

Ethyl Pyruvate Pretreatment Attenuates Concanavalin A-Induced Autoimmune Hepatitis in Mice

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Abstract

Pharmacological Relevance: Ethyl pyruvate (EP), a potent reactive oxygen species scavenger, has been reported to contribute to the inflammatory process. However, the protective effect of ethyl pyruvate on Concanavalin A (Con A)-induced autoimmune hepatitis have not been explored. Thus, the aims of this study are to investigate both the effects of ethyl pyruvate and its mechanism of protection on Con A-induced autoimmune hepatitis in mice.

Materials and Methods: Acute autoimmune hepatitis was induced by Con A (20 mg/kg) in Balb/C mice; ethyl pyruvate (40 mg/kg and 80 mg/kg) was administrated 1h prior to the Con A injection. At 3h, 6h and 24h post Con A injection, histological grading, proinflammatory cytokine levels and nuclear factor kappa B (NF- κ B) activity were determined.

Results: Following Con A challenge, cytokines TNF- α , IL-2, IL-1 β and IL-6 were expressed at 3h and 6h, and the level of HMGB1 significantly increased by 24h. Pretreatment with ethyl pyruvate ameliorated the pathological effects of Con A-induced autoimmune hepatitis and significantly decreased the levels of TNF- α , IL-2, IL-6 and IL-1 β at 3h and 6h and the level of HMGB1 at 6h and 24h post injection. Ethyl pyruvate blocked the degradation of κ B α and κ B β and decreased the expression of NF- κ B at 24h.

Conclusion: Taken together, these results indicated that ethyl pyruvate protected against Con A-induced autoimmune hepatitis by decreasing both early (TNF- α , IL-2, IL-1 β and IL-6) and late (HMGB1) cytokine expression in mice. The reduction of HMGB1 may correlate with the amelioration of NF- κ B activity.

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Introduction

Hepatitis seriously threatens human health and daily life due to a high incidence of transmitted disease. There are several types of hepatitis, including viral hepatitis, autoimmune hepatitis, and alcoholic hepatitis. With an understanding of the basic mechanism surrounding hepatitis, several treatments have been used, but current, therapeutics contribute little to the cure of hepatitis. Therefore, more effective therapies need to be explored to provide new applications in the clinic.

Concanavalin A (Con A) has the ability to activate T cells to secrete cytokines, resulting specifically in liver injury serving as a T cell mitogen. Pathological studies show the infiltration and accumulation of large quantities of lymphocytes in liver parenchyma, mainly CD4⁺ T cells [1]. Therefore, Con A is well known as an inducer of T cell-mediated hepatitis, especially in the model of autoimmune hepatitis. In addition to the infiltration of effector cells, mostly CD4⁺ T cells, kupffer cells, and natural killer T (NKT) cells, the secretion of proinflammatory cytokines such as

IL-1 β , IL-6, TNF- α and IFN- γ also play an important role in the early development of inflammation [2,3,4,5,6].

High-mobility group box 1 (HMGB1) is a highly conserved nuclear protein first purified from nuclei approximately 30 years ago [7]. HMGB1 is a non-histone protein and studies on function of it have shown a role in construction and stability of nucleosomes, regulation of gene transcription, and DNA repair [8]. The research of Wang et al. [9] revealed that HMGB1 can be released into the cytoplasm to induce inflammation. And HMGB1 has since attracted worldwide attention as a late cytokine. HMGB1 is widely found in the lymph tissue, liver, brain, spleen, kidney, and heart. HMGB1 can be either actively released by immune cells in response to stimulation by LPS, or passively released by damaged and necrotic cells. Once secreted into the cytoplasm, HMGB1 induces inflammation by engaging with multiple receptors, such as the Toll-like receptor-2 (TLR2), TLR4, TLR9, and the receptor for advanced glycation end products (RAGE) [10,11,12].

Recent studies have suggested that HMGB1 may be a mediator of inflammation, and has been implied in various diseases, such as

ischemia reperfusion injury and atherosclerosis [13,14]. Furthermore, HMGB1 has been implicated in acute liver injury [15] and Tong et al. [16] have reported that inhibition of HMGB1 activity alleviated liver injury in heatstroke. HMGB1 was also identified as an early mediator of injury and inflammation in hepatic ischemia-reperfusion injury, demonstrated by Watanabe et al. [17]. Blockade of HMGB1 by a neutralizing antibody inhibited proinflammatory cytokine production, NF- κ B activity, and as a result, attenuated Con A-induced hepatitis in mice, as reported by Gong et al. [18]. In addition to neutralizing antibodies, other agents have also been shown to be protective in inflammatory diseases, partly through decreasing systemic HMGB1 accumulation, such as quercetin, green tea and curcumin [19,20,21]. Therefore, HMGB1 has the potential to become a therapeutic target in treating inflammatory diseases. Ethyl pyruvate (EP) is a stable lipophilic ester derivative of pyruvate with the structural formula, $\text{CH}_3\text{COCOOCH}_2\text{-CH}_2$, and a molecular weight of 116.18 Da [22]. The exploration of ethyl pyruvate began with the study of the antioxidation of pyruvic acid. Pyruvic acid is the final product of the glycolytic cycle and the initiator of gluconeogenesis, and is the primary product in the process of energy metabolism. Pyruvic acid participates in the elimination of reactive oxygen species (ROS) to alleviate oxidative injury. However, the use of pyruvic acid as a therapeutic agent is limited by its aqueous instability. Ethyl pyruvate was first used as the succedaneum of pyruvic acid in animal experiments; it is more stable and less toxic compared to pyruvate acid. Ethyl pyruvate has now been used in various animal models [23]; for example, it has been shown to ameliorate acute pancreatitis and peritonitis [24]. Ethyl pyruvate also has protective effect after ischemia/reperfusion injury in heart, brain and kidney by reducing the release of HMGB1 [25]. Lu-Wen Wang et al. [26] demonstrated that ethyl pyruvate protects against experimental acute-onset chronic liver failure in rats. However, it is not known whether ethyl pyruvate attenuates Con A-induced hepatitis, an autoimmune disease model in Balb/c mice. In general, the mechanism of action of ethyl pyruvate has not been clearly determined.

In the present study, we probe the effects of ethyl pyruvate on Con A-induced autoimmune hepatitis and explore the mechanism of ethyl pyruvate action further.

Materials and Methods

2.1 Reagents

Ethyl pyruvate and Con A were purchased from Sigma-Aldrich (USA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-2, IL-6, TNF- α and IL-1 β were purchased from R&D systems (Minneapolis, MN, USA). HMGB1 ELISA kit was purchased from Shino-Test Corporation (Oonodai, Kanagawa, Japan). Antibodies used in this study include HMGB1 (Epitomics, CA), IL-2 (Biolegend, CA), IL-6 (Proteintech, CA), TNF- α (Santa Cruz, CA), NF- κ B (Proteintech, CA), IL-1 β (Biolegend, CA), I κ B α (Cell signal technology, CA) and I κ B β (Cell signal technology, CA).

2.2 Animals

Male Balb/c mice (6–8 weeks old, 22 ± 2 g) were purchased from Shanghai Laboratory Animal Co Ltd (SLAC, Shanghai, China). The mice were housed in an environment at a temperature of $25 \pm 2^\circ\text{C}$ with an alternating 12h light and dark cycle; they were permitted free access to standard laboratory food and water. All animal experiments were approved by the Animal Care and Use Committee of Shanghai Tongji University.

2.3 Experiment #1: Con A Treatment

Con A was dissolved in normal saline solution at a concentration of 20 mg/kg according to the prior report [23]. 100 mice were randomly divided into two groups: group A was injected with saline solution through the tail vein and group B was injected with Con A at 20 mg/ml. 5 mice randomly-selected from group A and group B were killed at designed time points: 0h, 2h, 4h, 6h, 8h, 10h, 12h, 14h, 18h and 24h after Con A injection. Serum and liver tissue samples were then collected and frozen at -80°C until analyzed for cytokine levels and liver enzymes.

2.4 Experiment #2: Pre-treatment of Con A-treated Mice with Ethyl Pyruvate

Based on the levels of IL-2, IL-6, TNF- α , IL-1 β and serum alanine transaminase (ALT) and aspartate transaminase (AST) observed in the first set of experiments, we set the time points at 3h, 6h and 24h. Next, we divided 72 mice randomly into four groups of 18 mice each. Each group received the following treatment.

Group I: normal control ($n = 18$); mice were injected in the tail vein with saline solution only.

Group II: model group ($n = 18$); mice were injected in the tail vein with 20 mg/kg Con A.

Group III: protected group ($n = 18$); mice were injected in the tail vein with ethyl pyruvate (40 mg/kg) 1h prior to Con A challenge.

Group IV: protected group ($n = 18$); mice were injected in the tail vein with ethyl pyruvate (80 mg/kg) 1h prior to Con A challenge.

Six mice from each group were randomly selected and were killed at time points 3h, 6h and 24h. All serum and liver tissue sample were collected and stored at -80°C .

2.5 Biochemical Analysis

2.5.1 Serum aminotransferase assay. After blood collection, serum was separated by centrifugation at 2000 rpm at room temperature for 10 min. To detect the level of hepatocellular injury following Con A challenge, serum ALT and AST were measured by an automated chemistry analyzer (Olympus AU1000, Japan).

2.5.2 Serum cytokine measurement. To assess the serum levels of IL-2, IL-1 β , IL-6, TNF- α and HMGB1, ELISA kits were used according to the manufacturer's instructions.

2.6 Histopathology

A portion of the liver tissue was preserved in 4% paraformaldehyde for at least 24 hours, and paraffin blocks were prepared according to the standard protocol [10]. Sections 3 μm thick were cut and stored at room temperature. The paraffin sections were then stained with hematoxylin and eosin (H&E) to observe the level of inflammation and tissue damage by light microscopy.

2.7 Immunohistochemistry

Prepared paraffin-embedded sections were dewaxed and rehydrated through a series of graded alcohols followed by heating in a baking oven at 60°C for 20 min. Antigen was recovered in citrate buffer incubated in a 95°C water-bath for 20 min and then endogenous peroxidase was blocked by incubating in 3% hydrogen peroxide for 20 min at 37°C . Membranes were ruptured with 0.2% triton at room temperature for 30 min and non-specific binding sites were blocked with 5% BSA at 37°C for 20 min followed by room temperature incubation for 10 min. The liver slices were then incubated overnight with rabbit anti-mouse

HMGB1 (1:500) and rabbit anti-mouse NF- κ B (1:50). On the second day, slices were incubated with secondary antibody (goat anti-rabbit) (Epitomics, CA) for 30 min at room temperature. The analysis of antibody binding was performed using a DAB kit. Afterwards, slides were counterstained with hematoxylin, dehydrated using graded ethanol and xylene, and mounted with Entellan. Slides were then observed by light microscopy. The assay was carried out by using Image-Pro Plus software 6.0 (Media Cybernetics, Silver Spring, MD, USA). The integrated optical density (IOD) of HMGB1 was calculated in our results. IOD is equal to the number of density (mean) multiply area, which can exactly indicate the amount of protein expressing in cytoplasm. And the rate of positive nucleus of NF- κ B was also calculated using Image-Pro Plus 6.0. Three different fields of vision were random selected in one slide, and the IOD of them were acquired with Image-Pro Plus 6.0. We calculated the average of these three IOD. The same method was administrated in other two mice random selected from the same group. The above method was applied in all groups.

2.8 Immunofluorescence

Fresh liver tissue from mice was fixed in 4% paraformaldehyde for 1h on ice. Fixed liver tissue was washed with PBS for 5 min three times on ice. The liver tissue was then dehydrated in 30% sucrose (dissolved in PBS) overnight at 4°C. Liver tissue was infiltrated in OCT for 2 hours on day 2. The liver tissue was then frozen and stored at -80°C. Sections of 5 μ m were cut with a freezing microtome and preserved at -20°C for preparation. Prepared sections were dried at room temperature for 5 min. OCT was then dissolved in PBS for 5 min, membranes were ruptured with 0.2% Triton at room temperature for 20 min, and non-specific antigen binding site was blocked by 5% BSA. Sections were then incubated with rabbit anti-mouse HMGB1 at 4°C overnight. Nuclear staining was performed by DAPI (1:1000) after incubating with goat anti-rabbit antibody for 30 min on day 2. All sections were observed with a fluorescence microscope.

2.9 Western Blot Analysis

Liver tissues were recovered from -80°C storage and rapidly ground in liquid nitrogen and then lysed with RIPA lysis buffer and protease inhibitor. The protein concentration was detected using the BCA method. Equivalent amounts of total protein (120 μ g) were boiled and subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) following standard methods. Non-specific binding was blocked with 5% non-fat milk (dissolved in PBS) for 1h and then blots were incubated overnight at 4°C with antibodies against rabbit anti-mouse IL-2 (1:500), rabbit anti-mouse IL-6 (1:500), rabbit anti-mouse TNF- α (1:500), mouse anti-mouse β -actin (1:1000), and rabbit anti-mouse HMGB1 (1:1000) diluted in 5% milk. β -actin was used as the internal reference for cytoplasmic proteins. All membranes were washed with PBST (1% Tween) and then incubated with a secondary goat anti-mouse or anti-rabbit antibody (1:1000), dissolved in PBST, for 45 min at 37°C. Finally, membranes were washed with PBST three times for 5 min each and proteins were detected using the Odyssey two-color infrared laser imaging system (detected with fluorescence).

2.10 Total RNA Isolation and Real-time Reverse-transcriptase Polymerase Chain Reaction (RT-PCR)

mRNA transcripts were detected and analyzed via quantitative RT-PCR of the liver tissue. Total RNA was extracted from frozen liver tissue using TRIzol reagent (TIANGEN Biotech, China) as

described by the manufacturer. To determine the expression of target genes in the liver, SYBR Green quantitative RT-PCR was performed using a 7900HT Fast Real-time PCR system (ABI, CA, USA) according to the instructions of SYBR Premix EX Taq (TaKaRa Biotechnology China). Primer sequences were as follows (shown in Table 1).

2.11 Statistical Analysis

All results are expressed as the mean \pm SD. The data of Real-time PCR and ELISA were analyzed using one-way analysis of variance (ANOVA). The results of ALT, AST, necrotic area, Western blot and Immunohistochemistry were analyzed using a Student's t test. In all comparisons, $p < 0.05$ were considered statistically significant. All statistical analyses were performed using SPSS 17.0 for Windows.

Results

3.1 Preliminary Study Results

The plasma ALT and AST levels from the first set of experiment are shown in Fig. 1. The levels of ALT and AST increased from 2h and reached peak levels at 6h, then decreased until 24h. The levels of IL-2, IL-6, TNF- α and IL-1 β showed similar trends as seen in Fig. 2; a significant change can be seen between 0h, 6h and 24h. Therefore, we used the time points 3h, 6h and 24h in the second set of experiments.

3.2 Ethyl Pyruvate Pretreatment Attenuates Con A-induced Liver Injury in Mice

It is known that Con A can induce liver injury in mice, imitating T-cell mediated liver disease, including autoimmune hepatitis. To determine the effect of ethyl pyruvate on Con A-induced hepatitis, mice were treated with ethyl pyruvate 1h before Con A was administrated. Serum and liver tissue were collected at 3h, 6h and 24h according to the experimental design. The level of ALT and AST in serum was determined as shown in Fig. 3A and Fig. 3B; ALT and AST levels were significantly increased at the three time points. However, the elevation of ALT and AST was clearly decreased with ethyl pyruvate pretreatment. This same result was demonstrated in the histopathological study. As shown in Fig. 3C, we found massive areas of necrosis in the Con A-induced group. In contrast, the ethyl pyruvate-treated group showed minor liver

Table 1.

Gene		Primer Sequence(5'→3')
TNF- α	Forward	CAGGCGGTGCCTATGTCTC
	Reverse	CGATCACCCGAAGTTCAGTAG
IL-2	Forward	TGAGCAGGATGGAGAATTACAGG
	Reverse	GTCCAAGTTCATCTTCTAGGCAC
IL-6	Forward	CTGCAAGAGACTTCCATCCAG
	Reverse	AGTGGTATAGACAGGTCTGTTGG
HMGB1	Forward	GCATCCTGGCTTATCCATTGG
	Reverse	GGCTGCTGTCTATCTGCTG
NF- κ B	Forward	ATGGCAGACGATGATCCCTAC
	Reverse	CGGATCGAAATCCCTCTGTT
β -actin	Forward	GGCTGTATCCCTCCATCG
	Reverse	CCAGTTGGTAAACATGCCATGT

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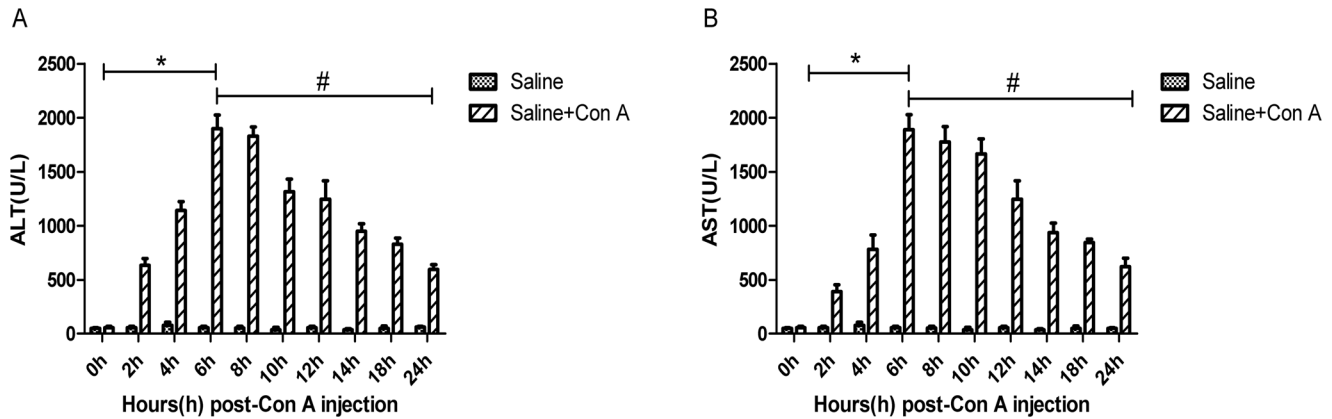


Figure 1. Preliminary study results. Mice ($n=5$ for each group) were injected with Con A (20 mg/kg) at 0h. And every group were sacrificed at time point of 0h, 2h, 4h, 6h, 8h, 10h, 12h, 14h, 18h and 24h after the injection of Con A. A, B separately show the plasma ALT and AST levels at each time point. Data are expressed as mean \pm SD ($n=5$, $*p<0.05$ for Con A/0h VS Con A/6h, $\#p<0.05$ for Con A/6h VS Con A/24h). Data analysis was performed by a student's test. doi:10.1371/journal.pone.0087977.g001

damage, indicating ethyl pyruvate pretreatment significantly reduced liver necrosis. Ethyl pyruvate administered at 80 mg/kg was more effective. According to the results analyzed with Image-pro Plus 6.0, it is obvious to find there exist statistical

significant among different groups. These results show that ethyl pyruvate pretreatment attenuates Con A-induced autoimmune hepatitis in mice.

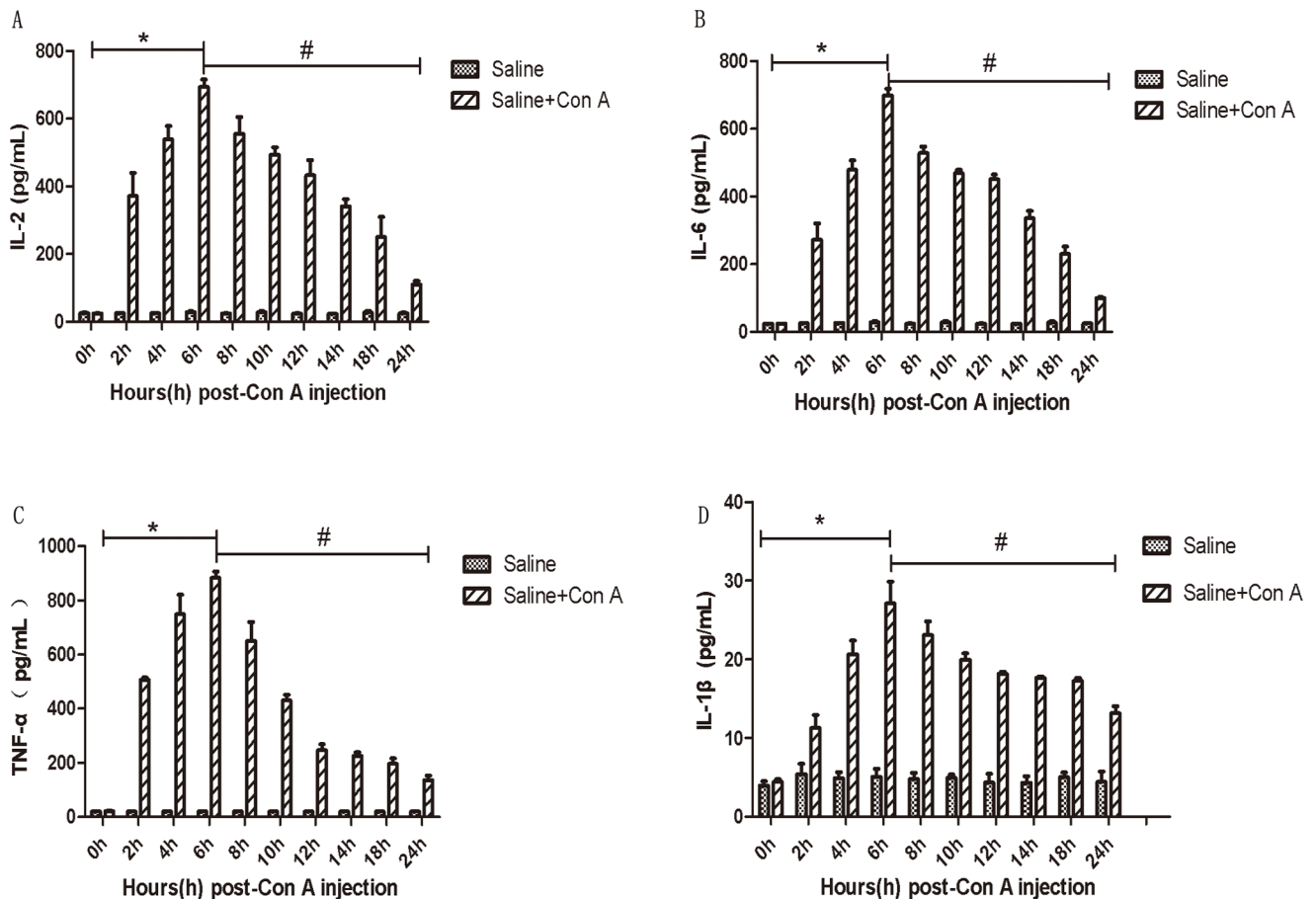


Figure 2. Preliminary study results. Mice ($n=5$ for each group) were injected with Con A (20 mg/kg) at 0h. And every group were sacrificed at time point of 0h, 2h, 4h, 6h, 8h, 10h, 12h, 14h, 18h and 24h after the injection of Con A. A, B, C and D separately show the level of IL-2, IL-6, TNF- α and IL-1 β in serum at each time point. Data are expressed as mean \pm SD ($n=5$, $*p<0.05$ for Con A/0h VS Con A/6h, $\#p<0.05$ for Con A/6h VS Con A/24h). doi:10.1371/journal.pone.0087977.g002

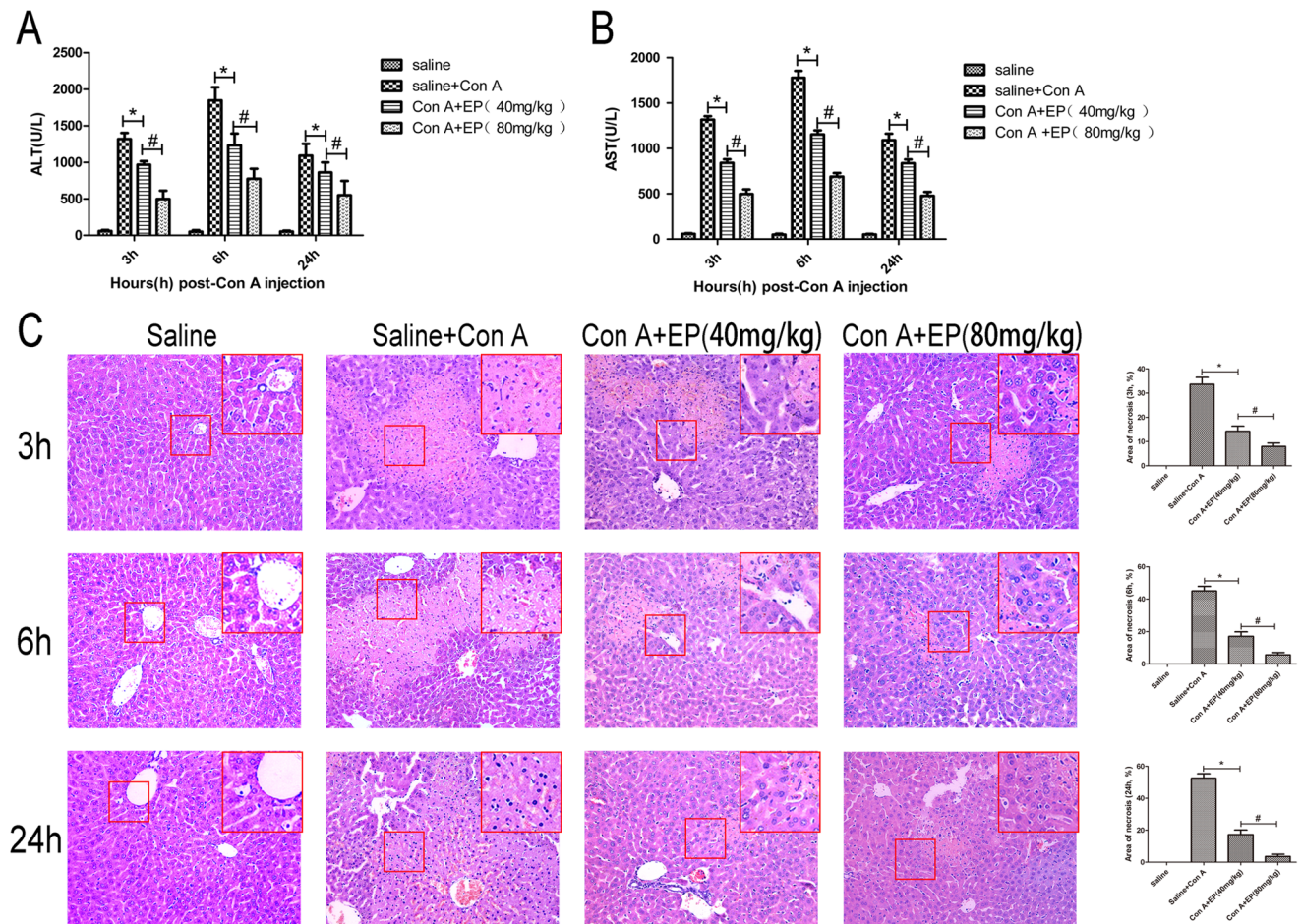


Figure 3. Ethyl pyruvate pretreatment attenuate Con A-induced autoimmune hepatitis. A, B. Effects of ethyl pyruvate on plasma ALT and AST levels at 3h, 6h, 24h after Con A injection in mice. Data are expressed as mean \pm SD [n=6, * p <0.05 for Saline+Con A vs. Con A+EP(40 mg/kg), # p <0.05 for Con A+EP(40 mg/kg) vs. Con A+EP(80 mg/kg)]. C. Hematoxylin and eosin staining of liver sections (Saline or Saline+Con A or Con A+EP groups at the time point of 3h, 6h and 24h). Original magnification: \times 200 and \times 400. The necrotic areas were analyzed with Image-pro Plus 6.0, indicating there existed statistical significance among different groups. [n=6, * p <0.05 for Saline+Con A vs. Con A+EP(40 mg/kg), # p <0.05 for Con A+EP(40 mg/kg) vs. Con A+EP(80 mg/kg)]. doi:10.1371/journal.pone.0087977.g003

3.3 Effect of Ethyl Pyruvate on Production of IL-2, IL-6, IL-1 β and TNF- α in Con-A Induced Hepatitis

It is a common perspective that the progress of hepatitis is associated with a series of proinflammatory cytokines such as IL-2, IL-6, IL-1 β and TNF- α . Therefore, the levels of IL-2, IL-6, IL-1 β and TNF- α in serum were determined by ELISA after Con A treatment, as shown in Fig. 4 (A, B, C, D). The levels of these cytokines increased after Con A induction and expressed most at 6h, and as expected, the production of IL-2, IL-6, IL-1 β and TNF- α were prevented with ethyl pyruvate pretreatment, as seen at 3h and 6h. Furthermore, to confirm our observations, mRNA expression of IL-2, IL-6, IL-1 β and TNF- α were detected by real-time PCR at the designed time points. Results (Fig. 5C) showed that mRNA expression of IL-2, IL-6, IL-1 β and TNF- α were significantly increased in the Con A-treated group and ethyl pyruvate pretreatment diminished the mRNA expression of IL-2, IL-1 β and TNF- α at all three time points and the expression of IL-6 obviously at 3h and 6h. In addition, IL-2, IL-6, IL-1 β and TNF- α were maximally expressed at 6h, indicating these four cytokines mainly expressed in the early phase of Con A-induced hepatitis. Finally, the protein level of IL-2, IL-6, IL-1 β and TNF- α were also assayed by western blot (Fig. 5A), where we observed that the

expression of these cytokines decreased in the ethyl pyruvate treatment group compared to the Con A-induced group. And the protein level of IL-2, IL-6, IL-1 β and TNF- α reached peak at 6h, similar to the mRNA expression (Fig. 5C). These results were analyzed with Quantity One, indicating there existed statistical significance among these changes (Fig. 5B). Thus, these results confirm that ethyl pyruvate pretreatment inhibits the production of proinflammatory cytokines, such as IL-2, IL-6, IL-1 β and TNF- α in the early phase of Con A-induced hepatitis, which is associated with preventing Con A-induced hepatitis.

3.4 Effect of Ethyl Pyruvate on the Expression of HMGB1 in Con A-induced Hepatitis in Mice

We have shown that ethyl pyruvate is able to prevent Con A-induced hepatitis by down-regulating the production of inflammatory cytokines such as IL-2, IL-6, IL-1 β and TNF- α . Here, we further explored the possible mechanism of ethyl pyruvate attenuation of Con A-induced hepatitis. It has been reported by Gong [18] that HMGB1 can exacerbate Con A-induced hepatitis. Therefore, we asked whether ethyl pyruvate acts by inhibiting HMGB1 in ConA-induced hepatitis. In one hand, the level of HMGB1 in serum was detected by ELISA, result showed that the

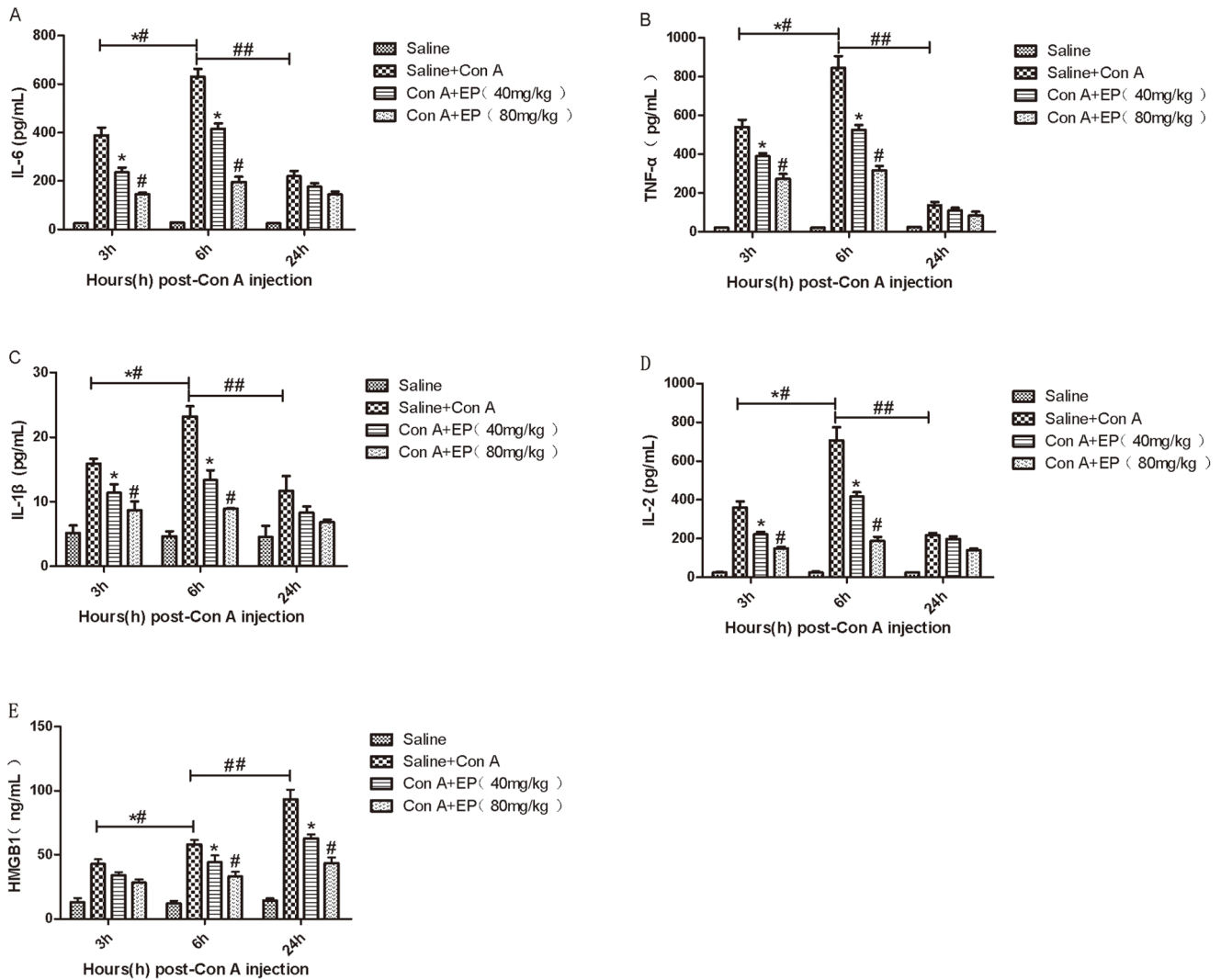


Figure 4. Effect of ethyl private on the release of IL-2, IL-6, TNF- α , IL-1 β and HMGB1 in Con A-treated mice. IL-2, IL-6, TNF- α and IL-1 β express maximally at 6h and HMGB1 expresses maximally at 24h. Ethyl pyruvate decreases the release of IL-2, IL-6, TNF- α and IL-1 β at 3h and 6h and the release of HMGB1 at 6h and 24h after Con A injection. Data are showed as mean \pm SD [$n=6$, * $p<0.05$ for Con A+EP(80 mg/kg) vs. Saline+Con A; # $p<0.05$ for Con A+EP(40 mg/kg) vs. Con A+EP(80 mg/kg); *# $p<0.05$ for Saline+Con A at 3h vs Saline+Con A at 6h; ## $p<0.05$ for Saline+Con A/6h vs Saline+Con A/24h].

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expression of HMGB1 was most at 24h, and ethyl pyruvate could successfully inhibit it at 6h and 24h (Fig. 4E). On the other hand, the expression of HMGB1 mRNA was detected by real-time PCR, as shown in Fig. 5C. The level of HMGB1 mRNA was significantly upregulated in the Con A-induced group and downregulated after ethyl pyruvate treatment. This result was also observed for protein expression of HMGB1, as determined by western blot. As shown in Fig. 5A, HMGB1 was expressed at all three time points, with higher expression at 24h than at 3h and 6h. As expected, all doses of ethyl pyruvate in the pretreatment group resulted in lower expression of HMGB1 compared to the Con A-induced group. This result was analyzed with Quantity One and agreed with the change in HMGB1 mRNA expression we observed. Based on these results, we conclude that ethyl pyruvate pretreatment may ameliorate ConA-induced hepatitis partly through downregulation of HMGB1.

3.5 Effect of Ethyl Pyruvate on HMGB1 Activation in Con A-induced Hepatitis

HMGB1 plays a key role in the progression of inflammation as a late proinflammatory cytokine. We observed HMGB1 mainly in the nucleus of normal liver tissue, as showed in Fig. 6A. Upon stimulation of the inflammatory signal, HMGB1 migrated from the nucleus to the cytoplasm to promote further inflammation. This transition was clearly shown by immunocytochemistry, as seen in Fig. 6B. In the normal control group, HMGB1 is mainly located in the nucleus and is hardly detected in the cytoplasm. As expected, HMGB1 was notably increased in the cytoplasm 24h after the injection of ConA partly in the area of necrosis, indicating that not only was HMGB1 released actively by inflammatory cells, but released passively due to injury and cell necrosis. Administration of ethyl pyruvate blocked the synthesis and migration of HMGB1, resulting in a significant decrease in the intensity of signal in the cytoplasm at 24h compared to the ConA-induced group. This result was analyzed with Image-pro Plus 6.0.

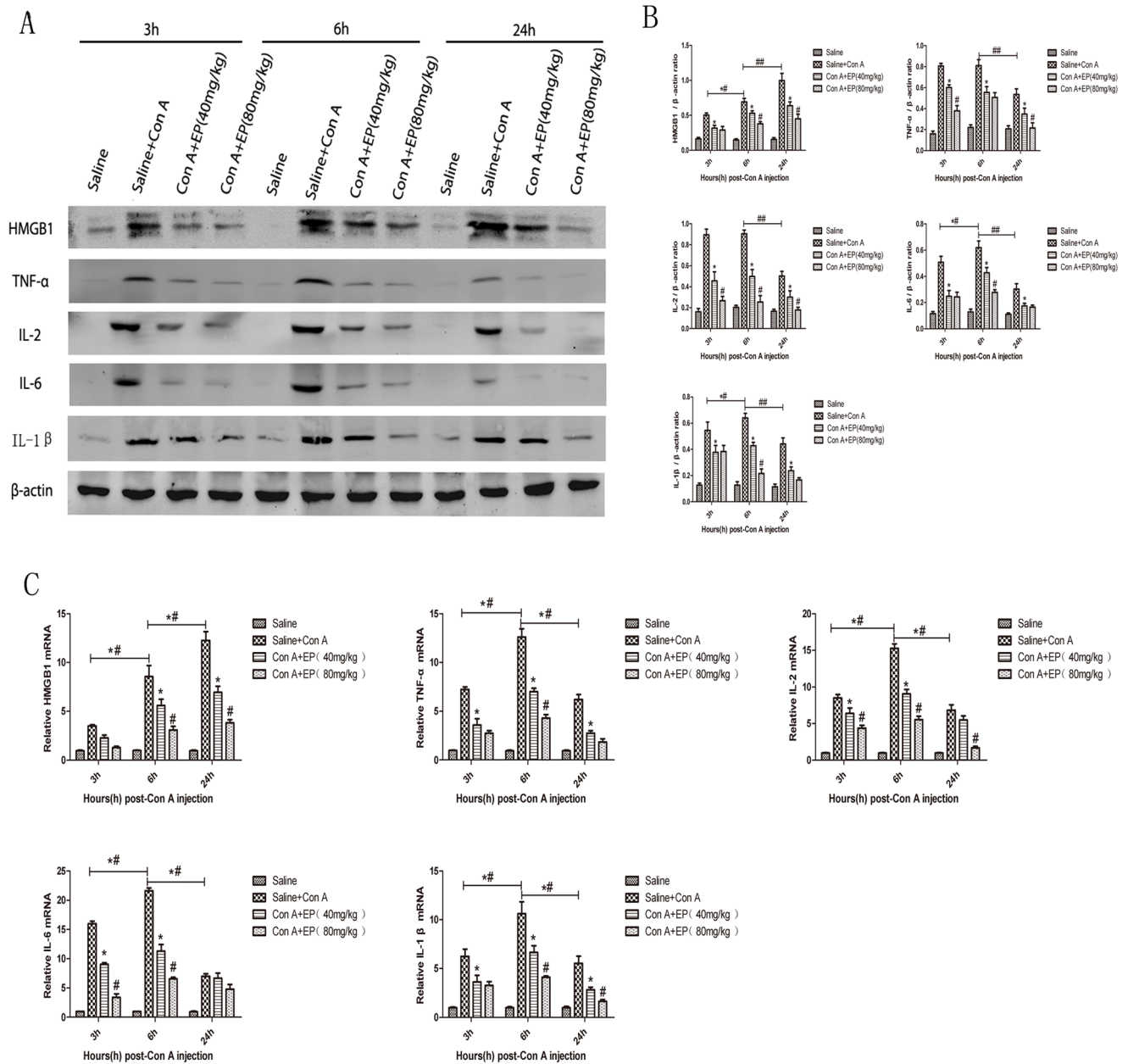


Figure 5. Ethyl pyruvate decreases the expression of IL-2, IL-6, TNF- α , IL-1 β and HMGB1. A. Ethyl pyruvate inhibits the protein level of IL-2, IL-6, TNF- α , IL-1 β and HMGB1 at all three time points detected by Western blot. HMGB1 significantly increased over time and other cytokines (IL-2, IL-6, TNF- α , IL-1 β) expressed most at 6h. B. The results of western blot were analyzed with Quantity one. [n=3, * p <0.05 for Con A+EP(80 mg/kg) vs. Saline+Con A; # p <0.05 for Con A+EP(40 mg/kg) vs. Con A+EP(80 mg/kg); ** p <0.05 for Saline+Con A at 3h vs Saline+Con A at 6h; ### p <0.05 for Saline+Con A/6h vs Saline+Con A/24h]. C. The mRNA expression of IL-2, IL-6, TNF- α , IL-1 β and HMGB1 were evaluated by Real time PCR. Data are showed as mean \pm SD[n=3, * p <0.05 for Con A+EP(80 mg/kg) vs. Saline+Con A; # p <0.05 for Con A+EP(40 mg/kg) vs. Con A+EP(80 mg/kg); ** p <0.05 for Saline+Con A at 3h vs Saline+Con A at 6h; ### p <0.05 for Saline+Con A/6h vs Saline+Con A/24h]. doi:10.1371/journal.pone.0087977.g005

3.6 Effect of Ethyl Pyruvate on NF- κ B Signal Pathway in Con A-induced Hepatitis in Mice

NF- κ B activation plays a key role in the induction of several proinflammatory mediators. To determine whether ethyl pyruvate down-regulated NF- κ B signal pathway during Con A-induced hepatitis in mice, we firstly detected the protein level of I κ B α and I κ B β with western blot analysis. Results showed the degradation of I κ B α and I κ B β was obviously blocked by ethyl pyruvate at 24h, not at 3h and 6h (Fig. 7B). Furthermore, we explored the

change of NF- κ B in our model. We found that ethyl pyruvate significantly decreased the expression of NF- κ B both in mRNA and protein level (Fig. 7A, Fig. 7B). We used Quantity One to analyze the result of western blot and demonstrated that these changes had statistical significant. Immunohistochemistry for NF- κ B was detected to differentiate the location of NF- κ B in different groups. As shown in Fig. 7C, we found that NF- κ B mostly expressed and located in nuclei in Con A-induced group compared to Saline group. And the expression of NF- κ B in nuclei

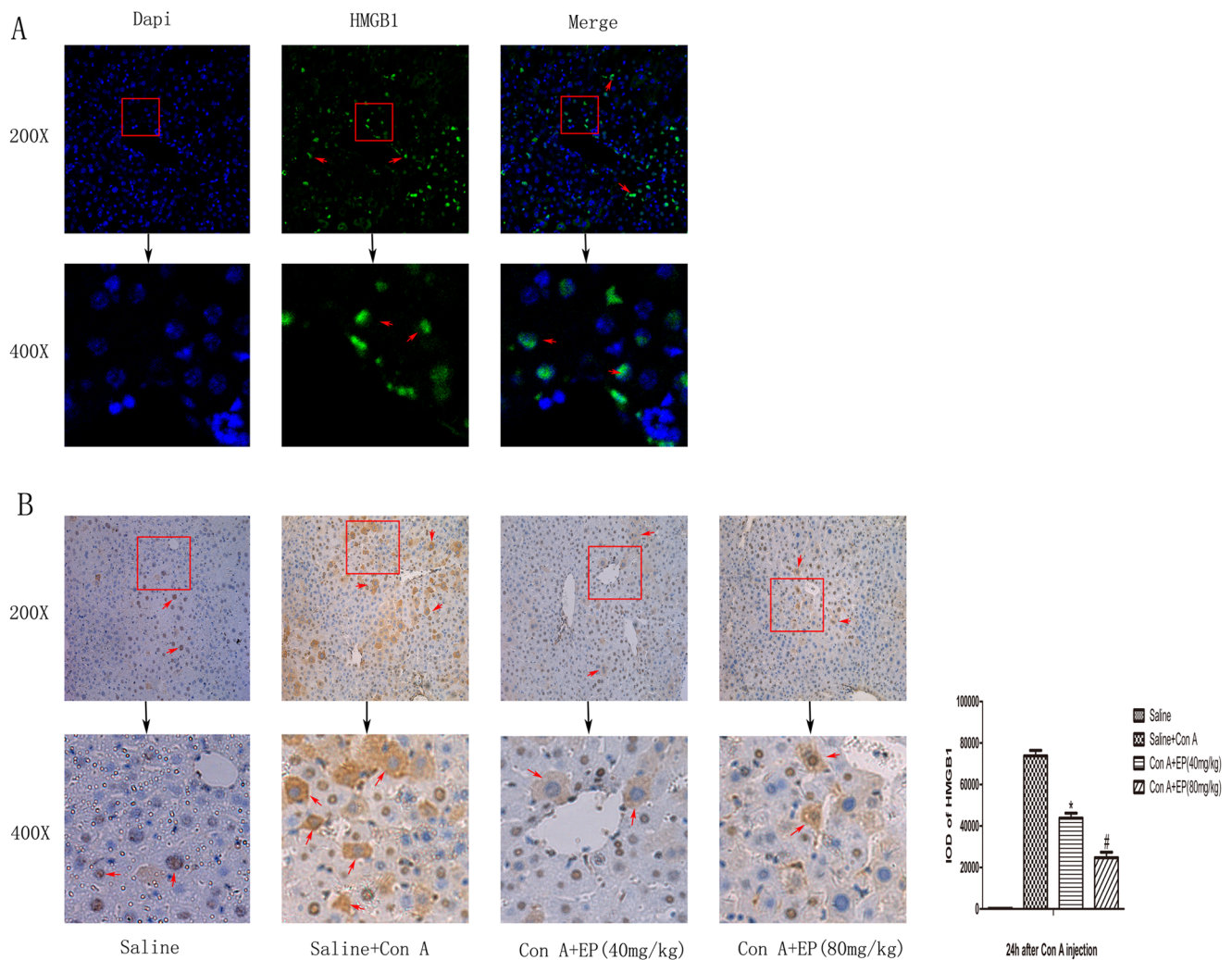


Figure 6. Ethyl pyruvate pretreatment significantly downregulated HMGB1 expression and translocation in Con A-induced mice. A. Location of HMGB1 in normal liver tissue collected at 24h was detected by immunofluorescence (original magnification: $\times 200$ and $\times 400$). Results showed HMGB1 expressed in nuclei indicated with red arrows. B. Liver tissues were collected 24h after Con A injection for immunohistochemical staining with anti-HMGB1 antibody (original magnification: $\times 200$ and $\times 400$). The IOD of HMGB1 in cytoplasm were analyzed by Image-Pro Plus 6.0. Results showed that ethyl pyruvate inhibited the expression and translocation of HMGB1 and had statistical significant. Data are showed as mean \pm SD [$n = 3$, $*p < 0.05$ for Saline+Con A vs. Con A+EP(40 mg/kg), $^{\#}p < 0.05$ for Con A+EP(40 mg/kg) vs. Con A+EP(80 mg/kg)]. The representative positive cells were indicated with red arrows. doi:10.1371/journal.pone.0087977.g006

was obviously decreased after ethyl pyruvate treatment at 24h. This result was analyzed with Image-pro Plus 6.0.

Discussion

Liver disease is really a series of common and detrimental diseases such as acute hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma; there is a relationship among these diseases. Beasley et al. [27] reported that the incidence of primary hepatocellular carcinoma among carriers of hepatitis B surface antigen was much higher than among non-carriers in a prospective general population study of 22,707 Chinese men in Taiwan. A large amount of evidence has been accumulated confirming the relationship between hepatitis and hepatocellular carcinoma. More effective drugs are urgently needed for intervention at the hepatitis stage to decrease the incidence of hepatocellular carcinoma. Ethyl pyruvate, as a stable lipophilic ester derivative of pyruvate, has potential health benefits for

humans and has attracted the attention of scientists. In this report, we demonstrated the effect of ethyl pyruvate on Con A-induced autoimmune hepatitis in mice.

Con A has the ability to activate T lymphocytes in vitro and cause T cell-dependent autoimmune hepatitis, and T lymphocytes are important contributors to the pathological process of hepatitis [1]. Recently, studies have reported that Con A-induced autoimmune hepatitis is largely mediated by the release of inflammatory cytokines such as IL-2, IL-4, IL-6, IL-10, IL-12, TNF- α and IFN- γ [28]. The timing of expression of these inflammatory cytokines plays an important role in the development of inflammation. IL-2, which has multiple effects on the immune system, is mainly released by activated T cells and participates in mediating inflammation and hemopoiesis. Sass et al. [28] explored the changes in cytokine expression in three mouse models of experimental hepatitis and found that the levels of IL-2 and IL-6 increased from 3h after the administration of Con A. It has also been reported that various concentrations of IL-2

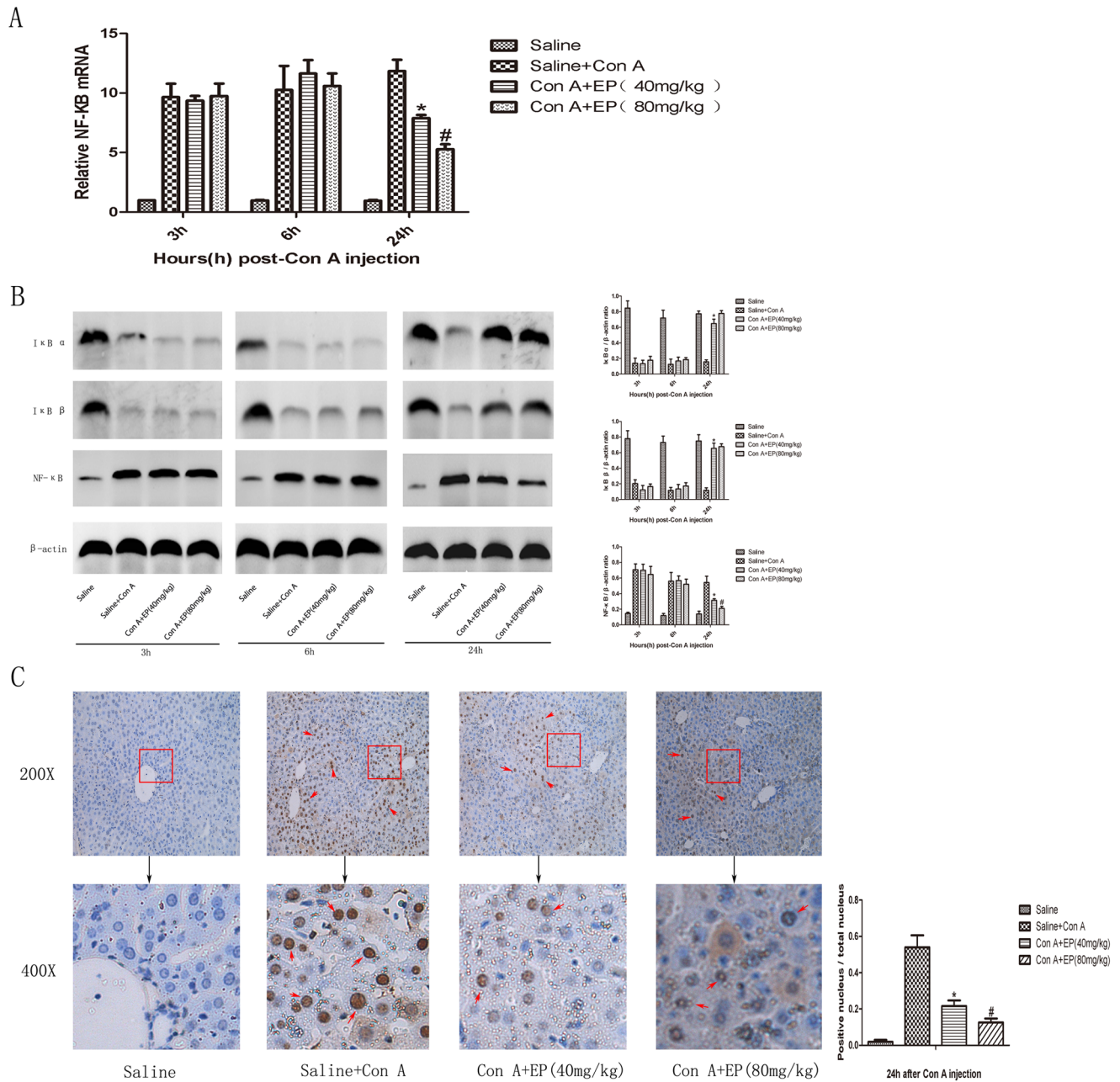


Figure 7. Ethyl pyruvate down-regulated NF- κ B signal pathway at 24h. A. Real-time PCR was performed to detect mRNA expression of NF- κ B. Compared to the Saline group, the expression of NF- κ B in EP treatment group (40 mg/kg) is significantly decreased ($*p < 0.05$), and the dose of 80 mg/kg has a more obviously effect ($\#p < 0.05$). B. The expression of I κ B α , I κ B β and NF- κ B was detected by western blot. The results were analyzed using Quantity One. [$n = 3$, $*p < 0.05$ for Saline+Con A vs. Con A+EP(40 mg/kg), $\#p < 0.05$ for Con A+EP(40 mg/kg) vs. Con A+EP(80 mg/kg)] C. The expression level of NF- κ B at 24h was detected by Immunohistochemistry with anti-NF- κ B antibody. Original magnification: $\times 200$ and $\times 400$. The positive cells were indicated with red arrows. The result was analyzed using Image-pro Plus 6.0. [$n = 3$, $*p < 0.05$ for Saline+Con A vs. Con A+EP(40 mg/kg), $\#p < 0.05$ for Con A+EP(40 mg/kg) vs. Con A+EP(80 mg/kg)]. doi:10.1371/journal.pone.0087977.g007

played different roles in Con A-induced autoimmune hepatitis; a low dose of IL-2 exacerbated Con A-induced liver injury as reported by Zhang et al. [29]. In addition, TNF- α plays a pivotal role in inflammatory diseases and has been identified as a critical mediator in the experimental T cell-dependent disease models. It has been demonstrated that pretreatment of mice by polyclonal sheep anti-mouse TNF- α antiserum or a TNF- α inhibitor protected against Con A-induced liver injury [30]. Furthermore, mice deficient in the TNF- α related receptors TNFR1 and

TNFR2 do not develop severe Con A-induced autoimmune hepatitis, as reported by Wolf et al. [31]. IL-2, IL-6, TNF- α and IL-1 β were released starting at 3h after administration of Con A, and inhibition of the release of these cytokines attenuates the Con A-induced autoimmune hepatitis. In our studies, we showed that the level of IL-2, IL-6, TNF- α and IL-1 β increased at 3h, reached peak levels at 6h, then diminished. We then showed that ethyl pyruvate pretreatment reduced the levels of IL-2, IL-6, TNF- α and IL-1 β at 3h and 6h in Con A treated mice; the corresponding

pathological features were also ameliorated in the ethyl pyruvate-treated group at 3h and 6h. Therefore, we consider that ethyl pyruvate may ameliorate Con A-induced liver injury through the reduction of inflammatory cytokines such as IL-2, IL-6, TNF- α and IL-1 β .

HMGB1, a highly conserved nuclear protein, not only mediates gene transcription and maintains the stability of the nucleosome structure, it is also regarded as a central mediator of inflammation. Andersson et al. [32] reported that recombinant HMGB1 could upregulate TNF mRNA and protein expression in human blood mononuclear cell cultures and, in vivo, the expression of HMGB1 increased in experimental severe acute pancreatitis, as shown by Yasuda et al. [33]. HMGB1 has also been studied in the clinic. Huang et al. [34] found plasma HMGB1 levels increased significantly in 338 patients of ischemic stroke and a similar study was also reported in clinical acute lung injury [12]. The release of HMGB1 is delayed relative to classical early cytokines such as TNF- α , IL-6, IL-2 and IL-1 β . Here, we observed that the levels of TNF- α , IL-6, IL-2 and IL-1 β were higher at 3h and 6h after Con A treatment than at 24h, both in serum and in tissue. On the other hand, the levels of HMGB1 increased gradually and reached peak levels at 24h post Con A treatment. This indicates that HMGB1 may serve a pivotal role in the later processes of Con A-induced hepatitis. When HMGB1 is activated, it migrates from the nucleus to the cytoplasm to increase inflammation. Here, we observed that HMGB1 expression was increased in the cytoplasm in Con A-induced mice. Because HMGB1 is involved in inflammation, HMGB1 is a potential target for inhibition of inflammation. Sawa et al. [35] reported that blockade of HMGB1 protein attenuated experimental severe acute pancreatitis. Recently, this result has been confirmed in animal models, such as traumatic brain injury, carotid artery injury, and liver injury in heatstroke [16]. Here, we explored whether ethyl pyruvate ameliorated inflammation by decreasing the expression of HMGB1 in Con A-induced autoimmune hepatitis in mice. We found that the level of HMGB1 in serum and tissue decreased significantly in the ethyl pyruvate pre-treatment group. It appears that ethyl pyruvate not only regulates the expression of newly synthesized HMGB1, but also suppresses HMGB1 transfer from the nucleus to the cytoplasm.

The mechanism behind how ethyl pyruvate decreases the expression of HMGB1 is not clear. It has recently been reported

by Wang et al. [36] that an NF- κ B inhibitor could inhibit HMGB1 expression in lung tissues of rats with COPD. Therefore, we firstly detected the expression of I κ B α and I κ B β in our model with western blot, results showed that the degradation of I κ B α and I κ B β was significantly blocked by ethyl pyruvate. Secondly, the expression of NF- κ B was also decreased by ethyl pyruvate. Taking these results, it can be demonstrated that ethyl pyruvate has the ability to down-regulate NF- κ B signal pathway. Therefore, it is possible that ethyl pyruvate may decrease the expression of HMGB1 by down-regulating NF- κ B signal pathway. There is also cross-talk between HMGB1 and other proinflammatory cytokines, such as TNF- α and IL-6; it has been reported that HMGB1 upregulated the level of TNF- α and IL-6 functioning as a later proinflammatory cytokine [30]. However, to a certain extent, these data appear to be in accordance with our results. Collectively, these data imply that there are other mechanisms involved in mediating the effects of ethyl pyruvate on HMGB1 in Con A-induced autoimmune hepatitis. We will focus on exploring these unknown mechanisms in the future.

Conclusions

The purpose of our study was to determine whether ethyl pyruvate could inhibit the expression and release of both early (TNF- α , IL-2, IL-6, IL-1 β) and late (HMGB1) cytokines in Con A-induced autoimmune hepatitis. Our results are as follows: (1) ethyl pyruvate attenuate Con A-induced autoimmune hepatitis in Balb/C mice; (2) ethyl pyruvate decreases the TNF- α , IL-2, IL-6, IL-1 β , and HMGB1 expression in vivo; (3) ethyl pyruvate may inhibit the release of HMGB1 through modulation of NF- κ B signal pathway. Although there are further mechanisms to be explored, our study provides a new approach for the treatment of acute hepatitis in the clinic.

Author Contributions

Conceived and designed the experiments: Y. Zhou CG. Performed the experiments: MS JL PC CL WD FW. Analyzed the data: MS CW Y. Zhang. Contributed reagents/materials/analysis tools: KC LX. Wrote the paper: MS.

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