

Original Article

Necrostatin-1 protects hepatocytes in rats with traumatic hemorrhagic shock via attenuating necroptosis

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Abstract: Objective: This study was designed to investigate the protecting mechanism of Nec-1 and identify how necroptosis triggers inflammation. Methods: This study used male Sprague-Dawley rats with traumatic hemorrhagic shock (mean blood pressure maintained at about 35 to 40 mmHg for 90 minutes) followed by fluid resuscitation. Rats in the control group received anesthesia and separation and ligation of blood vessels but not traumatic hemorrhagic shock and reperfusion. Rats in the Nec-1 group received 1 mg/kg Nec-1 5 minutes before reperfusion, while rats in the vehicle group received the same amount of solvent (0.5% dimethyl sulfoxide). Eight animals were sacrificed at 2 hours, 8 hours, 16 hours, and 24 hours post-reperfusion in each group. Results: Nec-1 ameliorated liver traumatic hemorrhagic injury, as indicated by lower serum aminotransferase levels, lower hepatic inflammatory cytokines (TNF- α and IL-1 β), and less severe traumatic hemorrhage-associated histopathologic changes. Traumatic hemorrhagic shock induced necroptosis in the liver, as indicated by an increase of RIP3, was inhibited by Nec-1. Furthermore, traumatic hemorrhagic shock increased the expression of damage-associated molecular patterns (DAMPs), whereas Nec-1 treatment decreased the release of DAMPs. Conclusions: The protective role might result from attenuation of proinflammatory responses (TNF- α and IL-1 β) and DAMPs (HMGB-1) in the liver.

Keywords: Damage-associated molecular patterns, hemorrhage, inflammation, necroptosis, Necrostatin-1, receptor-interacting protein 3

Introduction

Traumatic hemorrhagic shock (THS) is the leading cause of death for persons younger than 45 years in the United States [1]. The liver, a highly vascularized organ, is one of the organs most affected by THS; its central role in metabolism and homeostasis makes it a critical one for survival after severe injury [2, 3]. The extent of hepatic dysfunction reflects the severity of organ injury and is associated with morbidity and mortality [4]. Both apoptosis and necrosis can be found in the liver of rats with THS [5]. In 2005, Degterev et al [6] demonstrated that the Fas/TNFR receptor family can activate a common nonapoptotic death pathway in the absence of intracellular apoptotic signaling, a process they termed *necroptosis*. They also identified a specific potent small-molecule inhibitor of necroptosis, Necrostatin-1 (Nec-1), which can inhibit RIP1 kinase activity and necroptosis after ischemic brain injury [6, 7].

Necroptosis was used to denote necrotic cell death dependent on receptor-interacting protein kinase 3 (RIPK3) [8]. At the molecular level, necroptosis can be triggered by death receptors [9, 10], cell-surface Toll-like receptors [11], DNA-dependent activators of interferon regulatory factors (DAI) [12], and probably, other signals. Necroptosis leads to rapid plasma membrane permeabilization and the release of cell contents and exposure of damage-associated molecular patterns (DAMPs) [13]. Release of DAMPs enables cells to trigger inflammation.

Recent studies have demonstrated that necroptosis is of central pathophysiological relevance in ischemia-reperfusion injury [14, 15] and that Nec-1 can protect cells via attenuation of necroptosis. Based on these findings, we hypothesized that Nec-1 could protect against liver THS injury by reducing necroptosis, a process that could be regulated by RIP3. In this study, we aimed to investigate the protective

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Table 1. Characteristics of primers of selected genes

| Gene | Forward | Reverse |
|----------------|-----------------------------|--------------------------|
| TNF- α | 5'-GTTTCATCCGTTCTCTACC-3' | 5'-AGCGTCTCGTGTGTTTC-3' |
| IL-1 β | 5'-AGCGGTTTGTCTTCAAC-3' | 5'-TCAACTATGTCCCGACC-3' |
| β -actin | 5'-GCGTGACATTAAGAGAAGCTG-3' | 5'-AGAAGCATTGCGGTGCAC-3' |

mechanism of Nec-1 and identify how necroptosis triggers inflammation.

Materials and methods

Animal preparation

Ninety-six adult male specific antigen-free Sprague-Dawley rats weighing 250 ± 20 g, on average, obtained from the Academy of Military Medical Sciences Laboratory Animal (Beijing, China) were used in this study. All animal experiments were performed according to the guidelines of the Animal Welfare Act and The Guide for Care and Use of Laboratory Animals from the National Institutes of Health. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Tianjin First Center Hospital. All rats were caged individually in the animal house with controlled humidity $55\% \pm 1\%$, temperature $25 \pm 1^\circ\text{C}$, and lighting (light-dark cycle every 12 hours). The animals were given water and a basal diet, and they were allowed 1 week to adapt to the environment. Animals were fasted overnight but allowed free water access before the experiment. The rats were randomly classified into a control group, a vehicle group, and the Nec-1 group, with 32 rats in each group.

Experimental procedures

Rat TSH model: The rats were anesthetized via isoflurane inhalation (Baxter, Deerfield, MA, USA) and fixed on the operating table, and then a 5-cm midline laparotomy was performed to induce soft-tissue trauma. The abdominal wound was closed in layers after reperfusion. Polyethylene catheters were placed in the right femoral vein and the right carotid arteries via right inguinal and neck incision wounds (about 1 cm long). The wounds were bathed with 1% lidocaine (Otsuka Pharmaceuticals, Tianjin, China) throughout the operative procedure to reduce postoperative pain. About 0.1 to 0.2 mL 1% lidocaine was directly dropped into the operative wounds, including the midline abdo-

men and inguinal and neck areas. The animals were bled rapidly in 10 minutes to a mean arterial pressure of 35 to 40 mmHg. The mean pressure of 40 mmHg was maintained by removing more blood or infusing blood for 90 minutes. The volume of shed blood and Ringer's lactate was then infused for 60 minutes with a syringe pump. After reperfusion, all catheters were removed, and the wounds were closed in layers.

Drug administration

Rats in the control group received anesthesia and separation and ligation of blood vessels without THS and reperfusion. Rats in the Nec-1 group received 1 mg/kg Nec-1 (Sigma-Aldrich, St. Louis, MO, USA) through the femoral vein 5 minutes before reperfusion, while the rats in the vehicle group received the same volume of solvent. Nec-1 was dissolved in 0.5% dimethyl sulfoxide (DMSO) (Amresco, Solon, OH, USA). There were 32 rats in each group. Each group was classified into four subgroups after reperfusion (2 hours, 8 hours, 16 hours, and 24 hours).

Measurements

After reperfusion, eight rats in each group were humanely euthanized at 2 hours, 8 hours, 16 hours, and 24 hours after reperfusion. Five mL of blood were collected from the abdominal aorta using a disposable sterilized syringe, and then the blood samples were immediately centrifuged at 3000 revolutions per minute for 15 minutes. The plasma samples were collected and stored at -80°C . Part of the liver was fixed in 4.5% buffered formalin, and part was fixed in transmission electron microscopy fixative. Part of the liver was collected in microtubes and stored at -80°C .

Liver damage assessment

To assess the hepatocellular injury after THS, the serum alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were measured using the Automated Chemical Analyzer (Bayer, Leverkusen, Germany).

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Table 2. Comparisons of mortality among all groups

| | Survival results | Shock stage | 24 hours after reperfusion | Mortality rate |
|---------------|------------------|-------------|----------------------------|----------------|
| Control group | 32 | 0/32 | 0/32 | 0 |
| Vehicle group | 55 | 8/55 | 15/55 | 41.82% |
| Nec-1 group | 49 | 7/49 | 10/49 | 34.69% |

Histopathology

The liver tissue was fixed in 4.5% buffered formalin. Paraffin embedding was performed using standard techniques. Sections (4 μ m) were stained with hematoxylin and eosin and assessed for tissue damage.

Transmission electron microscopy

For electron microscopy, the liver tissues were fixed. Ultrathin sections were cut using a microtome and stained with uranyl acetate and lead citrate for viewing under an electron microscope (Tecnai G2 Spirit, FEI, Hillsboro, OR, USA). For quantification, 10 micrographs were randomly taken from each liver sample, and organelle structure was observed.

Enzyme-linked immunosorbent assay

Levels of high-mobility group protein B (HMGB)-1 in the serum were quantified using specific enzyme-linked immunosorbent assay (ELISA) kits for rats, according to the manufacturer's instructions (Biovol, Shanghai, China).

Western blot

A Western blot was performed to detect RIP3 and HMGB-1. Cytosol protein and total protein were extracted using a cytosol protein extraction kit (Boster, Wuhan, China) and total protein extraction kit (Solarbio, Beijing, China) according to the manufacturer's instructions. Equal amounts of protein (20 μ g) were separated by 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% nonfat milk for 2 hours at room temperature and then incubated overnight at 4°C with primary antibodies against RIP3 (1:400) (Santa Cruz Biotechnology, Dallas, TX, USA) and HMGB-1 (1:6000) (Abcam, Cambridge, UK).

After washing in tris-buffered saline 3 times for 10 minutes, the blots were incubated with

horseradish peroxidase-conjugated secondary antibodies (1:500) (Raleigh, NC, USA) for 2 hours at room temperature. An enhanced chemiluminescence detection kit (Merck Millipore, Temecula, CA, USA) was applied to detect immunoreactive bands, according to the manufacturer's instructions. GAPDH (1:1000) (R&D Systems, Minneapolis, MN, USA) was used as an internal control. The densities of bands on Western blot were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-PCR) was performed to detect TNF- α mRNA and IL-1 β mRNA. Total RNA was extracted from liver tissue with the Trizol kit (Takara Bio, Dalian, China). Complementary DNA (cDNA) synthesis was performed with a cDNA synthesis kit (Takara Bio, Dalian, China). PCR amplification was performed on an ABI 7300 quantitative PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The thermal cycling conditions consisted of a 5-minute template denaturation step at 95°C followed by 40 cycles at 95°C for 30 seconds and 60°C for 20 seconds. β -actin (Takara Bio, Dalian, China) was used to normalize gene expression. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method using samples from the control group as a calibrator (for primer sequences, see **Table 1**).

Statistical analysis

The data are expressed as mean \pm SD. Differences between groups were evaluated for significance using a one-way analysis of variance combined with an LSD post hoc test. All tests were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). A *P*-value below 0.05 was considered statistically significant.

Results

Nec-1 prolongs survival after THS

THS-induced necroptosis in the liver: The mortality of all group is shown in **Table 2**. Serum levels of ALT and AST were significantly lower at 8 hours, 16 hours, and 24 hours in the Nec-1 group than in the vehicle group ($P < 0.05$) (**Figure 1**). Nec-1 pretreatment decreased the eleva-

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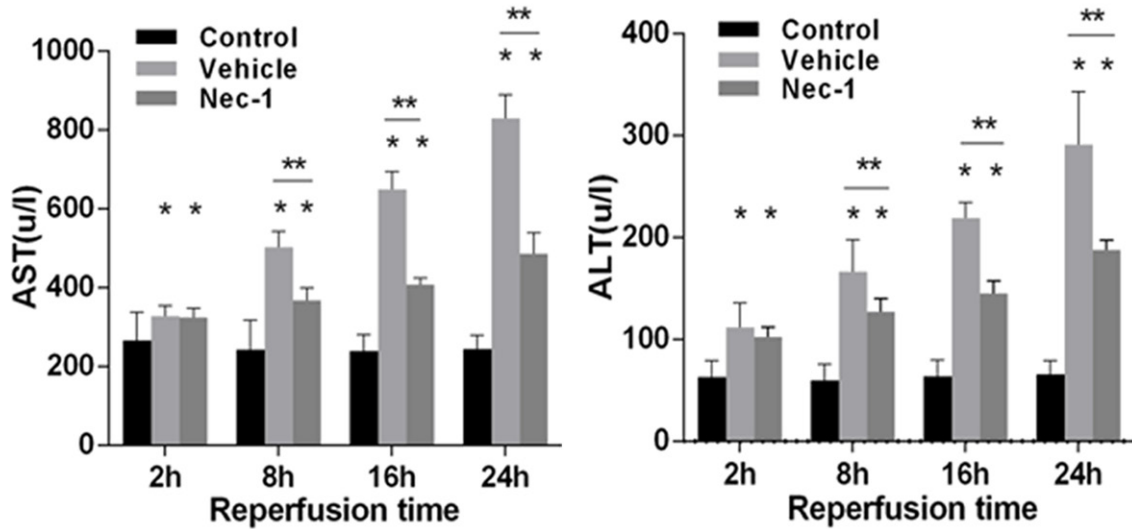


Figure 1. Serum alanine transaminase and aspartate aminotransferase levels were analyzed as a measure of hepatocellular injury. Data are shown as mean \pm SD (n = 8 per group). * P <0.05, significantly different from the control group, ** P <0.05, significantly different from the vehicle group.

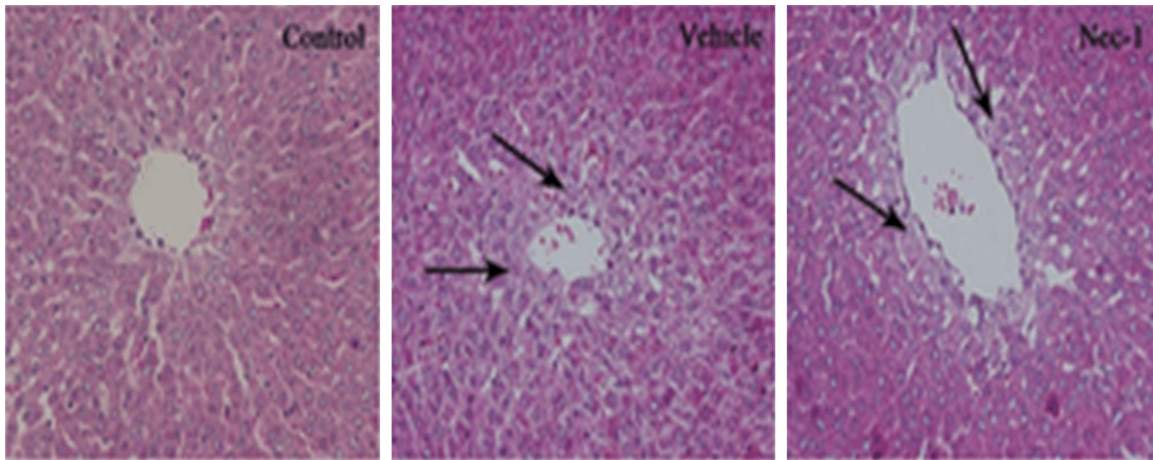


Figure 2. Routine histopathology was performed on formalin-fixed liver sections obtained from rats subjected to traumatic hemorrhagic shock 24 hours after reperfusion (original magnification, \times 400). The arrows denote hepatocellular necrosis. Representative images from 8 rats per group were selected.

tion of ALT and AST in THS rats. To assess cell necrosis in the liver, liver sections were stained with hematoxylin and eosin (Figure 2). Twenty-four hours after reperfusion, cell necrosis appeared throughout the liver. Apoptosis and necrosis could be found in the same liver sections (Table 2).

To eliminate the effect of apoptosis, TUNEL-Stained (Roche, Basel, Switzerland) was used to assess hepatocellular apoptosis in the liver. We used an immunohistochemical score to evaluate apoptosis degree [16]. The TUNEL

analysis showed that there was no significant difference between the vehicle and Nec-1 groups (Figure 3). For further study, transmission electron micrography (TEM) was used to observe organelle structure. The TEM analysis showed that Nec-1 pretreatment prevented THS-induced necrosis in the liver 24 hours after reperfusion (Figure 4). RIP3 was the main biochemical feature in necroptosis [8]. Western blot was used to detect RIP3 in the liver. After reperfusion (2 hours, 8 hours, 16 hours, and 24 hours), expression of RIP3 increased in the vehicle group and the Nec-1 group more than in

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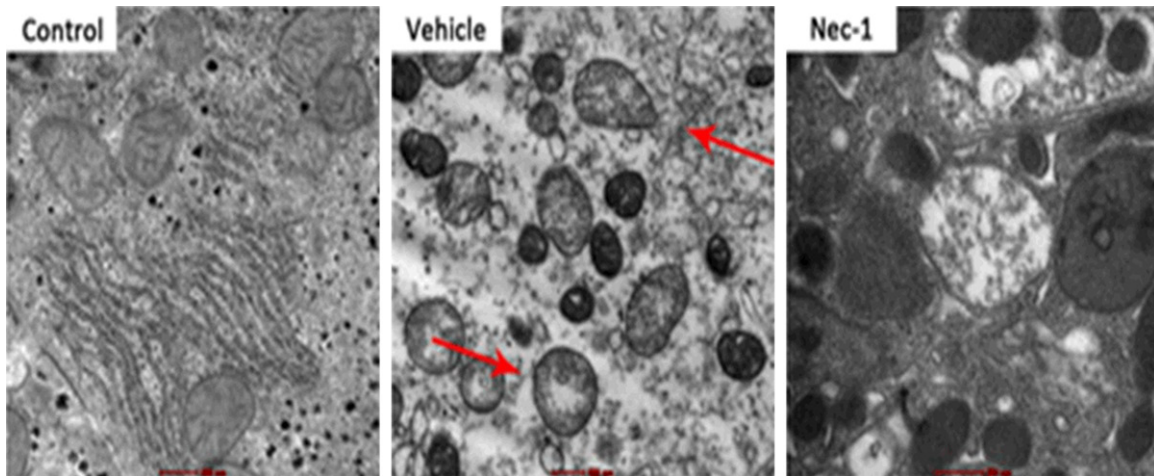


Figure 3. Representative electron microphotographs showing the mitochondria and endoplasmic reticulum in the hepatocyte 24 hours after reperfusion. The arrows denote structural failure of the mitochondria and endoplasmic reticulum. Representative transmission electron microscopic images from 8 per group were selected.

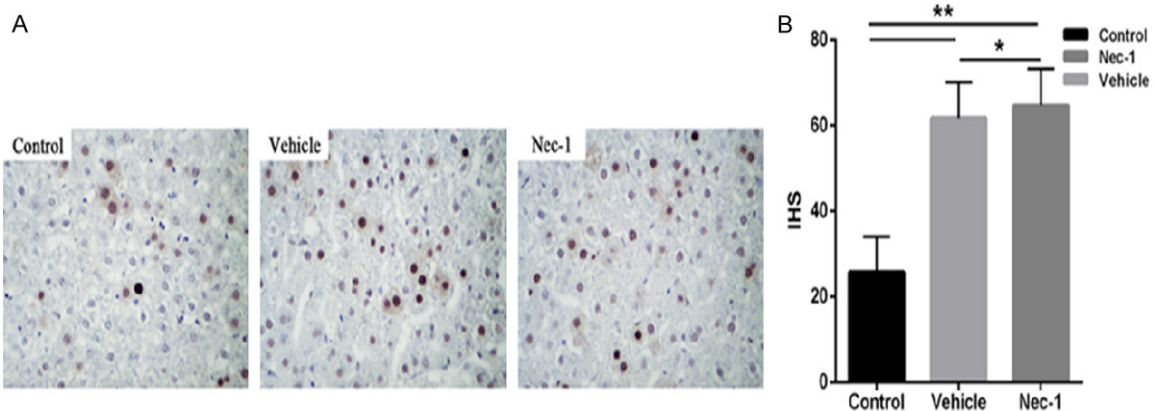


Figure 4. A. Representative images of TUNEL staining in liver 24 hours after reperfusion. IHS was used to evaluate degree of apoptosis. B. Data are shown as mean \pm SD (n = 8 per group). * P <0.05, significantly different from the control group. ** P <0.05, not significantly different from the vehicle group.

the control group (P <0.01) (Figure 5). It also indicated the degree of necroptosis in each group. The analysis showed that RIP3 decreased significantly more in the Nec-1 group than in the vehicle group (P <0.05).

Nec-1 protects hepatocyte during THS

To determine whether Nec-1 could attenuate hepatic injury after THS, PCR was performed for tumor necrosis factor- α (TNF- α) mRNA and interleukin-1 β (IL-1 β) mRNA. TNF- α and IL-1 β are classic inflammatory factors. Expression of TNF- α and IL-1 β increased at 2 hours, 8 hours, 16 hours, and 24 hours after reperfusion in the vehicle group and Nec-1 group compared with the control group (P <0.05) (Figure 6). It indicat-

ed that a serious inflammatory reaction occurred in the liver after THS. In addition, expression of TNF- α and IL-1 β in livers with Nec-1 were lower than in livers without Nec-1 after THS (P <0.05). Furthermore, Western blot was performed to detect HMGB-1 in the liver. HMGB-1 was a classic DAMP. First, we detected HMGB-1 in total protein (Figure 7). Western blot analysis revealed that Nec-1 treatment decreased HMGB-1 expression in response to THS. However, there was no obvious tendency to increase expression. Then Western blot was performed to detect HMGB-1 in cytosol protein. It revealed a rising tendency of HMGB-1 in the cytoplasm (Figure 8). The expression of HMGB-1 reached its peak 16 hours after reperfusion. Compared

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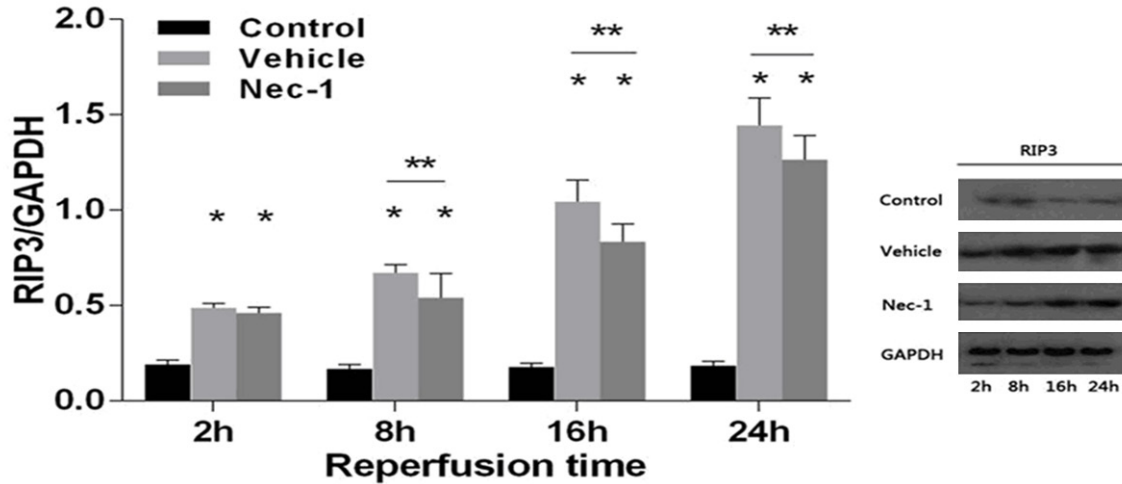


Figure 5. Expression of RIP3 in the liver after traumatic hemorrhagic shock was measured by Western blot analysis at different times after reperfusion to indicate the degree of necroptosis in the liver. Data are shown as mean \pm SD (n = 8 per group). * P <0.05, significantly different from the control group. ** P <0.05, significantly different from the vehicle group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

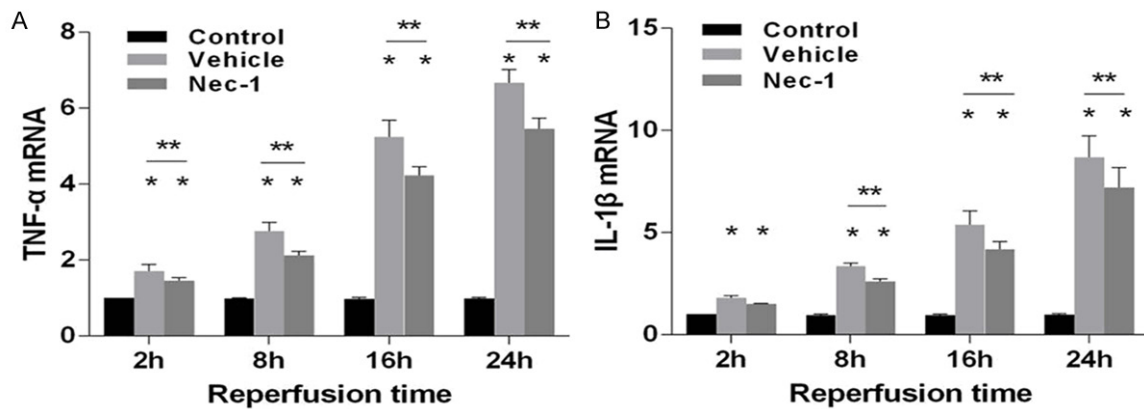


Figure 6. A. Hepatocellular TNF- α and IL-1 β . B. Messenger RNA (mRNA) assessed at 2 hours, 8 hours, 16 hours, and 24 hours after reperfusion in each group. Nec-1 treatment decreased liver expression of inflammatory mediators after THS. Data are shown as mean \pm SD (n = 8 per group). Compared with the control group, * P <0.05, significantly different from the control group, ** P <0.05, significantly different from the vehicle group.

with the vehicle group, Nec-1 treatment decreased the elevation of HMGB-1 in the cytoplasm (P <0.05). Similarly, an ELISA was performed to detect the level of HMGB-1 in the serum (Figure 9). It had acted similarly to the HMGB-1 in the cytoplasm.

Discussion

THS is always a concern because of its high mortality and disability rate. In this study, we demonstrated that that the expression of RIP3, which is increased after THS, can be inhibited by Nec-1 administration, preventing necrotic cell death and reducing liver damage. In addition,

a large number of TNF- α mRNA and IL-1 β mRNA were expressed following reperfusion, and the generation was decreased on Nec-1 administration, accompanied by a significant decrease in the release of HMGB-1. This also decreased the mortality rate over 24 hours.

RIP3 has emerged as a central player in necroptosis [17]. Each RIP family member encodes a unique C terminus. RIP3 has a C-terminal motif termed the *RIP homotypic interaction motif* [18]. RIP3 is recruited to the RIPK1 complex through direct interaction between the RHIM domains of RIPK1 and RIP3. If caspase-8 is inhibited after death receptor activation, RIP3

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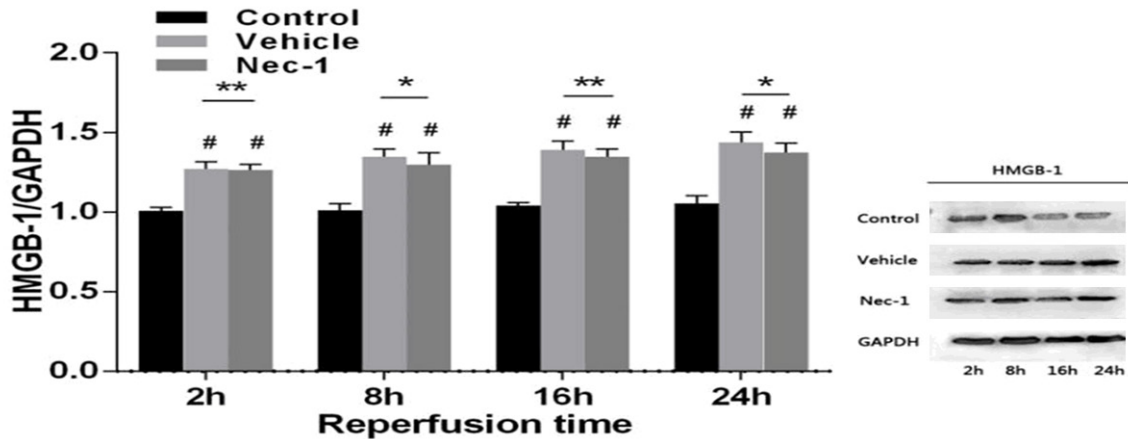


Figure 7. Nec-1 treatment decreased HMGB-1 expression and release after THS injury in total protein. HMGB-1 was measured with Western blot. Data are shown as mean \pm SD (n = 8 per group). # P <0.05, significantly different from the control group. ** P <0.05, significantly different from the vehicle group. * P <0.05, not significantly different from the vehicle group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

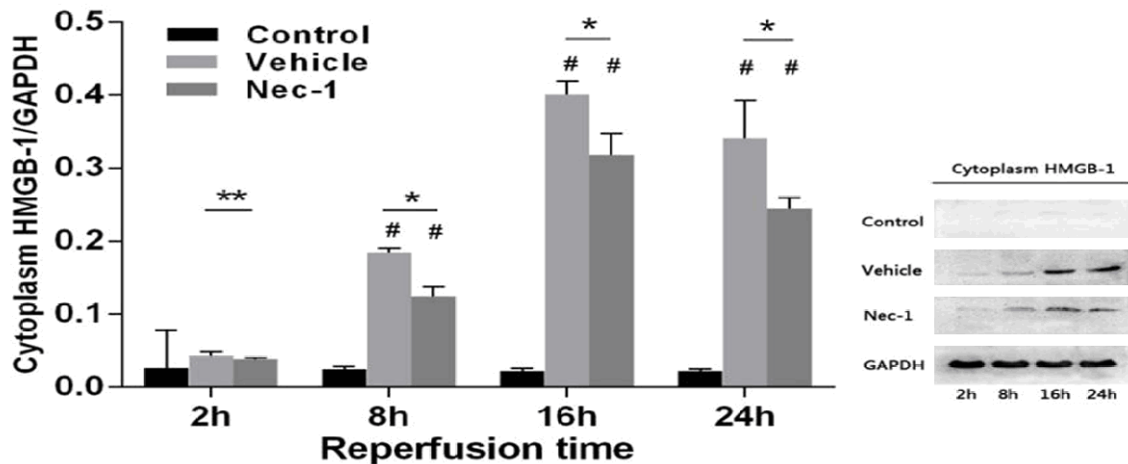


Figure 8. Nec-1 treatment decreased the elevation of HMGB-1 in the cytoplasm, as measured by Western blot. Data are shown as mean \pm SD (n = 8 per group). # P <0.05, significantly different from the control group. ** P <0.05, not significantly different from the control group. * P <0.05, significantly different from the vehicle group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

forms a stable complex with RIPK1/FADD/caspase-8 and transforms it to a pronecrosis complex [19], which leads to formation of Necrosome. Necrosome formation causes necrosis through the mitochondrial pathway, which is mediated by mixed-lineage kinase domain-like (MLKL), recruitment of phosphoglycerate mutase 5 long form (PGAM5L) and PGAM5S (short form), then PGAM5S recruitment of the mitochondrial fission factor Drp1 and activation of its GTPase activity [20].

PGAM5 and Drp1 mediate necrosis induced by mitochondrial fission, ROS, and calcium ionophore. In other words, the expression of RIP3

indicates the degree of necroptosis. As we have shown, Nec-1 treatment significantly reduces the amount of RIP3, suggesting that inhibition of RIP1/3 phosphorylation is clearly involved in Nec-1-induced protection in THS. A previous study showed that Nec-1 treatment reduced the amount of RIP3 in ischemia-reperfusion injury in vivo [14, 15]. We first demonstrated that the expression of RIP3 increased in the liver after THS, and Nec-1 administration reduced the expression of RIP3.

Cells that underwent necroptosis had swelling of organelles and plasma membrane rupture, leading to the release of cell contents and

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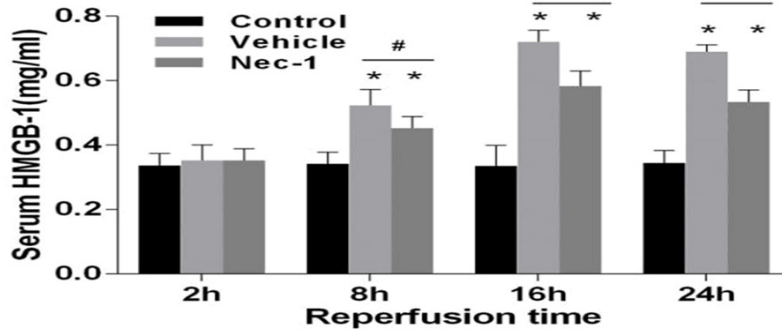


Figure 9. The release of HMGB-1 into serum was quantified with enzyme-linked immunosorbent assay. Data are shown as mean \pm SD (n = 8 per group). # P <0.05, significantly different from the vehicle group. * P <0.05, significantly different from the control group.

exposure of DAMPs [13] and provoking local and systemic liberation of both pro- and anti-inflammatory cytokines such as IL-1 β , IL-2, IL-4, and TNF- α [21, 22]. Thus, severe and developing THS ultimately results in systematic inflammatory response syndrome or multiple organ failure and even death [23]. Therefore, release of DAMPs is significant in the pathophysiology of necroptosis. Our study demonstrated that TNF- α and IL-1 β were passively released after reperfusion and hepatocytes protected by TNF- α and IL-1 β were decreased by Nec-1 administration.

HMGB-1, a classic DAMP, is an abundant component of the cell nucleus, and, when present in the extracellular space, signals tissue damage. Extracellular HMGB-1 has been reported to engage multiple receptors, including the receptor for advanced glycation end products (RAGE) and Toll-like receptors 2 (TLR2) and 4 (TLR4). RAGE has been reported to activate MAPKs. Both RAGE and Toll-like receptors activate NF- κ B [24, 25], leading to enhanced expression of pro-inflammatory cytokines. In earlier research, HMGB-1 was a late mediator of endotoxin lethality [26]. Recent research indicated that HMGB-1 activated the innate immune system in acute sterile inflammation as an early mediator [27, 28]. Our study indicated that the expression of HMGB-1 in cytoplasm increased 2 hours after reperfusion, reaching a peak at 16 hours. It demonstrated that HMGB-1 is released early in rats with THS.

We detected HMGB-1 with Western blot in total protein and the cytoplasm. An obvious temporal trend was found in the cytoplasm after

reperfusion, and a significant temporal trend in total protein was also identified. This finding indicated possible generation mechanisms of HMGB-1: one was release from the nucleus to the cytoplasm (the primary way), and another was synthesis of proteins. Compared with the vehicle group, no significant difference was found in the Nec-1 group at 8 hours and 24 hours after reperfusion in total protein, which demonstrated that Nec-1 has

no effect on synthesis of HMGB-1. The result in the cytoplasm indicated that Nec-1 effectively reduced the release of HMGB-1 from the nucleus to the cytoplasm. In apoptotic cells, HMGB-1 is bound firmly to chromatin because of generalized underacetylation of histone, while it is passively released by necrotic or damaged cells [29]. After the plasma membrane ruptured, HMGB-1 overflowed from the nucleus to the cytoplasm in necrotic cells. Thus, Nec-1 reduced the release of HMGB-1 from the nucleus to the cytoplasm via inhibition of necroptosis.

Conclusions

In conclusion, administration of Nec-1 attenuated hepatocyte impairment in rats with THS by reducing necroptosis. This protective role might result from attenuation of proinflammatory responses (TNF- α and IL-1 β) and DAMPs (HMGB-1) in the liver. Our study suggests that Nec-1 has antinecroptosis effects and may be a potential therapy.

Ethical approval: All animal experiments were performed according to the guidelines of the Animal Welfare Act and The Guide for Care and Use of Laboratory Animals from the National Institutes of Health. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Tianjin First Center Hospital.

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Disclosure of conflict of interest

None.

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