The Protective Effect of Ethyl Pyruvate on Liver Injury in Rats with Hemorrhagic Shock and Its Potential Mechanisms

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Abstract: To investigate the protective effects and underlying mechanisms of ethyl pyruvate (EP) on acute liver injury induced by hemorrhagic shock in rats. An acute liver injury model induced by hemorrhagic shock in rats was established. The intervention was performed with EP, an HMGB1 release inhibitor, to observe its protective effects on this injury. Thirty-two male Wistar rats were randomly divided into four groups: normal control group (Normal group, N=8), hemorrhagic shock group (Shock group, N=8), hemorrhagic shock and resuscitation control group (Shock+actated Ringer's solution group, N=8), and EP intervention group (Shock+EP intervention group, N=8). Rats that died during the experiment were immediately dissected, and their livers were collected. Venous blood samples were collected at 1 hour, 6 hours, and 12 hours after resuscitation. After 12 hours of resuscitation, rats were euthanized, and their livers were collected. Levels of total bilirubin (TBIL), direct bilirubin (DBIL), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in rat serum were determined using an automatic biochemical analyzer. Liver specimens were stained with hematoxylin-eosin (HE) for pathological examination. The levels of acetylated high mobility group protein B1 (AC-HMGB1), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), malondialdehyde (MDA), and the activity of superoxide dismutase (SOD) in liver tissues were detected using ELISA. Compared with the normal control group, rats in the hemorrhagic shock group exhibited significantly elevated levels of TBIL, DBIL, ALT, and ALP in serum ($p<0.05$). Liver tissues showed extensive infiltration of inflammatory cells and localized hemorrhagic necrosis. Expression of AC-HMGB1, IL-6, TNF-α, and MDA in liver tissues was significantly increased ($P<0.05$), while SOD was significantly decreased ($p<0.05$). Compared with the hemorrhagic shock group, rats in the EP intervention group showed significantly decreased levels of TBIL, DBIL, ALT, and ALP in serum ($p<0.05$), along with improved pathological changes in liver tissues. Additionally, expression of AC-HMGB1, IL-6, TNF-α, and MDA in liver tissues was significantly reduced ($p<0.05$), while SOD was significantly increased ($p<0.05$). EP effectively ameliorates acute liver injury in rats induced by hemorrhagic shock, potentially through its inhibition of inflammatory response, antioxidant properties, and reduction of AC-HMGB1 expression in liver tissues.

***Keywords*:** Hemorrhagic Shock; Acute Liver Injury; Ethyl Pyruvate (EP); HMGB1

1. Introduction

Hemorrhagic shock is a significant global health challenge, contributing to approximately 1.9 million deaths annually[1]. The substantial loss of blood in the body results in a marked reduction in effective blood volume within the vasculature, leading to insufficient oxygen delivery to tissues, subsequently causing cellular metabolic disruptions and multi-organ dysfunction[2]. Acute liver injury commonly accompanies hemorrhagic shock and serves as an important indicator of multi-organ dysfunction in affected individuals. Research has highlighted the critical role of HMGB1 in mediating various liver diseases and pathological processes[3], with its acetylation modification being crucial for extracellular release and biological effects[4]. However, the precise molecular mechanisms underlying acute liver injury triggered by hemorrhagic shock remain unclear. Therefore, this study established an acute liver injury model in rats induced by hemorrhagic shock and intervened with ethyl pyruvate (EP) to investigate its impact on liver injury in model rats and explore underlying mechanisms. The objective is to offer new research directions for the treatment of acute liver injury induced by hemorrhagic shock.

2. Experimental animals and grouping

Thirty-two male Wistar rats of clean grade, weighing between 200-250 g, were selected from the Experimental Animal Center of Anhui Medical University. They were provided with standard pellet feed ad libitum and had unrestricted access to water. The ambient temperature was maintained between 22-25°C with a relative humidity of (50±5)%, and a 12-hour light-dark cycle was maintained. The rats were fasted for 12 hours prior to the experiment but were allowed free access to water. The 32 rats were randomly assigned to four groups using a random number table: the Normal Control group (N=8), the

Hemorrhagic Shock Group (Shock group, N=8), the Hemorrhagic Shock and Resuscitation Control Group (Shock+actated Ringer's solution group, N=8), and the EP Intervention Group (Shock+EP intervention group, N=8).

3. Main reagents and instruments

3.1 Primary reagents

EP was sourced from Sigma-Aldrich in the United States. The assay kit for acetylated high mobility group protein B1 was obtained from Shanghai Fusheng Biotechnology Co., Ltd. Malondialdehyde (MDA) assay kit was sourced by Hepeng Biotechnology (Shanghai) Co., Ltd. EDTA stabilizer was purchased from Shanghai Jingke Chemical Technology Co., Ltd. HE staining solution supplied by Aobo Biotechnology Co., Ltd.

3.2 Primary instruments

Experimental instruments include a BS-240 Vet automatic biochemical analyzer (Mindray). TU-1810 UV-visible spectrophotometer (Beijing Persee General Instrument Co., Ltd.). XTL-3400F optical microscope (Shanghai Batuo Instrument Co., Ltd.). Electric constant temperature water bath (Shanghai Bluepard Instruments Co., Ltd.). Centrifuge (Eppendorf, Germany). Multifunctional microplate reader (Thermo Fisher Scientific, USA), along with pipettes of various specifications from Eppendorf in Germany.

4. Experimental method

4.1 Preparing the rat model of hemorrhagic shock

Establishing the rat model of hemorrhagic shock[5]: Rats were secured on a fixed platform in a supine position and anesthetized using 10% chloral hydrate administered intraperitoneally at a dose of 0.3mL/100g. After preparing and disinfecting the neck area, the right carotid artery was exposed through a midline neck incision and cannulated. The cannula was then connected to a pressure sensor and transducer via a three-way connector to facilitate invasive blood pressure monitoring. Electrodes were placed on the limbs of the rats according to the standard limb lead for electrocardiogram (ECG) monitoring. A temperature probe was inserted into the anus of the rats approximately 5cm deep to record rectal temperature. The skin of the thigh on the same side was prepared, and both the femoral artery and femoral vein were isolated. Cannulation was performed in both the arteries and veins, with the cannula for the femoral artery connected to a constant flow pump for blood withdrawal and the cannula for the femoral vein utilized for blood transfusion and drug administration. Animals were kept warm with a warming blanket. Sodium heparin at a dose of 1U/kg was intravenously administered for systemic heparinization.

4.2 Handling of animal models

The rat model of hemorrhagic shock was established by blood withdrawal from the femoral artery, with continuous monitoring of arterial blood pressure to maintain mean arterial pressure within 35-40 mmHg (1 mmHg = 0.133 kPa) for 60 minutes through either continued blood withdrawal or partial blood reinfusion[5]. Apart from abstaining from blood withdrawal and infusion procedures, rats in the normal control group received the same treatment as the hemorrhagic shock group. Specifically, rats in the normal control group underwent no surgical procedures and received no drug treatment before resuscitation; rats in the hemorrhagic shock group underwent all surgical procedures for the hemorrhagic shock model and received no drug treatment during resuscitation at any time point; rats in the EU intervention group (Shock+EP intervention) underwent complete surgery for the hemorrhagic shock model and, during resuscitation, received intraperitoneal injection of 40 mg/kg EU and reinfusion of the withdrawn blood along with an equal volume of normal saline at a rate of <2 ml/min, completing fluid resuscitation within 30 minutes; rats in the hemorrhagic shock and resuscitation control group (Shock+actated Ringer's solution group) underwent all preparation surgeries for the hemorrhagic shock model and, during resuscitation, received intraperitoneal injection of Ringer's acetate solution equivalent in volume to the EP solution and reinfusion of withdrawn blood along with an equal volume of normal saline at a rate of <2 ml/min, completing fluid resuscitation within 30 minutes.

4.3 Specimen collection

Venous blood samples were collected at 1, 6, and 12 hours post-resuscitation. After 12 hours of resuscitation, the rats were euthanized under anesthesia, and liver tissue samples were collected and placed on ice. Approximately 1-2 pieces from the same area of the samples (about 0.5 cm3 each) were fixed in 10% formalin buffer for paraffin sectioning. The remaining liver tissue was stored at -80°C for further analysis.

The animal handling procedures in this study adhered to ethical standards in animal research.

4.4 Tests of the serum liver function

TBIL, DBIL, ALT, and ALP in serum samples from each group were measured using an automatic biochemical analyzer.

4.5 Histological examination of liver tissues by group

Liver tissue sampling → dehydration fixation → embedding in paraffin → slicing, mounting, and drying → staining → sealing → microscopic examination.

4.6 Measurement of AC-HMGB1, TNF-a, IL-6, MDA, and SOD in liver tissues

Liver tissue weighing 100 mg underwent homogenization, and the supernatant was collected. Enzyme-linked immunosorbent assay (ELISA) was employed to determine AC-HMGB1, TNF-a, IL-6, MDA, and SOD in liver tissues.

5. Statistical Analysis

Data analysis was performed using SPSS 25.0, and Rstudio 4.1.2 was used for plotting. The Shapiro-Wilk (SW) test was employed to verify the normality of the data distribution. For normally distributed data, results were presented as mean ± standard deviation ($\bar{x} \pm s$). Repeated measures data underwent repeated measures analysis of variance (ANOVA), followed by LSD-t tests for multiple comparisons. Single-factor analysis of variance was applied for comparisons among multiple groups, with further multiple comparisons conducted using the Student-Newman-Keuls (SNK-q) method. A significance level of $P<0.05$ was considered statistically significant.

6. Results

6.1 Comparison of serum biochemical indices by group

Compared to the normal control group, TBIL, DBIL, ALT, and ALP in the serum from the hemorrhagic shock group exhibited significant elevation ($p<0.05$), with these indices progressively increasing over time ($p<0.05$). Except for the normal control group, the indices just mentioned from other groups continued to rise over time and were mostly significant ($p<0.05$). However, in the hemorrhagic shock and resuscitation control group, there was no significant difference in TBIL and ALT at 1 and 6 hours post-injury and DBIL at 6 and 12 hours post-injury. In the EP intervention group, TBIL exhibited no significant difference at 1 and 6 hours post-injury and at 6 and 12 hours post-injury, while DBIL showed no significant difference at 6 and 12 hours post-injury.

In the comparison of TBIL, DBIL, ALT, and ALP among different groups, the differences were all significant ($p<0.001$). Almost consistently across all time points, the hemorrhagic shock group > the hemorrhagic shock and resuscitation control group > EP intervention group > normal control group, with statistically significant differences ($p<0.05$). However, at 1 hour post-injury, there was no significant difference in TBIL between the hemorrhagic shock group, the hemorrhagic shock and resuscitation control group, and the EP intervention group; at 6 hours post-injury, there was no significant difference in TBIL between the hemorrhagic shock and resuscitation control group and the EP intervention group; at 1 and 12 hours post-injury, there was no significant difference in DBIL between the hemorrhagic shock and resuscitation control group and the EP intervention group.

Additionally, there was an interaction effect of time and group on the changes in serum TBIL, DBIL, ALT, and ALP, indicating different trends in the changes of these liver biochemical indices over time. There was no significant difference in the trend of TBIL changes between the normal control group and the EP intervention group, and no significant difference in the trend of DBIL changes between the hemorrhagic shock and resuscitation control group and the EP intervention group (Table 1 and Figure 1).

Table 1. Changes in serum levels of TBIL, DBIL, ALT, and ALP in each group (x±s, n=8)

Group	n	TBIL			DBIL			ALT			ALP		
		1h	6h	12h	1h	6h	12h	1h	6h	12h	1h	6h	12h
Normal Control Group	8	26.53±4.12	23.14±3.94	25.52±3.08	7.49±0.80	7.91±1.35	6.29±0.87	23.85±1.07	24.84±1.40	23.75±1.32	31.55±0.46	31.39±0.43	31.80±0.44
Hemorrhagic Shock Group	8	40.64±6.20③	59.89±8.59①③	82.77±9.87①②③	12.53±1.58③	18.08±2.76①③	33.88±9.58①②③	64.64±4.02③	70.00±2.45①③	102.44±3.08①②③	50.12±0.53③	51.18±0.54①③	66.10±0.92①②③

Group	n	TBIL			DBIL			ALT			ALP		
		1h	6h	12h	1h	6h	12h	1h	6h	12h	1h	6h	12h
Hemorrhagic Shock and Resuscitation Control Group	8	45.90±6.66③	40.98±4.04③④	63.23±6.20①②③④	10.81±2.00③④	17.29±1.74①③	19.98±5.60①③④	52.82±2.37③④	52.07±2.11③④	79.30±1.82①②③④	44.26±0.59③④	46.63±0.75①③④	49.80±0.52①②③④
EP Intervention Group	8	42.17±4.84③	45.90±10.01③④	50.66±8.04①③④⑤	10.01±1.12③④	11.43±2.53③④⑤	12.78±0.76①②③④	36.89±3.34③④⑤	39.68±1.93①③④⑤	57.73±1.83①②③④⑤	38.05±0.95③④⑤	42.01±0.75①③④⑤	43.72±1.42①②③④⑤
F time/P time		74.622/<0.001			45.637/<0.001			993.386/<0.001			920.427/<0.001		
F group/P group		79.616/<0.001			19.472/<0.001			1604.945/<0.001			3132.452/<0.001		
F interaction/P interaction		23.567/<0.001			59.192/<0.001			143.857/<0.001			276.145/<0.001		

Note: Compared to the 1-hour time point within the group, ① P<0.05; compared to the 6-hour time point within the group, ② P<0.05; compared to the normal control group at the same time point, ③ P<0.05; compared to the hemorrhagic shock group at the same time point, ④ P<0.05; compared to the hemorrhagic shock and resuscitation control group at the same time point, ⑤ P<0.05.

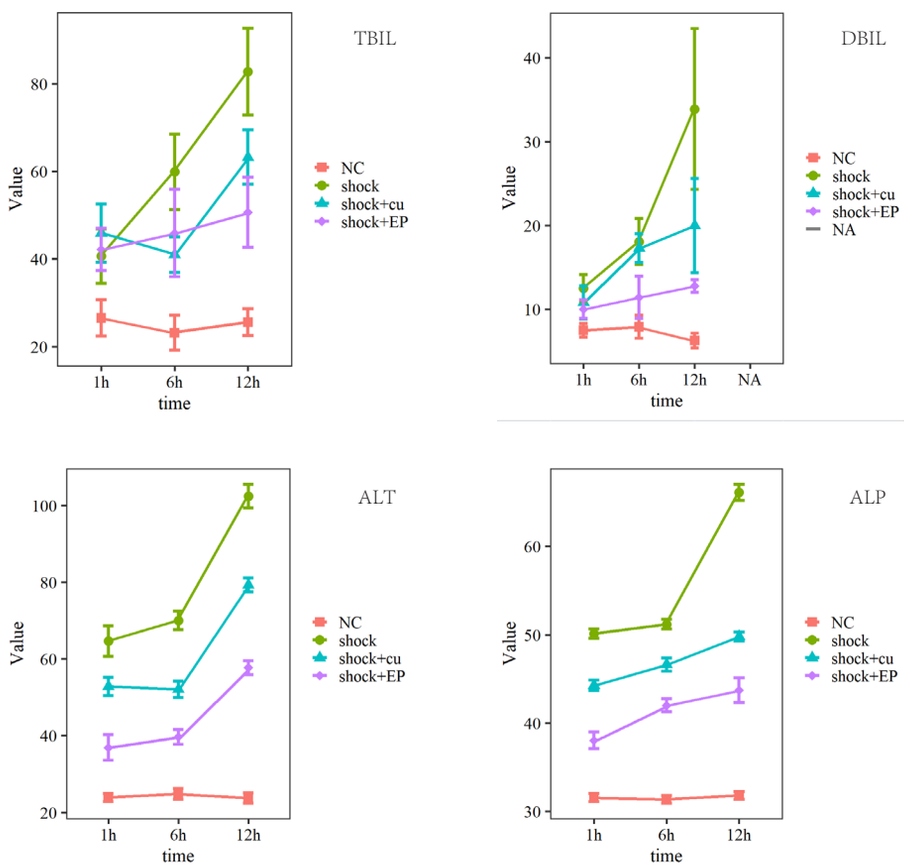

Figure 1. The variations in serum levels of TBIL, DBIL, ALT, and ALP across different groups(note:different trends in the changes of these liver biochemical indices over time)

6.2 Histopathological changes in liver tissue among different groups

In the normal control group, hepatic plates are intact and arranged in single rows, with no signs of inflammatory cell infiltration, apoptosis, or necrosis. The hemorrhagic shock group exhibits extensive liver necrosis, hepatocyte degeneration, and necrosis within hepatic plates, along with widened hepatic sinusoids and increased infiltration of inflammatory cells. Both the hemorrhagic shock and resuscitation control group and the EP intervention group show reduced inflammatory cell infiltration and lessened damage in liver tissues, with the EP intervention group displaying particularly notable effects (Figure 2).

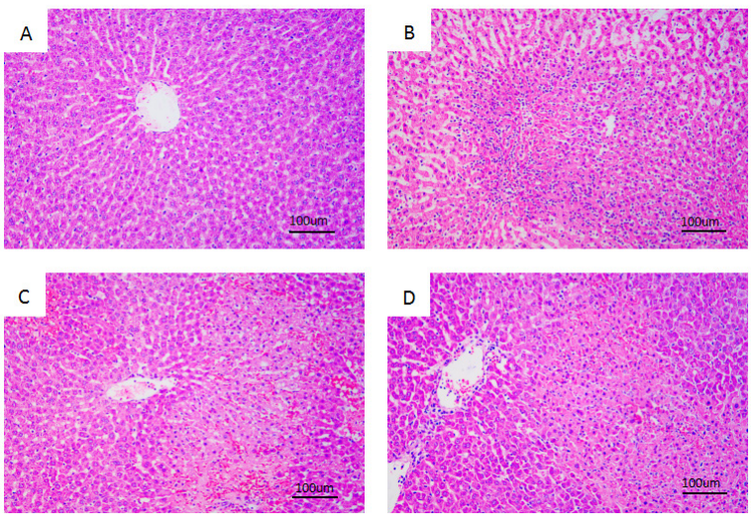

Figure 2. Histopathological slices of liver tissues from different groups (HE×200)

Note: A represents the normal control group, B represents the hemorrhagic shock group, C represents the hemorrhagic shock and resuscitation control group, and D represents the EP intervention group.

6.3 Comparison of AC-HMGB1, TNF-a, IL-6, MDA, and SOD in liver tissues across different groups

Compared to the normal control group, the hemorrhagic shock group displays significantly elevated levels of AC-HMGB1, TNF-a, IL-6, and MDA ($P<0.05$), alongside decreased SOD levels ($p<0.05$). Compared to the hemorrhagic shock group, both the hemorrhagic shock and resuscitation control group and the EP intervention groups exhibit significantly decreased expression of AC-HMGB1, TNF-a, IL-6, and MDA in liver tissues ($P<0.05$), coupled with significantly increased SOD levels ($P<0.05$); the EP intervention group exhibits more pronounced changes in these indicators (Table 2).

Table 2: Levels of AC-HMGB1, TNF-a, IL-6, MDA, and SOD in liver tissues ($\bar{x}\pm s$, n=8).

Group	n	AC-HMGB1	TNF-a	IL-6	MDA	SOD
Normal Control Group	8	14.42±0.30	35.20±1.38	16.93±0.61	6.53±0.31	150.40±13.36
Hemorrhagic Shock Group	8	34.66±1.55 ①	82.06±2.69 ①	40.06±1.16 ①	13.12±0.25 ①	90.44±8.70 ①
Hemorrhagic Shock and Resuscitation Control Group	8	31.56±2.03 ①②	67.84±2.59 ①②	31.68±0.65 ①②	10.58±0.34 ①②	109.56±6.06 ①②
EP Intervention Group	8	20.98±0.51 ①②③	52.16±2.21 ①②③	24.45±0.53 ①②③	8.97±0.44 ①②③	133.74±6.01 ①②③
F value		765.617	629.284	1284.461	525.165	68.232
P value		<0.001	<0.001	<0.001	<0.001	<0.001

Note: Compared to the normal control group, ① $P<0.05$; compared to the hemorrhagic shock group, ② $P<0.05$; compared to the hemorrhagic shock and resuscitation control group, ③ $P<0.05$.

7. Discussion and Conclusion

Hemorrhagic shock poses a significant global health threat, annually claiming the lives of up to 1.9 million people worldwide[1]. Its pathophysiological progression begins with the loss of substantial blood volume, thus leading to a drastic reduction in effective blood volume and an imbalance between oxygen supply and demand. This ultimately results in microcirculatory disturbances, inflammatory responses, and multi-organ dysfunction[6-7]. As a highly vascularized organ, the liver is often among the first to be affected following hemorrhagic shock, making acute liver injury one of the common and high-mortality conditions in critical care medicine. Acute liver injury typically manifests as structural changes in the liver and abnormal elevation of serum biochemical markers such as transaminases and bilirubin. Currently, the precise mechanisms underlying hemorrhagic shock-induced acute liver injury remain unclear. However, studies suggest that various factors, including the release of inflammatory mediators and oxygen free radicals, ischemia-reperfusion injury, and accelerated apoptosis, may collectively contribute to this process. Therefore, thorough research into the pathophysiological

mechanisms of acute liver injury resulting from hemorrhagic shock and the identification of effective preventive and therapeutic measures are of utmost clinical importance.

The findings of this study revealed extensive necrosis in the liver tissue of rats in the hemorrhagic shock group, accompanied by hepatocyte degeneration and necrosis within the hepatic plates, along with widened liver sinusoids and significant infiltration of inflammatory cells. Moreover, serum levels of TBIL, DBIL, ALT, and ALP were significantly elevated. Furthermore, with prolonged shock duration, both the histopathological and serological parameters of liver injury deteriorated further, indicating a close relationship between hemorrhagic shock-induced acute liver injury and the duration of shock.

Hemorrhagic shock results in a mismatch between oxygen delivery and metabolism, leading to increased oxygen debt and accumulation of anaerobic metabolites such as lactate, thereby triggering the generation of Damage Associated Molecular Patterns (DAMPs)[8]. Among these, High Mobility Group Box-1 (HMGB1), a typical DAMP molecule, serves as a crucial regulator of infection, injury, and inflammatory responses. In the early stages of hemorrhagic shock, a plethora of inflammatory mediators, including HMGB1, Tumor Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1), and Interleukin-6 (IL-6), are released into the bloodstream. The acetylation of HMGB1 forms the basis for its extracellular release and biological activity[4]. Recent studies have demonstrated that reducing acetylation levels of HMGB1 can significantly mitigate oxidative stress damage and airway inflammation in hormone-resistant asthma[9], ameliorate the severity of alcoholic liver disease[10], ameliorate the severity of alcoholic liver disease[11], among other effects. As an immune injury marker of drug-induced liver damage, AC-HMGB1 exhibits better prognostic accuracy with lower concentrations compared to ALT and can predict the clinical outcomes of patients more precisely[12]. This study found a significant increase in AC-HMGB1 expression in the liver tissue of the hemorrhagic shock group compared to the normal control group, suggesting a close association between AC-HMGB1 and the development of acute liver injury induced by hemorrhagic shock.

As a derivative of pyruvic acid, EP finds wide application in animal studies. Serving as a drug that inhibits HMGB1 release, EP reduces HMGB1 synthesis and its acetylation levels, thus inhibiting its release and alleviating systemic inflammation, thereby exerting organ-protective effects[16]. Numerous studies have demonstrated that EP significantly ameliorates ischemia-reperfusion injury in myocardial and cerebral tissues via animal models, reduces mortality in models of liver fibrosis induced by carbon tetrachloride, and exerts potent anti-inflammatory and antioxidant effects[13]. However, there are limited reports on the impact of EP on acute liver injury induced by hemorrhagic shock. The findings of this study suggest that, compared to the hemorrhagic shock group, after EP intervention, there exists a significant reduction in pathological liver tissue damage in rats, along with markedly improved serum biochemical indicators and reduced AC-HMGB1 levels, indicating that EP may alleviate acute liver injury caused by hemorrhagic shock by diminishing AC-HMGB1 levels in liver tissue. Nevertheless, within the EP intervention group, there were no significant differences in TBIL and DBIL between 1 hour and 6 hours post-injury and between 6 hours and 12 hours post-injury, suggesting that this may be associated with the dosing regimen, timing, concentration, and frequency of EP administration. Further studies are required to explore the specific reasons.

TNF-α is an inflammatory mediator with broad biological activity, and it significantly enhances the synthesis of pro-inflammatory cytokines such as IL-1 and IL-6 and the expression of adhesion molecules. TNF-α plays an important role in the development of various inflammatory diseases, including COVID-19[17] and drug-induced liver injury[18]. Studies have shown that by administering TNF-α inhibitor CC-5013, effective alleviation of the pathological damage on liver and kidney in rat models of acute liver and kidney failure is achieved, along with reduced levels of inflammatory factors. This study found that TNF-α and IL-6 levels in the liver tissue of the hemorrhagic shock group were significantly elevated, consistent with previous studies. However, following EP intervention, TNF-α and IL-6 levels were significantly reduced, suggesting that EP may mitigate acute liver injury in rats caused by hemorrhagic shock through its anti-inflammatory effects.

In liver tissue, SOD and MDA levels reflect the extent of oxidative damage from free radicals and lipid peroxidation. The results of this experiment indicate that MDA levels increased and SOD activity decreased in the liver tissue of rats in the hemorrhagic shock group. However, after EP intervention, MDA levels decreased, and SOD activity increased, suggesting that EP can alleviate acute liver injury in rats caused by hemorrhagic shock through antioxidant mechanisms.

In conclusion, hemorrhagic shock-induced acute liver injury in rats is associated with increased AC-HMGB1 expression. EP reduces AC-HMGB1 levels in liver tissues and mitigates systemic inflammatory responses and oxidative stress damage, thereby reducing the severity of acute liver injury caused by hemorrhagic shock. AC-HMGB1 may potentially serve as an immune injury indicator for acute liver injury caused by hemorrhagic shock, aiding clinicians in assessing prognosis and providing new avenues for identifying therapeutic targets.

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Conflict of Interests

The authors declared no conflict of interests.

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