Alcohol-induced attenuation of post-traumatic inflammation is not necessarily liver-protective following trauma/hemorrhage

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Received November 19, 2018; Accepted June 4, 2019

DOI: 10.3892/ijmm.2019.4259

Abstract. Due to their high prevalence, blunt chest trauma (TxT) and hemorrhagic shock have a significant influence on the outcomes of trauma patients, causing severe modulations of the immune system and high mortality rates. Alcohol consumption in trauma patients has a high clinical impact. Studies investigating the timing of alcohol intoxication prior to trauma are limited, although there are two typical scenarios regarding alcohol consumption: Acute ('drink and drive scenario') and sub-acute ('evening binge drinking'). Therefore, the present study investigated the influence of either an acute or sub-acute alcohol-drinking scenario in an *in vivo* model of TxT and hemorrhagic shock, focusing on liver inflammation and outcomes. At 12 h (sub-acute) or 2 h (acute) before the experiment, female Lewis rats received a single oral dose of alcohol (ethanol, EtOH) or saline (NaCl, ctrl), followed by TxT, hemorrhagic shock (35±3 mm Hg) and resuscitation (H/R). The animals were either sacrificed 2 h later or their survival was determined for 72 h. The results revealed that EtOH induced significant fatty changes in the liver. TxT + H/R-induced increases in the gene expression of interleukin (IL)-6 and intercellular adhesion molecule-1 and the protein expression of tumor necrosis factor (TNF)-α and IL-1β were significantly reduced in both EtOH groups compared with those in the corresponding TxT + H/R ctrl groups. The local presence of IL-10-expressing cells in the liver was significantly increased following TxT + H/R in all groups, although the sub-acute EtOH TxT + H/R group had a significantly higher proportion of IL-10-positive cells compared with all other groups. Stimulating peripheral whole blood with lipopolysaccharide led to significantly lower levels of TNF-α release in the sub-acute EtOH group compared with the levels in all other groups. Significant TxT + H/R-induced increases in liver transaminases and liver damage were most prominent in the sub-acute EtOH group. The TxT + H/R EtOH group exhibited the lowest levels of glucose. There were no significant differences in mortality rate among the TxT + H/R groups. The data obtained indicates that the severity of liver damage following TxT + H/R may depend on the timing of alcohol consumption and severity of trauma, but also on the balance between pro- and anti-inflammatory responses.

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Key words: alcohol, blunt chest trauma, hemorrhagic shock, inflammation, liver

Introduction

Due to their high prevalence, blunt chest trauma (TxT) and hemorrhagic shock have significant effects on the outcomes of trauma patients, causing severe modulations of the immune system and high mortality rates (1-4). Early mortality is mainly caused by marked blood loss, traumatic brain injuries or non-controllable organ damage within the first minutes and during the first hours following trauma, whereas later mortality is mainly caused by secondary complications of the respiratory system or, for example, the development of sepsis during the first days to weeks of admission leading to multiple organ failure and multiple organ dysfunction syndrome (MODS) (1). Here, an essential factor during the pro-inflammatory reaction of the immune system is involved in the release of pathogen-associated molecular patterns and/or damage-associated molecular patterns for the development of MODS (1,5,6). The immune system reacts to trauma with a substantial release of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β, and the expression of intercellular adhesion molecule (ICAM)-1. Activation of the transcription factor NF-κB serves a key role in the production, regulation and release of these mediators (7-9).

Alcohol consumption in trauma patients constitutes an extra variable influencing the post-traumatic immune response with high clinical impact. Literature on the positive or negative influence of alcohol is inconsistent. Our previous clinical and *in vivo* studies found an immune-modulating influence of alcohol, and even reduced mortality rates following

isolated hemorrhagic shock in rats (2,10,11). Phelan *et al* and Zambell *et al* also confirmed the influence of alcohol on the immune system, including modulated neutrophil function and cytokines TNF-α and IL-10 in an *in vivo* hemorrhage model (12,13). In contrast to our results, in the studies of Phelan *et al* and Zambell *et al*, the changes due to alcohol consumption aggravated outcomes. In addition to the volume or concentration of alcohol, which is known to be of importance regarding either the positive or negative effects of alcohol consumption (14,15), the timing of alcohol admission is another relevant clinical question for affecting healthcare and outcomes for trauma patients. With regard to the metabolic changes caused by alcohol consumption, the timing of its consumption serves a decisive role (16,17). This is partly explained by the influence of alcohol on gluconeogenesis, as alcohol-induced inhibition of gluconeogenesis can lead to hyperlactacidemia, hypoglycemia and liver damage (16). However, few studies have examined the timing of alcohol intoxication prior to trauma or the time-dependent effects on the inflammatory response and organ damage following trauma/hemorrhage.

Therefore, the present study investigated the influence of either an acute ('drink and drive scenario') versus a sub-acute ('evening binge drinking') alcohol-drinking scenario in an *in vivo* model of TxT and hemorrhagic shock, with particular focus on the hepatic inflammatory response and survival rates. The study aimed to test the hypothesis that acute and subacute alcohol intake has a beneficial influence on local inflammatory response and liver injury in a clinically relevant double hit trauma.

Materials and methods

Animals and experimental model. The present study was approved by the Veterinary Department of the Regional Council in Darmstadt, Germany [Regierungspraesidium (RP) Darmstadt, Veterinaerswesen', Hessen, Germany] and designed in accordance with the ARRIVE guidelines (18). Animals were always handled by group members holding a certificate of the Federation of European Laboratory Animal Science Associations.

The experimental setting was as described previously (17). Prior to experimental procedures, female Lewis rats (age: 12-14 weeks, 190-240 g, provided by Janvier Labs, France) remained in the facility for at least 7 days for acclimation with a maximum of four animals per cage. The animals were fasted over night before beginning the surgical experimentation. Water was available *ad libitum* during the whole housing. Animals were kept under standardized temperature (21±2˚C), relative humidity (50%), and artificial light-dark cycles (14 h light, 10 h dark). Depending on the group assignment, the animals either received a single dose of alcohol (5 g/kg, 30% ethanol, EtOH) or control solution (sodium chloride, NaCl 0.9%, ctrl) via oral gavage either 2 h (acute) or 12 h (sub-acute) before experimentation. Previous studies using the same dose of EtOH clearly demonstrated alcohol-dependent changes in the liver, including decreased graft survival, disturbance of hepatic microcirculation and increased triglyceride content (19-21). This is consistent with our previous studies, in which fatty liver was induced by this dose and concentration of alcohol (2,22). Taken together, these previous data provided a valuable basis for the use of alcohol at a dose of 5 g/kg body weight (30%) to examine the effects of acute intoxication under inflammatory conditions induced by hemorrhage. Prior to our previously reported studies (2,22), preliminary experiments were performed to establish the model of acute alcohol-induced fatty liver using 20% EtOH, rather than 30%. Although an EtOH concentration of 20% was initially used, a decrease in the gavage volume was required as repeated aspirations occurred. Therefore, a decision was made to increase the concentration to 30%. Transaminase release and the level of hepatic fat deposition were compared using both concentrations following H/R and a sham procedure and resulted in no differences. The 30% concentration was used in another previous study (23) in addition to our own previously published study (17). For anesthesia, isoflurane (1.2-3.0%), buprenorphine (0.05 mg/kg body weight) and a local anesthesia to the incision sites (0.25% Carbostesin) were applied. During anesthesia initiation, a 3.0% isoflurane oxygen mixture was used, the vessels were cannulated with a 2.0-2.5% isoflurane oxygen mixture depending on individual responses to pain stimulation, the induction of trauma was performed with a concentration of 2% isoflurane following a short stabilization period and in the reperfusion period. At the end of experimentation, the isoflurane concentration was reduced in a step-by-step manner. The right femoral artery was cannulated with polyethylene tubing to measure blood pressure, and a bilateral lung contusion was induced, as described previously (24,25). Briefly, a Mylar polyester film (0.190-mm, DuPont Teijin Films, Luxembourg) was fixed in a cylinder 6 cm above the chest of the animals, and a standardized pressure wave was then applied to rupture the film and induce the lung contusion. Subsequently, the left jugular vein and the right carotid artery were cannulated to induce hemorrhagic shock, as described previously (26,27). Here, blood was withdrawn into a heparinized syringe via the carotid artery to maintain a target mean arterial blood pressure (MABP) of 35±3 mm Hg. For the regulation of the MABP at this level, further withdrawal or recirculation of withdrawn blood was performed for 60 min. The MABP was monitored using a blood pressure analyzer (Sirecust 960, Siemens AG). Reperfusion over 30 min was conducted with 60% of the withdrawn blood volume plus 50% Ringer's lactate (RL) solution of the maximum removed volume via the jugular vein. The hemorrhagic shock model was established in our laboratory of the Department of Trauma, Hand and Reconstructive Surgery, University Hospital Frankfurt, Goethe University, 10 years ago with a resuscitation protocol of retransfusing 60% of the withdrawn blood to simulate blood loss and reperfusion in traumatized patients. As our previous studies were performed with a resuscitation of 60% of the withdrawn blood, the present study was also performed according to this established protocol to ensure comparability of studies (26,27). Following removal of the catheters, the wounds were sutured. At the end of the surgical/trauma phase, the animals were monitored. During the entire experimentational procedure, temperature was monitored. The animals subsequently had free access to water and food. Postoperative analgesia was performed via application of buprenorphine (0.05 mg/kg body weight) twice a day.

Depending on group allocation, the animals were sacrificed either 2 h following the end of the experiment or the overall survival was monitored for 72 h. The sacrifice was performed, as briefly described, by withdrawing blood via the aorta using the same isoflurane oxygen concentration as

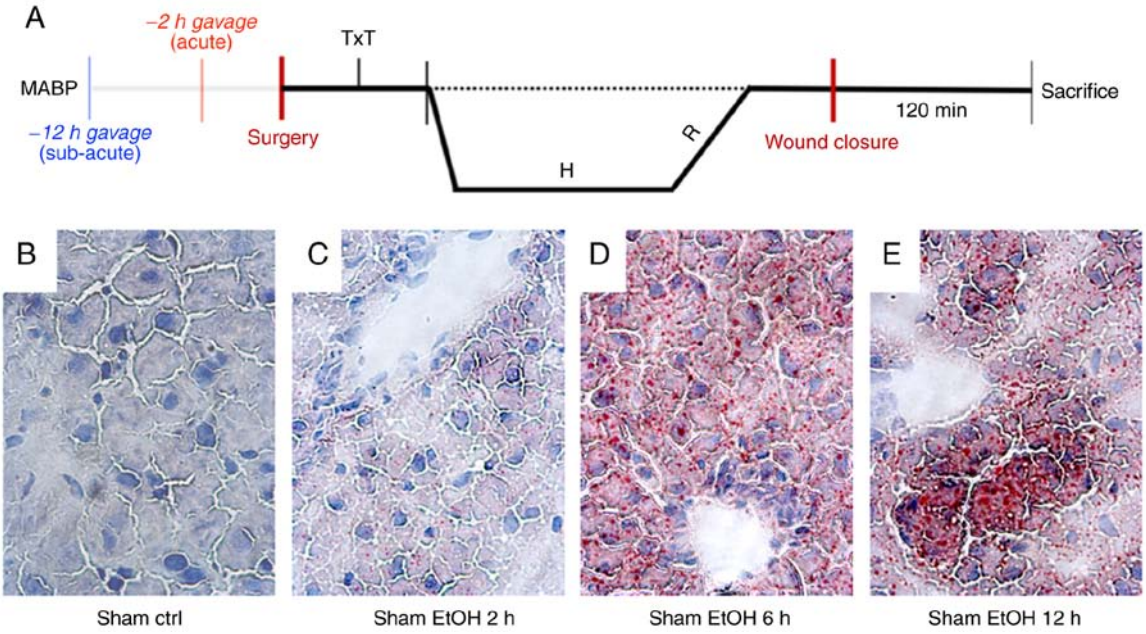

Figure 1. Lipid accumulation. (A) Schematic timeline of the experimental design. Acute gavage: EtOH or NaCl were gavaged 2 h before the beginning of trauma preparation, sub-acute gavage: EtOH or NaCl were gavaged 12 h before the beginning of trauma preparation. The Sham group underwent all surgical procedures without TxT + H/R. Representative lipid accumulation in Oil red O-stained liver sections (40X objective) from (B) sham ctrl, (C) sham EtOH animals after 2 h, (D) sham EtOH animals after 6 h and (E) sham EtOH animals after 12 h. H, hemorrhagic shock; MABP, mean arterial blood pressure; R, resuscitation; TxT, blunt chest trauma; ctrl, control.

during the preparation period and flushing the abdominal organs with RL. Subsequently, the left liver lobe was ligated, removed and snap-frozen in liquid nitrogen. The remaining liver was flushed with 20 ml 10% buffered formalin solution and removed for further handling for (immuno) histology.

During the survival evaluation, frequent and close examination of the condition of each animal was performed. In the occurrence of any cut-off criteria, which were defined in accordance with the guidelines of the Ethics Committee of the RP Darmstadt, animals were sacrificed and their mortality rate evaluated. Fig. 1A shows the experimental overview.

Group allocation. A total of 48 animals were randomly assigned to the sub-acute or acute EtOH or NaCl groups (n=6; Table I). Animals in the sham groups also received oral gavage of EtOH or NaCl and all surgical procedures were performed without TxT or hemorrhagic shock and resuscitation (H/R). For survival analysis, four animals in the sham groups, and eight animals in the TxT + H/R groups were included (Table II).

Examination of EtOH-induced hepatic fat accumulation. The hepatic frozen sections (3-μm) were fixed with 10% buffered formalin. Lipids were stained at room temperature for 50-60 min with with 2% (w/v) Oil red O working solution (0.07 g Oil red O dissolved in 25 ml 100% methanol mixed with 10 ml 1 M NaOH), counterstained with hematoxylin (5 g/l) for 10 min. The images were captured by using the Zeiss Axio Observer Z1 microscope (40X objective, Zeiss AG).

Ribonucleic acid (RNA) isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from the snap-frozen liver samples using the RNeasy-system (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The residual remaining DNA were removed using the RNase-free DNase set, according to the manufacturer's instructions (Qiagen GmbH), and the RNA samples were stored at -80˚C. The Affinity script QPCR-cDNA synthesis kit (Stratagene; Agilent Technologies, Inc., La Jolla, CA, USA) was used for RT-qPCR analysis using 100 ng of total RNA for reverse transcription according to the manufacturer's instructions. The mRNA expression levels of *IL-6, IL-1β* and *ICAM* were determined on a Stratagene; MX3005p QPCR system (Stratagene; Agilent Technologies, Inc.) using gene-specific primers (rat *IL-6*: NM_012589, cat. no. PPR06483B; *IL-1β*: NM_031512, cat. no. PPR06480B and rat *ICAM*: NM_012967, cat. no. PPR42235A; all SABiosciences, SuperArray, Frederick, MD, USA). As a reference gene, the expression of *GAPDH* (rat *GAPDH*: NM_017008, cat. no. PPR06557B, SABiosciences) was measured. The PCR protocol was set up with 1X RT^2 SYBR Green/Rox qPCR Master mix (SABiosciences) in a 25 μl volume according to manufacturer's instructions and as described previously (22,28). The relative mRNA expression of each target gene was calculated using the comparative threshold cycle method ($2^{-\Delta\Delta Cq}$ method). Briefly, the quantity of target mRNA in each sample was normalized to *GAPDH*, to give ΔCq, and then to the samples from the sham groups. The relative mRNA expression is presented as % calculated in relation to sham following normalization to *GAPDH* (29).

Quantification of cytokine protein levels. The concentrations of the protein levels of TNF-α and IL-1β were determined using a rat TNF-α ELISA set (Diaclone) and rat IL-1β Tissue Culture ELISA Ready-Set-Go!® (eBioscience; Thermo Fisher Scientific, Inc.) according to manufacturer's instructions.

Table I. Overview of the groups for analysis post-sacrifice.

Group	Treatment protocol	Number of animals
1	Sham, sub-acute NaCl gavage	6
2	Sham, acute NaCl gavage	6
3	Sham, sub-acute EtOH gavage	6
4	Sham, acute EtOH gavage	6
5	TxT + H/R, sub-acute NaCl gavage	6
6	TxT + H/R, acute NaCl gavage	6
7	TxT + H/R, sub-acute EtOH gavage	6
8	TxT + H/R, acute EtOH gavage	6

The animals received either EtOH or NaCl 2 h (acute) or 12 h (sub-acute) before the beginning of experiments. Animals in the sham group underwent all surgical procedures without the induction of TxT + H/R. EtOH, ethanol; TxT, blunt chest trauma; H/R, hemorrhagic shock with resuscitation.

Table II. Overview of the groups for survival analysis.

Group	Treatment protocol	Number of animals
9	Sham, sub-acute NaCl gavage	4
10	Sham, acute NaCl gavage	4
11	Sham, sub-acute EtOH gavage	4
12	Sham, acute EtOH gavage	4
13	TxT + H/R, sub-acute NaCl gavage	8
14	TxT + H/R, acute NaCl gavage	8
15	TxT + H/R, sub-acute EtOH gavage	8
16	TxT + H/R, acute EtOH gavage	8

Animals received either EtOH or NaCl 2 h (acute) or 12 h (sub-acute) before the beginning of the experiments. Animals in the sham group underwent all surgical procedures without induction of TxT + H/R. EtOH, ethanol; TxT, blunt chest trauma; H/R, hemorrhagic shock with resuscitation.

ELISA was performed using the Infinite M200 microplate reader (Tecan, Männedorf, Switzerland).

Staining of IL-10. The paraffin-embedded liver samples were sectioned (3-μm), deparaffinized, rehydrated and stained with anti-IL-10 antibody. Following deparaffinization, epitope recovery was performed under a humidified atmosphere using R-Universal epitope recovery buffer (Aptum, Kassel, Germany) for 1 h (Retriever 2010, Prestige Medical). Following washing with water and PBS, mouse monoclonal antibody against IL-10 (Cloud-Clone-Corp., MAA056Ra21, 1:30) was applied as a primary antibody. Following incubation for 1 h at room temperature, and a subsequent washing procedure, a secondary AlexaFluor568 donkey anti-mouse antibody (1:100, Invitrogen; Thermo Fisher Scientific, Inc., A10037) was applied to detect specific binding. After 1 h at room temperature, the sections were washed, and mounted using fluorescent mounting medium containing DAPI nucleic acid stain (Vectashield HardSet Antifade Mounting Medium with DAPI, Vector Laboratories, Ltd., Cambridge, UK). Fluorescence was visualized using a Zeiss inverted fluorescence microscope AXIO Observer Z1 (Carl Zeiss AG, Oberkochen, Germany). Representative images were captured from five random fields. IL-10-positive cells were quantified by counting their number in a total of five high-power fields per liver section in a blinded manner. Data from each tissue section were pooled to determine mean values.

Ex vivo in vitro whole blood stimulation for the TNF-α production assay. Blood samples (50 μl) were withdrawn prior to the onset of TxT and were diluted in 450 μl RPMI-1640 (Seromed, Berlin, Germany) in a polypropylene tube (BD Biosciences, Franklin Lakes, NJ, USA) supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.), and 20 mM HEPES buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The samples were then stimulated with lipopolysaccharide (LPS, 10 μg/ml, *E. coli* 0127:B8, Sigma-Aldrich; Merck KGaA) and incubated at 37˚C and 5% CO_2. After 24 h, the samples were centrifuged for 15 min at 2,100 g at room temperature, and the supernatant was collected and stored at -80˚C. As a reference, corresponding blood samples were incubated as described above without adding LPS for stimulation. The concentration of TNF-α was determined using the rat TNF-α ELISA kit (Diaclone) according to the manufacturer's instructions.

Examination of liver injury. Plasma was stored at -80˚C for later analysis of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) using the Spotchem EZ SP-4430 device (Arkray, Inc., Kyoto, Japan). The determination of histological damage was performed by an independent veterinary pathologist, who allocated the hematoxylin-eosin-stained liver sections to the various experimental groups in a blinded manner. Cell enlargement and nuclear dissolution in hepatocytes were characterized as necrotic. The pathologist investigated the following: Hemorrhage, necrosis, congestion, single cell degeneration/necrosis or individualization, zonal necrosis (perivenous), vacuolization of hepatocytes (periportal), extramedullary hematopoiesis and granulated cytoplasm/mineralized mitochondria.

Glucose determination. Glucose was determined prior to the onset of TxT (baseline) using GEM Premier 4000 (Instrumentation Laboratory GmbH, Kirchheim, Germany; BGA Set Optimedical Comfort Sampler Basic kit).

Statistical analysis. Differences between groups were determined by one-way analysis of variance using Kruskal-Wallis with Dunn's post hoc test. Changes in target gene expression were analyzed by Wilcoxon matched-pair analysis. Data are presented as the mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA).

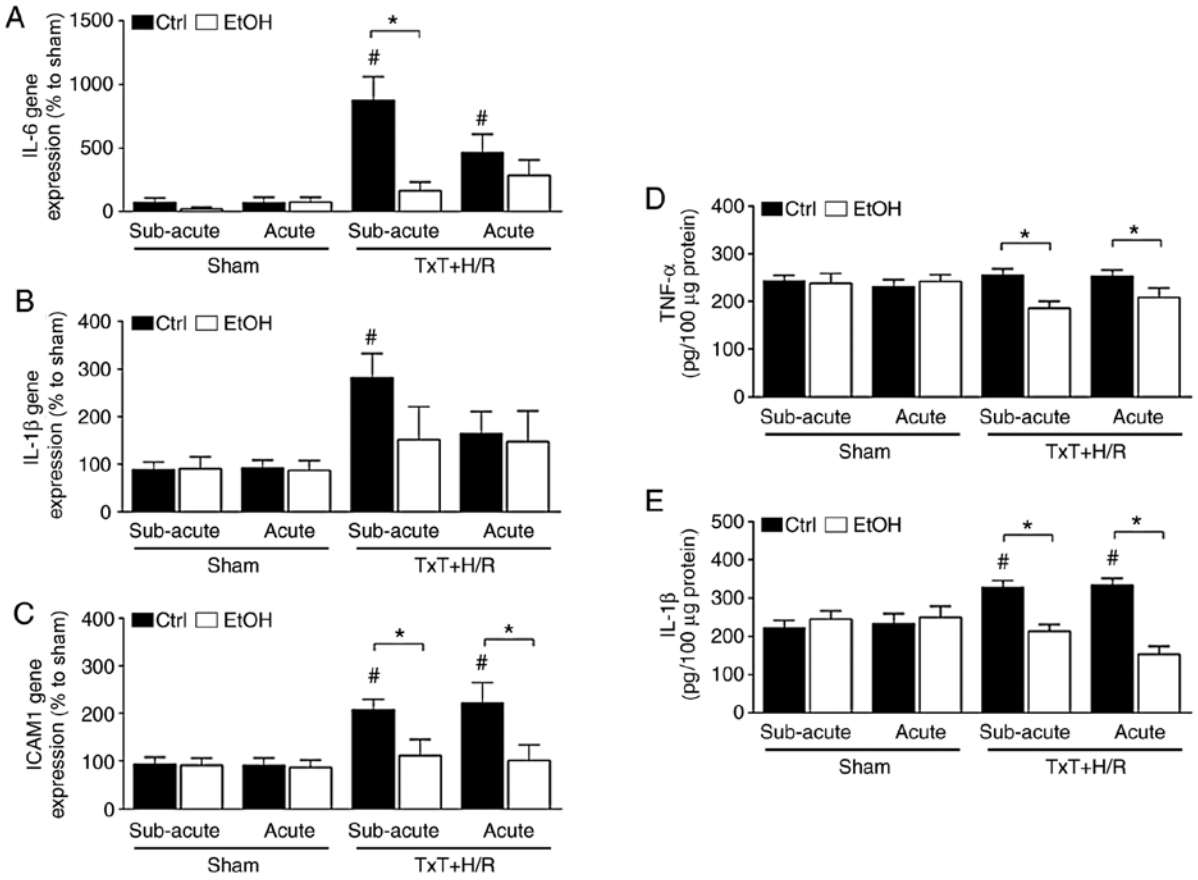

Figure 2. Inflammatory changes. Gene expression levels of (A) IL-6, (B) IL-1β and (C) ICAM-1, and protein expression levels of (D) TNF-α and (E) IL-1β are shown. At 12 h (sub-acute) or 2 h (acute) before the experiment, female Lewis rats received a single oral dose of EtOH or saline (ctrl), followed by TxT + H/R. The sham group underwent all surgical procedures without TxT + H/R. *P<0.05 between indicated groups; #P<0.05 vs. sham group. IL, interleukin; ICAM-1, intercellular adhesion molecule-1; TNF-α, tumor necrosis factor-α; EtOH, alcohol; NaCl, saline; ctrl, control; TxT, blunt chest trauma; H/R, hemorrhagic shock with resuscitation.

Results

Lipid deposition following EtOH gavage. Lipid deposition was determined 2, 6 and 12 h following EtOH or ctrl gavage by staining of lipid accumulation with Oil red O. At 2 h post-EtOH gavage, an accumulation of lipid droplets was present, with the most distinct occurrence after 6 h. At 12 h post-EtOH gavage, the accumulation of lipid droplets remained clearly visible, but regenerative fat reduction was observed (Fig. 1B-E).

Local inflammatory changes. TxT + H/R induced a significant increase in the gene expression of IL-6 in the liver of both ctrl groups compared with that in the corresponding sham groups. Both EtOH trauma groups exhibited lower gene expression of IL-6 compared with the corresponding ctrl groups, however, only in the sub-acute EtOH group was the difference significant following TxT + H/R (ctrl: 877.3±182.5 vs. EtOH: 165.1±68.2% to sham, P<0.05, Fig. 2A). The gene expression of IL-1β was only significantly increased in the sub-acute ctrl group following TxT + H/R compared with the that in the corresponding sham group (Fig. 2B). The gene expression pattern of ICAM-1 was comparable to that observed for IL-6. A significant increase in the gene expression of ICAM-1 was induced by TxT + H/R in both ctrl groups compared with that in the corresponding sham groups (sub-acute ctrl: 207.6±21.7 vs. 92.7±14.8 and acute: 221.7±41.8 vs. 91.4±14.9% to sham, P<0.05, Fig. 2C). By contrast, both EtOH groups exhibited significantly lower gene expression levels of ICAM-1 compared with that in the corresponding ctrl group following TxT + H/R (sub-acute EtOH: 110.8±34.2 and acute EtOH: 101.3±32.5% to sham, P<0.05, Fig. 2C).

In terms of the hepatic protein expression of TNF-α, no significant changes were observed following TxT + H/R compared with the sham. However, there were significant reductions in the sub-acute and acute EtOH TxT + H/R groups compared with the corresponding TxT + H/R ctrl groups (sub-acute EtOH vs. ctrl: 185.6±14.5 vs. 254.9±13.2 pg/100 μg protein and acute EtOH vs. ctrl: 208.3±20.0 vs. 252.9±13.0 pg/100 μg protein, P<0.05, Fig. 2D).

The hepatic protein expression of IL-1β was significantly enhanced in both ctrl groups following TxT + H/R exposure compared with the corresponding sham groups (sub-acute ctrl vs. sham ctrl: 328.8±16.9 vs. 223.2±19.4 pg/100 μg protein; acute ctrl vs. sham ctrl: 334.5±17.0 vs. 233.8±25.4 pg/100 μg protein, P<0.05, Fig. 2E). Both EtOH trauma groups had significantly lower protein levels of IL-1β than the corresponding ctrl groups following TxT + H/R exposure (P<0.05, Fig. 2E).

The number of positively stained cells was comparable in all sham groups (Fig. 3A). The presence of IL-10-expressing

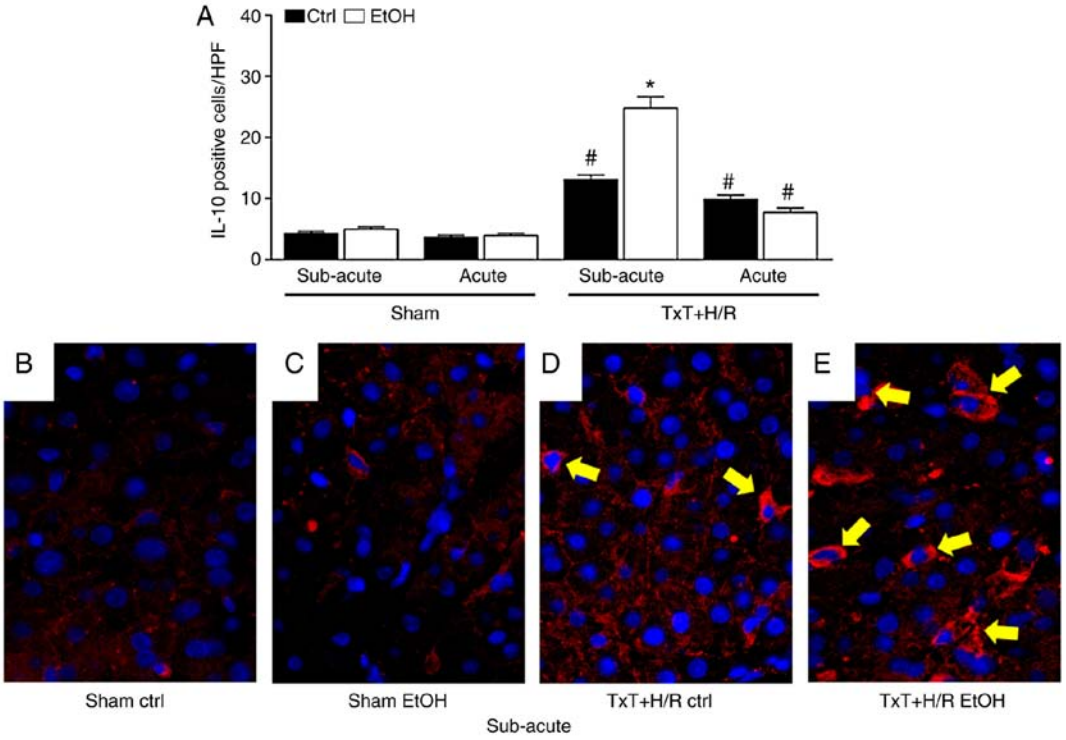

Figure 3. IL-10 levels. (A) IL-10-positive cells. At 12 h (sub-acute) or 2 h (acute) before the experiment, female Lewis rats received a single oral dose of EtOH or saline (ctrl), followed by TxT + H/R. The sham group underwent all surgical procedures without TxT + H/R. *P<0.05 vs. all groups; #P<0.05 vs. sham group. Representative immunohistological images (40X objective) show IL-10-positively stained cells 2 h following reperfusion in the (B) sham ctrl, (C) sham EtOH, (D) TxT + H/R ctrl and (E) Txt + H/R EtOH groups. Exemplary IL-10-positively stained cells are marked with arrows. IL, interleukin; EtOH, alcohol; ctrl, control; TxT, blunt chest trauma; H/R, hemorrhagic shock with resuscitation.

cells in the liver was significantly increased in all trauma groups compared with each corresponding sham group (P<0.05, Fig. 3A). In the sub-acute EtOH group, a significantly higher number of IL-10-positive cells were found following TxT + H/R exposure compared with that in the sub-acute ctrl group (24.8±1.9 vs. 13.1±0.7, respectively, P<0.05, Fig. 3A). Representative immunohistological images show the IL-10-positively stained cells at 2 h post reperfusion (Fig. 3B-E).

Whole-blood stimulation assay. The release of TNF-α upon stimulation with LPS levels was determined in a whole-blood stimulation assay in order to analyze whether there is an immunosuppression/immunotolerance prior to the onset of trauma/hemorrhage. A significantly lower level of TNF-α was found in the sub-acute EtOH group compared with the ctrl group (60.7±4.1 vs. 134.4±14.4 pg/ml, respectively, P<0.05, Fig. 4).

Cell and liver damage. To evaluate hepatocellular damage, AST and ALT were determined. TxT + H/R caused a significant increase in the levels of AST and ALT in all trauma groups compared with each corresponding sham group (P<0.05, Fig. 5A and B). The AST and ALT levels were further increased in the sub-acute EtOH trauma group compared with the sub-acute ctrl trauma group, and this increase was significant (P<0.05, Fig. 5A and B). In terms of general cell damage, as indicated by systemic LDH levels, TxT + H/R induced a significant increase in LDH in all trauma groups compared with the corresponding sham groups (P<0.05, Fig. 5C).

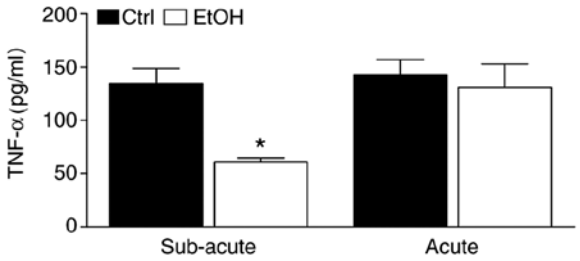

Figure 4. TNF-α levels. At 12 h (sub-acute) or 2 h (acute) before the experiment, female Lewis rats received a single oral dose of EtOH or saline (ctrl). Release of TNF-α from peripheral whole blood upon stimulation with lipopolysaccharide was determined prior to the onset of trauma/hemorrhage. *P<0.05 vs. all groups. TNF-α, tumor necrosis factor-α; EtOH, alcohol; ctrl, control.

However, there were no significant differences among the trauma groups.

Histological analysis of the liver sections from the acute, sub-acute EtOH and both ctrl TxT + H/R groups revealed significant liver damage at 2 h post-resuscitation compared with sections from the sham groups (Fig. 6A-G). In accordance with the data from liver transaminases, in the sub-acute EtOH TxT + H/R group, liver damage was significantly higher following TxT + H/R compared with that in the sub-acute ctrl group (Fig. 6A-G).

Hypoglycemia in the sub-acute EtOH group. Regarding glucose levels, a significant decrease was observed in the sub-acute EtOH prior to the onset of TxT compared with the

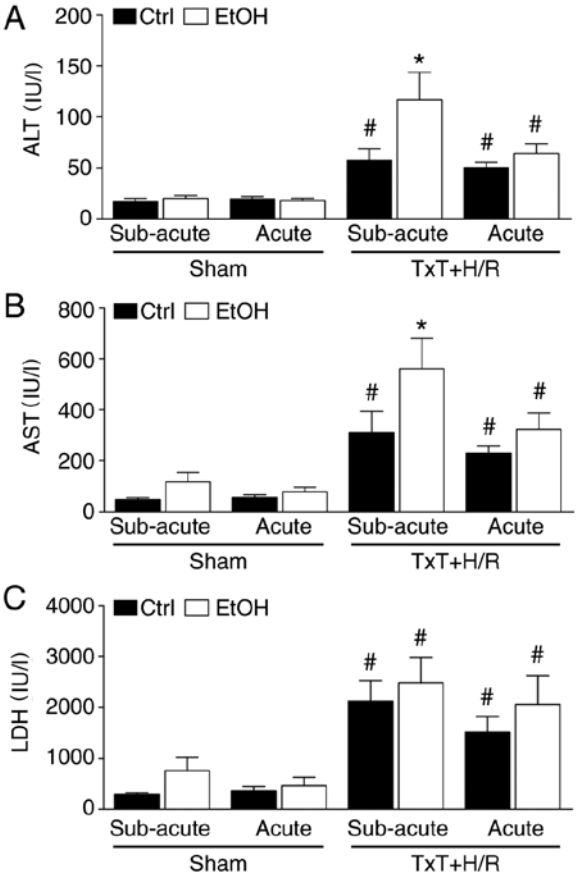

Figure 5. Cell and liver damage. (A) ALT, (B) AST and (C) LDH levels are shown. At 12 h (sub-acute) or 2 h (acute) before the experiment, female Lewis rats received a single oral dose of EtOH or saline (ctrl), followed by TxT + H/R. The sham group underwent all surgical procedures without TxT + H/R. *P<0.05 vs. all groups; #P<0.05 vs. sham group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; EtOH, alcohol; ctrl, control; TxT, blunt chest trauma; H/R, hemorrhagic shock with resuscitation.

sub-acute ctrl group (137.8±12.8 vs. 212.6±10.3 mg/dl, respectively, P<0.05). There was no significant difference between the acute EtOH group and the acute ctrl group.

Survival analysis. Survival was assessed after 72 h. All animals in the sham groups survived. TxT + H/R led to significantly lower survival rates in the sub-acute ctrl and acute EtOH TxT + H/R groups compared with the sham groups (P<0.05), whereas the number of animals that died did not differ significantly among the trauma groups (sub-acute TxT + H/R ctrl vs. EtOH: 4/8 (50%) vs. 2/8 (25%); acute TxT + H/R ctrl vs. EtOH: 2/8 (25%) vs. 4/8 (50%) (Fig. 7).

Discussion

In the present study, the influence of alcohol in an *in vivo* double-hit model of blunt chest trauma and hemorrhagic shock was investigated, with particular interest in the liver inflammatory response, organ damage and overall mortality. Sub-acute and acute exposure to alcohol induced significant anti-inflammatory changes in the local immune response to trauma/hemorrhage. However, histological analysis of the liver tissues revealed that no protective influence of alcohol was evident. In addition, increased damage of the liver was present upon sub-acute exposure to alcohol. Of note, increased tissue damage was associated with an enhanced presence of IL-10-expressing cells in the liver. These findings were paralleled by an increased accumulation of fat in the liver and a significant hypoglycemia. However, overall mortality rates among did not differ significantly among groups following trauma/hemorrhage.

The liver is susceptible to the development of organ damage following hemorrhage (2,30) and, due to its potent immunomodulatory potential, has an important role in the outcome of patients. The negative effects of chronic alcohol consumption on the liver are well known, whereas the effects of acute alcohol consumption on the liver and patient outcomes in trauma patients constitute an interesting subject of intensive research due to the currently controversial results. In daily routine clinical practice, patients frequently present either with acute alcohol intoxication, with residual alcohol levels following evening consumption, or with chronic alcohol intoxication. Consequently, in the present study, an experimental setting covering the two most likely scenarios of acute alcohol consumption, acute 'drink and drive scenario' and sub-acute 'evening binge drinking' (17), was used. In our previous study, the influence of sub-acute alcohol intoxication on rats with hemorrhagic shock was examined, in which significantly higher ALT values were found compared with those in the control group during the time course, with a later decrease in ALT (2). The increased liver damage that was observed underlying sub-acute alcohol consumption was in line with the results of Hu *et al* (31). Rats received 5 g/kg i.v. EtOH followed by volume-controlled hemorrhagic shock, and the highest levels of AST and ALT were found in the intoxicated hemorrhagic shock group (31). However, in our previous study, sub-acute alcohol consumption has also exhibited hepatoprotective potential in an *in vivo* model of isolated hemorrhagic shock (2). These data are not fully in line with the results of the present study, however, there is an important difference in the model used. In the present study, the additional influencing factor of TxT was included in order to mimic a more realistic clinical scenario, as severe bleeding does not occur in isolation but rather occurs in combination and as consequence of additional tissue trauma. In terms of such combinatory trauma models including alcohol intoxication, limited data exist and, to the best of our knowledge, no studies have been performed regarding specifically blunt chest trauma and hemorrhagic shock with previous alcohol intoxication. A study by Desiderio examined the influence of intragastric and intravenous EtOH application in blunt cardiac trauma *in vivo*, and found significantly higher mortality rates in the intoxicated group, which was caused by electrical-mechanical dissociation (32,33). However, liver damage was not addressed in their study. In the present study, significant liver damage was detected in the NaCl and the EtOH groups. Of note, the highest levels of ALT and AST were found in the sub-acute EtOH group. This group also exhibited the most pronounced damage histologically, which was higher than in the corresponding reference group following trauma/hemorrhage. A trend in increased transaminase values and liver damage was also observed in the acute EtOH group, although the difference with the corresponding reference group following trauma/hemorrhage

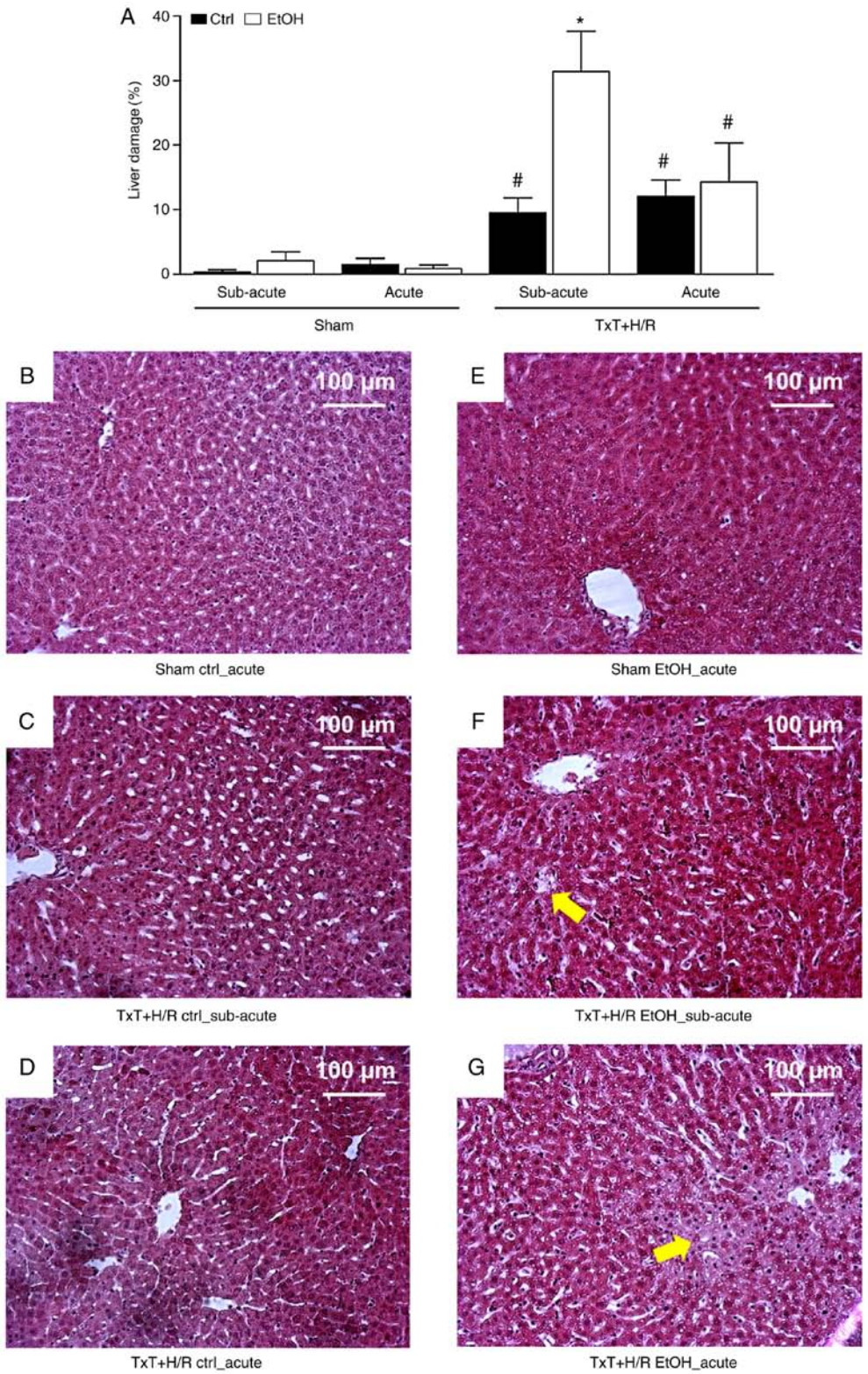

Figure 6. Liver damage. (A) Liver damage in the treatment groups. At 12 h (sub-acute) or 2 h (acute) before the experiment, female Lewis rats received a single oral dose of EtOH or saline (ctrl), followed by TxT + H/R. The sham group underwent all surgical procedures without TxT + H/R. *P<0.05 vs. ctrl TxT + H/R; #P<0.05 vs. sham group. Representative liver sections (10X objective) of the (B) sham ctrl acute, (C) sham EtOH acute, (D) TxT + H/R ctrl sub-acute, (E) Txt + H/R EtOH sub-acute groups, (F) TxT + H/R ctrl acute and (G) TxT + H/R EtOH acute groups are shown. Scale bar, 100 μm. Cell enlargement and nuclear dissolution in hepatocytes as signs of necrosis were more pronounced in the TxT + H/R groups than in the sham groups. Arrows point to exemplary necrotic regions. EtOH, alcohol; ctrl, control; TxT, blunt chest trauma; H/R, hemorrhagic shock with resuscitation.

was not prominent. A possible explanation for the contradictive results regarding the potentially hepatoprotective effects shown in previous studies and the aggravation of liver damage as represented shown in the present study following sub-acute EtOH consumption may lie in the increased trauma severity. The present model, with the addition of TxT over isolated

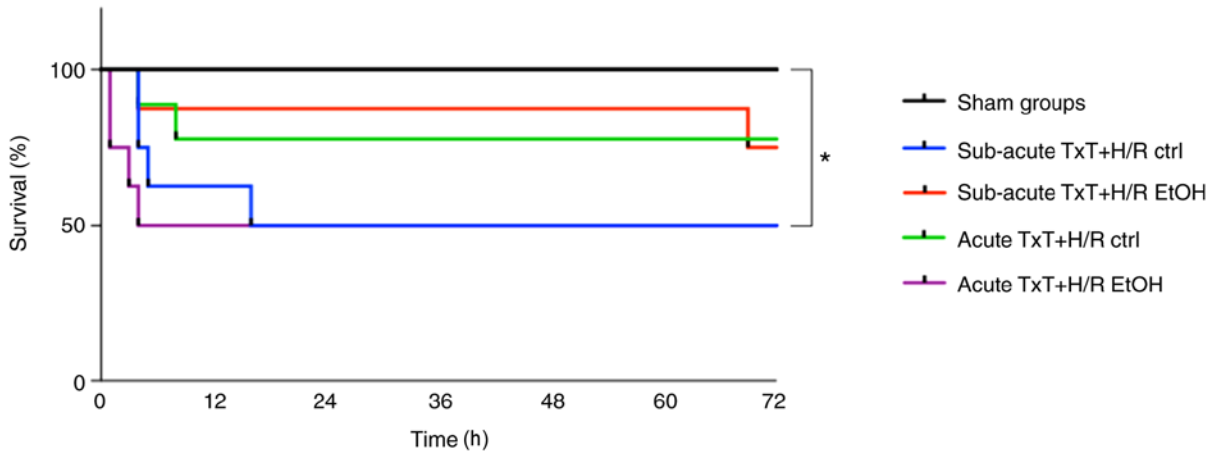

Figure 7. Survival of the experimental groups. At 12 h (sub-acute) or 2 h (acute) before the experiment, female Lewis rats received a single oral dose of EtOH or saline (ctrl), followed by TxT + H/R. The sham group underwent all surgical procedures without TxT + H/R. *P<0.05 vs. indicated groups. EtOH, alcohol; ctrl, control; TxT, blunt chest trauma; H/R, hemorrhagic shock with resuscitation.

hemorrhagic shock, may cause an increased vulnerability of the total organism to a trauma/hemorrhage-induced immune response and subsequent tissue damage. In addition, the influence of trauma severity on the metabolism of EtOH elimination may be a possible explanation. However, the exact relevance of trauma severity and the timing of alcohol consumption on the difference between the EtOH trauma groups remains to be elucidated in future investigations. The following inflammatory changes may provide an explanation approach.

The impact of TxT and H/R on the immune response has been addressed previously. Seitz *et al* examined the inflammatory response in a model of TxT, hemorrhagic shock or the combination of both (34). The highest levels of TNF-α, IL-6 and IL-10 were found in Kupffer cells in the trauma/hemorrhage group (34). Similarly, in the present study, a profound inflammatory reaction in the liver with elevated gene expression levels of IL-6, IL-1β and ICAM-1 was found in TxT + H/R control groups. By contrast, either sub-acute or acute exposure to EtOH markedly attenuated pro-inflammatory gene expression following TxT + H/R, data that is consistent with our previous report addressing the immunosuppressive potential of acute alcohol consumption following isolated hemorrhagic shock (2). In general, findings regarding the immunosuppressive effect of alcohol are contradictory. Sato *et al* investigated the dose-dependent influence of alcohol in an *in vivo* setting of hemorrhagic shock, and found no significant difference between the alcohol groups and the control group regarding plasma levels of TNF-α and IL-1β (35). They identified increased mortality in the alcohol groups, which was associated with the inhibition of norepinephrine, epinephrine and vasopressin (35). As described above, Hu *et al* observed elevated serum levels of TNF-α and IL-6 in a group of alcohol-intoxicated animals compared to a control group following hemorrhagic shock (31). Mathis *et al* also investigated the influence of alcohol upon hemorrhagic shock and detected an attenuation of the trauma-induced increase in lung IL-6 and TNF-α in the alcohol group, although no alterations in inflammatory parameters were observed in the spleen or in lung IL-1 and IL-10 (36). When interpreting these results, the limited comparability of the studies due to model differences must be considered. The experiments differ in terms of the alcohol dose, but also in the timing and the mode of administration. Trauma severity and the analyzed inflammatory parameters also differ. However, the anti-inflammatory potential of alcohol consumption in the underlying model has also been confirmed by reduced protein expression of TNF-α and IL-1β following trauma/hemorrhage. In accordance to these changes, the sub-acute EtOH group in the present study exhibited the highest level of IL-10-positive cells in response to trauma. The marked anti-inflammatory potential of sub-acute EtOH consumption was evidently not only limited to local liver response, but was also exerted in the reduced systemic release of TNF-α upon LPS stimulation. In the acute setting of EtOH consumption, the TxT + H/R-induced inflammatory response reduction was limited to the gene expression level and did not markedly alter IL-10-positive cells in the liver or systemic TNF-α release. These differences between the two EtOH TxT + H/R groups may explain why the tissue damage was differentially modulated in these groups. Miller *et al* examined the inflammatory reaction and liver damage in IL-10-knockout mice in a setting of alcoholic and non-alcoholic steatohepatitis; and observed increased liver inflammation, as expected (37). These results are in line with the findings of the present study, showing that the enhanced presence of IL-10-expressing cells is closely associated with the reduced expression and release of pro-inflammatory markers. Additionally, IL-10-knockout mice exhibited less steatosis and hepatocellular damage following alcohol exposure, an effect that is underlined by the findings of the present study showing damage to tissue was higher with a higher presence of IL-10-expressing cells. Furthermore, increases in IL-6 and hepatic signal transducer and activator of transcription 3 (STAT3) have been shown in IL-10-knockout mice. In double knockout mice with additional elimination of IL-6 or STAT3, liver damage and steatosis were re-established (37). Therefore, it was concluded that the protective effects on the liver were associated with hepatic IL-6/STAT3 activation, and that the inflammatory balance between liver-protective and harmful cytokines determines the effect in a positive or negative

direction (37). Previous studies have already documented that pro-inflammatory cytokines TNF-α and IL-6, but also anti-inflammatory cytokines, including IL-10, are important in promoting liver regeneration (38-43). The data obtained in the present study does not reject the hypothesis that IL-10, as an anti-inflammatory cytokine, may negatively regulate liver regeneration via suppressing the pro-inflammatory response. Therefore, IL-10 may act as a repressor of tissue regeneration, which may have led to the observed differences among the two EtOH groups with regard to tissue damage following TxT + H/R. The exact role of IL-10 remains to be further elucidated.

Regarding the underlying mechanism, the activation of NF-κB serves a key role in the production, regulation and release of cytokines following trauma (7-9). In a previous *in vitro* study, it was confirmed that the anti-inflammatory effects of acute alcohol exposure are regulated via the canonical NF-κB pathway (44). In addition, Mandrekar *et al* confirmed the influence of alcohol on NF-κB and its subunits (45). By contrast, in the setting of chronic alcohol exposure, an increased activation of NF-κB has been detected, combined with the release of pro-inflammatory cytokines (46). In addition, Wang *et al* reported the augmented activation of NF-κB in patients with human hepatocellular carcinoma and chronic alcohol consumption (47). It appears that acute alcohol exposure leads to the inhibition of NF-κB, in contrast to chronic alcohol exposure. There are two questions that remain unanswered. The first is whether acute and sub-acute exposure to alcohol potentially both reduce NF-κB, and if this is the case, whether the exposure strategy influences NF-κB signaling in the same way regarding the canonical or non-canonical pathway. The answers to these questions may be key to explaining the present data, and requires elucidation in further investigations using specific inhibitors of the canonical and non-canonical NF-κB pathway.

In addition to the above-mentioned anti-inflammatory potential of EtOH, the sub-acute group exhibited low blood glucose levels. Hypoglycemia can continue 24 h after alcohol consumption, therefore, it is reasonable that this effect was observed in the sub-acute group. This may be a result of the liver having to remove EtOH from the blood, rather than managing blood glucose levels, or by the body increasing insulin release in response to acute alcohol consumption.

In terms of the limitations of the present study, it is important to mention that clinical reality can only be reproduced in a reduced form using *in vivo* experimentation. Existing medication and co-existing disease have important effects on daily routine in the treatment of trauma patients, and the possible influence of anesthesia in the frame of the model should be considered. Furthermore, in the present experimental setting, sacrifice was performed 2 h following the end of experimentation with subsequent removal of organs and blood, or survival was assessed for 72 h. Collecting the blood at the defined time of sacrifice grants group comparability, however, the dynamic development of values is not represented. Furthermore, gene and protein expression from all parameters were not consistently measured. In addition, regeneration of the liver and the influence of alcohol on NF-κB were not elaborated and require further investigation. Finally, a high alcohol dose was used; it is important in interpreting results to be aware that alcohol and its metabolites may act dose-dependently. Therefore, a focus on ethanol metabolism is required in future studies, particularly regarding the extent to which elimination is influenced through trauma severity. To mimic clinical reality in more detail, a fracture and a traumatic brain injury may be added to the model in the future, as clinical data show that TxT is not the leading injury in patients intoxicated with alcohol (48-50). In addition, if applying a multiple trauma model within a 'drink-and-drive' scenario, fracture should be applied additionally, as applying brain injury to this model is difficult. In the present study, two factors were crucial for selecting an experimental setting with TxT. First, our long standing established hemorrhagic shock model required further expansion, and adding more than one additional injury type risks the misinterpretation of results as it would be unclear which of the additional injuries caused the different results compared with previous studies. Second, due to our particular interest on post-traumatic inflammation, additional injury with a marked influence on the immune system was selected. Seitz *et al* and Weckbach *et al* underlined the key role of TxT regarding the induction of the immune response (34,51). However, the limitations described above must be addressed in future studies.

In conclusion, the data obtained in the present study indicate that the severity of liver damage may be dependent on the timing of alcohol intoxication, severity of trauma and/or the balance between pro- and anti-inflammatory responses. The data clearly indicate the requirement for further investigations regarding metabolic changes and the relevance of the pro- and anti-inflammatory balance for tissue susceptibility to injury, and a greater number of animals per group are required to evaluate mortality.

Acknowledgements

The authors would like to thank Mrs. Katrin Jurida, Mrs. Kerstin Kontradowitz and Mr. Alexander Schaible from the Department of Trauma, Hand and Reconstructive Surgery, University Hospital Frankfurt, Goethe University, for their technical assistance.

Funding

The study was supported by grants from the Deutsche Forschungsgemeinschaft (grant nos. DFG RE 3304/5-1 and PE 908/3-1).

Availability of data and materials

The authors can provide relevant data on request.

Authors' contributions

NW performed the experiments, wrote the manuscript and contributed to the organization of the project. SD and NF contributed to the experimental procedure. KK performed the histological analysis. MP contributed to the funding and intellectual realization. IM contributed to the intellectual realization and the final manuscript. BR was the head of the project, performed the statistical analysis and contributed to the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Veterinary Department of the Regional Council in Darmstadt, Germany (Regierungspraesidium Darmstadt, Veterinaerswesen', Hessen, Germany) and designed in accordance with the ARRIVE guidelines (18).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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