Assessment and preservation of liver function in hepatic ischemia and reperfusion
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Citation for published version (APA):
Heijnen, B. H. M. (2003). Assessment and preservation of liver function in hepatic ischemia and reperfusion

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Influence of acidosis and hypoxia on liver ischemia and reperfusion injury in an in vivo rat model.

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*J Appl Physiol* 93: 319-323, 2002
Abstract

The contribution of acidosis to the development of reperfusion injury is controversial. In this study we examined the effects of respiratory acidosis and hypoxia in a frequently used in vivo liver ischemia and reperfusion (I/R) injury rat model. Rats were anesthetised with intraperitoneal anesthetics and subjected to partial liver ischemia (70%) for 60 min and subsequent reperfusion for 90 min under the following conditions: 1) no acidosis and normoxia, maintained by controlled ventilation, 2) acidosis and normoxia, maintained by passive supply with oxygen, 3) no acidosis and hypoxia, maintained by bicarbonate administration without respiratory support and 4) acidosis and hypoxia, i.e. without respiratory support or pH correction. Changes in plasma AST and ALT levels were measured as parameters of hepatocellular injury and bile secretion was monitored. AST and ALT levels were lowest in the ventilated rats and highest in the bicarbonate treated rats. No differences in bile secretion were found between groups. Our results suggest that respiratory acidosis significantly enhanced liver I/R injury under normoxic conditions whereas respiratory acidosis significantly reduced liver I/R injury under hypoxic conditions.

Keywords: ventilation, respiration, hepatocellular injury, bicarbonate, hypercapnia.
Introduction

Normothermic ischemia and reperfusion (I/R) injury of the liver has been the focus of a large number of animal studies. Temporary cross clamping of the pedicle to the left lateral and median lobes is a frequently used in vivo model of liver I/R. Although it is considered a reliable model, little attention has been paid to the influence of the method of anesthesia on the results produced with this model in the rat. Different anesthetic procedures, especially the use of respiratory support, may influence both pH and oxygen tension in the blood, producing potential differences in the extent of I/R injury. Although the influence of acidosis on hepatocytes and endothelial cells have been studied in vitro (2;4), no data exist on the influence of acidosis on liver I/R injury in vivo. Previous experiments in our laboratory showed development of marked acidosis and hypoxia during liver I/R in non-ventilated rats (unpublished observations). Although the use of respiratory support by intubation and ventilation is not commonly practiced, this may strongly influence the extent of liver I/R injury and thus the interpretation of results. This study was undertaken to examine liver I/R injury in acidic and non-acidotic rats under normoxic and hypoxic conditions. We hypothesized, based on in vitro results in literature (2), that acidosis would have a protective effect on liver I/R injury under hypoxic conditions and no effect under normoxic conditions in an in vivo rat model of partial liver I/R.

Materials and methods

Animal preparation

This study was approved by the Animal Experiment Committee of the Academic Medical Center, University of Amsterdam, The Netherlands. Male Wistar rats (325-375 g; Broekman, Someren, The Netherlands) were allowed to acclimatize to the laboratory environment for 7 days with free access to water and standard laboratory chow (Hope Farms, Woerden, The Netherlands). Rats were housed under standard environmental conditions with a 12-hour light/dark cycle. Before use in experiments, rats were fasted overnight with free access to water.

Anesthesia

All rats were anesthetised by intraperitoneal (i.p.) injection of 3 ml/kg of a mixture of 0.4 ml midazolam (0.5 mg/ml; Dormicum®, Roche Nederland B.V., Mijdrecht, The Netherlands), 1 ml of fentanyl citrate and fluanisone (0.315 mg/ml and 10 mg/ml resp.; Hypnorm®, Janssen-Cilag Ltd., Saunderton, High Wycombe, Buckinghamshire, United Kingdom) and 2.6 ml of NaCl (0.9%).
Anesthesia was maintained by 0.1 ml Hypnorm® i.p. every 45 min. A temperature probe (HP temperature module M 1029A, Agilent Technologies Netherlands B.V., Amstelveen, The Netherlands) was inserted up to 1.5 cm in the rectum after induction of anesthesia. Rectal temperature of 37°C was maintained during the procedure and controlled by keeping the animals in supine position on a heating pad with the additional use of a heating lamp (8).

Experimental design
Twenty-four Wistar rats received i.p. anesthesia and were subjected to partial liver ischemia under four different conditions. In the ventilated group, rats were endotracheally intubated (14G Venflon®) and ventilated (Zoovent ventilator, Instruvet, Amerongen, The Netherlands) with a mixture of oxygen and air (1:1 vol/vol, 2 l/min) without the use of positive end-expiratory pressure (PEEP). Adequate ventilation was confirmed by continuous monitoring of end-tidal CO₂. In this group we aimed at maintaining a physiological pH without development of hypoxia. In the second group, rats received oxygen and air (1:2 liter) via a mask while rats were breathing spontaneously without further respiratory support. These rats should develop acidosis, but not hypoxia. In the bicarbonate group, rats received bicarbonate infusion (2.1%, 3 ml/h) via the tail vein while respiratory support was not provided. These rats should develop hypoxia without acidosis. In the control group, rats were breathing spontaneously without any form of respiratory or metabolic support. These rats will develop acidosis and hypoxia.

Surgical procedure
In all animals a silicone catheter (Ø 0.9 mm) was inserted into the right carotid artery for assessment of hemodynamic parameters during surgery and for collection of blood samples. After midline laparotomy, the common bile duct was cannulated with a polyethylene catheter (Ø 0.4 mm) for collection of bile. Bile was continuously collected before ischemia (15 min), during ischemia (60 min) and during reperfusion (90 min) as parameter of hepatocyte function. After dissection of the falciform ligament, the afferent vessels to the median and left lateral lobes were exposed by evertting the hepatic lobes upward. An atraumatic vascular clip was applied to these vessels to induce partial hepatic ischemia (70%) during 60 min. At the end of the ischemic period, the clip was removed and subsequent reperfusion was initiated. After 90 min of reperfusion liver biopsies were collected, fixed in 4% buffered formaldehyde and routinely processed for haematoxylin and eosin staining of paraffin sections (4 μm).
Blood sampling

Blood samples (500 µl) were collected prior to induction of ischemia, at the end of the ischemic period and after 90 min of reperfusion in microtainer® tubes containing lithium heparin (Becton Dickinson and Company, Franklin Lakes, New Jersey). Samples were centrifuged (10 min, 3000 rpm, 4°C) and plasma was collected for the assessment of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as parameters for hepatocellular injury. Activity of AST and ALT in plasma was determined by routine laboratory testing. Pre-ischemia, at the end of the ischemic period and after 90 min of reperfusion, routine arterial blood gas analysis was performed (ABL™ 505 and OSM 3 hemoximeter®, Radiometer, Copenhagen, Denmark).

For calculation of mean and significant differences, pH values were converted to blood hydrogen ion concentrations and expressed as mean ± standard error of the mean (SEM). However for clarity, also mean pH values are mentioned in table 1.

Histopathology

Light microscopic evaluation of liver I/R injury was performed on H&E stained sections of liver biopsies after 60 min of ischemia and 90 min reperfusion in all groups. After liver I/R, vascular congestion in the sinusoidal space, presence of polymorphonuclear leukocytes, cell death (karyorhexis, pyknosis, karyolysis or cytolysis) and vacuolisation were semi-quantitatively assessed in ten high power fields (40x objective) per section. Semi-quantitative scores for each phenomenon ranged from 0 to 5, depending on the frequency of the phenomenon (0, never; 1, seldom (frequency in less than 1% of the cells); 2, occasionally (1-10% of the cells scattered throughout the liver section); 3, regularly (in 1-10% of the liver parenchyma); 4, often (10-50% of the liver parenchyma); 5, very often (>50% of the liver parenchyma).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). A two-way ANOVA and repeated measurements testing were performed using SPSS® version 10.1 for Windows®. A p-value <0.05 was considered significant.
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Results

Hemodynamics
Mean arterial pressures (MAP) returned to pre-ischemic values within five to ten minutes after vascular inflow occlusion and splanchnic congestion was not observed, owing to partial occlusion of the afferent liver vessels. Animals in all groups, except for the non-supported control group, showed stable hemodynamics during the entire liver I/R procedure. At the end of the ischemic period and after 90 min of reperfusion, rats in the control group showed a lower MAP when compared to all other groups. After 90 min of reperfusion, the MAP in the control group was significantly decreased when compared to the pre-ischemic values (table 1).

pH
Ventilated rats and rats in the bicarbonate group showed a physiological blood pH during the entire procedure. Rats in the control group developed acidosis during the ischemic period with pH values lower than ventilated rats and bicarbonate treated rats. Rats with an oxygen mask showed the lowest pH (table 1).

HCO$_3^-$
Blood bicarbonate (HCO$_3^-$) levels were lowest in ventilated rats and highest in rats treated with bicarbonate. Bicarbonate levels remained stable in all rats during the experiment, except in bicarbonate treated rats (table 1).

pCO$_2$
All non-ventilated rats showed increased blood pCO$_2$ levels, whereas normal blood pCO$_2$ levels were maintained in the ventilated rats. Blood pCO$_2$ levels were highest in rats in the mask group, followed by pCO$_2$ levels in rats in the control group and the bicarbonate group (table 1).

pO$_2$
Although rats in the mask group showed lower blood oxygen pressures after ischemia when compared to ventilated rats, blood oxygen pressures in both groups were sufficient to maintain adequate organ oxygen supply during the experiment. In contrast, both rats in the bicarbonate and control group showed low oxygen pressures immediately after induction of anesthesia. After ischemia, oxygen pressures dropped to critically low levels in both the bicarbonate and the control group (table 1).
### Table 1

Mean arterial blood pressure (MAP), arterial pH, pCO₂ and pO₂ values measured before induction of partial liver ischemia (pre-I), at the end of 60 min ischemia (end-I) and after 90 min of reperfusion (90 min R). Control rats showed a decrease in MAP at the end of ischemia and after 90 min of reperfusion. Physiological pH was maintained in ventilated and bicarbonate treated rats, whereas mask and control rats developed acidosis (see comments in ‘materials and methods’). An accumulation of CO₂ was observed in all rats, except for ventilated rats. Bicarbonate (HCO₃⁻) levels were different between groups. All rats showed a stable bicarbonate concentration during the experiment, except in bicarbonate treated rats, pO₂ values in bicarbonate and control rats were significantly lower than in ventilated rats and rats with an oxygen mask. Values represent means ± SEM. Significant differences exist between values tagged with equal fonts (p<0.05).

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Ventilated</th>
<th>Mask</th>
<th>Bicarbonate</th>
<th>Control</th>
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</thead>
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<tr>
<td><strong>MAP (Torr)</strong></td>
<td></td>
<td></td>
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<tr>
<td>pre-I</td>
<td>67 ± 2</td>
<td>68 ± 1</td>
<td>66 ± 2</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>end-I</td>
<td>62 ± 2 A</td>
<td>67 ± 2 B</td>
<td>64 ± 2 C</td>
<td>56 ± 2 ABC</td>
</tr>
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<td>90 min R</td>
<td>66 ± 2 D</td>
<td>68 ± 2 E</td>
<td>64 ± 2 F</td>
<td>55 ± 4 DEFG</td>
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<tr>
<td><strong>H⁻ (nM)</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>pre-I</td>
<td>38.9 ± 1.3</td>
<td>{7.41}</td>
<td>39.0 ± 1.3</td>
<td>43.2 ± 1.9</td>
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<tr>
<td>end-I</td>
<td>38.8 ± 1.7 AD</td>
<td>{7.41}</td>
<td>39.3 ± 2.5 BE</td>
<td>53.9 ± 1.5 CDEG</td>
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<tr>
<td>90 min R</td>
<td>38.2 ± 1.1 HK</td>
<td>{7.42}</td>
<td>36.1 ± 1.2 LL</td>
<td>55.7 ± 1.9 JKLN</td>
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<td><strong>pCO₂ (Torr)</strong></td>
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<tr>
<td>pre-I</td>
<td>35.6 ± 1.4 A</td>
<td>{7.41}</td>
<td>44.7 ± 2.1 M</td>
<td>43.7 ± 2.9 N</td>
</tr>
<tr>
<td>end-I</td>
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<td>{7.41}</td>
<td>49.6 ± 3.5 CE</td>
<td>52.4 ± 4.0 DF</td>
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<tr>
<td>90 min R</td>
<td>31.6 ± 1.3 GHI</td>
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<td>52.1 ± 1.5 HJM</td>
<td>51.2 ± 2.1 IKN</td>
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<tr>
<td><strong>HCO₃⁻ (mmol/L)</strong></td>
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<td></td>
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<tr>
<td>pre-I</td>
<td>22.1 ± 0.4 ABC</td>
<td>{7.41}</td>
<td>27.6 ± 0.7 BDE</td>
<td>24.6 ± 0.9 CE</td>
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<tr>
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<td>20.2 ± 1.2 FGH</td>
<td>{7.41}</td>
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<td>22.8 ± 0.5 HJ</td>
</tr>
<tr>
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<td>20.1 ± 0.8 KL</td>
<td>{7.42}</td>
<td>35.2 ± 1.0 LMN</td>
<td>22.9 ± 0.6 N</td>
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<td><strong>pO₂ (Torr)</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>pre-I</td>
<td>221 ± 11 AB</td>
<td>195 ± 17 CDG</td>
<td>87 ± 4 ACP</td>
<td>76 ± 11 BDG</td>
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<tr>
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<td>133 ± 17 EHI</td>
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<td>58 ± 2 GIK</td>
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<td>90 min R</td>
<td>197 ± 3 L MN</td>
<td>127 ± 14 LQ</td>
<td>49 ± 2 MP</td>
<td>49 ± 3 NQ</td>
</tr>
</tbody>
</table>
Hepatocellular injury and function

AST and ALT levels at the end of ischemia and after 90 min of reperfusion showed significant elevation in rats of all groups when compared to pre-ischemic values (figure 1). After 90 min of reperfusion, plasma AST and ALT levels were lower in the ventilated rats when compared to all other groups. AST and ALT levels of rats in the bicarbonate group were higher when compared to rats in the mask and control group.

**Figure 1**
Plasma aspartate aminotransferase (AST, top) and alanine aminotransferase (ALT, bottom) measured before induction of partial liver ischemia (pre-I), at the end of 60 min ischemia (end-I) and after 90 min of reperfusion (90 min R) in ventilated rats (hatched bars), rats with and oxygen mask (grey bars), bicarbonate treated rats (open bars) and control rats (closed bars). AST and ALT levels were lowest in ventilated rats and highest in bicarbonate treated rats. Bars represent means ± SEM.
* Significantly different from ventilated rats.
# Significantly different from bicarbonate rats.
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Overall, bile secretion was significantly reduced during liver ischemia and during 90 min reperfusion when compared to pre-ischemic levels in all rats. None of the animals showed a significant recovery of bile secretion after 90 min of reperfusion. Furthermore, bile secretion was not significantly different between rats in all groups (figure 2).

**Figure 2**
Bile secretion measured during 15 min before induction of partial ischemia (pre-I), during ischemia (during-I) and during 90 min of reperfusion (90 min R) in ventilated rats (hatched bars), rats with an oxygen mask (grey bars), bicarbonate treated rats (open bars) and control rats (closed bars). All groups showed significant reduction in bile secretion during ischemia and reperfusion when compared to pre-ischemic bile secretion (significance not shown). Bars represent means ± SEM.

**Histopathology**

In H&E-stained paraffin sections of the I/R liver lobes (illustrated in figure 3), the semi-quantitative scoring of liver injury corresponded to the degree of parenchymal injury indicated by the plasma AST and ALT levels found in all groups (mean scores, ventilated rats 2.5, mask group 3.0, bicarbonate group 4.2 and control group 3.2), while the non-I/R control lobes showed none of the investigated alterations (see material & methods).

**Figure 3**
Microphotograph of a H&E stained paraffin section of rat liver after 60 min of ischemia and 90 min of reperfusion in a bicarbonate treated rat showing congestion of erythrocytes (A), extensive multifocal pericentral and midzonal parenchymal necrosis with hepatocellular cytolysis, vacuolization, pyknosis (B) and karyorhexis (C). CV, central vein; PV, portal vein.
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Discussion

The results of this study emphasize that the development of acidosis and/or hypoxia during in vivo liver I/R experiments has substantial impact on the extent of liver I/R injury. Temporary cross clamping of the pedicle to the left lateral and median lobes of the liver is a frequently applied in vivo model for partial (70%) liver I/R in the rat. The absence of severe splanchnic congestion is a major advantage of this model, since splanchnic congestion, when prolonged for more than 20 min, will result in circulatory shock with intestinal ischemia, unless a concomitant decompressing portal venous-systemic shunt is created (6). Because venous return to the heart is not compromised in this model, only minor temporary changes in blood pressure were observed directly after selective vascular inflow occlusion.

When reviewing literature on the use of the partial liver ischemia rat model, anesthesia appears to be induced and maintained with numerous drugs (chloralhydrate, pentobarbital, atropine, ketamine, xylazine, diazepam, Hypnorm®, urethane, isoflurane, halothane, metofane, ether and multiple combinations). Drugs are administered via intra-muscular and intra-peritoneal injections as well as by inhalation and by ventilation. Our findings suggest that comparison between published results using a 70% partial liver I/R rat model should take into account the method of anesthesia applied and, accordingly, should be made with caution.

In this study, non-ventilated rats with bicarbonate infusion retained a physiologic pH, whereas other non-ventilated rats developed acidosis. Also, non-ventilated rats developed hypoxia when oxygen was not supplied. Ventilated rats maintained normocapnia, a physiological pH and mild hyperoxia. Hypercapnia and hypoxia in the non-ventilated rats are evidently caused by inadequate gas exchange in the alveoli due to inadequate respiration, leading to respiratory acidosis. At the end of the ischemic period, when the hepatic circulation was not yet restored, liver-derived lactic acid unlikely played a role in the development of acidosis. Moreover, differences in pH between rats with an oxygen mask and ventilated rats already existed before induction of ischemia. During reperfusion, pH remained stable in all groups (as did the pCO₂ and HCO₃⁻ levels), which makes the contribution of lactic acid to the prolongation of acidosis less likely.

The upper pO₂ limit in the ventilated rats was 200 Torr, which is higher than normal O₂ pressures (ca. 94 Torr). It has been reported that hyperoxia (induced by exposure to 100% oxygen under normobaric conditions) can increase reactive oxygen species formation in different organs after at least 24 hours of exposure (1;12;13). Because in our study ventilated rats were exposed to
Influence of acidosis and hypoxia

high O₂ pressures for a maximum of 3 hours, hyperoxia is not thought to contribute to the development of liver I/R injury.

Although rats with an oxygen mask showed increased respiratory insufficiency (i.e. increased pCO₂ levels) and significantly lower blood O₂ pressure after ischemia compared to ventilated rats, O₂ pressures were still above physiological levels. This suggests that the differences in arterial O₂ pressures unlikely contributed to the differences in liver I/R injury between rats with an oxygen mask and ventilated rats.

Rats with an oxygen mask showed significantly enhanced hepatocellular I/R injury when compared to ventilated rats. This suggests that hypercarbic acidosis during normoxic reperfusion has an adverse effect on liver I/R injury. In our experiments, pCO₂ levels did not exceed 65 Torr and therefore, only mild acidosis developed. Although it has been reported that mild acidosis can be protective whereas severe acidosis can enhance hypoxic injury in isolated hepatocytes (7), the present experiments show opposing results under normoxic conditions. Our data suggest that enhanced hepatocellular I/R injury despite normoxic reperfusion in rats with an oxygen mask was due to respiratory acidosis.

In contrast, rats of the control group with mild acidosis and severe hypoxia during liver I/R showed significantly less hepatocellular injury when compared to bicarbonate treated rats with hypoxia only. The protective effect of acidosis under hypoxic conditions is well documented in literature (2;4). Some studies suggest that the supply of bicarbonate may well restore blood pH values, but leads to a paradoxical decrease of intracellular pH and hence to impaired cellular function (9;10;17). This assumption is still highly controversial and other studies suggest that the role of this phenomenon is minimal (5;11;14) and transient (15) in vivo.

The critically low pO₂ during liver I/R in non-ventilated control- and bicarbonate rats actually prolonged the hypoxic period of the liver during reperfusion despite restoration of blood flow. Prolonged hypoxia during reperfusion, reportedly, severely augments liver I/R injury (16). As was mentioned before, mild acidosis in hypoxic, non-ventilated control rats attenuated liver I/R injury during hypoxia when compared to hypoxic, non-ventilated bicarbonate-treated rats with physiologic pH. Mild acidosis did not completely protect the liver from the adverse effects of hypoxic reperfusion, since plasma AST and ALT levels in non-ventilated, hypoxic control rats with acidotic pH were still significantly higher when compared to ventilated, normoxic rats with physiologic pH.

Bile secretion is considered to be a good indicator of hepatocyte function (3). However in this study during the entire period of liver I/R, bile secretion was not significantly altered by
acidosis and/or hypoxia, despite large differences in hepatocellular I/R injury. Although prolonged observation may reveal larger differences between the groups, previous studies in our laboratory showed incomplete recovery of bile secretion also after 24 hours of reperfusion (data not shown).

In conclusion, the results of this study suggest that respiratory acidosis significantly enhanced liver I/R injury under normoxic conditions whereas respiratory acidosis significantly reduced liver I/R injury under hypoxic conditions. Clearly, standardisation of methods used in I/R studies is crucial for comparison and validation of animal models. This should include, as is our conclusion from the data presented here, controlled respiratory support of the animal during ischemia and reperfusion of the liver.
References


