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RESEARCH PAPER

Acute alcohol intoxication reduces mortality, inflammatory responses and hepatic injury after haemorrhage and resuscitation *in vivo*

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BACKGROUND AND PURPOSE

Haemorrhagic shock and resuscitation (H/R) induces hepatic injury, strong inflammatory changes and death. Alcohol intoxication is assumed to worsen pathophysiological derangements after H/R. Here, we studied the effects of acute alcohol intoxication on survival, liver injury and inflammation after H/R, in rats.

EXPERIMENTAL APPROACH

Rats were given a single oral dose of ethanol (5 g·kg^{-1}, 30%) or saline (control), 12 h before they were haemorrhaged for 60 min and resuscitated (H/R). Sham groups received the same procedures without H/R. Measurements were made 2, 24 and 72 h after resuscitation. Survival was assessed 72 h after H/R.

KEY RESULTS

Ethanol increased survival after H/R three-fold and also induced fatty changes in the liver. H/R-induced liver injury was amplified by ethanol at 2 h but inhibited 24 h after H/R. Elevated serum IL-6 levels as well as hepatic IL-6 and TNF-α gene expression 2 h after H/R were reduced by ethanol. Ethanol enhanced serum IL-1β at 2 h, but did not affect increased hepatic IL-1β expression at 72 h after H/R. Local inflammatory markers, hepatic infiltration with polymorphonuclear leukocytes and intercellular adhesion molecule 1 expression decreased after ethanol compared with saline, following H/R. Ethanol reduced H/R-induced IκBα activation 2 h after H/R, and NF-κB-dependent gene expression of MMP9.

CONCLUSIONS AND IMPLICATIONS

Ethanol reduced H/R-induced mortality at 72 h, accompanied by a suppression of proinflammatory changes after H/R in ethanol-treated animals. Binge-like ethanol exposure modulated the inflammatory response after H/R, an effect that was associated with NF-κB activity.

Abbreviations

ALT, alanine aminotransferase; BAC, blood alcohol concentrations; H/R:, haemorrhagic shock and resuscitation; ICAM-1, intercellular adhesion molecule 1; MODS, multiple organ dysfunction syndrome; MOF, multiple organ failure; PMNL, polymorphonuclear leukocytes

Introduction

Alcohol consumption is associated with one-third of all traumatic injury deaths each year (Li *et al.*, 1997; Rehm *et al.*, 2003). Almost 50% of trauma victims have positive blood alcohol concentrations (BACs), among them 35% with a BAC greater than 1 mg·mL^{-1} (Reyna *et al.*, 1985; Rivara *et al.*, 1993; Madan *et al.*, 1999; Hadfield *et al.*, 2001). Alcohol-intoxicated

trauma victims have an increased risk for subsequent complications such as pneumonia, sepsis or multiple organ failure (MOF); some studies report a greater morbidity and mortality in these patients (Faunce *et al.*, 1997; Bagby *et al.*, 1998; Ruiz *et al.*, 1999; Boe *et al.*, 2001; 2003; Messingham *et al.*, 2002; Zhang *et al.*, 2002). Other studies report divergent results showing that acute alcohol intoxication does not affect the outcome and is even associated with decreased 24 h mortality after trauma (own unpublished data). The source of complications in trauma victims after alcohol consumption is multifactorial and depends primarily on the injury severity, genetic predisposition of the patient and the history of alcohol abuse and use (chronic vs. acute alcohol abuse respectively) (Madan *et al.*, 1999; von Heymann *et al.*, 2002; Spies *et al.*, 2004; Lau *et al.*, 2009). After H/R, the phagocytic and oxidative burst capacity of polymorphonuclear leukocytes (PMNL) are suppressed in alcohol-intoxicated rodents (Molina *et al.*, 2004). Acute alcohol intoxication suppresses innate immunity and production of inflammatory cytokines, such as TNF-α, IL-1β and IL-6, in human and animals (Gluckman and MacGregor, 1978; Nelson *et al.*, 1989; Verma *et al.*, 1993; Szabo *et al.*, 1999; Boe *et al.*, 2001; 2003). Both chronic and acute alcohol abuses result in significant alterations in haemodynamic stability, metabolic regulation and immune function.

Haemorrhagic shock induces an early proinflammatory response, which may lead to multiple organ dysfunction syndrome (MODS) or MOF that may ultimately lead to a greatly increased mortality rate after trauma (Sauaia *et al.*, 1995; Moore *et al.*, 1996; Baue *et al.*, 1998; Treggiari *et al.*, 2004). Haemorrhage followed by fluid resuscitation (H/R) disturbs the microcirculation and activates immune cells [monocytes and PMNL)] to subsequent excessive responsiveness to inflammatory stimuli (Partrick *et al.*, 1996; Hietbrink *et al.*, 2006). These changes result in damage of cell membranes, DNA and proteins, and subsequent organ damage (Redl *et al.*, 1993; Botha *et al.*, 1995; Partrick *et al.*, 1996; Akgur *et al.*, 2000; Holzer *et al.*, 2005; Passos *et al.*, 2007). The increase of proinflammatory cytokines is strongly associated with an increased mortality in a model of H/R (Cai *et al.*, 2009). Furthermore, elevated cytokine levels contribute to the up-regulation of adhesion molecules, apoptotic and/or necrotic changes of endothelial, parenchymal and immune cells and accompany compromised organ function after H/R, notably in the liver (Yamakawa *et al.*, 2000; Cockerill *et al.*, 2001; Liaw *et al.*, 2005; Shimizu *et al.*, 2007; Lehnert *et al.*, 2008; Relja *et al.*, 2009; 2010). NF-κB is involved in the regulation of inflammatory and immune processes, and is activated in the liver following H/R (Meng *et al.*, 2001). Its activation requires removal and degradation of its endogenous inhibitor IκBα (Zingarelli, 2005). Once activated, NF-κB translocates from the cytosol to the nucleus and regulates gene transcription of several proinflammatory cytokine genes, such as IL-6, IL-1, TNF-α, and intercellular adhesion molecule 1 (ICAM-1) (Tak and Firestein, 2001; nomenclature follows Alexander *et al.*, 2011).

Thus, we studied the influence of a binge-like exposure to alcohol (using a single high dose of ethanol) upon mortality, hepatic injury and inflammation after H/R in rats.

Methods

Animals and experimental model

All animal care and experimental protocols were approved by the Veterinary Department of the Regional Council in Darmstadt, Germany. As effects of alcohol exposure vary with gender, and several studies have reported a pronounced susceptibility for alcohol-induced inflammation and organ damage in females, in both humans and animals (Loft *et al.*, 1987; Emanuele *et al.*, 2001; Nanji *et al.*, 2001), we used female rats (180–250 g, Harlan, Borchen, Germany). Also induction of an acute fatty liver after ethanol gavage is highly reliable in Lewis rats, this strain was chosen for our study (Zhong *et al.*, 1999). Twelve hours before H/R, rats were gavaged with a single dose of ethanol (30% solution, equivalent to 5 $g \cdot kg^{-1}$; . . . .); referred to as H/R+EtOH (Sigma-Aldrich, Steinheim, Germany) or an equal volume of saline (H/R+Ctrl), before induction of the H/R procedure. Using a 20% solution of ethanol in preliminary studies, we found the risk of aspiration due to high gavage volume was substantial and we reduced the volume administered by increasing the concentration to 30%. Using this concentration, no aspiration occurred, and hepatic fat accumulation and hepatic damage both after sham and H/R procedures were comparable (data not shown; Kozan *et al.*, 2009). The BAC at 12 h after ethanol gavage was measured using an Ethanol Assay Kit (BioVision, Heidelberg, Germany) according to the manufacturer's instructions. After an overnight fast, rats were anaesthetized with isoflurane (1.5%; Baxter, Unterschleißheim, Germany), and the right carotid artery, the right femoral artery and the left jugular vein were cannulated with polyethylene tubing. Then, haemorrhage was induced over 5 min by withdrawing blood from the right carotid artery into a heparinized syringe (10 U) to a mean blood pressure of 30 ± 2 mmHg. Systemic blood pressure was monitored in the right femoral artery using a blood pressure analyzer (BPA 400, Digi-Med, Louisville, KY, USA). Constant pressure was maintained by further withdrawal of small volumes of blood as necessary for 60 min. Then, rats were resuscitated by transfusion of 60% of the shed blood plus a volume of lactated Ringer's solution corresponding to 50% of the shed blood volume with a syringe pump over 30 min via the left jugular vein (Relja *et al.*, 2009). After the end of resuscitation, catheters were removed, the vessels were occluded and the wounds were closed. Sham groups underwent surgical procedures without H/R (sham+Ctrl and sham+EtOH groups). At euthanasia, the vena cava was punctured, blood was collected and tissue samples were collected. For each rat, the two right dorsal liver lobes were snap-frozen in liquid nitrogen. The remaining liver was flushed with normal saline, perfused and fixed with 10% buffered formalin through the portal vein, embedded in paraffin and subsequently sectioned and stained with haematoxylin–eosin. Body temperature was measured in the colon and maintained at 37°C throughout the experiment with a heating pad. Thirty-two animals were observed for 72 h to assess survival.

Group allocation

Survival study. Thirty-two female Lewis rats (180–250 g) were randomly allocated to receive either saline gavage (Ctrl), or ethanol gavage before either sham or the H/R procedure

($n = 7$ and $n = 9$ per group, respectively). Survival was assessed at 72 h.

Organ injury and inflammatory changes. Twenty-eight animals were randomly allocated to four groups and treated in the same way as described above ($n = 7$ per each group) and tissues samples were collected at 2 h after resuscitation. Twelve animals, also randomly allocated to four groups ($n = 3$ per each group) were killed at 24 h after resuscitation. The animals from the survival group and additional animals in the H/R+Ctrl group were killed at 72 h after resuscitation ($n = 7$).

Examination of ethanol-induced hepatic fat accumulation

Hepatic 5 μm frozen sections were fixed with 10% buffered formalin. Lipids were stained with Oil red O, counterstained with haematoxylin, and photographs were taken.

Examination of tissue injury

Sera were stored at –80°C for later analysis of alanine aminotransferase (ALT) and LDH using the Vitros 250 device (Ortho-Clinical Diagnostics, Neckargemünd, Germany). Determination of histological damage was performed by an independent examiner who allocated the haematoxylin–eosin-stained liver sections to the various experimental groups, without knowledge of the treatments, as described by Meng *et al.*, (2001).

Detection of PMNL

Analysis of the hepatic infiltration with PMNL was performed by chloroacetate esterase staining (4% pararosanilin, 4% sodium nitrite and naphthol solution) for 30 min at room temperature. Sections were counterstained with haematoxylin. PMNL were determined by counting the number of chloroacetate esterase-positive cells in a total of 25 high-power (400×) fields per liver section per rat, without knowledge of the treatments. Data from each tissue section were pooled to determine means.

Quantification of cytokine levels

Concentrations of serum IL-6, TNF-α and IL-1β level were determined using a Quantikine Rat IL-6, Rat TNF-α and Rat IL-1β/IL-1F2 ELISA kit of R&D Systems (Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instuctions. ELISA was performed using Infinite M200 microplate reader (Tecan, Männedorf, Switzerland).

Western blotting for intracellular signalling

Liver tissue was homogenized in lysis buffer at 4°C, followed by centrifugation for 30 min at 4°C at 20 000× *g*. Supernatants were stored at –80°C for later analysis. Lysates (50 μg protein) were separated by electrophoresis on 12% polyacrylamide SDS gels and transferred to nitrocellulose membranes (Amersham-Buchler, Braunschweig, Germany). Phosphorylated IκBα (phospho-IκBα) was detected using rabbit polyclonal phospho-IκBα (phospho-Ser^{32} + Ser^{36}) antibody, and IκBα using rabbit polyclonal IκBα antibody (Abcam, Cambridge, UK). Determination of β-actin with anti-β-actin antibody (Sigma, Taufkirchen, Germany) served as a loading control. Blots were blocked (10% non-fat dry milk in 1 mM Tris, 150 mM NaCl, pH 7.4) for 1 h, incubated for 1 h at room temperature with primary antibody (diluted according to manufacturer's instructions in blocking buffer with 0.5% Tween 20 and 0.5% BSA) and then incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1000 in blocking buffer with 0.5% Tween 20 and 0.5% BSA at room temperature. Proteins were detected with ECL™ Western blot detection reagents (GE Healthcare, Munich, Germany). Films were digitized, and the integrated density of individual bands was determined using the software Multi-analyst (Biorad, Munich, Germany). By densitometric measurements using the same software, the amount of protein expression was normalized to β-actin. The activation state of IκBα was calculated as the ratio of phosphorylated and total (phosphorylated plus non-phosphorylated) protein values of densitometry results in per cent (phospho/total*100).

Staining of ICAM-1

Cryosectioned liver samples (5 μm) were air dried for 10 min, fixed in acetone 10 min at room temperature and air dried for 60 min at room temperature. Sections were washed with PBS and water. Then, endogenous peroxidase activity was blocked with hydrogen peroxide (2% for 10 min, room temperature). Hepatic sections were washed and incubated with blocking solution (2% BSA in PBS) for 1 h at room temperature. Mouse anti-rat CD54 monoclonal antibody (BD Pharmingen, Heidelberg, Germany) diluted 1:150 in PBS (pH 7.4) containing 1% BSA was used as primary antibody (overnight incubation, 4°C). Anti-mouse horseradish peroxidase-linked secondary antibody (30 min, room temperature) and diaminobenzidine (Peroxidase EnVision Kit, DakoCytomation, Hamburg, Germany) were used to detect specific binding. Sections were counterstained with haematoxylin. The immunostained tissue sections were captured at 400× and analysed without knowledge of the treatments. The extent of labelling in the liver lobule was defined as the percentage of the field area within a preset colour range determined by the software (Adobe Photoshop 7.0, Adobe Systems Inc., San Jose, CA, USA). Data from each tissue section (10 fields per section) were pooled to determine mean values.

RNA isolation, quantitative reverse transcription (RT)-PCR

Total RNA of the snap-frozen liver lobes was isolated using the RNeasy-system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The residual amounts of DNA remaining were removed using the RNase-Free DNase Set according to the manufacturer's instructions (Qiagen). The RNA was stored immediately at –80°C. Quality and amount of the RNA were determined photometrically using the NanoDrop ND-1000 device (NanoDrop Technologies, Wilmington, DE, USA).

RNA was subsequently used for quantitative RT-PCR. In brief, 100 ng of total hepatic RNA was reversely transcribed using the Affinity script QPCR-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. To determine the mRNA expression of IL-6,

TNF-α, IL-1β and MMP9, qRT-PCR was carried out on a Stratagene MX3005p QPCR system (Stratagene) using gene-specific primers for rat Il6 (NM_012589, UniGene#: Rn.9873, Cat#: PPR06483A), rat Il1b (NM_031512, UniGene#: Rn.9869, Cat#: PPR06480A), rat Tnf (NM_012675, UniGene#: Rn.2275, Cat#: PPR06411E) and rat Mmp9 (NM_031055, UniGene#: Rn.10209, Cat#: PPR44728B) purchased from SABiosciences (SuperArray, Frederick, MD, USA). As reference gene, the expression of *GAPDH* with rat Gapdh (NM_017008, UniGene#: Rn.91450, Cat#: PPR06557A, SABiosciences, SuperArray) was measured. Sequences of these primers are not available. PCR reaction was set up with 1× RT^2 SYBR Green/Rox qPCR Master mix (SABiosciences) in a 25 µL volume according to the manufacturer's instructions. A two-step amplification protocol consisting of initial denaturation at 95°C for 10 min followed by 40 cycles with 15 s denaturation at 95°C and 60 s annealing/extension at 60°C was chosen. A melting-curve analysis was applied to control the specificity of amplification products.

Relative expression of each target genes, mRNA level was then calculated using the comparative threshold-cycle (CT) method ($2^{-\Delta\Delta CT}$ method). In brief, the amount of target mRNA in each sample was first normalized to the amount of *GAPDH* mRNA, to give ΔCT and then to a calibrator consisting of samples obtained from the sham+Ctrl group. The relative mRNA expression of target genes is presented as fold increase calculated in relation to sham+Ctrl after normalization to *GAPDH*.

Statistical analysis

Differences between groups were determined by one-way ANOVA using a multiple comparison procedure (Student–Newman–Keuls *post hoc*). Changes in target gene expression were analysed by Wilcoxon matched-pair analysis followed by Bonferroni correction. A *P*-value of less than 0.05 was considered significant. Data are given as mean ± SEM. Kaplan–Meier test followed by log-rank test was used for analysis of survival data. All statistical analyses were performed employing GraphPad Prism 5 (Graphpad Software, Inc., San Diego, CA).

Results

Lipid deposition in the liver after ethanol administration

Lipid deposition representing ethanol-induced fatty liver was demonstrated by staining of lipid accumulation with Oil red O, 12 h after ethanol or saline gavage. Two representative histological pictures demonstrate ethanol-induced accumulation of lipid droplets in both periportal and pericentral zones of liver lobules (Figure 1B). In livers of saline-gavaged rats, no lipid deposition was detected (Figure 1A). However, the BAC at 12 h after ethanol gavage was almost not detectable (10–15 mM, data not shown).

Survival rates of ethanol-intoxicated rats after H/R

In order to reveal the effect of acute ethanol intoxication on survival after H/R, rat survival at 72 h after H/R was evaluated. In both sham-operated groups, no deaths occurred. Haemorrhagic shock followed by resuscitation induced a mortality of 80% at 72 h after H/R in our model. Acute ethanol gavage before H/R strikingly attenuated the mortality to 19% after H/R ($P < 0.001$, Figure 2).

Histopathological changes in hepatic tissue after H/R

Liver sections from saline-pretreated (Ctrl) rats (Figure 3, left row) revealed areas of coagulative necrosis at 2 h after H/R (Figure 3B). H/R-induced necrosis was increased 24 h after resuscitation as indicated by cellular enlargement and nuclear dissolution in predominantly midzonal and pericentral areas of liver lobules from ctrl rats (Figure 3C). Necrotic areas were reduced 72 h after H/R, in H/R+Ctrl rats (Figure 3D). In ethanol-intoxicated rats (Figure 3, right row), hepatocytes with cell enlargement and nuclear dissolution were most pronounced at 2 h after resuscitation, with a marked reduction of necrosis at 24 h and 72 h after H/R respectively (Figure 3F–H). These changes were not detected after sham operation (Figure 3A and E).

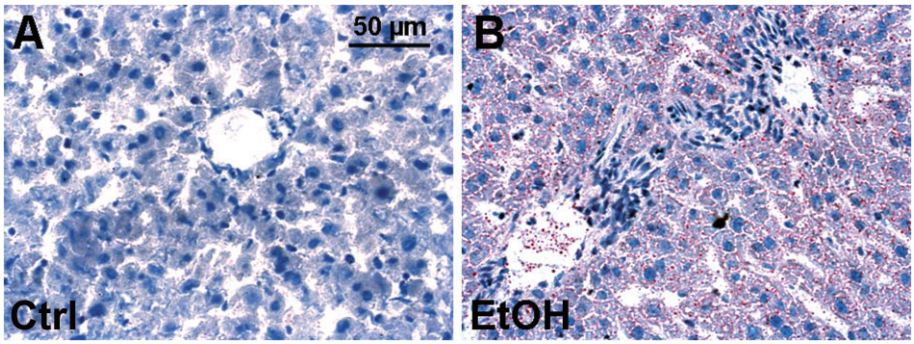

Figure 1

Oil red O staining of lipid droplets after acute alcohol exposure. Lipid deposition after ethanol gavage was analyzed by Oil red O staining. Left figure (A) depicts livers of saline-gavaged rats (ctrl), right figure (B) depicts livers of ethanol-gavaged rats (EtOH). Tissues were collected 12 h after saline or ethanol gavage. Representative Oil red O stained liver sections are shown; bar is 50 µm.

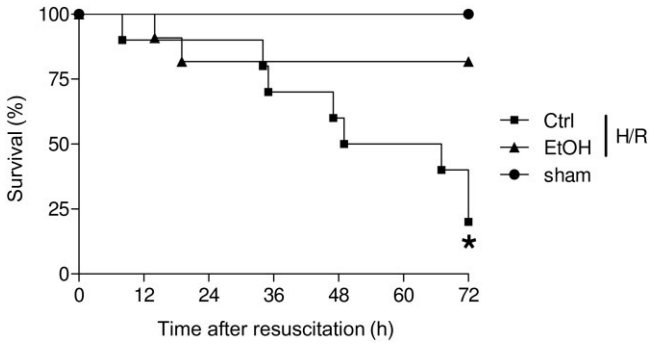

Figure 2

Acute ethanol (EtOH) ingestion reduces mortality after H/R. Mortality is shown as survival (% group size) after 72 h. Group sizes were: sham+Ctrl and and sham+EtOH, each $n = 7$; H/R+EtOH, $n = 9$; H/R+Ctrl, $n = 9$. *$P < 0.001$ vs. other groups; Kaplan–Meier test followed by log-rank test.

Cell damage after H/R

H/R induced an increase of serum ALT, a marker of hepatocellular damage at 2 h after H/R, (compare H/R+Ctrl group value with the corresponding sham value (131 ± 16 IU·L^{-1}; $P < 0.01$, Figure 4A). The sham values did not vary between 2, 24 and 72h and are shown as a single point in Figure 4A. Twenty-four hours after H/R, ALT increased further ($P < 0.05$, Figure 4A) and then fell by 72 h after resuscitation. Pretreatment with ethanol enhanced ALT release at 2 h after H/R (compare H/R+EtOH values with H/R+Ctrl) ($P < 0.05$, Figure 4A). In contrast to the saline-gavaged rats, ALT decreased in rats that received an ethanol gavage before H/R at 24 and 72 h.

LDH, an indicator of general cell damage, was clearly raised at 2 h in the H/R+Ctrl group compared with the sham+Ctrl rats (320 ± 120 IU·L^{-1}, $P < 0.001$, Figure 4B); the sham+Ctrl values did not vary between 2, 24 and 72 h and are shown as a single point in Figure 4B. The raised LDH in the H/R+Ctrl groups decreased at 24 h and 72 h after resuscitation to levels comparable with the corresponding sham+Ctrl rats. In ethanol-intoxicated rats, LDH release increased at 2 h after resuscitation, compared with sham+EtOH (163 ± 16 IU·L^{-1}, $P < 0.001$, Figure 4B; one point is shown). LDH decreased in ethanol-intoxicated rats 24 and 72 h after resuscitation. Ethanol gavage itself did not increase ALT or LDH values after sham operation compared with saline-gavaged rats (Figure 4A and B).

Systemic proinflammatory changes after H/R – serum IL-6, IL-1β and TNF-α levels

Haemorrhage followed by resuscitation induces a systemic immune response, which was assessed at 2, 24 or 72 h after H/R by serum IL-6, IL-1β and TNF-α levels. Compared with the sham+Ctrl group, serum IL-6 levels increased in the H/R+Ctrl group, 2 h after resuscitation ($P < 0.05$, Figure 5A). IL-6 levels in ethanol-gavaged rats, at 2 h after resuscitation, were markedly reduced ($P < 0.05$, Figure 5A), comparing H/R+EtOH with H/R+Ctrl groups. After sham operation, serum IL-6 levels did not differ between sham+Ctrl and sham+EtOH groups (Figure 5A). 24 h after resuscitation, IL-6 decreased further in both H/R groups without significant differences, followed by a decrease to basal and partly undetectable levels at 72 h in all groups (Figure 5A).

Serum IL-1β levels (Figure 5B) were not increased after H/R in the H/R+Ctrl group at 2 h, compared with both sham groups (4 ± 2 and 0 pg mL^{-1}). IL-1β levels in ethanol-treated rats increased markedly after H/R, compared with all other groups ($P < 0.05$, Figure 5B). At 24 and 72 h, the initial systemic increase in IL-1β was lost and levels were comparable to the results obtained for sham groups at these time points (Figure 5B).

Serum TNF-α concentrations were not detectable at any time (2, 24 and 72 h) in any group.

Hepatic gene expression of local proinflammatory markers after H/R – IL-6, IL-1β and TNF-α

The real-time PCR showed an increase of IL-6 and TNF-α gene expression at 2 h after resuscitation in liver samples obtained from saline treated (H/R+Ctrl) animals, compared with all other groups ($P < 0.05$, Figure 6A and C). The expression of IL-1β was initially increased at 72 h after H/R in both saline and ethanol pre-treated groups ($P < 0.05$, Figure 6B).

Local proinflammatory changes after haemorrhage and resuscitation – ICAM-1 expression and hepatic neutrophil accumulation

The expression of ICAM-1, as revealed by immunohistology, increased markedly 2 h after resuscitation as compared with sham-operated ctrl rats in the saline-gavaged group (Figure 7A and B). A typical sinusoidal lining pattern was present. ICAM-1 was strongly expressed in livers of ctrl animals also at 24 h after H/R with a decline of ICAM-1 expression at 72 h after H/R (Figure 7C and D). Hepatic ICAM-1 expression was markedly reduced in the group of ethanol-gavaged animals at 2 h after resuscitation, as compared with H/R+Ctrl_2 h group, and declined further at 24 h after H/R (Figure 7B, F and G). At 72 h after H/R, in the ethanol group, hepatic ICAM-1 expression was comparable with both sham groups (Figure 7H, A and B).

Hepatic neutrophil infiltration increased at 2 and 24 h after resuscitation, respectively, compared with values after sham operation (1 ± 1 cells per high-power field, $P < 0.05$, Figure 8). The sham values did not vary between 2, 24 and 72 h and are shown as a single point. By 72 h after H/R, PMNL infiltration was markedly reduced (Figure 8). Ethanol markedly diminished hepatic neutrophil infiltration after H/R (Figure 8) compared with the corresponding saline-gavaged group (H/R+Ctrl) to levels not different from those in the sham+EtOH animals (2 ± 1 cells per high-power field, Figure 8; sham values did not vary between 2, 24 and 72 h and are shown as a single point). Ethanol gavage did not increase ICAM-1 expression or PMNL infiltration after sham operation compared with saline-gavaged rats.

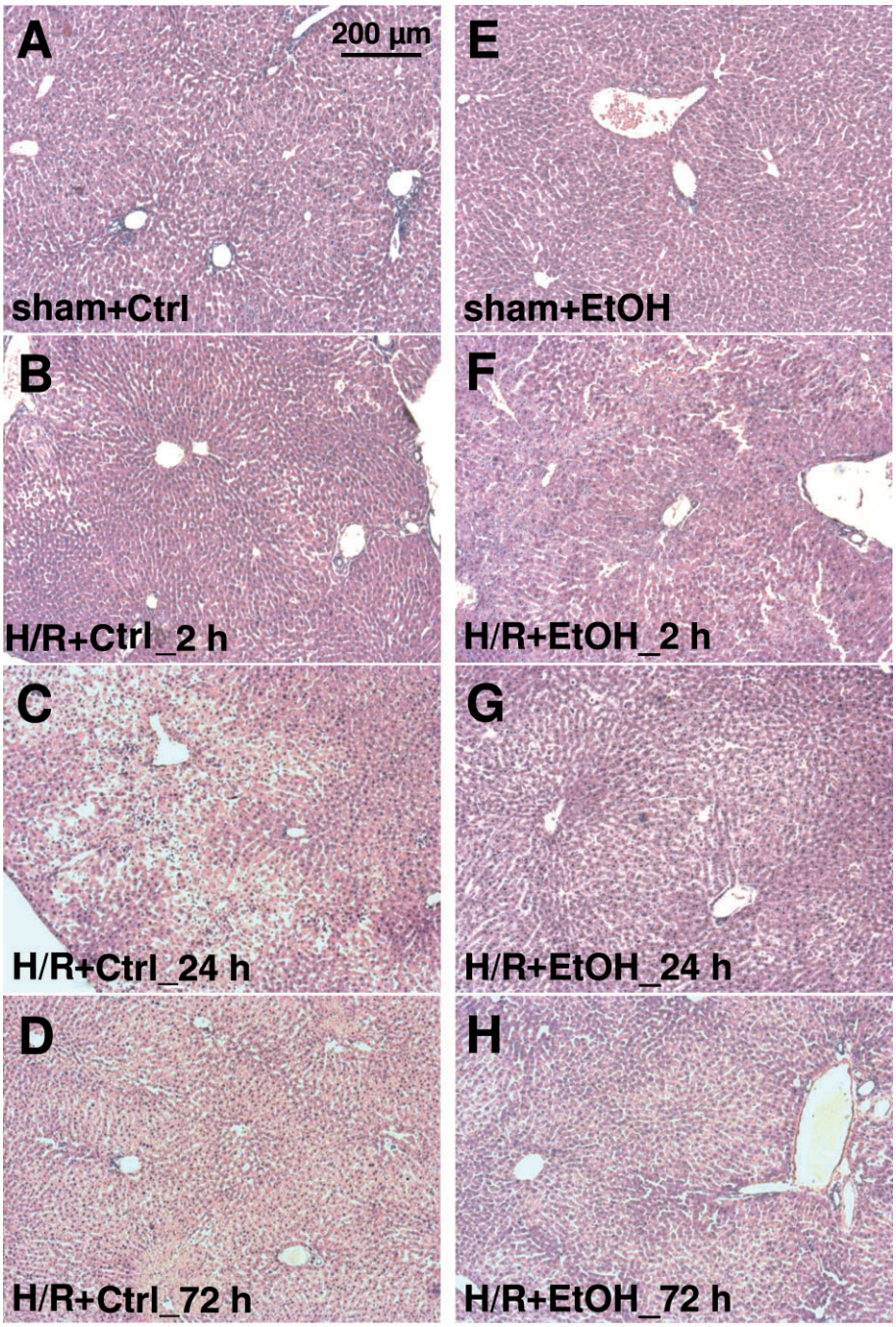

Figure 3

Histological liver necrosis after acute ethanol ingestion after at 2 h, 24 h and 72 h after H/R. Rats were gavaged with saline (Ctrl) or ethanol (5 g·kg^{-1}, 30% EtOH), 12 h before H/R. Sham-operated animals underwent the same surgical procedures but H/R was not carried out. Representative haematoxylin- and eosin- stained liver lobes from saline-treated (Ctrl) (A: after sham operation, B, C and D, 2, 24 and 72 h after H/R, respectively) or ethanol-treated (E: after sham operation, F, G and H, 2, 24 and 72 h after H/R) rats are shown. Bar is 200 µm.

Analysis of the NF-κB inhibitor IκBα and NF-κB-dependent expression of MMP9 after H/R

To analyze how ethanol attenuates inflammation after H/R, phosphorylated and non-phosphorylated IκBα was determined by Western blots in liver homogenates collected at 2 h after resuscitation. Densitometric analysis of relative protein expression related to β-actin and subsequent ratio of phosphorylated to total protein indicated an increase in IκBα phosphorylation after H/R, compared with the ratio in sham-operated rats ($P < 0.05$, Figure 9A and B). Pre-treatment with

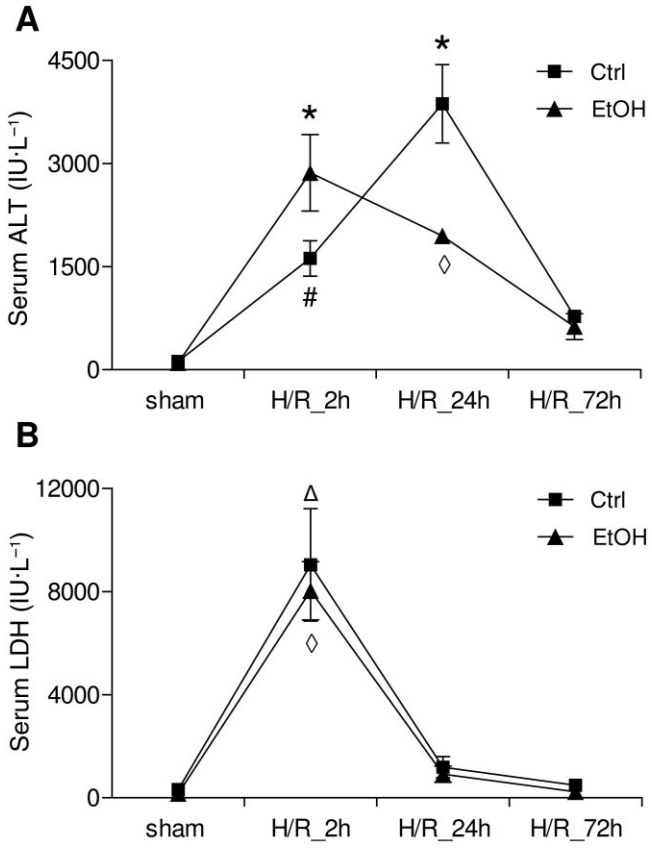

Figure 4

Serum ALT and LDH levels after acute ethanol ingestion and H/R in rats. 12 h before H/R, rats were gavaged with saline (Ctrl) or ethanol (EtOH).Blood samples were collected at 2, 24 and 72 h after H/R for measurement of ALT (A) and LDH (B) enzyme activity. *$P < 0.05$ vs. all, $^{\#}P < 0.05$ vs. all except H/R+EtOH 24 h group, $\diamond P < 0.001$ vs. all except H/R+Ctrl 2 h, $\Delta P < 0.001$ vs. all except H/R+EtOH 2 h, $n = 3$ for 24 h groups, and $n = 7$ for other groups.

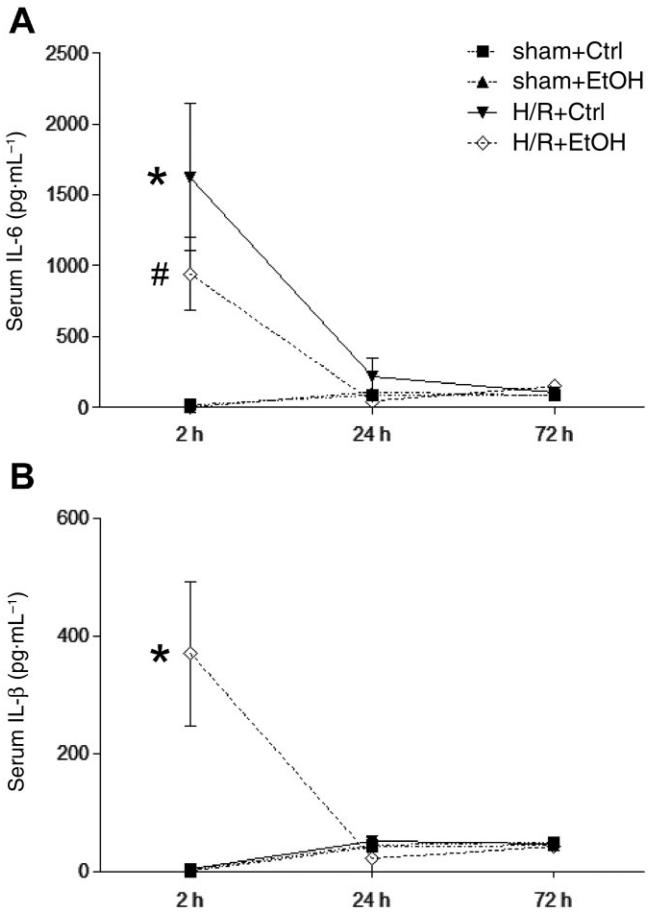

Figure 5

Acute ethanol ingestion affects serum IL-6 and IL-1β after H/R. 12 h before H/R, rats were gavaged with saline (Ctrl) or ethanol (EtOH). Blood samples were collected at 2, 24 and 72 after resuscitation. Serum concentrations of IL-6 (A) and IL-1β (B) were determined by ELISA. Sham-operated animals underwent the same surgical procedures as the H/R groups but H/R was not carried out (*$P < 0.05$ vs. all, $^{\#}P < 0.05$ vs. H/R+Ctrl group 2 h group, $n = 7$ for 2 h group, $n = 3$ for 24 h group, $n = 3$ for 72 h group).

ethanol prevented the increase in the amount of phosphorylated IκBα protein after H/R ($P < 0.05$, Figure 9A and B).

The real-time PCR showed an increase of NF-κB-dependent MMP9 expression at 2 h after resuscitation in liver samples obtained from saline-treated animals (H/R+Ctrl), compared with all other groups ($P < 0.05$, Figure 9C). The expression of MMP9 was not increased at any time in the H/R+EtOH (Figure 9C).

These results indicate that H/R induced NF-κB activation and expression of NF-κB-dependent MMP9. These effects were strongly influenced by ethanol that inhibited NF-κB activation and NF-κB-mediated expression of MMP9 after H/R.

Discussion

In the present study, we evaluated the effect of a binge-like ethanol treatment, 12 h before the onset of H/R. Previous studies using doses of 5 g·kg^{-1} of ethanol clearly demonstrated ethanol-dependent changes in the liver, that is, decreased graft survival and disturbance of hepatic microcirculation as well as increased triglyceride content (Ylikahri *et al.*, 1972; Zhong *et al.*, 1996; Enomoto *et al.*, 2000) This is consistent with our study where a fatty liver was induced by this dose of ethanol (Figure 1). Taken together, these data provide a valid basis for using ethanol at a dose of 5 g·kg^{-1} to study the effects of an acute fatty liver in inflammatory conditions, that is, with the H/R procedure. Interestingly, the BAC showed an early peak 1 and 2 h after alcohol gavage with a rapid decline to almost normal levels by 10–12 h after the gavage, results that correspond to BAC measurements in the present study (Wendell and Thurman, 1979; Rivera *et al.*, 1998). Our results showed that alcohol intoxication reduced mortality after H/R from 78% in the saline-treated group to 22% (Figure 2). Hepatocellular damage at 2 h after H/R, was aggravated by ethanol, with subsequent hepatocellular recovery (Figures 3 and 4). In addition, the ethanol-intoxicated

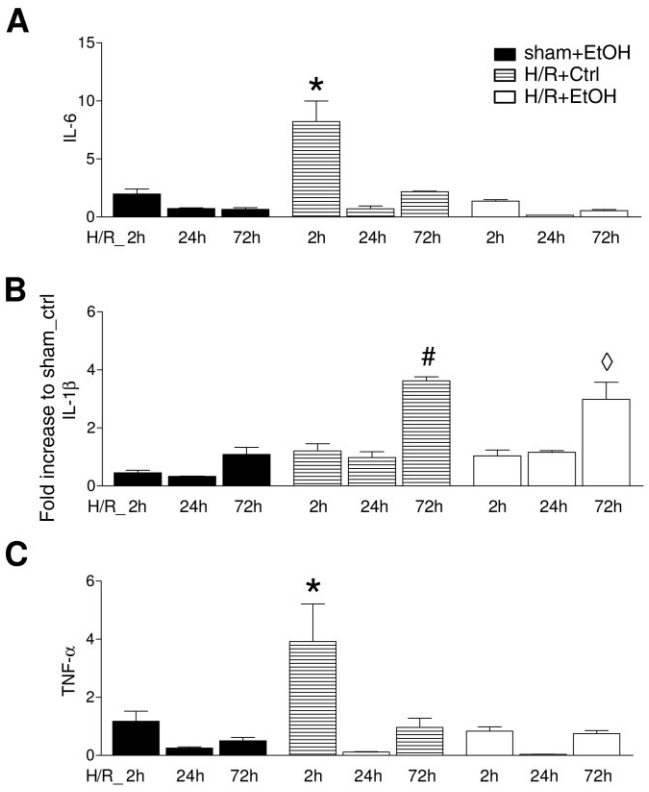

Figure 6

Acute ethanol ingestion modifies gene expression of cytokines (IL-6, TNF-α and IL-1β) in liver at 2 h after H/R. 12 h before H/R, rats were gavaged with saline (Ctrl) or ethanol (EtOH). Hepatic gene expression of IL-6 (A), IL-1β (B) and TNF-α (C) was analysed, at 2, 24 and 72 h after H/R, Sham-operated animals underwent the same surgical procedures but H/R was not carried out. After normalization to GAPDH expression, gene expression was measured as fold increase compared with the sham+Ctrl group (*$P < 0.05$ vs. all, $^{\#}P < 0.01$ vs. all except H/R+EtOH 72 h group, ◇$P < 0.05$ vs. all except H/R+Ctrl 72 h, $n = 3$ for 24 h groups, and $n = 7$ for other groups).

group showed enhanced systemic IL-1β levels, while IL-6 levels were reduced 2 h after H/R (Figure 5). Ethanol also reduced hepatic gene expression of local proinflammatory markers IL-6 and TNF-α as well as the hepatic expression of ICAM-1 and hepatic PMNL infiltration as compared with the saline-treated group early after H/R (Figures 6–8). These beneficial effects are associated with an ethanol-dependent inhibition of the activation of NF-κB (Figure 9).

Trauma victims are frequently intoxicated with alcohol (Madan *et al.*, 1999; Hadfield *et al.*, 2001). Several studies report an association of alcohol intoxication with an increased risk of secondary complications due to an impaired host immune response (Faunce *et al.*, 1997; Messingham *et al.*, 2002; Zhang *et al.*, 2002; Boe *et al.*, 2003; Zambell *et al.*, 2004; Greiffenstein *et al.*, 2007). However, consequences of acute alcohol intoxication on the inflammatory changes after H/R have not been systematically assessed. Both acute and chronic alcohol abuse and H/R have been shown to induce hepatocellular dysfunction. With regard to the liver, an additive effect of acute alcohol intoxication and H/R on liver function, leading to increased risk for hepatic failure after injury, has been suggested (Phelan *et al.*, 2002). In parallel to these findings, we also showed enhanced elevation of the liver enzyme ALT, as well as increased necrotic areas, an effect that was confined to the early post-resuscitation period, at 2 h after completion of resuscitation, in ethanol-gavaged animals (Figure 4), suggesting that acute ethanol intoxication contributes to early liver injury. Of note, the early resolution of markers of liver injury was associated with the lower inflammatory response after binge-like ethanol treatment before H/R. This contrasted with the late rise in hepatocellular damage at 12 and 24 h after H/R in saline-gavaged animals, effects associated with a stronger inflammatory response. However, although H/R increased serum LDH as a marker of general cellular damage, this was not affected by ethanol consumption combined with H/R. Taken together, these data indicate that acute ethanol ingestion was a significant contributor to early hepatic damage after H/R.

H/R-induced hepatic injury was accompanied by elevated circulating and hepatic cytokine expression. During haemorrhage, NF-κB is activated to promote tissue injury and augment the inflammatory cascade, modulating IL-6 and TNF-α production (Rushing and Britt, 2008). With regard to the liver, NF-κB is activated after haemorrhagic shock followed by resuscitation, and this activation is associated with increased IL-6 gene expression (Hierholzer *et al.*, 1998; Gaddipati *et al.*, 2003). Consistent with these observations, we detected an activation of the NF-κB inhibitor IκBα at 2 h after resuscitation in the liver. The increase in hepatic MMP9 expression confirmed the NF-κB activation with subsequent production of NF-κB-dependent proteins (Figure 9). Furthermore, early after H/R, both circulatory IL-6 and hepatic IL-6, as well as TNF-α mRNA levels were increased, suggesting an augmented inflammatory response after H/R in our model (Figures 5 and 6). IL-6 and TNF-α are early response cytokines not only after haemorrhagic shock (Bahrami *et al.*, 1997; Lehnert *et al.*, 2008; Lee *et al.*, 2009; Relja *et al.*, 2010). Down-regulation of IL-6 by anti-IL-6 antibodies and in IL-6 knockout mice resulted in diminished liver damage and improved bile production after trauma/haemorrhage in rats undergoing H/R (Meng *et al.*, 2001; Toth *et al.*, 2004). Treatment with anti-TNF-α antibodies reduced organ injury and improved survival in rats after haemorrhagic shock followed by resuscitation (Bahrami *et al.*, 1997). Ethanol inhibited cytokine responses under acute inflammatory conditions such as induction of LPS shock or after H/R (Nelson *et al.*, 1989; Phelan *et al.*, 2002). Consistent with these findings, we found that ethanol markedly reduced serum IL-6 levels as well as hepatic IL-6 and TNF-α mRNA expression. Furthermore, the ethanol-dependent anti-inflammatory effects after H/R were associated with decreased hepatic IκBα activation and MMP-9 expression, both indicating an inhibition of NF-κB activation in our model (Figures 7–9). Interestingly, early serum IL-1β levels were enhanced in ethanol-intoxicated rats after H/R (Figure 5). Given the transient increase in IL-1β, this may be due to an altered post-translational processing of this cytokine into its active form by the caspase-1 inflammasome. However, we studied the activity of circulating immune cells by evaluating LPS-stimulated IL-1β production in whole blood obtained 12 h after oral gavage with EtOH or

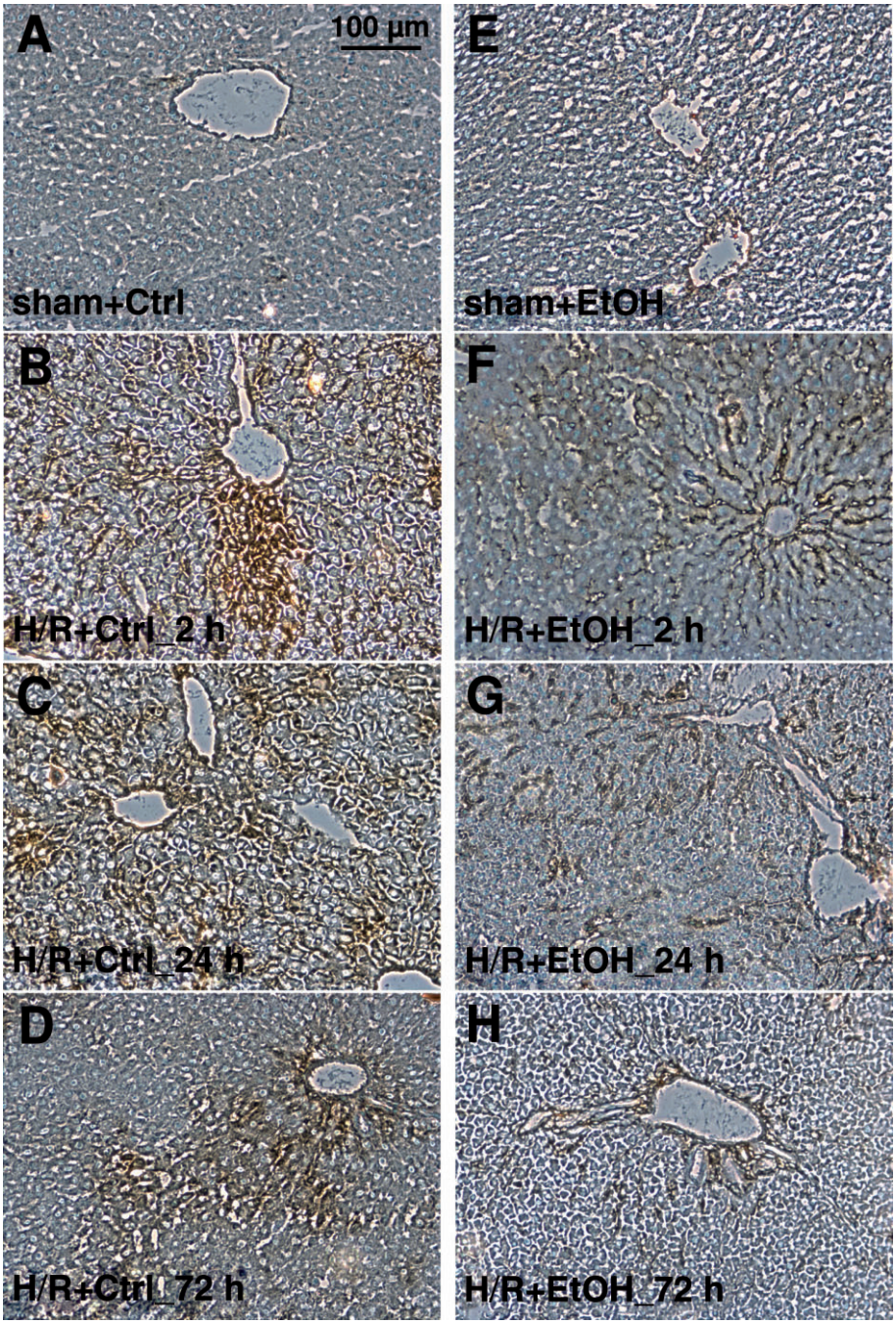

Figure 7

Acute ethanol ingestion decreases ICAM-1 expression after H/R. Hepatic ICAM-1 expression was assessed by immunhistological staining with anti-CD54 antibody (brown). 12 h before H/R, rats were gavaged with saline (Ctrl) or ethanol (EtOH). Sham-operated animals underwent the same surgical procedures without blood withdrawal. Representative liver sections from Ctrl groups (A: after sham operation, B, C and D, 2, 24 and 72 h after H/R, respectively) and EtOH groups (E: after sham operation, F, G and H, 2, 24 and 72 h after H/R) rats are shown. Bar is 100 µm.

saline (stimulation with 10 ng/ml LPS for 24 h). Whole blood from saline-treated animals released significantly more IL-1β compared with whole blood from ethanol-gavaged rats. Moreover, ethanol markedly reduced IL-1β production in whole blood stimulated with LPS (data not shown). Nevertheless, hepatic IL-1β mRNA expression increased 72 h after H/R and was not diminished by ethanol exposure, probably because this time was beyond the duration of action of ethanol in this model (Figure 6). These data demonstrate that ethanol affects various cytokines differentially, resulting in an early anti-inflammatory effect of acute ethanol intoxication.

Several studies attribute the anti-inflammatory effects of alcohol to a decreased chemokine production and neutrophil

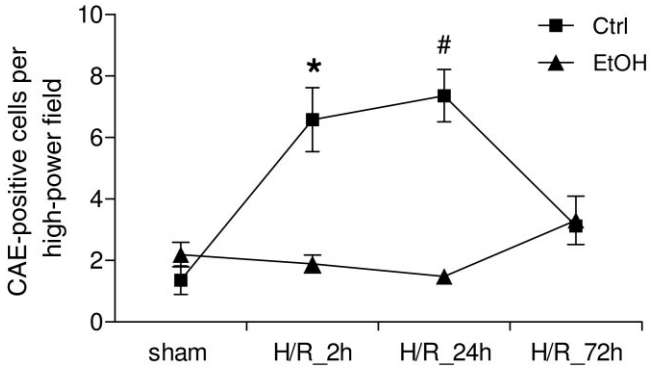

Figure 8

Acute ethanol (EtOH) ingestion reduces hepatic neutrophil infiltration after H/R. Saline (Ctrl) or EtOH-gavaged rats were subjected to H/R or sham operation. Liver samples were collected at 2, 24 and 72 h after H/R and neutrophils in liver sections were identified by chloroacetate esterase (CAE) cytochemistry and counted in 25 high-power fields as described previously (Lehnert *et al.*, 2008). $^{*}P < 0.05$ vs. all except H/R+Ctrl 24 h, $^{\#}P < 0.05$ vs. all except H/R+Ctrl 2 h group, $n = 3$ for 24 h groups, and $n = 7$ for other groups.

recruitment (Szabo and Mandrekar, 2009). ICAM-1 expression is necessary for transendothelial migration and adherence of neutrophils to parenchymal cells. Neutrophil infiltration in the liver plays an important role in hepatic injury after H/R, hepatic ischaemia/reperfusion and LPS shock (Xu *et al.*, 1994). Furthermore, ICAM-1 production depends on NF-κB activation (Zingarelli, 2005). In our study, release of proinflammatory cytokines and the upregulation of ICAM-1 after H/R correlated with increased hepatic adhesion and infiltration with neutrophils and enhanced hepatic damage (Figures 3–8). Acute ethanol ingestion was associated with reduced activation of NF-κB and down-regulation of ICAM-1 expression with subsequently decreased neutrophil infiltration in the liver (Figures 7–9).

Besides acute alcohol intoxication itself, an acute withdrawal syndrome occurring 8–16 h after alcohol intoxication ('hangover') is associated with a reduced proinflammatory response in animals and humans (Szabo *et al.*, 1994; Gottesfeld *et al.*, 2002; Kim *et al.*, 2003; Greiffenstein and Molina, 2008; Zhang *et al.*, 2009). In accordance with these results, we also found 12 h after ethanol gavage, a decreased IL-1β production in LPS-stimulated whole blood (data not shown). Therefore, a 'hangover' may influence the pathophysiology after H/R via anti-inflammatory effects and counteract the proinflammatory changes induced by H/R. Further studies should evaluate potential differences in the activation status of the immune system prior to an experimental insult in a 'hangover' state.

Taken together, we have demonstrated a reduction in mortality and early systemic and local inflammation in acutely ethanol-intoxicated rats, undergoing resuscitated blood loss, accompanied by a faster resolution of liver damage. Furthermore, the anti-inflammatory effects of ethanol were also manifested in reduced ICAM-1 expression and neutrophil infiltration and were associated with the stabilization of the NF-κB inhibitor IκBα.

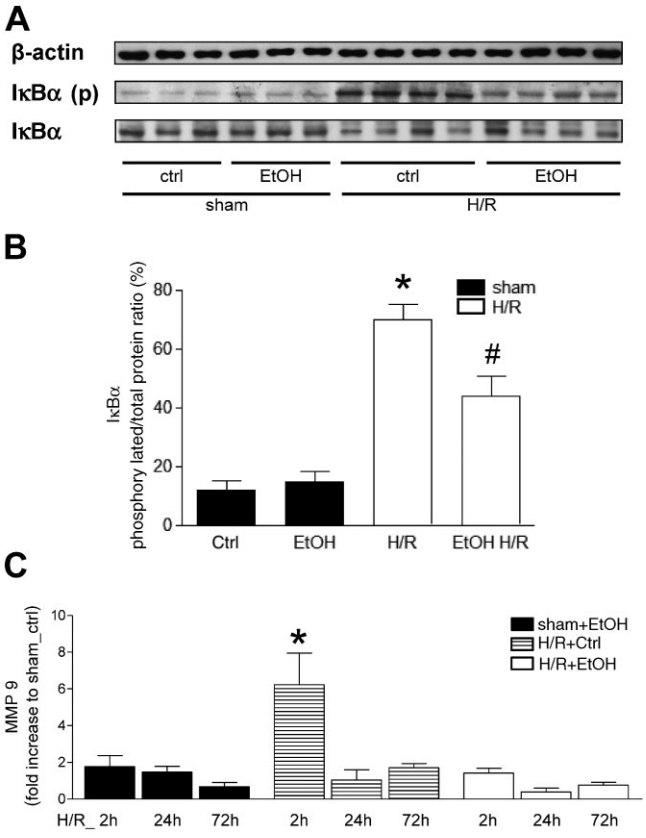

Figure 9

Acute ethanol (EtOH) ingestion reduces hepatic IκBα phosphorylation and MMP9 gene expression at 2 h after H/R. Saline- (Ctrl) or EtOH-gavaged rats were subjected to H/R or sham operation. Two hours after the end of resuscitation, liver tissue was collected and Western blot for IκBα, IκBα-phospho and β-actin was performed. A: 1–6 were liver protein extracts from rats after sham operation (sham, lane 1–3: Ctrl, lane 4–6: EtOH gavage) or H/R (lanes 7–10: Ctrl, lane 11–14: EtOH gavage). In B, ratio of phosphorylated IκBα and total protein after densitometric measurements and normalization to β-actin are represented ($^{*}P < 0.05$ vs. all, $^{\#}P < 0.05$ vs. H/R+Ctrl, $n = 6$, representative gel from 3 experiments is shown). At 2, 24 and 72 h after resuscitation, hepatic gene expression of MMP (C) was analysed. After normalization to GAPDH expression, gene expression was measured as fold increase compared with sham+Ctrl group ($^{*}P < 0.05$ vs. all, $n = 7$).

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Conflicts of interest

The authors declare no conflicts of interest.

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