mainly expressed on dendritic cells and to lesser extent on KC [98]. Furthermore, dendritic cells and KC also express TLR-4 [99]. This hints to an important, but at present unclear, function of dendritic cells during IR injury of the liver.

This complex communication of the described networks is responsible to initiate and propagate IR injury.

6. Conclusions

The understanding of the molecular mechanisms underlying cell death in hepatic IR injury will provide the basis for the development of new strategies for inhibition of liver injury and improvement of survival of the graft. The initial phase of IR injury involves the release of ROS and proinflammatory mediators by KC. ROS lead to oxidative damage, induction of p53, apoptosis and necrosis of hepatocytes and endothelial cells. The late phase (6–48 hours after reperfusion) is characterized by neutrophil-mediated inflammatory responses. Thus, proteins regulating the cellular redox equilibrium, p53-dependent apoptosis and cellular death receptors represent potential targets for novel pharmaceutical interventions to protect hepatocytes from IR injury-induced cell death.

Author's Contribution

Peter Schemmer and Martina Müller equally contributed to this work.

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Review Article

Anesthetic Considerations in Hepatectomies under Hepatic Vascular Control

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Background. Hazards of liver surgery have been attenuated by the evolution in methods of hepatic vascular control and the anesthetic management. In this paper, the anesthetic considerations during hepatic vascular occlusion techniques were reviewed. *Methods*. A Medline literature search using the terms "anesthetic," "anesthesia," "liver," "hepatectomy," "inflow," "outflow occlusion," "Pringle," "hemodynamic," "air embolism," "blood loss," "transfusion," "ischemia-reperfusion," "preconditioning," was performed. *Results*. Task-orientated anesthetic management, according to the performed method of hepatic vascular occlusion, ameliorates the surgical outcome and improves the morbidity and mortality rates, following liver surgery. *Conclusions*. Hepatic vascular occlusion techniques share common anesthetic considerations in terms of preoperative assessment, monitoring, induction, and maintenance of anesthesia. On the other hand, the hemodynamic management, the prevention of vascular air embolism, blood transfusion, and liver injury are plausible when the anesthetic plan is scheduled according to the method of hepatic vascular occlusion performed.

1. Introduction

Hepatectomy is one of the therapies available for benign and malignant liver disease. Although liver resections have been associated with high mortality and morbidity rates, recent advances in anesthetic and surgical management have significantly reduced the operative risk. The techniques of vascular control during hepatectomy are highly demanding and should be performed under special anesthetic considerations.

Hepatic vascular control methods can be categorized as those involving occlusion of liver inflow and those involving occlusion of both liver inflow and outflow. They can be summarized as following.

(1) Inflow vascular occlusion.

  (A) Hepatic pedicle occlusion:

    (a) Continuous Pringle maneuver (CPM),
    (b) intermittent Pringle maneuver (IPM).

  (B) Selective inflow occlusion.

(2) Inflow and outflow vascular exclusion

  (A) Total hepatic vascular exclusion (THVE),
  (B) inflow occlusion with extraparenchymal control of the major hepatic veins: with selective hepatic vascular exclusion (SHVE).

When performing these techniques, the conduct of anesthesia should take into account hemodynamic management, risks of vascular air embolism, ischemia reperfusion liver injury, intraoperative blood loss, and the need for transfusion, factors which usually complicate hepatic vascular control methods. Special attention should also be paid to the preoperative assessment and induction of anesthesia, as patients undergoing liver resection usually have a compromised health status. Careful selection of the anesthetic drugs can minimize the effects of hepatic blood flow decrease induced by the surgical technique adopted.

2. Methods

A comprehensive literature search was performed. Our objective was to identify the anesthetic considerations in techniques of hepatic vascular control methods. Articles were selected by a Medline literature search, according to the following criteria.

(1) All prospective randomized studies were thoroughly evaluated and presented, as they are the most important source of information on the outcomes of surgical and anesthetic manipulations.

(2) Large retrospective studies were also included. Few case reports and smaller studies are mentioned, given the fact that they highlight special anesthetic aspects.

3. Results

3.1. Preoperative Assessment. Healthy patients undergo a routine preoperative assessment including a full blood count and a standard biochemical and coagulation test.

Preexisting hepatic impairment is a risk factor, even for nonhepatic surgery, with higher blood transfusion requirements, a longer hospital stay, a higher number of complications, and increased mortality rates of 16.3% in cirrhotic patients as compared to 3.5% in controls [1]. Estimating the health status of patients presenting for hepatectomy is quite challenging: coagulopathy, volume and electrolyte disturbances, viral infections (Hep C), hepatorenal [2–4] and hepatopulmonary [3] syndrome, portopulmonary hypertension, and low cardiovascular reserve capacity can occur in patients with chronic liver disease.

The identification of patients at risk to develop postoperative hepatic or renal failure is important and, ideally, involves many related disciplines such as surgery, anesthesia, and intensive care. Although vascular occlusion techniques have minimized hepatic bleeding, the risk for postoperative liver and/or renal failure remains high for patients of advanced age and those with steatosis and cirrhosis, on preoperative chemotherapy and with small remnant liver volumes [5]. Slankamenac et al. [6] have developed and validated a prediction score for postoperative acute liver failure following liver resection based on the preoperative parameters of cardiovascular disease, chronic liver failure, diabetes, and ALT levels, which seems to be an easily applicable and attractive tool in clinical practice.

Vascular control techniques during hepatectomy require optimization of the cardiac and pulmonary function [7]. Hepatic ischemia and reperfusion on subsequent liver dysfunction is associated with unexpected responses to surgical stress [7–9] and poor prognosis [10]. Patients with end-stage liver disease have a characteristic hemodynamic profile: increased cardiac output with blunted response to painful stimuli, splanchnic vasodilatation and central hypovolemia. As a result, silent moderate-to-severe coronary artery disease cannot be easily recognized. Currently, there are no specific guidelines for the identification of coronary artery disease in patients with advanced liver disease [11, 12]. Preoperative invasive assessment of preexisting cardiovascular dysfunction is indicated only for high risk patients, provided that any coagulopathy is corrected [11]. In the noninvasive assessment of coronary artery disease in patients with cirrhosis, dobutamine stress echocardiography has failed as a screening tool [12]. Furthermore, beta blockade discontinuation in order to permit adequate cardiac function assessment may be hazardous in patients with advanced liver disease [12]. Beta blockers reduce portal hypertension, decrease cardiac workload, and their use seems to be beneficial to both the liver and the heart in the setting of hepatectomy.

In general, the preoperative assessment needs to be adapted to the individual patient to minimize the perioperative liver insults of hepatic vascular control.

3.2. Induction and Maintenance of Anesthesia. Liver resections are usually performed under general anesthesia with tracheal intubation and controlled ventilation. Patients with ascites undergo rapid sequence induction [13]. Cis-atracurium is the nondepolarizing muscle relaxant of choice in patients with liver disease as it is hydrolyzed by Hoffman elimination. Moreover, it is haemodynamically stable due to its scarce release of histamine [14]. Atracurium can provide stable neuromuscular blockade, as its requirements remained unchanged during exclusion of the liver from the circulation [15].

An intravenous hypnotic is used for induction and a halogenated volatile agent in air-oxygen mixture is used for maintenance [16]. Hepatic vascular control techniques depress cardiovascular function in addition to the depression caused by general anesthesia. Careful selection of the volatile agent is required. Most commonly used volatile anesthetics for maintenance are isoflurane and sevoflurane. Isoflurane has mild cardiodepressive effects but maintains hepatic oxygen supply, due to vasodilatation in the hepatic artery and portal vein [17]. Both isoflurane and sevoflurane upregulate heme-oxygenase-1, release iron and carbon monoxide, and thus decrease portal vascular resistance in rats [18]. In humans, sevoflurane decreases portal vein blood flow but increases hepatic artery blood flow [19]. In addition, Beck-Schimmer et al., in a randomized controlled trial on patients undergoing liver surgery [20], showed that ischemic preconditioning with sevoflurane before inflow occlusion limited postoperative liver injury, even in patients with steatosis. Although various inhalational anesthetics are used in liver surgery, no optimal anesthetic technique has been established for the maintenance of anesthesia. Desflurane appears

Table 1: Hemodynamic changes on clinical series of hepatectomies induced by hepatic vascular occlusion techniques.

	Technique	Haemodynamic changes		
		Heart rate	Mean arterial blood pressure	Cardiac index
Inflow and outflow occlusion	THVE*			
	Redai et al.[a] [16]	↑ 25%	↓ 17,64%	↓ 50%
	Smyrniotis et al.[a] [123]	↑ 21%	↓ 23%	↓ 50%
	Figueras et al.[a] [124]	↑ 18,75%	↓ 20,48%	↓ 60%
	Smyrniotis et al. [54]	↑ 29%	↑ 22%	↓ 50%
	SHVE**			
	Figueras et al.[a] [124]	↑ 2,46%	↑ 3,79%	N/A
	Smyrniotis et al. [54]	↑ 5%	↑ 5,55%	↓ 10%
Inflow occlusion	Pringle			
	Redai et al.[a] [16]	↑ 6.25%	↑ 15%	↓ 10%
	Smyrniotis et al.[a] [123]	↑ 12%	↑ 16%	↓ 10%
	Figueras et al.[a] [124]	↑ 8.83%	↑ 13.85%	N/A

[a]Values expressing % change of heart rate, mean arterial blood pressure, and cardiac index during clamping and uclamping of hepatic vessels.
*THVE: total hepatic vascular exclusion.
**SHVE: selective hepatic vascular exclusion.
↑: increase.
↓: reduction.

to have no greater liver toxicity than currently used volatile anesthetic agents [21]. Additionally, desflurane undergoes only minor biodegradation (it is metabolized at a ratio of 0.02%) and in fact it may cause less hepatocellular damage due to its reduced metabolism [21]. Ko et al. [22], comparing the effects of desflurane and sevoflurane on hepatic and renal functions after right hepatectomy in living donors reported better postoperative hepatic and renal function tests with desflurane as compared to sevoflurane at equivalent doses of 1 MAC without, however, being able to validate the clinical importance of their study. Arslan et al. [23] comparing the effects of anesthesia with desflurane and enflurane on liver function, showed that during anesthesia with desflurane, liver function was well preserved; glutathione-S-transferase and aspartate aminotransferase levels were significantly lower in the desflurane group. On the other hand, Laviolle et al. [24] suggested that propofol has an early protective effect against hepatic injury compared with desflurane after partial hepatectomy under inflow occlusion.

It is now generally accepted that anesthesia reduces hepatic blood flow. However, few studies on the effects of general anesthesia during hepatectomies under vascular control techniques are available in patients with significant comorbidities.

3.3. Hemodynamic Management

3.3.1. Inflow Vascular Occlusion. CPM, IPM, and selective inflow occlusion share common hemodynamic management. Portal triad clamping increases systematic vascular resistance by up to 40% and reduces cardiac output by 10%. Mean arterial pressure increases about 15% (Table 1). Following unclamping, hemodynamic parameters gradually return to baseline values [25–28]. However, the systemic circulation in patients with cirrhosis is hyperdynamic and dysfunctional, with increased heart rate and cardiac output, decreased systemic vascular resistance, and low or normal arterial blood pressure. Thus, maintaining adequate organ perfusion may be difficult to achieve and preoperative optimization of the patient is required.

The anesthetic management is dictated by the surgical approach and the patient's health status. For healthy patients, routine monitoring is used. Monitoring can even be limited to just peripheral vein catheters [29]. Invasive monitoring provided by a central venous line or pulmonary catheterization is reserved for patients with poor cardiovascular status or when prolonged vascular occlusions are performed.

A low CVP (between 2 and 5 mmHg), while aiming at euvolemia, reduces blood loss during liver surgery and improves survival [30, 31]. A low CVP can be achieved by limitation of intravenous fluids administration pre- and intraoperatively. Maintenance fluids and crystalloids to stabilize blood pressure >90 mmHg and ensure diuresis of at least 0.5 mL/kg/h can be used safely with minor hemodynamic disturbance [32]. If fluid restriction is ineffective to keep a low CVP, vasoactive agents are used. Nitroglycerin reduces CVP to the desired level during the resection phase or when excessive oozing is observed from the resected surface [13, 16]. Intravenous morphine has also been used for its hypotensive effect.

CPM with a CVP of 5 mmHg or less is associated with minor blood loss and a shorter hospital stay [33]. IPM may result in fluctuations of systemic blood pressure. If, however, it is applied under a low CVP during transection, blood loss and hemodynamic changes are minimal [34–37]. In an experimental animal study, Sivelestat, a neutrophil elastase inhibitor, reduced hepatic injury and stabilized hemodynamics after ischemia-reperfusion following IPM [38].

The advantages of a low CVP must be weighed against inadequate perfusion of the vital organs and loss of volemic reserve in case of bleeding and/or air embolism. A 15° Trendelenburg position protects against air embolism. Melendez et al. [34] support that in low CVP anesthesia during liver resection, the incidence of perioperative renal failure does not increase significantly.

3.3.2. Inflow and Outflow Vascular Occlusion

(1) Total Hepatic Vascular Exclusion (THVE). In THVE, rapid hemodynamic changes (Table 1) are frequent due to surgical events such as caval clamping, sudden blood loss, and hepatic reperfusion. Cross-clamping of the inferior vena cava and portal vein result in a 40–60% reduction of venous return and cardiac output, with a compensatory 80% increase in systemic vascular resistance and a 50% increase in heart rate. Although systemic vascular resistance and heart rate increase, the cardiac index is reduced by half, secondary to a preload reduction. Unclamping is followed by an increase in cardiac index and a significant reduction in systemic vascular resistance [39].

The anesthetist should take prompt steps to manage the preload reduction and the sudden decrease in cardiac output evoked by the inferior vena cava and portal vein clamping. Intraoperative monitoring includes ECG, pulse oximetry, $ETCO_2$ tension, invasive blood pressure monitoring through an arterial line, and CVP monitoring through a large bore central venous line. Patients with pulmonary hypertension require pulmonary artery catheterization. In addition, the presence of a pulmonary artery catheter allows the tailored administration of vasopressors in case of massive hemorrhage due to vena cava injury. The Vigileo, an uncalibrated arterial pulse contour cardiac output monitoring system, has been proved to be unreliable in cirrhotic patients with hyperdynamic circulation undergoing major liver surgery [40].

Before THVE, colloids can be administered to prevent the abrupt decrease in cardiac output. Colloids, beyond correcting volume deficits [33], improve splanchnic circulation, displace fluid into the blood compartment, and reduce bowel edema. Blood pressure and circulatory support is achieved by aiming at a CVP of at least 14 mmHg [16]. Vasopressin or norepinephrine are administered if volume loading is inadequate to maintain blood pressure following clamping of the vena cava [7].

There is no standard approach to the use of vasoactive agents in THVE. Most studies have mainly been performed in septic patients or in animal models. Vasoactive agents should be used carefully, as they improve cardiac output at the expense of microcirculatory blood flow. During vascular isolation of the liver in eight pigs, norepinephrine infusion (0.7 μg/kg/min) decreased hepatic vascular capacitance by activation [41]. In a recent study in septic patients, Krejci et al. [42] showed that norepinephrine increased systemic blood flow but reduced microcirculatory blood flow on liver's surface.

Vasopressin on the other hand, is known to rapidly restore blood pressure during septic shock. However, in an experimental study [43], vasopressin proved to be inferior to norepinephrine in terms of improving hepatosplanchnic blood flow. The response to both norepinephrine and vasopressin is blunted in patients with cirrhosis [44, 45].

Preventing renal impairment is another important consideration for the anesthesiologist. Renal autoregulation ceases below a renal perfusion pressure of 70 to 75 mmHg, below which, flow becomes pressure dependent. Perioperative fluid shifts, intravascular hypovolemia, and sympathetic activation during THVE result in a reduction of renal blood flow. Mannitol, furosemide, and "low dose dopamine" have been used with the aim of preventing intraoperative renal injury without evidence of substantial benefit [46]. Fenoldopam had beneficial effects [47] on postoperative creatinine levels and creatinine clearance of critically ill patients [48]. Recently, terlipressin along with volume expansion have been shown to improve renal function, without, however, improving survival [49].

Hemodynamic intolerance to THVE or ischemia under THVE exceeding 30 or 60 minutes, require venovenous bypass [50, 51]. THVE should be limited to selected cases, as hemodynamic intolerance has been observed in 10–20% of patients, as well as increased morbidity and hospital stays (Table 2).

(2) Selective Hepatic Vascular Exclusion (SHVE). SHVE is a flexible technique that can be applied in a continuous or intermittent manner. Should accidental tears of major hepatic veins occur, rapid conversion to THVE must be undertaken. The literature suggests that many institutions favor SHVE as one of the standard methods of vascular control because it provides a bloodless surgical field and it is tolerated by most patients. No special anesthetic considerations regarding the hemodynamic management of SHVE are referred, as this method diminishes blood pressure and heart rate fluctuations during liver resection (Table 1).

In a cohort study [52] among 246 patients, hemodynamic tolerance to SHVE was excellent with only a slight increase in systemic and pulmonary resistance during clamping. No deaths were reported and the mean hospital stay was 9.6 days.

SHVE is the method of choice in cases when CVP cannot be lowered (i.e., right heart failure, poor cardiovascular status) [53–56]. In a retrospective study on 102 patients, SHVE was shown to be unaffected by CVP levels and the authors concluded that it should be used whenever CVP remains high despite adequate anesthetic management [57]. Although the performance of SHVE requires significant surgical expertise, it is tolerated by most patients and has a hemodynamic profile similar to that of CPM [53, 54]. Furthermore, it controls backflow bleeding of the hepatic veins. In a large clinical study [58], SHVE proved to be more effective than CPM in controlling intraoperative bleeding, preventing blood loss, and reducing postoperative complications and mortality rates (Table 2). Combined SHVE and perioperative fluid restriction has also been suggested as a liver and renal protective procedure in partial hepatectomy. Moug et al. [59] demonstrated that active preoperative dehydration of the

Table 2: Clinical series of hepatectomies performed under vascular occlusion techniques.

Technique-study	No. of patients	Type of hepatectomy[a]	Clamp time (min)	Morbidity/mortality (%)	Transfusions (%)	CVP (mmHg)
I.P[b]						
Torzilli et al. [36]	329	Major 71%	69	26/0	3.9	N/A
Nuzzo et al. [125]	120	Major 38%	39	N/A	60	<5
Omar Giovanardi et al. [126]	72	Major 81%	N/A	24/7	57	N/A
THVE[c]						
Smyrniotis et al. [54]	18	Major	32	33/0	30	N/A
Figueras et al. [124]	39	N/A	41	N/A	4	6.4
SHVE[d]						
Smyrniotis et al. [54]	20	Major	38	25/0	15	<5
Zhou et al. [58]	125	N/A	21.7	39.2/0	32	4.4
Fu et al. [127]	246	Major	N/A	24.8/0	24	2–5
Figueras et al. [124]	41	N/A	47	N/A	6	7.2
Pringle-IPM[e]						
Wang et al. [98]	114	N/A	N/A	N/A	13.1	5–10
Zhou et al. [58]	110	N/A	22.5	51.8/1.8	80.9	4.6
Ishizaki et al. [128]	380	Major 39.4%	62	23.9/0	34	N/A

[a] Major hepatectomy is defined as resection of more than two segments according to Couinaud's classification.
[b] I.P: ischemic preconditioning.
[c] THVE: total hepatic vascular exclusion.
[d] SHVE: selective hepatic vascular exclusion.
[e] IPM: intermittent pringle maneuver.

patient, low CVP anesthesia and SHVE resulted in minimal blood loss, low morbidity, and zero mortality in patients undergoing partial liver resection.

In conclusion, SHVE which is not associated with cardiorespiratory and hemodynamic alterations is well tolerated by the majority of patients and requires shorter hospitalization times [54].

3.4. Vascular Air Embolism. Although the relative risk of air embolism in hepatic surgery is low (<5%) [60], several cases have been reported during liver vascular control techniques. Factors predisposing to vascular air embolism during liver resections include: (a) surgical technique, (b) size and place of the tumor, (c) blood loss, and (d) low CVP anesthesia.

Clinical signs of vascular air embolism during anesthesia with respiratory monitoring are: a decrease in end-tidal carbon dioxide and decreases in both arterial oxygen saturation (SaO_2) and tension (PO_2), along with hypercapnia. From the cardiovascular system monitoring, tachyarrhythmias, electromechanical dissociation, pulseless electrical activity as well as ST-T changes can be noted. Major hemodynamic manifestations such as sudden hypotension may occur before hypoxemia becomes present.

When performing techniques of inflow vascular occlusion (CPM, IPM, selective inflow occlusion), air embolism may be observed during parenchymal transection under low CVP anesthesia or during reperfusion, due to mobilization of air bubbles trapped in opened veins. Resection of large tumors situated in the right lobe [61], close to the inferior vena cava or the cavohepatic junction, put the patient at risk of venous air embolism. Those tumors should therefore be resected under THVE or SHVE if possible. Recent clinical trials assessing the efficacy of SHVE and Pringle maneuver in preventing vascular air embolism showed that embolism occurred in three out of 2100 patients or in one out of 29 patients of the Pringle group, following massive blood loss during tumor resection. Air embolism did not occur in any case of the SHVE group [62–64].

Massive bleeding (>5000 mL) and subsequent air embolism can even result in intraoperative death in patients undergoing major liver resections [65]. The morbidity and mortality of air embolism depend on the volume and rate of air accumulation [66]. From case reports of accidental intravascular delivery of air, the adult lethal volume has been described as between 200 and 300 mL or 3–5 mL/kg [67, 68]. Low CVP further enhances the negative pressure gradient at the surgical field compared to the right atrium and increases the possibility of air embolism.

Currently, the most sensitive monitoring devices for vascular air embolism are transesophageal echocardiography and precordial Doppler ultrasonography, detecting as little as 0.02 mL/kg and 0.05 mL/kg of air, respectively [69–71].

The consequences of air embolism can be minimized by placing the patient in a 15 degree Trendelenburg position [72–74]. However, recent literature has questioned the efficacy of Trendelenburg position on improving hemodynamics [75]. Furthermore, Moulton et al. [75] in a small study among ten patients, showed that patient positioning alone during liver surgery does not affect the risk of venous air embolism. Thus, the beneficial effects of low CVP in

liver resections must be carefully weighed against adequate hydration and volume status optimization.

Vascular air embolism is a potentially hazardous complication. Additionally, cirrhotic patients undergoing hepatectomy have pulmonary abnormalities including intrapulmonary shunting, pulmonary vascular dilatation, and arteriovenous communications. In these patients, air can pass into the systemic circulation (paradoxical air embolism), even if cardiac abnormalities (patent foramen ovale) are not present, evoking fatal consequences [76].

Recent literature suggests that SHVE prevents vascular air embolism and provides operative tolerance. However, recognizing the risk for vascular air embolism and planning the appropriate level of monitoring and treatment is the key to patient safety.

3.5. Blood Loss and Transfusion. Liver resections may result in significant blood loss and subsequent transfusion of RBC (red blood cells) in about 25%–30% of patients [77]. The two main sources of bleeding during a liver resection are (a) the inflow system (hepatic artery and portal vein) and (b) the outflow system (backflow bleeding from the hepatic veins). Bleeding may also occur during liver mobilization, hepatic transection, and dissection of biliary structures.

Blood loss has been linked to morbidity and mortality since 1989 [8], whereas RBC transfusions are associated with multiple disadvantages, risks, and side effects. Furthermore, operative blood loss independently predicts recurrence and survival after resection of hepatocellular carcinomas [78]. Operative mortality in patients refusing blood transfusions was 7.1% for patients with hemoglobin levels >10 g/dL and 61.5% for patients with hemoglobin levels <6 g/dL [79, 80].

The refinement of inflow and outflow occlusive techniques as well as the appropriate anesthetic management has reduced intraoperative bleeding and the need for blood transfusions. The surgical approach to hepatic resection is of major importance in preventing blood loss. Study of the literature reveals the following results regarding bleeding with different vascular occlusion techniques: Pringle maneuver has been shown to be effective in reducing blood loss during parenchyma transection [81]. Portal triad clamping is associated with less bleeding compared with no clamping [82]. In procedures of liver ischemia time < one hour, CPM is equal to IPM. Belghiti et al. [9], in a prospective study of IPM versus CPM, found no difference in total blood loss or the volume of blood transfused between the two groups, despite higher blood loss during parenchyma transection. Man et al., in two prospective studies of IPM versus no use of vascular control at all, showed lower total blood loss and fewer transfusions in the IPM group [83–85]. Hemihepatic vascular clamping was shown superior to IPM and to no application of vascular control, with reduced both blood loss and transfusion requirements [86]. SHVE provides a bloodless surgical field similar to THVE, but is better tolerated by patients. Many authors favor SHVE as one of the standard methods of vascular control, as it substantially prevents massive blood loss and diminishes transfusion needs.

From an anesthetic standpoint, a low CVP level plays an important role in reducing intraoperative blood loss and transfusion rates [30, 57, 87]. Maintaining a CVP < 5 mmHg by volume restriction and intravenous infusion of nitroglycerine and a systolic blood pressure above 90 mmHg by intravenous infusion of dopamine (4–6 μg/kg) has dramatically reduced bleeding and transfusion requirements [88]. The anesthetist should also provide normothermic conditions to the patient undergoing liver resection, because hypothermia reduces blood coagulation, especially platelet function, and increases intraoperative blood loss.

Alternative methods of diminishing blood loss have been investigated. Of the pharmacological methods, desmopressin, although used in treating hemophilia, was not effective in reducing blood loss and transfusion needs in patients undergoing liver resection. In a randomized clinical trial, the use of recombinant factor VIIa in major liver resections failed to reduce the number of units transfused [89]. A significant reduction in blood transfusion needs in liver resections has been shown with the use of aprotinin. Aprotinin was found to reduce intraoperative blood loss by 25% and transfusion requirements by 50% [81]. Redai et al. [16] used half dose aprotinin (10^6 KIU followed by 2.5×10^5 KIU/hour infusion) during hepatic transplantation in patients who have a significant coagulopathy or portal hypertension and in those who had previous abdominal surgery. However, Lentschner et al. [90] cautioned against the routine use of aprotinin due to the incidence of life threatening allergic reactions, thrombotic potential, and renal failure. Currently, there is no scientific support for the routine use of aprotinin in patients undergoing partial hepatectomy, whereas its efficacy in liver transplantation is well established [91]. Tranexamic acid has also been shown to reduce blood requirements in liver resection surgery but safety concerns have been raised and require further investigation [92, 93]. In the future, two artificial oxygen carriers (hemoglobin solutions and perfluorocarbons) may become essential in reducing the need for allogeneic RBC transfusions [94–96]. Artificial oxygen carriers improve O_2 delivery and tissue oxygenation as well as the function of organs with marginal O_2 supply. More studies examining their efficacy in ischemic liver during hepatectomy need to be performed.

Undoubtedly, the improvement of vascular control techniques during hepatectomy has permitted an aggressive approach for liver resections with low mortality rates (4%) [52]. In addition, anesthesia orientated towards an almost transfusion free setting has also improved mortality and morbidity following liver surgery. To this direction, Pulitanò et al. [97] proposed a score predicting blood requirements in liver surgery. A transfusion risk score, including variables of: (a) preoperative hemoglobin concentrations below 12.5 g/dL, (b) largest tumor more than 4 cm, (c) need for exposure of the vena cava, (d) need for an associate procedure, and (e) cirrhosis, accurately predicted the likelihood of blood transfusions in liver resections.

Recently, Cescon et al. [52], in a retrospective review assessing the outcome of 1500 consecutive patients who underwent hepatic resection, estimated overall mortality and morbidity at 3% and 22.5%, respectively. Their multivariate

analysis revealed that blood transfusions, primary liver tumors, and additional procedures were associated with an increased risk of postoperative complications, whereas blood transfusions, cirrhosis, biliary malignancies, and extended hepatectomy were associated with an increased risk of postoperative mortality. Wang et al. [98], evaluating the long-term outcomes of liver resection for hepatocellular carcinoma, estimated that 86.9% of the patients did not require perioperative blood transfusion and that Pringle maneuver and RBC transfusions are independent prognostic factors influencing survival.

Blood transfusions are well known to carry the risk of transmitted infections, acute or delayed reactions and "wrong blood" incidents. In liver resections, blood transfusions are associated with suppression of the immune system. There is strong evidence that blood transfusions have an impact on tumor recurrence for patients with early stages of hepatocellular carcinoma. However, no such effect could be demonstrated for patients undergoing partial liver resection for late stages of hepatocellular carcinoma, colorectal metastasis, or cholangiocarcinoma [99]. Transfusion evoked immunosuppression is also responsible for TRALI (transfusion-related acute lung injury). Dyspnea, hypotension, fever, and bilateral noncardiogenic pulmonary edema, present within 6 h of transfusion and complicate the postoperative outcome of patients following major liver surgery [100]. Patients with chronic liver disease have the greatest risk of developing TRALI, in comparison to other populations [101, 102]. Although all blood products can lead to this life-threatening situation, plasma-containing products were responsible for the majority of cases in patients undergoing liver transplantation [101]. Recent studies suggest that TRALI fatalities followed plasma transfusion components were linked to multiparous female donors with leukocyte antibodies [103, 104]. Therefore, the establishment of new strategies in blood donation excluding multiparous women as donors, as potential carriers of TRALI-inducing antibodies, is expected to eliminate this entity.

In conclusion, given the influence of blood loss and transfusions on the surgical outcome, techniques of liver vascular control and anesthetic management should be adjusted to the individual patient. The tumor location, the underlying liver disease and the patient's cardiovascular status should therefore be taken into account, in order to minimize blood loss and transfusion requirements.

3.6. Ischemia-Reperfusion Injury and Preconditioning. Ischemia/reperfusion (I/R) injury is a serious complication of liver surgery, especially after extended hepatectomies [105]. It causes a local and systemic inflammation response and its clinical manifestations may vary from transient arrhythmias to multiorgan dysfunction and death [106]. Reperfusion injury is mediated via reactive oxygen species which damage cellular membranes, stimulate leukocyte activation and endothelial adhesion, and activate the complement. All these pathophysiological changes lead to microcirculatory failure. Hepatic I/R injury affects patient recovery after major surgery and bears a risk of poor postoperative outcome [107]. In liver surgery, ischemic preconditioning (IP) and intermittent clamping are the only established methods to provide protection against tissue damage due to ischemia during inflow occlusion [98, 108].

IP is defined as a process in which a short period of ischemia, separated by intermittent reperfusion, renders an organ more tolerant to subsequent episodes of ischemia [107, 109]. It was initially described for a canine heart by Murry et al. in 1986 [110]. As far as the liver is concerned, the beneficial effect of IP was first demonstrated in a rodent model by Lloris-Carsi et al. [111]. Clavien et al. provided the first clinical evidence of benefit in patients undergoing hemihepatectomy [112]. It leads to improvement of hepatic microcirculation, reduction in tissue apoptosis, and improvement of survival. Experimental data suggest that generation of adenosine, activation of adenosine A_2 receptors with subsequent generation of NO and release of NO cause vasodilation and prevent the increase in endothelins, thus protecting the liver from reperfusion injury [107]. IP stimulates adenosine receptors on Kupffer cells in nonischemic lobes to produce oxygen radicals, leading to the promotion of liver regeneration after partial hepatectomy [113]. In a clinical study of 61 patients undergoing liver surgery performed by Heizmann et al., the absence of preconditioning was found to be an independent risk factor for postoperative complications [114]. The benefit of ischemia is restricted by old liver [109]. It has been stated that IP might also be less beneficial during extended liver resections, due to hyperperfusion-induced derangement in hepatic microcirculation. Similarly, the effect of preconditioning was lost in patients undergoing tissue loss above 50% [115]. In small liver remnants of about 30%, it may in fact have detrimental effects. This is because the small remaining tissue suffers from shear stress-associated microvascular injury. Ischemic preconditioning seems to attenuate the apoptotic response of hepatic cells in major hepatectomies performed under SHVE [115]. On the other hand, Azoulay et al. found that IP failed to protect human liver against IR injury after major hepatectomy under continuous vascular occlusion with preservation of caval flow [116]. Other strategies should be used to induce protection in this setting. Combined IP and salvialonic acid-B have been shown to possess synergistically protective effects in rats, mediated through reduction of postischemic oxidative stress, higher ATP levels and reduction in hepatocellular apoptosis [105].

The severity of IR injury is related to the duration of vascular occlusion. The preconditioning effect fades away when the ischemic time is prolonged [108]. In this case, intermittent vascular occlusion, although more complex surgically, seems to be the method of choice. Van Wagensveld et al. demonstrated that prolonged intermittent vascular inflow occlusion in pig liver surgery caused less microcirculation impairment and hepatocellular necrosis compared with continuous occlusion and recommend it when a prolonged period of vascular inflow occlusion is expected [117]. It has been found that when ischemia persists for more than 40 minutes, intermittent vascular occlusion offers better protection of liver cells, demonstrated by lower AST values, lower apoptotic activity and reduced capsase-3 activation [108].

In several animal models, pharmacological preconditioning with a volatile anesthetic has been proven to provide protection against ischemic injury. Beck-Schimmer et al. evaluated the effects of sevoflurane preconditioning before liver ischemia and concluded that this particular volatile anesthetic limited the postoperative increase of serum transaminase levels by 261 U/L for the ALT and by 239 U/L for the AST. The sevoflurane group had less major complications (such as sepsis, bilioma, bleeding, and infection) than the control (propofol) group. The protective effects were more pronounced in patients with liver steatosis [20]. However, according to Wang et al., propofol also seems to have the ability to protect human hepatic L02 cells from H_2O_2-induced apoptosis [118]. Intraportal administration of L-arginine, a precursor of NO, has been recently studied in pigs and appears to reduce cell damage during the early phase of reperfusion, by downregulating capsase-3 activty and by preserving mitochondrial structure. Clinically, it resulted in a reduction of AST and an increase in bile production [119]. In another animal study, simvastatin (5 mg/kg) protected the rat liver from I/R injury by regulating the inflammatory response and by improving microvascular flow [120]. Prostaglandins have also been found to have protective effects on I/R-injured livers by inhibiting the generation of reactive oxygen species, preventing leucocyte migration, improving hepatic insulin and lipid metabolism and regulating the production of inflammatory cytokines. They are also essential after hepatectomy because they promote hepatocyte proliferation [121].

Finally, Ramalho et al. reported that angiotensin II type I receptor (AT1R) antagonist increased regeneration in nonsteatotic livers, while in the presence of steatosis both AT1R and AT2R antagonists increased liver regeneration [122].

4. Conclusions

Hepatic vascular occlusion techniques require anesthetic expertise. Intolerance to THVE is not unusual and this method should be reserved for patients in need for extensive reconstruction of the inferior vena cava. SHVE has the most favorable intraoperative and postoperative hemodynamic profile. Inflow occlusion techniques, although simple and effective, require specific anesthetic manipulations to reduce liver injury and prevent backflow bleeding.

Every method of hepatic vascular control applied under a carefully selected anesthetic plan can improve the outcome of patients undergoing hepatectomy. The surgeon and anesthesiologist must work together effectively. Anesthetic vigilance along with thorough knowledge of the surgical manipulations promotes team-based health care in the operative room.

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Research Article

Small-for-Size Liver Transplantation Increases Pulmonary Injury in Rats: Prevention by NIM811

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Pulmonary complications after liver transplantation (LT) often cause mortality. This study investigated whether small-for-size LT increases acute pulmonary injury and whether NIM811 which improves small-for-size liver graft survival attenuates LT-associated lung injury. Rat livers were reduced to 50% of original size, stored in UW-solution with and without NIM811 (5 μM) for 6 h, and implanted into recipients of the same or about twice the donor weight, resulting in half-size (HSG) and quarter-size grafts (QSG), respectively. Liver injury increased and regeneration was suppressed after QSG transplantation as expected. NIM811 blunted these alterations >75%. Pulmonary histological alterations were minimal at 5–18 h after LT. At 38 h, neutrophils and monocytes/macrophage infiltration, alveolar space exudation, alveolar septal thickening, oxidative/nitrosative protein adduct formation, and alveolar epithelial cell/capillary endothelial apoptosis became overt in the lungs of QSG recipients, but these alterations were mild in full-size and HSG recipients. Liver pretreatment with NIM811 markedly decreased pulmonary injury in QSG recipients. Hepatic TNFα and IL-1β mRNAs and pulmonary ICAM-1 expression were markedly higher after QSG transplantation, which were all decreased by NIM811. Together, dysfunctional small-for-size grafts produce toxic cytokines, leading to lung inflammation and injury. NIM811 decreased toxic cytokine formation, thus attenuating pulmonary injury after small-for-size LT.

1. Introduction

Pulmonary complications including acute lung injury and acute respiratory distress syndrome frequently occur after liver transplantation (LT) and contribute significantly to perioperative and postoperative morbidity and mortality [1–4]. The frequency of pulmonary complications is reported as high as 75% in some studies [1], and the mortality rate for acute respiratory distress syndrome reaches 50%–80% [2, 5]. Prolonged cold storage, retrieval procedures, intraoperative transfusion of plasma-containing blood products, ischemia/reperfusion- (I/R-) induced graft injury, proinflammatory cytokine and chemokine formation, leukocyte recruitment and release of neutrophil elastase, pulmonary endothelial barrier disruption, and vascular hyperpermeability possibly play critical role in the development of posttransplantation acute lung injury [1, 2, 5–8]. Since primary liver graft failure is often associated with pulmonary injury, prevention and treatment of pulmonary complications could improve the outcome of LT.

Due to severe shortage of donor organs, partial LT has increased rapidly in recent years [9–11]. In adult-to-adult living donor and split LT, small-for-size syndrome occurs when the ratio of liver graft volume is less than 30–40% of the standard liver volume of recipient [9, 12]. Such small-for-size grafts are associated with increased graft injury, inhibited liver regeneration, poor graft function, more severe posttransplantation complications, and increased mortality [9, 12]. Mechanisms of small-for-size liver graft failure remain unclear but are most likely multifactorial. Energy

supply is crucial for cell survival and proliferation. Therefore, compromised energy supply could lead to liver graft injury and suppressed regeneration. Our previous studies showed that free radical production in small-for-size liver grafts leads to mitochondrial dysfunction [13–15]. Mitochondrial depolarization occurring in small-for-size liver grafts is related to opening of high conductance mitochondrial permeability transition (MPT) pores [16]. MPT pore opening collapses mitochondrial membrane potential, leading to failure of oxidative phosphorylation. NIM811, a non-immunosuppressive cyclosporine A derivative, inhibits MPT pore opening by binding to cyclophilin D, a component of the pore [17]. In small-for-size liver grafts, NIM811 protects against mitochondrial depolarization, thus decreasing injury and improving liver regeneration and functional recovery [16].

Whether small-for-size LT increases pulmonary complications remains unclear. A previous report showed that liver splitting procedures cause leukocyte recruitment in the lung tissue of donor [18]. Rapid onset of acute respiratory distress syndrome is also observed after major hepatectomy [19]. Hemodynamic alterations occurring after small-for-size LT could also lead to pulmonary complications [20, 21]. Moreover, previous studies showed that hepatic I/R promotes remote organ injury, including leukocyte infiltration and parenchymal cell damage in the lung [22]. Accordingly, this study investigated whether pulmonary complications occur after small-for-size LT and whether protection against small-for-size liver graft dysfunction by NIM811 prevents postoperative acute lung injury.

2. Methods

2.1. Liver Transplantation. Male Lewis rats (170–200 g) were used for orthotopic LT [14, 23]. Briefly, livers were explanted after flushing *in situ* with 5 mL ice-cold UW cold storage solution (Barr Laboratories, Pomona, NY) via the portal vein and removed. In ice-cold UW solution, cuffs prepared from 14-gauge *i.v.* catheters were placed over the subhepatic vena cava and the portal vein. Liver mass was reduced *ex vivo* to ∼50% of original size by removing the left lateral lobe, the left portion of the median lobe, and the anterior and posterior caudate lobes after ligation with 4–0 silk suture [23]. Explants were stored in UW solution at 0-1°C for 6 h and rinsed with room-temperature-lactated Ringer's solution (Abbott Laboratories, North Chicago, IL) just prior to implantation. NIM811 (5 μM, Novartis Pharma Ltd., Switzerland) was added to the storage and rinse solutions. Reduced-size liver explants were implanted into recipients of similar (170–200 g) or greater body weight (350–420 g), which results in a graft weight/standard liver weight (defined as 4% body weight) of ∼50% (half-size graft, HSG) and ∼25% (quarter-size graft, QSG), respectively. Unreduced livers were implanted into recipients of similar body weights (170–200 g) as full-size grafts (FSG). The hepatic artery and bile duct were reconstructed as described previously [14]. The ratios of graft weight/standard liver weight were not significantly different between QSG with or without NIM811 treatment ($P > 0.1$ by Students' t-test). All animals were given humane care in compliance with institutional guidelines using protocols approved by the Institutional Animal Care and Use Committee.

2.2. Serum Alanine Aminotransferase (ALT). To access liver graft injury, serum ALT was measured from blood samples collected from the vena cava at 38 h after implantation using analytical kits from Sigma Chemical (St. Louis, MO). To adjust for graft size and recipient blood volume, serum ALT was normalized by multiplying by the recipient's standard blood volume (6.4% of body weight) and dividing by graft weight [24].

2.3. Pulmonary and Hepatic Histology. Under pentobarbital (50 mg/kg, *i.p.*) anesthesia at various times after implantation (5, 18, and 38 h), the lung and liver were harvested and fixed with 4% paraformaldehyde in Dulbecco's phosphate buffered saline (Invitrogen Corp. Grand Island, NY) [25, 26], imbedded in paraffin and processed for histology. In sections stained with hematoxylin and eosin (H&E), lung and liver images were acquired using a Universal Imaging Image-1/AT image acquisition and analysis system (West Chester, PA) incorporating an Axioskop 50 microscope (Carl Zeiss, Inc., Thornwood, NY) and using 20x and 10x objective lenses, respectively. Alveolar septal wall thickness was quantified by image analysis of 5 randomly selected alveolar septa per field in 10 randomly selected fields per slide using an IPlab 3.7v software (BD Biosciences, Rockville, MD). Relative alveolar septal thickness was expressed as the ratios between the average thicknesses of different transplantation groups to the sham-operation group. Liver necrosis was quantified by image analysis of 10 randomly selected fields per liver in a blinded manner using the same software and calculated by dividing the necrotic areas by the total cellular area [26].

2.4. Immunohistochemical Staining for 5-Bromo-2′-Deoxyuridine, Myeloperoxidase, ED1, and Intracellular Adhesion Molecule-1. To assess liver regeneration, 5-bromo-2′-deoxyuridine (BrdU, 100 mg/kg *i.p.*) was injected 1 h prior to liver harvesting to detect cells synthesizing DNA. BrdU incorporation in liver sections was determined by immunohistochemical staining as described elsewhere [27, 28]. For immunohistochemistry of leukocytes and adhesion molecules in the lung tissue, pulmonary sections were deparaffinized with xylene (Mallinckrodt Baker, Paris, Kentucky) and taken through a graded series of alcohol/water mixtures to rehydrate the tissue. To stain for myeloperoxidase (MPO), an indicator of neutrophil infiltration, lung sections were immersed in 10 mM citrate acid (pH 6), heated in microwave for antigen retrieval, and then exposed to rabbit anti-MPO polyclonal antibodies (DAKO Corp., Carpinteria, CA) at a concentration of 1 : 200 in 0.1 M phosphate buffer-0.5% Tween 20 for 30 min at room temperature followed by a 20 min incubation with peroxidase-conjugated anti-rabbit IgG_1 antibody (DAKO Corp., Carpinteria, CA) at room temperature. 3,3′-Diaminobenzidine chromagen was then added as the peroxidase substrate. After the immunostaining

procedure, a light counterstain of Meyer's hematoxylin was then applied. MPO-positive cells were counted in 10 random fields per slide in a blind manner using a 40x objective lens [29]. Immunohistochemistry of ED1, a marker of monocytes/macrophages, was performed using specific antibody (Serotek, Raleigh, NC) at a dilution of 1 : 150 for 30 min at room temperature. To stain for intracellular adhesion molecule 1 (ICAM-1) in the lungs, slides were treated in microwave as described previously for antigen retrieval and then exposed to rabbit anti-ICAM-1 polyclonal antibodies (BD Biosciences Pharmingen, San Diego, CA) at a concentration of 1 : 200 overnight at 4°C.

2.5. Immunoblotting. Liver tissue was homogenized in 0.1 M phosphate buffer (pH 7.2) containing 0.1% SDS, 1% IGEPal, 1% protease, and 1% phosphatase inhibitor cocktails (Sigma, St. Louis, MO) and centrifuged at 14,000 ×g for 15 min at 4°C. Aliquots of supernatant (40 μg of protein) were separated on NuPAGE 4–12% Bis-Tris gels, transferred onto nitrocellulose membranes, and immunoblotted with primary antibodies specific for proliferating cell nuclear antigen (PCNA; Dako, Glostrub, Denmark) at 1 : 1000 and actin (ICN, Costa Mesa, CA) at 1 : 3000 over night at 4°C. Horseradish peroxidase-conjugated secondary antibodies were applied, and detection was by chemiluminescence (Pierce Biotec., Rockford, IL).

2.6. Detection of Interleukin-1β and Tumor Necrosis Factor-α mRNAs by Quantitative Real-Time PCR. Total RNA was isolated from liver tissue with Trizol (Invitrogen, Grand Island, NY). Single stranded cDNAs were synthesized from RNA (2 mg) from liver tissue using a Bio-Rad iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) [30]. The primer sequences are listed in Table 1. qPCR was conducted using a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The abundance of mRNAs was normalized against hypoxanthine phospho-ribosyl-transferase (HPRT), a house-keeping gene, using the $\Delta\Delta Ct$ method.

2.7. Statistical Analysis. Groups were compared using ANOVA plus a Student-Newman-Keuls post hoc test. Data shown are means ± S.E.M. Group sizes were 4 livers in each group for all parameters, as indicated in Section 3 and corresponding figure legends. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Increased Liver Injury and Suppressed Regeneration of Small-for-Size Liver Grafts: Reversal by NIM811. Previously, we reported that all recipients of FSG survived after transplantation [14]. Survival was decreased slightly to 80% after transplantation of HSG and markedly to 30% in QSG recipients [14]. Inhibition of the MPT by NIM811 decreased injury, improved liver regeneration, and increased survival of small-for-size liver grafts from 30% to 81% [16]. Consistent with the early work, in the present study, no pathological changes were observed in liver tissue at 5 h (data not shown) and 38 h after sham operation (Figure 1(a)), and necrosis was minimal in FSG and HSG. At 5 h after transplantation, necrosis was barely detectable in QSG (data not shown). By contrast, necrosis increased at 38 h after implantation of QSG, mainly in periportal and midzonal regions of liver lobules. NIM811 decreased necrosis in QSG ($n = 4$ per group). Serum ALT (Figure 1(c)) was ~0.09 U/g liver before transplantation. ALT increased at 5 h after transplantation of QSG, peaked at about 18 h, and then remained at high levels (not shown). At 38 h after implantation, ALT was ~14 U/g in rats receiving QSG but was only 0.3 U/g liver and 1.3 U/g liver in rats receiving FSG and HSG, respectively, indicating more severe injury in QSG. NIM811 decreased ALT to 2.9 U/g in QSG recipients ($n = 4$ per group).

Table 1: Primers for Real-Time PCR.

mRNAs		Primers
IL-1β	Forward:	5′-AGCAGCTTTCGACAGTGAGGAGAA-3′
	Reverse:	5′-TCTCCACAGCCACAATGAGTGTGACA-3′
TNF-α	Forward:	5′-CAGACCCTCACACTCAGATCATCTT-3′
	Reverse:	5′-CAGAGCAATGACTCCAAAGTAGACCT-3′
HPRT	Forward:	5′-TCGAAGTGTTGGATACAGGCCAGA-3′
	Reverse:	5′-TACTGGCCACATCAACAGGACTCT-3′

IL-1β: interleukin-1β; TNF-α: tumor necrosis factor; HPRT: hypoxanthine phospho-ribosyl-transferase.

Liver regeneration was evaluated by BrdU incorporation (Figure 1(b)) and expression of PCNA (Figure 1(d)). BrdU-positive cells were barely detectable at 5 h (not shown) and 38 h after sham-operation and in FSG (Figure 1(b)). In HSG, BrdU labeling was undetectable at 5 h, increased slightly after 18 h (not shown), and increased sharply at 38 h (Figure 1(b)). Proliferating cells were predominantly hepatocytes [13]. In contrast, BrdU-positive cells were rare in QSG at all time points ($n = 4$ per group). PCNA was barely detectable in sham-operated livers and FSG but increased substantially in HSG (~160-fold) at 38 h, consistent with cell proliferation. PCNA expression increased only ~4.6-fold in QSG ($n = 4$ per group). These results show suppression of cell proliferation in QSG, which NIM811 largely reversed.

3.2. Pulmonary Leukocyte Infiltration and Injury Is Greater after Small-for-Size Liver Transplantation. Pulmonary leukocyte infiltration and injury were evaluated at 5, 18, and 38 h after LT. At 5 h after transplantation of QSG, very mild perivascular edema was observed. However, alveolar septal thickness was not significantly increased (Figures 2(b) and 2(i)). ED1-positive monocytes/macrophages increased slightly (Figures 2(f) and 2(j)), but neutrophils were not increased (Figure 2(k)). At 18 h after transplantation, the alveolar septa were slightly thickened compared to 5 h, but leukocyte infiltration remained at low levels (Figures 2(c), 2(g), 2(i), 2(j), and 2(k)). At 38 h after transplantation of QSG, alveolar septa were markedly thickened (4.1-fold) with increased cellularity (Figures 2(d) and 2(i)). Numerous leukocytes, including neutrophils and mononuclear cells, were seen in the peribonchial spaces, the lumina of blood vessels, and the perivascular as well as intra-alveolar space.

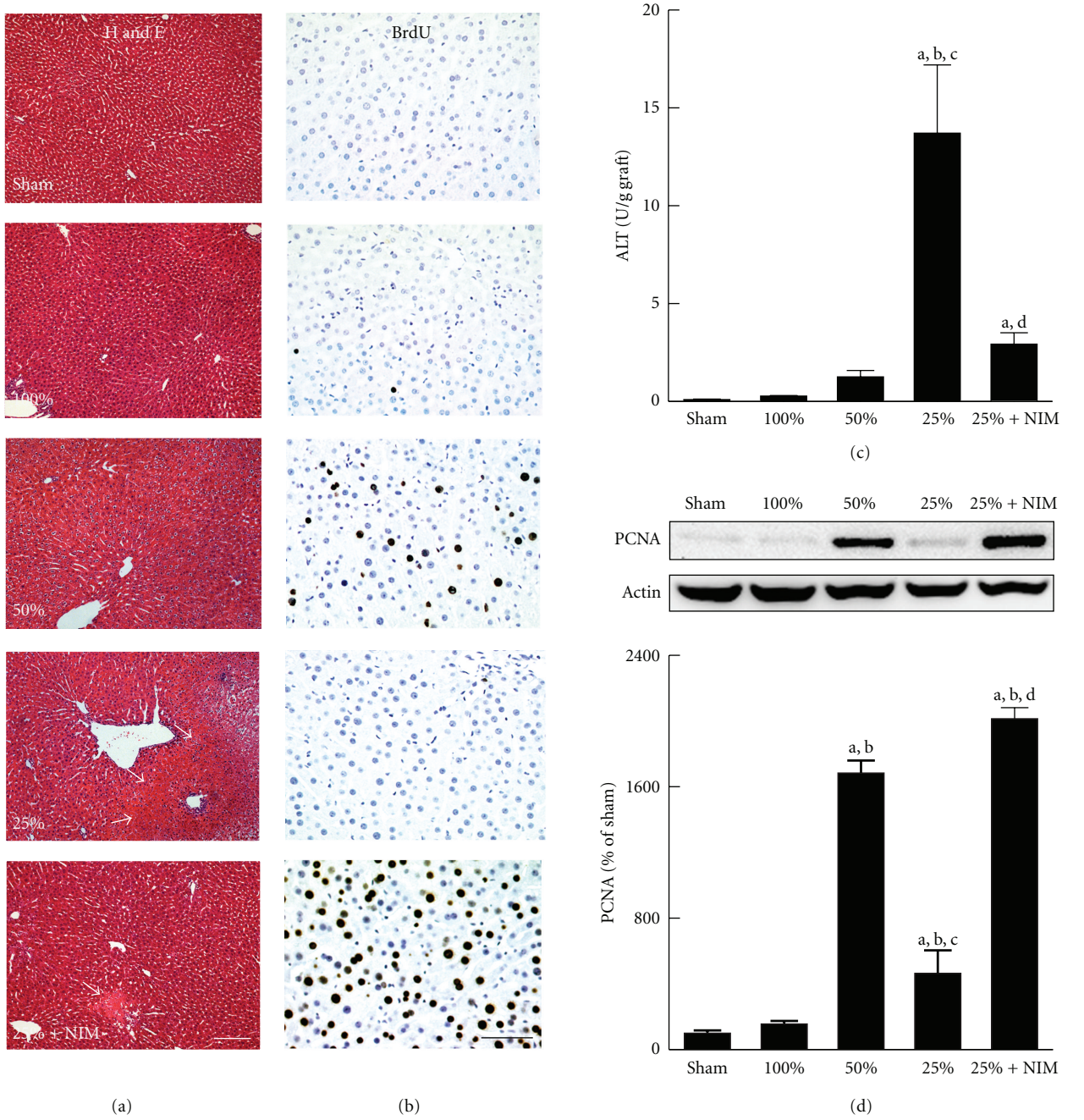

Figure 1: NIM811 protects against graft injury and promotes liver regeneration after transplantation of small-for-size liver grafts. Full-size and reduced-size rat livers were transplanted, as described in Section 2. In some experiments, NIM811 (NIM, 5 μM) was added to the storage and poststorage lactated Ringer's rinse solutions. In (a) and (b), liver grafts were harvested at 38 h after transplantation for H&E staining ((a), bar is 100 μm) or BrdU immunohistochemistry ((b), bar is 50 μm). Representative images are shown. Arrows identify necrotic areas. Panels are as follow: *1st row*, liver from a sham-operated rat; *2nd row*, FSG (100%); *3rd row*, HSG (50%); *4th row*, QSG (25%); *5th row*, QSG treated with NIM811. In (c), blood samples were collected at 38 h after transplantation for ALT measurement. In (d), proliferating cell nuclear antigen (PCNA) expression in liver tissue was detected by immunoblotting and quantified by densitometry. Values are means ± S.E.M. Group sizes were 4 per group: (a) $P < 0.05$ versus sham operation; (b) $P < 0.05$ versus FSG (100%); (c) $P < 0.05$ versus HSG (50%); (d) $P < 0.05$ versus QSG (25%).

At 38 h, ED1-positive cells (monocytes/macrophages) increased ~6.5-fold and MPO-positive cells (neutrophils) increased ~47-fold (Figures 2(j) and 2(k)) ($n = 4$ per group for all parameters). Together, these findings show that lung pathological changes became marked at ~38 h after transplantation of small-for-size grafts. At earlier times after QSG transplantation, infiltration was slight and composed predominantly of mononuclear cells.

Lung injury was compared among groups at 38 h after transplantation. After transplantation of FSG, alveolar septa

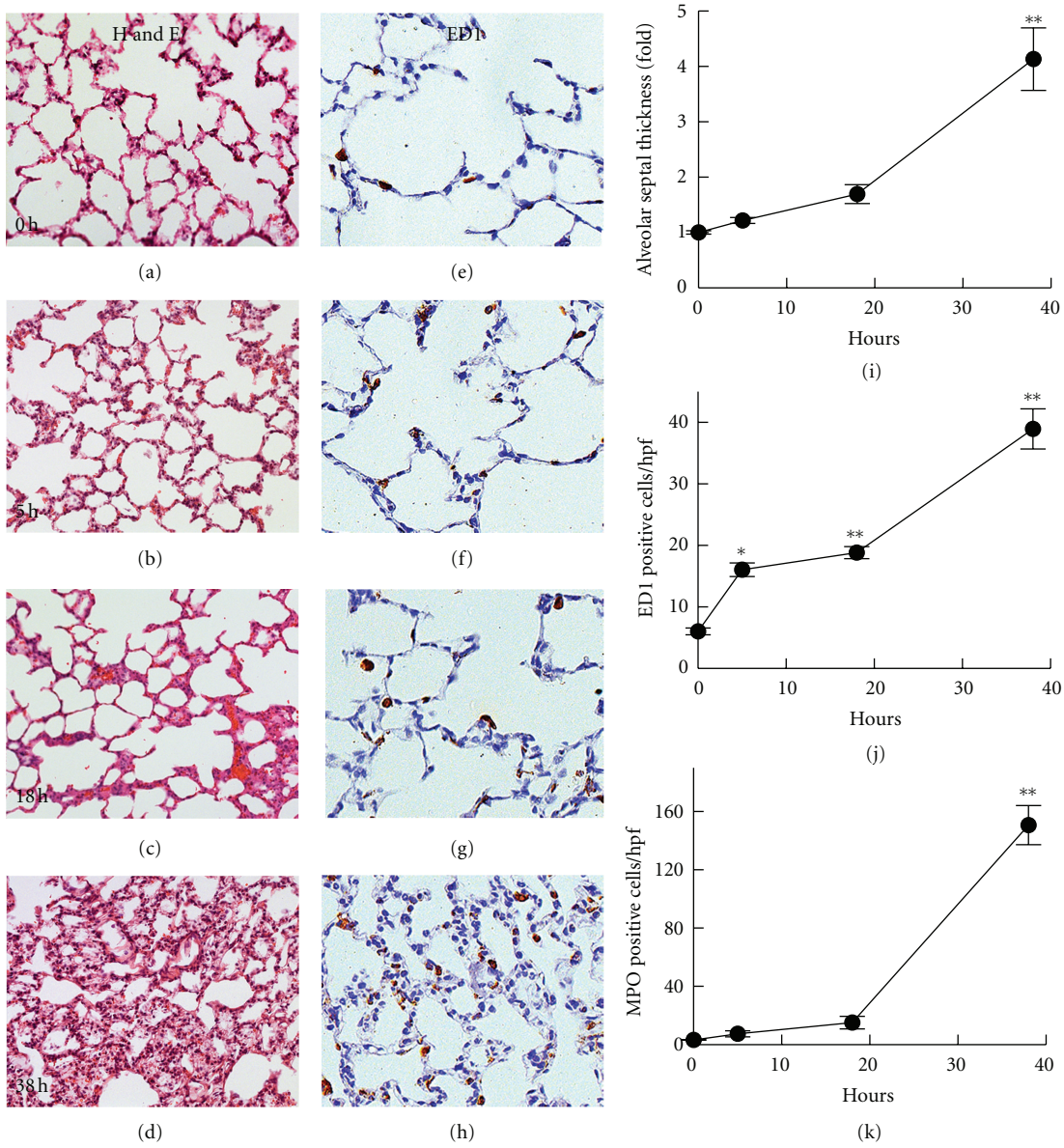

Figure 2: Time course of pulmonary histopathological changes after transplantation of small-for-size liver grafts. In (a)–(d), lungs were harvested at 5, 18, and 38 h after transplantation of QSG. Lung sections were stained with H&E. In (e)–(h), representative images of ED1 staining ($n = 4$ per group) are shown. In (i), alveolar septal thickness was quantified by image analysis of 5 random alveolar septa per image and 10 random images per slide using IPlab 3.7v software. Relative alveolar septal thickness was expressed as the ratio between the thicknesses of different transplantation groups to the sham-operation group. In (j) and (k), ED1 (j) and myeloperoxidase (MPO, (k)) positive cells per high power field (hpf) were counted in 10 random fields using a 40x objective lens in a blinded manner. Values are means ± S.E.M. Group sizes are 4 per group: $^{*}P < 0.05$ and $^{**}P < 0.01$ versus 0 h.

were not thickened, and neutrophils were not increased, but monocytes/macrophages increased ~3-fold (Figure 3). After transplantation of HSG, alveolar septa thickened slightly (1.9-fold), and monocyte/macrophages increased ~3-fold, but neutrophils were not increased. Overall, after transplantation of QSG, alveolar septal thickening, increased cellularity, monocyte/macrophage, and neutrophil sequestration were substantially more severe compared to recipients of FSG and HSG (Figure 3) ($n = 4$ per group).

3.3. NIM811 Attenuates Lung Injury after Transplantation of Small-for-Size Liver Grafts. Since NIM811 decreased hepatic injury after small-for-size LT, we examined the effects of NIM811 on lung injury. Compared to vehicle, NIM811 decreased alveolar septal thickening by 52%, monocyte/macrophage infiltration by 44% and neutrophil infiltration by 51% in QSG recipients (Figure 3) ($n = 4$ per group). Overall, alveolar septal thickening and monocyte/macrophage infiltration after NIM811 treatment of

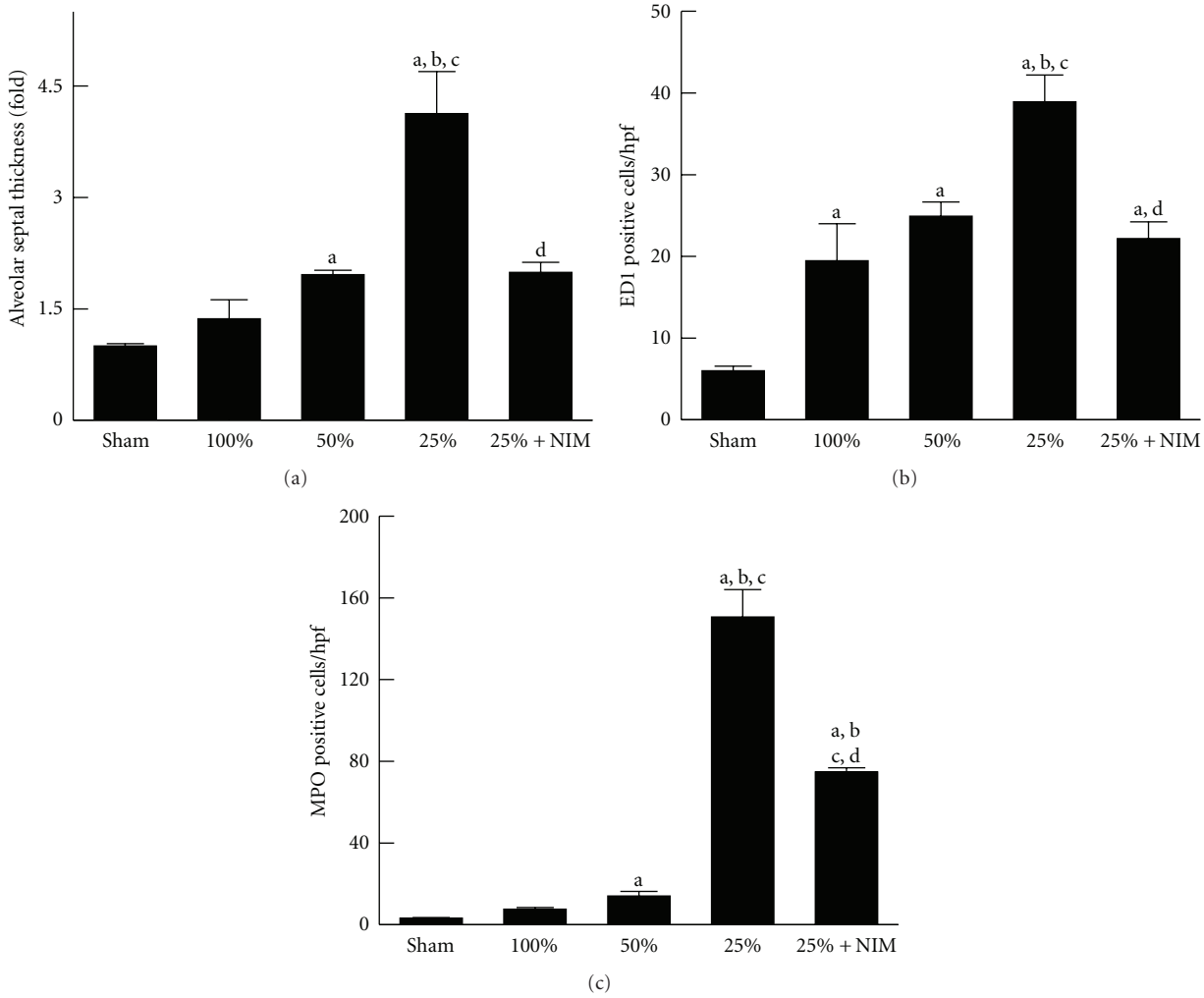

Figure 3: NIM811 decreases lung inflammation after small-for-size liver transplantation. Full-size and reduced-size rat livers were transplanted, as described in Section 2. Lungs were harvested 38 h after transplantation. Alveolar septal thickness (a) was quantified by image analysis of 5 random alveolar septa per image from 10 random images per H&E-stained slide using a IPlab 3.7v software. ED1 positive cells (b) and myeloperoxidase (MPO) positive cells (c) after immunohistochemical staining were counted in 10 random fields per slide using a 40x objective lens. *Sham*: lungs from sham-operated rats; 100%: lungs from FSG recipients; 50%: lungs from HSG recipients; 25%: lungs from QSG recipients; 25% + NIM: lungs from recipients of QSG treated with NIM811. Values are means ± S.E.M. Group sizes were 4 per group: (a) $P < 0.05$ versus sham operation; (b) $P < 0.05$ versus FSG (100%); (c) $P < 0.05$ versus HSG (50%); (d) $P < 0.05$ versus QSG (25%).

QSG returned to levels close to those observed after transplantation of FSG and HSG. Neutrophil infiltration, although diminished, remained increased relative to FSG and HSG.

3.4. Oxidative/Nitrosative Stress and Apoptosis after Transplantation of Small-for-Size Liver Grafts: Protection by NIM811. Previous studies show that oxidative and nitrosative stresses occur in pulmonary injury after hepatic I/R [22, 31]. Infiltrating leukocytes can produce reactive oxygen and nitrogen species, leading to oxidative and nitrosative stresses and cell death in the lung. Oxidative and nitrosative stresses were evaluated by 4-hydroxynonenal and 3-nitrotyrosine adduct formation, respectively (Figure 4) ($n = 4$ per group) [32]. 4-Hydroxynonenal and 3-nitrotyrosine adducts were barely detectable in the lung tissue after sham operation and full-size LT (Figure 4). At 38 h after implantation of HSG, 4-hydroxynonenal and 3-nitrotyrosine adducts in the lung increased slightly. By contrast, 4-hydroxynonenal and 3-nitrotyrosine immunostaining became marked after transplantation of QSG (Figure 4). Staining occurred in some leukocytes, vascular endothelial cells and alveolar epithelial cells. NIM811 partially decreased hydroxynonenal and nitrotyrosine protein adducts in the lung after transplantation of QSG (Figure 4).

Pulmonary apoptosis was revealed by TUNEL staining. TUNEL-positive cells (shown by red nuclear staining) were rare in pulmonary tissue after sham operation and full-size LT. TUNEL-positive cells increased slightly after transplantation of HSG and markedly after transplantation of QSG

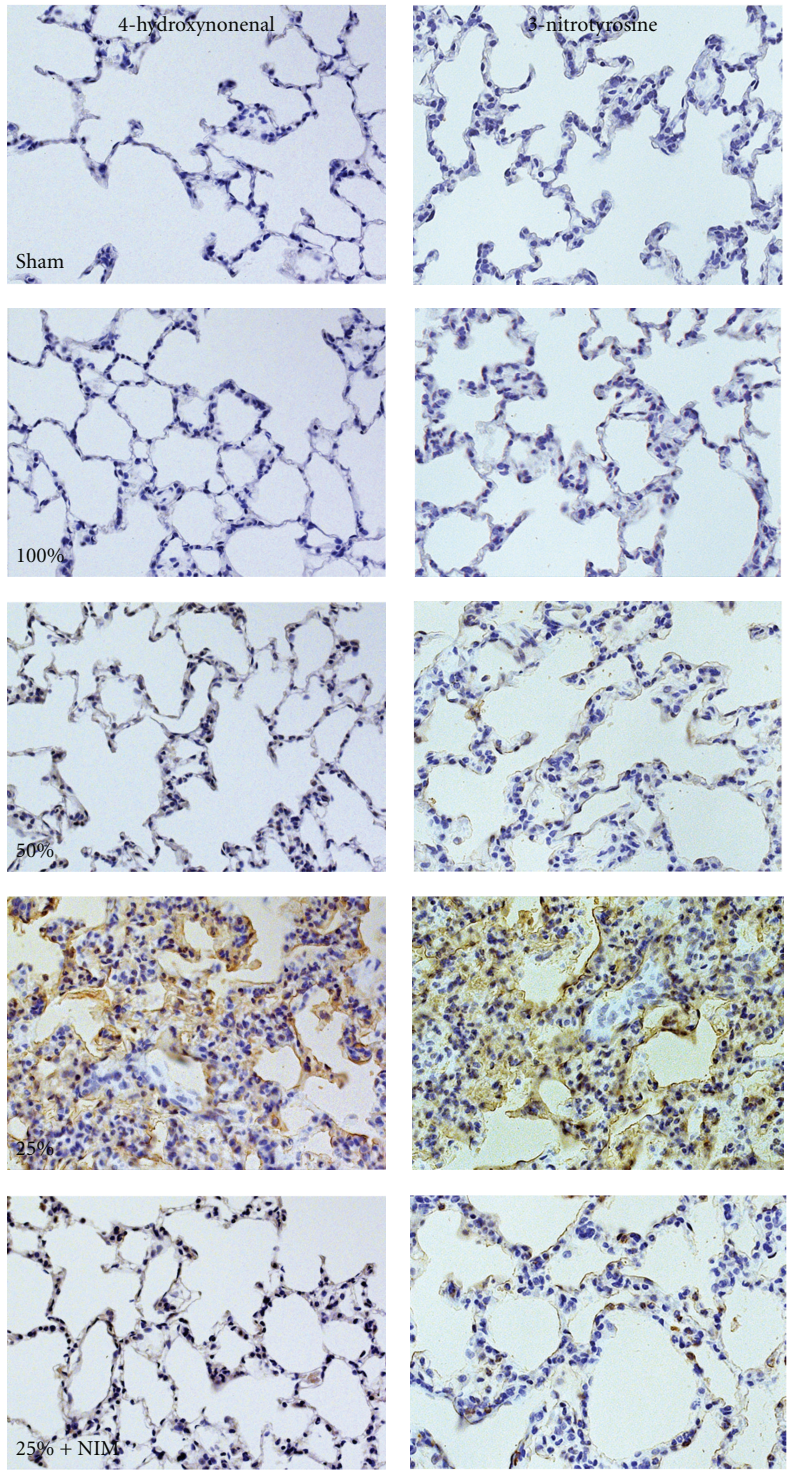

Figure 4: Increased pulmonary oxidative and nitrosative adduct formation after transplantation of small-for-size liver grafts: Prevention by NIM811. Lungs were harvested 38 h after transplantation. Representative images of pulmonary slides after immunohistochemical staining for 4-hydroxynonenal (*left column*) and 3-nitrotyrosine (*right column*) are shown. Panels are as follow: *1st row*, lung from a sham-operated rat; *2nd row*, lung from a FSG recipient (100%); *3rd row*, lung from a HSG recipient (50%); *4th row,* lung from a QSG recipient (25%); *5th row*, lung from a recipient of a QSG treated with NIM811. Group sizes were 4 per group.

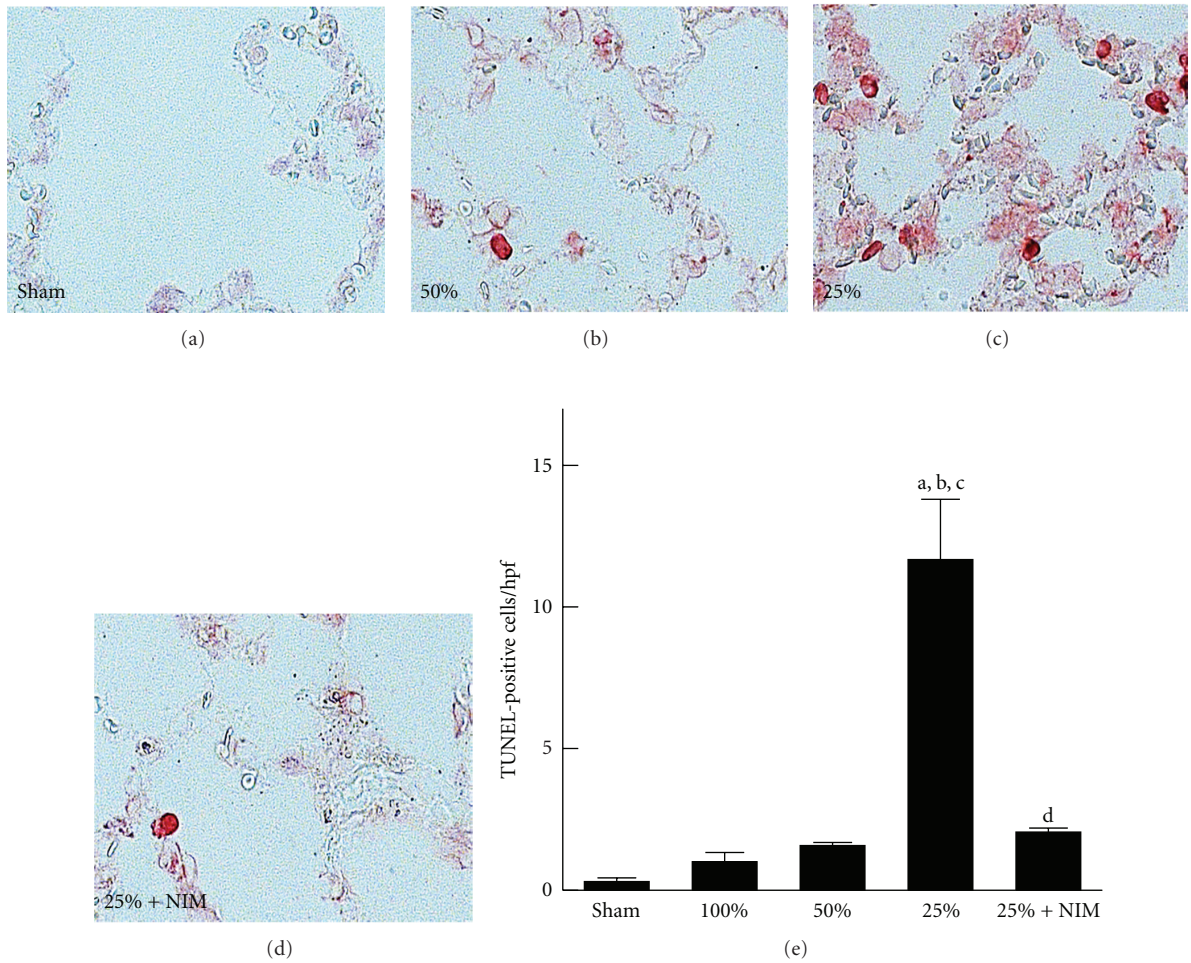

Figure 5: NIM811 protects against apoptosis in the lung after transplantation of small-for-size liver grafts. Lungs were harvested 38 h after transplantation. Representative images of pulmonary slides ($n = 4$ per group) after TUNEL staining are shown in (a)–(d). TUNEL-positive cells were counted in 10 random fields per slide using a 20x objective lens (e). *Sham*: lungs from sham-operated rats; 100%: lungs from FSG recipients; 50%: lungs from HSG recipient; 25%: lungs from QSG recipients; 25% + NIM: lungs from recipients of QSG treated with NIM811. Values are means ± S.E.M. Group sizes were 4 per group. (a) $P < 0.05$ versus sham operation; (b) $P < 0.05$ versus FSG (100%); (c) $P < 0.05$ versus HSG (50%); (d) $P < 0.05$ versus QSG (25%).

(Figure 5). Apoptotic cells were primarily vascular endothelial cells, and/or alveolar epithelial cells. Pretreatment of liver grafts with NIM811 decreased apoptosis in the lung after transplantation of QSG (Figure 5) ($n = 4$ per group).

3.5. NIM811 Decreased Toxic Cytokine Formation in Small-for-Size Liver Grafts. Failing liver grafts possibly produce toxic, inflammatory cytokines, resulting in inflammation in remote organs. Accordingly, we measured hepatic cytokine expression after transplantation ($n = 4$ per group for all cytokines). Tumor necrosis factor alpha (TNFα) mRNA did not increase in FSG and increased only slightly (1.7-fold) in HSG (Figure 5(a)). By contrast after transplantation of QSG, TNFα mRNA increased ~4-fold. NIM811 treatment of QSG decreased TNFα mRNA by 43% (Figure 6(a)). Similar changes occurred for interleukin-1β (IL-1β). IL-1β mRNA increased slightly (~2.5-fold) in both FSG and HSF but increased 8.1-fold in QSG (Figure 6(b)). NIM811 blunted the increase of IL-1β mRNA in QSG by 32%.

3.6. NIM811 Decreased Adhesion Molecule Expression in the Lung after Transplantation of Small-for-Size Liver Grafts. Proinflammatory cytokines released from the failing grafts may promote adhesion molecule expression in remote organs, thus promoting leukocyte infiltration and inflammation in these organs. Accordingly, we investigated intercellular adhesion molecule 1 (ICAM-1) expression in the lung ($n = 4$ per group). ICAM-1 was barely detectable in lungs of sham-operated rats and recipients of FSG (Figures 6(c) and 6(d)). ICAM-1 expression increased slightly after transplantation of HSG and substantially after transplantation of QSG (Figures 6(e) and 6(f)). ICAM-1 expression increased in vascular endothelial cells and alveolar epithelial cells (Figure 6), as well as bronchial epithelial cells (not shown). NIM811

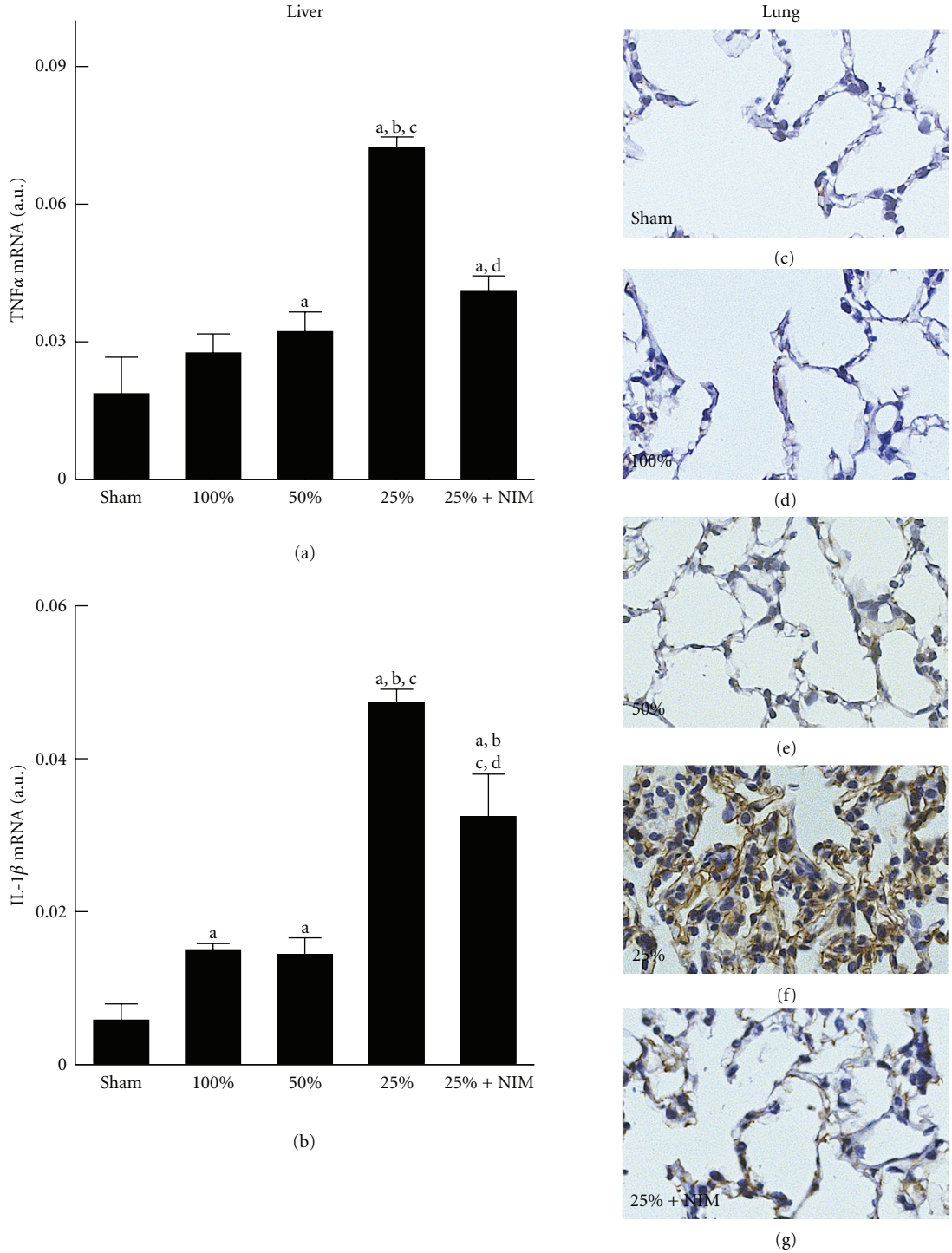

Figure 6: Hepatic toxic cytokine formation and pulmonary expression of adhesion molecules increased after transplantation of small-for-size liver grafts: Reversal by NIM811. Liver grafts and lungs were harvested at 38 h after transplantation. TNFα (a) and IL-1β (b) mRNAs were detected by real-time PCR. Values are means ± S.E.M. Group sizes were 4 per group. (a) $P < 0.05$ versus sham operation; (b) $P < 0.05$ versus FSG (100%); (c) $P < 0.05$ versus HSG (50%); (d) $P < 0.05$ versus QSG (25%). In (c)–(g) pulmonary ICAM-1 expression was detected immunohistochemically, and representative images are shown ($n = 4$ per group): (c) lung from a sham-operated rat; (d) lung from a FSG recipient (100%); (e) lung from HSG graft recipient (50%); (f) lung from QSG recipient (25%); (g) lung from a recipient of QSG pretreated with NIM811.

blunted pulmonary IACM-1 expression after transplantation of QSG (Figure 6(g)).

4. Discussion

4.1. Acute Lung Injury Increases after Transplantation of Small-for-Size Liver Grafts. Pulmonary complications are severe and life-threatening conditions that adversely affect the clinical outcomes of LT, leading to high mortality [1, 3, 5]. Many factors during transplantation may cause pulmonary complications. LT often involves substantial blood loss, necessitating blood transfusion, and large fluid shifts, which can lead to pulmonary edema [1, 3, 5]. Liver I/R injury can cause damage to remote organs, such as lung and kidney [22]. Prolonged cold storage aggravates damage to donor livers and leads to acute lung injury after LT [6]. Pulmonary complications also occur frequently in patients with fulminant hepatic failure [33], suggesting interactions of liver and pulmonary functions. Clearly, prevention of pulmonary complications is crucial for increasing survival after LT.

With more frequent application of partial LT, prevention and treatment of the small-for-size syndrome become increasingly important for improving the clinical outcomes. Therefore, in this study we investigated whether transplantation of small-for-size liver grafts increases the risk of acute lung injury after transplantation. After transplantation of FSG, lung injury was minimal (Figure 3). After transplantation of HSF, pathological changes in the lung were modest (Figure 3). In contrast, transplantation of QSG resulted in overt pathological changes in the lung, including infiltration of inflammatory cells, increased alveolar septal cellularity and thickness, exudates in alveoli, oxidative/nitrosative adduct formation, and vascular endothelial/pulmonary epithelial cell death (Figures 2, 3, 4, and 5). These results indicate that small-for-size LT increases lung injury. Interestingly, lung injury occurred at a relatively late stage after transplantation (38 h), at a time point when mortality occurs [14]. These observations indicate that pulmonary complications are, at least in part, responsible for high mortality after transplantation of small-for-size liver grafts.

4.2. Protection of Liver Mitochondria by NIM811 Prevents Pulmonary Complications after Transplantation of Small-for-Size Liver Grafts. Lung edema could occur due to massive blood transfusion or fluid shift. Transfusion-related lung edema usually occurs early after transplantation. In this study, all recipients received similar amounts of lactated Ringer's solution during transplantation. In addition, no overt edema was detected at 5–18 h after transplantation in any groups. Therefore, the pulmonary injury observed in this study is unlikely due to transfusion. By contrast, lung injury occurred at a later stage (38 h) after transplantation and predominantly in the recipients of small-for-size liver grafts. Previous studies showed that in these small grafts, mitochondrial dysfunction occurs, leading to decreased ATP production, more severe graft injury, suppressed regeneration, and poorer liver function [13, 14, 16]. Mitochondrial dysfunction in these small-for-size grafts is not due to upregulation of uncoupling proteins but is related to onset of the MPT [16]. Opening of nonselective, highly conductive permeability transition pores in the mitochondrial inner membrane causes onset of the MPT [34]. MPT onset collapses the mitochondrial membrane potential, uncouples oxidative phosphorylation, and leads to necrotic cell death from ATP depletion [35, 37, 38]. Moreover, the MPT causes release of cytochrome *c* from the intermembrane space, which triggers activation of caspases and apoptosis [35, 36]. Growing evidence supports a critical role of the MPT in cell necrosis and apoptosis in I/R injury [37, 38]. NIM811, a MPT inhibitor, prevented hepatic mitochondrial dysfunction, decreased injury, and improved regeneration of small-for-size liver grafts [16]. This treatment increases survival after small-for-size liver grafts from 30% to 81% [16]. Here, we show that NIM811 also substantially decreases lung inflammation and injury after transplantation of QSG. Since NIM811 was added only to the cold storage solution and the poststorage rinse solution, actions of NIM811 are predominantly in the liver. Therefore, protection by NIM811 on the lung is most likely secondary to decreases in liver injury and improvement of liver graft function. A direct effect of NIM811 on lung appears unlikely, since only a small amount of NIM811 in the vasculature of quarter-size grafts enters the circulation and is distributed to other organs of the recipient. Studies will be performed in the future to determine pulmonary NIM811 concentrations at various times after transplantation of small-for-size grafts and to assess whether treatment of recipients with NIM811 at similar levels could protect against pulmonary inflammation.

4.3. Role of Toxic Cytokine Release from Small-for-Size Liver Grafts in Lung Injury. How small-for-size liver grafts cause lung injury remains unclear. We tested the hypothesis that toxic cytokines released from failing small-for-size liver grafts result in lung injury. Leukocytes increased markedly in the lungs of QSG recipients but only mildly after transplantation of FSG and HSG which do not fail (Figure 3). Liver I/R injury can cause inflammatory responses in remote organs, including the lung [22]. Pulmonary neutrophil infiltration and release of elastase result in acute lung injury after LT, and this injury can be attenuated by an elastase inhibitor [39]. Prolonged cold storage increases hepatic production of proinflammatory cytokines TNFα and IL-1β, resulting in pulmonary NFκ-B activation and subsequent inflammatory responses and acute lung injury [6]. A previous study showed that transplantation of 60%-liver grafts does not change liver TNFα and IL-1β [25]. In the present study after transplantation of FSG, TNFα and IL-1β mRNA did not increase in the liver grafts, most likely due to the short cold storage time (Figure 6). Moreover, hepatic TNFα and IL-1β expression increased only slightly in HSG. By contrast, expression of these cytokines increased markedly in QSG (Figure 6). In parallel, pulmonary expression of ICAM-1 and leukocyte recruitment increased only modestly in the lungs of HSG recipients but substantially in QSG recipients (Figures 3 and 6). NIM811, which prevents injury to QSG, reduced hepatic

TNFα and IL-1β expression, pulmonary ICAM-1 expression, and leukocyte recruitment. These results are consistent with the conclusion that failing small-for-size grafts produce toxic cytokines that promote inflammation and injury in the lung.

Surgical trauma during liver splitting also increases leukocyte sequestration into donor lungs [18]. Partial LT requires more complicated surgical procedures, and organ manipulation during liver harvest increases graft injury and activates Kupffer cells, which are the major resources of toxic cytokines in the liver [40–43]. Destruction of Kupffer cells with gadolinium chloride prevents pulmonary injury after hepatic I/R [44]. However, in this study HSG and QSG were exposed to virtually identical surgical procedures. Nonetheless, toxic cytokine formation was substantially higher in QSG than that in HSG after transplantation. Therefore, higher toxic cytokine production in QSG is unlikely to be solely due to surgical trauma. Liver injury was more severe in small-for-size liver grafts, which may stimulate inflammatory responses and toxic cytokine formation. Diminishing small-for-size graft injury by NIM811 may then decrease subsequent toxic cytokine formation.

ROS production increases markedly in small-for-size liver grafts [14]. ROS trigger opening of MPT pores [36, 45–47], and uncoupling of oxidative phosphorylation caused by the MPT further increases oxidative stress [45, 48], thus causing a vicious cycle. ROS are well known triggers of toxic cytokine formation [49, 50]. Therefore, breaking the vicious cycle of ROS production by MIN811 could also decrease toxic cytokine formation. In addition to proinflammatory cytokine production, poor liver function causes hyperbilirubinemia. Although bilirubin has antioxidant properties [51], bilirubin also causes mitochondrial toxicity and has a detrimental effect on lung surfactant surface tension properties [52–54]. Hyperbilirubinemia due to small-for-size LT may also contribute, in part, to lung injury in the recipients. It seems likely that these detrimental factors act together to promote lung injury.

5. Conclusions

Taken together, this study shows that acute lung injury occurs after transplantation of small-for-size liver grafts, possibly due to increased proinflammatory cytokine formation from injured and/or failing grafts with subsequent inflammatory changes in the lung. Protection of the small-for-size grafts by NIM811 diminishes this lung injury. These results also suggest that anti-inflammatory treatment can be effective in prevention of lung injury, thus improving the outcome of small-for-size LT.

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