

Original Article

Erythropoietin improves hepatic fibrosis via suppression of hepatic stellate cells and macrophage activation

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Abstract: Oxidative stress is one of the key factors responsible for occurrence and development of hepatic fibrosis. Nuclear factor erythroid 2-related factor 2 (Nrf2) serves as a major regulator of the cellular defense system against oxidative stress. The current study was designed to test whether rhEPO therapy improves CCl₄-induced liver fibrosis through Nrf2/HO-1 signaling pathways. Rats were subcutaneously injected with 50% sterile CCl₄ (a mixture of pure CCl₄ and olive oil, 0.3 mL/100 g) twice a week for 6 weeks. EPO (500 and 1000 U/kg three times a week, sc) was administered in the last 2 weeks. In CCl₄-treated rats, treatment of rhEPO could improve hepatotoxicity, alleviate hepatic stellate cell (HSC) activation, and decrease the extent of hepatic fibrosis. Furthermore, data supports that rhEPO significantly improved hepatic oxidative stress and inflammation. It was found that HO-1 expression and nuclear translocation of Nrf2 were significantly increased by rhEPO. Subsequent *in vitro* experiments found that rhEPO-induced inhibition in HSCs and Raw264.7 cell activation was significantly reversed by ZnPP. Data indicates that EPO has protective effects on CCl₄ induced oxidative stress, inflammation, and liver fibrosis in rats. The protective effects of rhEPO may be dependent on inhibition of HSCs and macrophage activation via Nrf2/HO-1 induction.

Keywords: EPO, hepatic fibrosis, CCl₄, HO-1, Nrf2

Introduction

Liver fibrosis is a scar repair process response to hepatic damage. With the passage of time, this process can lead to liver cirrhosis and liver function deterioration. Measures for prevention of liver fibrosis are particularly important. Erythropoietin (EPO), a kind of low molecular glycoprotein hormone, has additional multiple non-hematopoietic protective effects on a variety of diseases, including brain ischemia, myocardial infarction, and sepsis [1-3]. A previous study indicated that pretreatment with recombinant human erythropoietin (rhEPO) decreases carbon tetrachloride (CCl₄)-induced hepatic fibrosis [4]. However, mechanisms concerning the beneficial effects of EPO on hepatic fibrosis have not been completely demonstrated.

Nuclear factor erythroid 2-related factor 2 (Nrf2) activation promotes expression of anti-oxidative enzymes, such as heme oxygenase 1 (HO-1), which play important roles in anti-in-

flammation and anti-oxidation, according to previous studies [5]. Many studies have recently confirmed that HO-1 has protective effects on the liver under several pathological conditions, such as ischemia/reperfusion injury, alcoholic liver disease, drug-induced liver injury, and so forth [6-8]. In chronic liver disease, induction of Nrf2 and HO-1 is also important in preventing the development of liver fibrosis [9, 10]. Previous studies have indicated that EPO plays a role in cardiovascular and renal protection through HO-1 signaling pathways [11]. Therefore, it can be postulated that rhEPO might mitigate liver fibrosis through Nrf2/HO-1 signaling pathways.

Hepatocellular damage leads to the release of pro-fibrotic factors, such as transforming growth factor (TGF- β), cytokines, and chemokines, secreted by infiltrating inflammatory cells, especially macrophages. This results in the activation of hepatic stellate cells (HSCs) [12]. Activated HSCs are major targets for antifibro-

tic treatment. In addition, macrophages are found close to activated HSCs and play a key role in the development of hepatic fibrosis [12, 13]. The current study was designed to demonstrate whether rhEPO therapy improves CCl₄-induced liver fibrosis through Nrf2/HO-1 signaling pathways. It was hypothesized that Nrf2/HO-1 signaling pathways play dual roles by regulating both HSCs and macrophage activation in liver fibrosis.

Material and methods

Animals

Male Sprague-Dawley rats, weighing 200 to 250 g, were used in the study after 7 days of acclimatization. They were housed in the animal facilities with a 12-hour light/dark cycle. They were allowed access to standard rat chow and water ad libitum. All procedures were carried out in accordance with "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985).

Experimental design

Animals were randomly assigned to the control group (n = 6), CCl₄ group (n = 10), CCl₄-Epo500 group (n = 10), and CCl₄-Epo1000 group (n = 10). CCl₄-treated animals were subcutaneously injected with 50% sterile CCl₄ (a mixture of pure CCl₄ and olive oil, 0.3 mL/100 g) twice a week for 6 weeks. For rhEPO treatment (Kirin Holdings Company, Limited, Japan), 500 U/kg body weight rhEPO for the CCl₄-Epo500 group or 1,000 U/kg body weight rhEPO for the CCl₄-Epo1000 group was subcutaneously administered, three times a week, for last 2 weeks before the study date. All rats were anesthetized and sacrificed at 6 weeks. Animals in the control group received olive oil subcutaneous injections and 0.9% NaCl subcutaneous injections, respectively.

On the study date, all rats were anesthetized. Blood was aspirated from the inferior vena cava for serum biochemical analysis. The liver was removed and either snap-frozen in liquid nitrogen or fixed in phosphate-buffered formalin.

Biochemical assessment

Blood samples were centrifuged and serum was collected for biochemical testing, including

alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AKP), and total bile acid (TBA) assays at the laboratory.

Histologic scoring

Histopathology was evaluated by light microscopy on hematoxylin and eosin (H&E) and Masson's stained tissue sections. The scoring system for parenchymal damage, inflammatory response, and fibrosis was evaluated according to a score proposed by Thompson [14]. Scores concerning parenchymal damage are as follows: Score 1 (focal hepatocyte loss), Score 2 (zonal hepatocyte loss-patchy large/continuous thin), and Score 3 (zonal hepatocyte loss-large/confluent). Scores concerning inflammation are as follows: Score 1 (scanty cells present at junction of necrotic zone), Score 2 (cells regularly present), Score 3 (predominantly neutrophils present), and Score 4 (predominantly mononuclear present). Scores concerning the extent of fibrosis are as follows: Score 1 (slender septa present), Score 2 (slender septa linking hepatic veins), Score 3 (broad/well-developed septa), and Score 4 (cirrhosis).

Oxidative stress measurement

Protein concentrations of tissue extracts were assayed using the BCA assay kit from Bio-Rad (Hercules, CA, USA). Lipid peroxidation was evaluated by measuring the production of malondialdehyde (MDA) with a lipid peroxidation MDA assay kit (Beyotime, Shanghai, China). Catalase (CAT) activity was assayed, as previously described [15]. Glutathione (GSH) was determined by the spectrophotometric method, based on the use of Ellman's reagent [16]. Levels of SOD were measured, according to manufacturer instructions (Beyotime, Shanghai, China).

RNA extraction and RT-PCR analysis

Real time PCR was performed with a slightly modified procedure [17]. Briefly, total RNA was extracted from hCMEC/D3 cells using TRIzol Reagent (Invitrogen), according to manufacturer protocol. Complimentary DNA (cDNA) was synthesized from 100 to 500 ng of total RNA using the ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Gene expression in the samples was determined by qPCR using SYBR green

Table 1. Sequences of primers for RT-PCR

Gene	Sequence (S; sense)	Sequence (A; anti-sense)
<i>HO-1</i>	S: 5'-CAGAAGAGGCTAAGACCGCC-3'	A: 5'-CTGAGTGTGAGGACCCATCG-3'
<i>Nrf2</i>	S: 5'-GACTTGGAATTGCCACCGCC-3'	A: 5'-TCCTGTTCTTCTGGAGTTGC-3'
<i>IL-6</i>	S: 5'-AGTTGCCTTCTTGGGACTGA-3'	A: 5'-ACTGGTCTGTTGTGGGTGGT-3'
<i>TNF-α</i>	S: 5'-TTCCGTCCCTCTCATACACTG-3'	A: 5'-AGACACCGCCTGGAGTTCT-3'
<i>IL-1β</i>	S: 5'-GCCAACAAAGTGGTATTCTCCA-3'	A: 5'-TGCCGTCTTTCATCACACAG-3'
<i>iNOS</i>	S: 5'-ATCCCGAAA CGCTTCACT T-3'	A: 5'-CGGCTG GACTTCTCACTC-3'
<i>TGF-β1</i>	S: 5'-TTGCTTCAGCTCCACAGAGA-3'	A: 5'-TGGTTGTAGAGGGCAAGGAC-3'
<i>COL1A1</i>	S: 5'-CATGTTCACTTTGTGGACCT-3'	A: 5'-GCAGCTGACTTCAGGGATGT-3'
<i>COL1A1</i>	S: 5'-TCCCCTGGAATCTGTGAATC-3'	A: 5'-TGAGTCGAATTGGGGAGAAT-3'
<i>Fibronectin</i>	S: 5'-TGTCACCCACCACCTTGA-3'	A: 5'-CTGATTGTTCTTCAGTGCGA-3'
<i>GAPDH</i>	S: 5'-GGTGAAGGTCGGTGTGAACG-3'	A: 5'-CTCGCTCCTGGAAGATGGTG-3'

based fluorescence method. Thermocycling was performed as follows: 95°C for 10 minutes; 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds; and 72°C for 10 seconds. PCR primer sequences are shown in **Table 1**. Target gene expression (based on Ct) in each sample was normalized against glyceraldehyde-3-phosphate dehydrogenase. All reactions were performed in triplicate and data were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Proteins were loaded into 10% SDS-polyacrylamide gels and electro-transferred. PVDF membranes were blocked, incubated with the primary antibody at 4°C overnight, and incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody (1:20000, Sigma-Aldrich) for 1 hour at room temperature. After washing, the immune complexes were detected using ECL (Pierce Chemical Co, Thermo Scientific). Primary antibodies used for Western blotting were: α -SMA (1:2000; Sigma), HO-1 (1:1000, Abcam), Nrf2 (1:1000; CST), Lamin B (1:1000, Abcam), and GAPDH (1:1000, Abcam). Protein expression of each molecule was expressed as relative intensity and normalized to GAPDH or Lamin B (for nuclein).

Cell culturing and treatment

HSC-T6 and Raw264.7 cells were purchased from Shanghai Institutes for Biological Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% FBS, 100 U/mL of penicillin, and 100 U/mL streptomycin. For all experiments, cells were allowed to grow for 24 hours in the

culture medium prior to treatment. HSC-T6 cells were treated with rhEPO (0, 5, 10, and 20 U/mL) solutions for 24 hours. To test the roles of HO-1 in rhEPO exerted protective effects, HSC-T6 cells were treated with rhEPO in the presence or absence of ZnPP (10 μ M; Sigma-Aldrich), an inhibitor of HO-1. Raw264.7 cells were pre-treated with 20 U/mL rhEPO for 2 hours and with 100 ng/mL LPS (Sigma) for another 8 hours. To test the roles of HO-1 in rhEPO exerted anti-inflammatory effects, Raw264.7 cells were treated with rhEPO and LPS in the presence or absence of ZnPP (10 μ M).

Immunofluorescence staining

Cells were fixed in 2% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, washed, blocked with 10% FBS in PBS, and incubated with an anti- NF- κ B p65 primary antibody in 1.5% FBS overnight at 4°C. This was followed by incubation with a FITC (488 nm)-conjugated secondary antibody for 1 hour in 6% BSA. Cells were stained with 1 μ g mL⁻¹ of DAPI for 5 minutes. Stained cells were washed with PBS and visualized using a verted fluorescence microscope.

Detection of intracellular ROS

HSC-T6 and Raw264.7 cells were plated in 6-well plates and treated with rhEPO at indicated concentrations. Cells were harvested and suspended in 500 μ L DCFH-DA (10.0 μ M) for 30 minutes for detection of ROS. This was analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA).

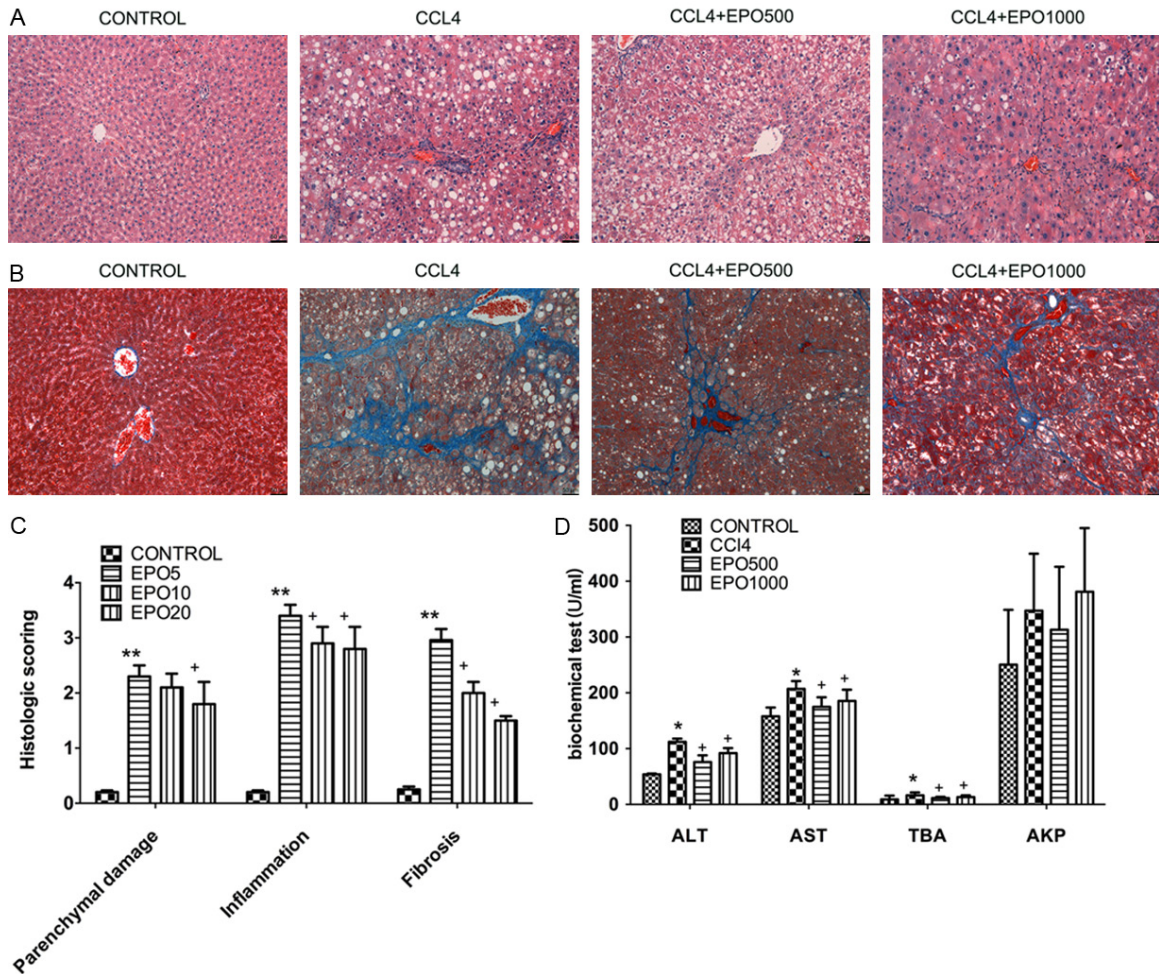


Figure 1. Effects of rhEPO on hepatotoxicity and hepatic fibrosis following CCl₄ treatment. A. Representative photomicrographs of liver histology (H&E) from each group. B. Representative photomicrographs of liver histology (Masson's trichrome staining) from each group. C. Scores of parenchymal damage, inflammatory scores, and fibrosis scores in each group. D. Serum parameters in control rats and CCl₄ rats receiving rhEPO or vehicle treatment. *P < 0.05, compared with control, **P < 0.01, compared with control. +P < 0.05, compared with CCl₄ group.

Statistical analyses

Data are reported as the mean \pm 1.0 SD, as appropriate. The statistical significance of differences among groups was analyzed by one-way ANOVA, followed by Newman-Keuls test. Comparisons between the two groups were performed using unpaired Student's t-test. All analyses were conducted with SPSS13.0 (SPSS Inc., Chicago, IL). Statistical significance is indicated by P < 0.05.

Results

Effects of rhEPO on CCl₄-induced liver injury and fibrosis

According to liver H&E staining examinations (Figure 1A, 1C), rats in the Control group had

no pathological changes. However, CCl₄ group rats had degenerative changes and centrilobular necrosis in the liver, including ballooning of hepatocytes, deposition of lipid droplets in hepatocytes, and infiltration of inflammatory cells, as well as collagen deposition. In accord with results concerning serum aminotransferase activities (Figure 1D), severe hepatic lesions induced by CCl₄ were mitigated by the administration of rhEPO, to some extent. Results showed alleviation of the degree of hydropic degeneration, inflammation, and necrosis.

Hepatic fibrosis was evaluated by Masson's trichrome staining (Figure 1B). Obvious bridging fibrosis was observed in the CCl₄ group, which showed masses of collagen deposition surrounding the portal area and formation of many

false lobules. Liver fibrosis and corresponding fibrosis scores were obviously reduced in rhEPO treated groups (**Figure 1C**). Present results demonstrate that rhEPO may improve CCl₄-induced hepatotoxicity, to some extent.

Effects of rhEPO on CCl₄-induced HSCs activation

HSCs play a crucial role in hepatic fibrosis and have become a major therapeutic target in prevention and treatment of liver cirrhosis [12]. The α -SMA is a marker of HSCs activation. TGF- β acts as a triggering factor to activate HSCs and fibroblasts, which accelerates the accumulation of extracellular matrix (ECM) proteins. Moreover, mRNA levels of TGF- β 1 were significantly increased in livers of the CCl₄ group, compared to the control group, and were significantly decreased by rhEPO co-treatment (**Figure 2A**). Western blot analysis showed that α -SMA levels were greater in the CCl₄ group than in the control group (**Figure 2B**). However, rhEPO-treated rats had significantly decreased α -SMA levels. In addition, levels of hepatic hydroxyproline were gradually increased in the CCl₄ group, but were markedly reduced by rhEPO (**Figure 2C**). Moreover, the effects of rhEPO against CCl₄-induced HSCs activation were further confirmed by mRNA levels of COL1A1, COL3A1, and fibronectin (**Figure 2A**).

Effects of rhEPO on CCl₄-induced liver oxidative stress and inflammation

The involvement of oxidative stress can be demonstrated in fundamental events of hepatic fibrogenesis, including activation of HSCs [12, 13]. Levels of MDA were detected to evaluate the effects of rhEPO on CCl₄-induced lipid peroxidation. MDA levels in the CCl₄ group were significantly higher than those in the control group. Moreover, rhEPO treatment significantly decreased MDA levels. GSH plays pivotal roles in the scavenging of free radicals and prevention of liver damage caused by ROS. GSH levels in CCl₄ groups were lower than those in the control group and GSH levels in rhEPO groups were higher than those in the control group. Similar to GSH, CAT activity and SOD levels were significantly decreased in CCl₄ groups, but mildly reserved by rhEPO (**Figure 3A-D**).

Expression and synthesis of this inflammatory cytokine are modulated through redox-sensitive reactions [18]. Thereby, the current study determined mRNA levels of inflammatory cyto-

kines, such as TNF- α , IL-6, IL-1 β , and iNOS. Results showed that liver mRNA levels of TNF- α , IL-6, IL-1 β , and iNOS were significantly elevated in the CCl₄ group, compared to the control group. These increased levels of pro-inflammatory cytokines were downregulated in rhEPO group (**Figure 3E-H**).

Effects of rhEPO on HO-1 expression and Nrf2 nuclear localization

It was hypothesized that the protective effects of rhEPO against CCl₄ induced oxidative stress are mediated by the induction of antioxidant gene HO-1 through Nrf2. EPO treatment did not significantly increase liver Nrf2 mRNA expression in the CCl₄ group (**Figure 4A**). However, Western blotting showed that rhEPO significantly increased the nuclear translocation of Nrf2, as evidenced by the accumulation of Nrf2 in the nucleus (**Figure 4C**). Additionally, as shown in **Figure 4B**, compared with the CCl₄ group, rhEPO treatment significantly increased HO-1 mRNA expression. In correspondence with mRNA expression, this study also found that protein levels of HO-1 were also significantly increased by rhEPO (**Figure 4D**). These data support the present hypothesis that rhEPO-induced Nrf2 translocation promotes the transcription of antioxidant gene HO-1.

Roles of Nrf2/HO-1 signaling in rhEPO-induced effects on HSCs

Cellular formation of reactive oxygen species (ROS) is involved in HSCs activation [12]. HSC-T6 cell proliferation was inhibited by rhEPO treatment and 20 U/mL of rhEPO showed better effects (**Figure 5A**). Compared with control cells, rhEPO treatment significantly increased HO-1 mRNA expression and the highest expression was observed in the 20 U/mL group (**Figure 5B**). Western blotting showed that rhEPO significantly increased the nuclear translocation of Nrf2, proven by the accumulation of Nrf2 in the nucleus (**Figure 5C**). Compared with control HSC-T6 cells, rhEPO treatment significantly increased HO-1 expression and decreased α -SMA levels.

To determine whether upregulation of Nrf2/HO-1 signaling pathways is involved in the protective effects of rhEPO, cells were treated with ZnPP and rhEPO. The intervention concentration of 20 U/mL was chosen for the following study. It was demonstrated that rhEPO-induced decreases in ROS levels and α -SMA levels were

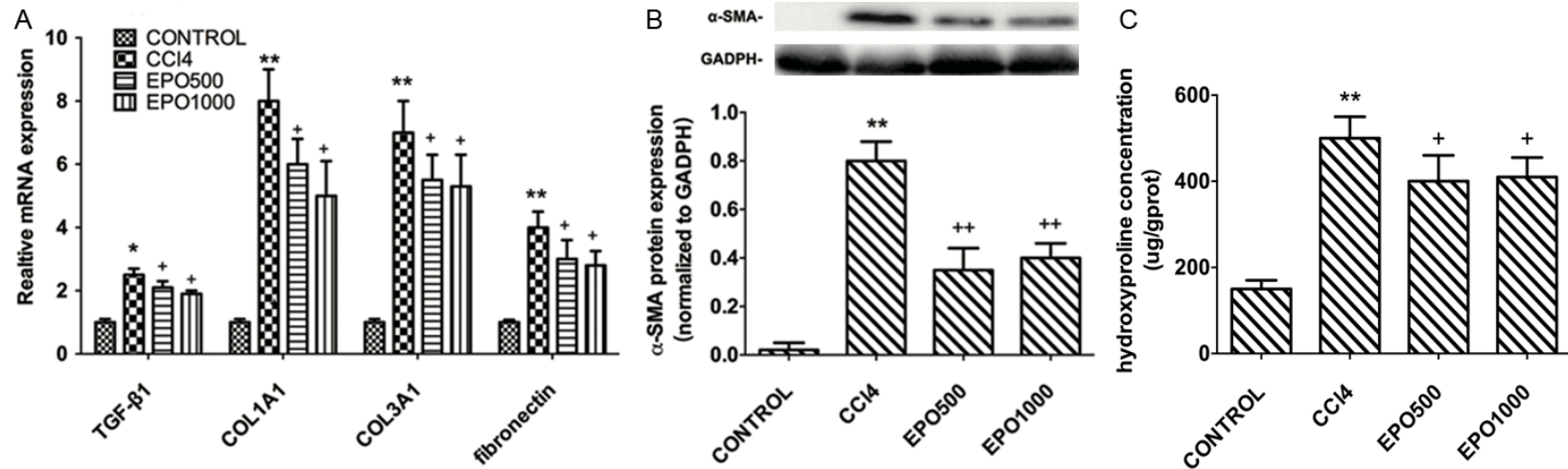


Figure 2. Effects of rhEPO on HSCs activation *in vivo*. A. Effects of rhEPO on mRNA levels of TGF-β1, COL1A1, COL3A1, and fibronectin in the rats. B. Effects of rhEPO on α-SMA levels, as determined by Western blot analyses. C. Effects of rhEPO on hydroxyproline levels in rat livers. *P < 0.05, compared with control, **P < 0.01, compared with control. +P < 0.05, compared with CCl₄ group, ++P < 0.01, compared with CCl₄ group.

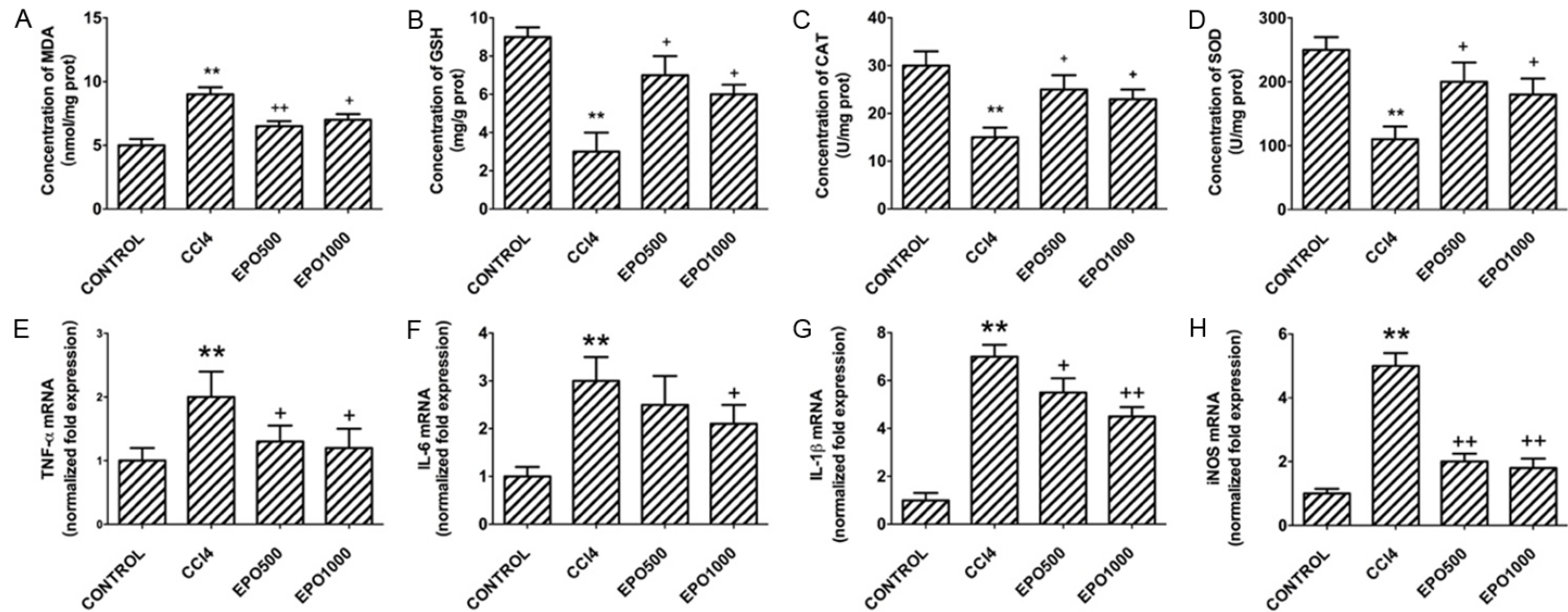


Figure 3. Effects of rhEPO on hepatic oxidative stress and inflammation. Data are shown as follows: (A) MDA levels, (B) GSH levels, (C) CAT activities, (D) SOD activities, (E-H) Hepatic TNF- α , IL-6, IL-1 β , and iNOS mRNA as determined by Rt-PCR. *P < 0.05, compared with control, **P < 0.01, compared with control. +P < 0.05, compared with CCl₄ group, ++P < 0.01, compared with CCl₄ group.

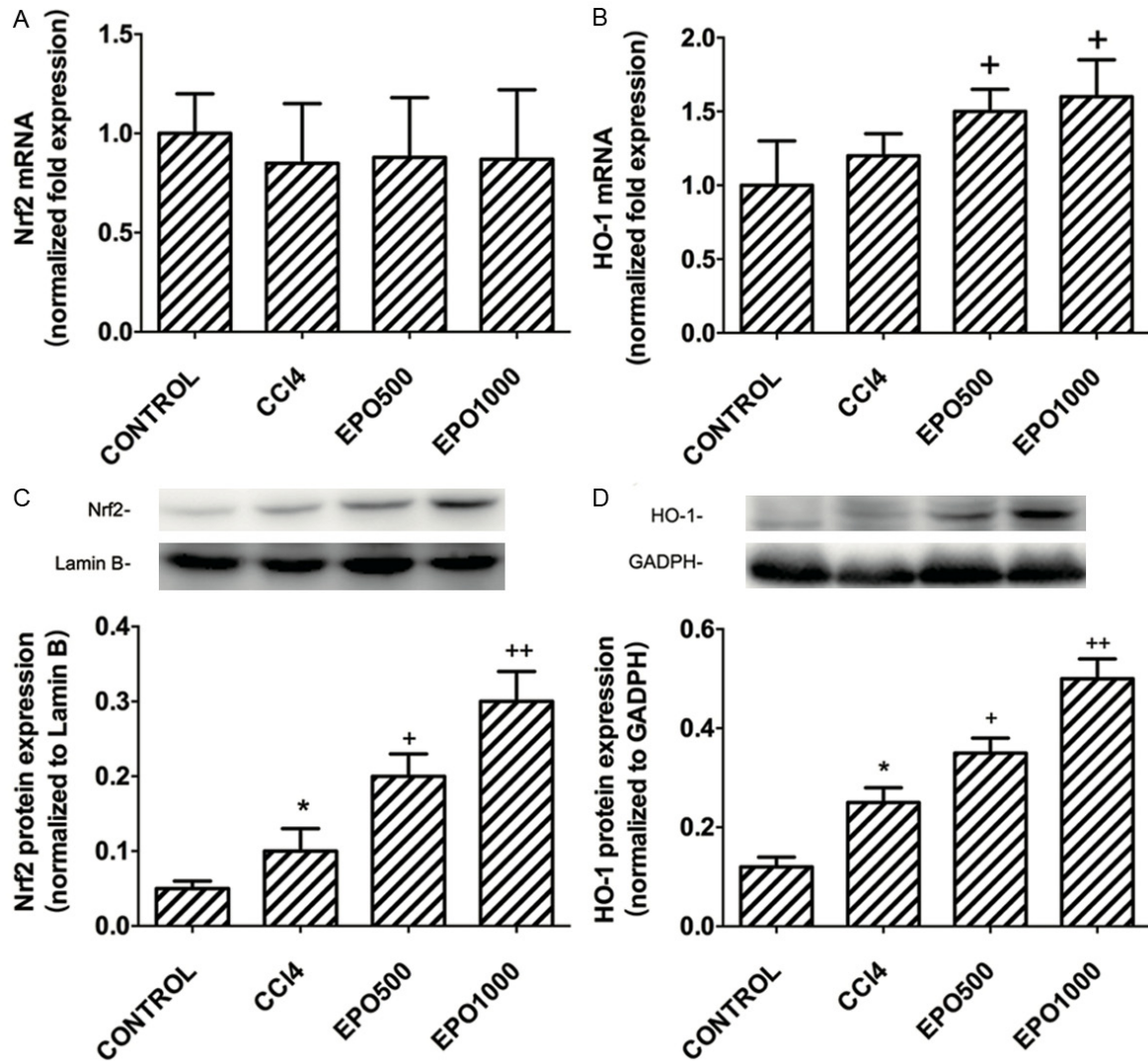


Figure 4. Effects of rhEPO on HO-1 expression and Nrf2 nuclear localization. (A) Hepatic Nrf2 mRNA and (B) HO-1 mRNA, as determined by Rt-PCR. (C) Nuclear Nrf2 expression and (D) total HO-1 expression in liver tissue, as determined by Western blot analyses. *P < 0.05, compared with control, **P < 0.01 compared with control. +P < 0.05, ++P < 0.01 compared with CCl₄ group.

significantly reversed by ZnPP treatment (**Figure 5D, 5E**). Results indicate that Nrf2/HO-1 signaling pathways are involved in rhEPO-induced protective effects against HSCs activation.

Roles of Nrf2/HO-1 signaling in rhEPO-induced effects on macrophage activation

A previous study showed that EPO inhibits the production of proinflammatory genes induced

by activated macrophages, which is mechanistically attributable to blockage of nuclear factor (NF)- κ B p65 activation by EPO [19]. Cytokines from infiltrating inflammatory cells, especially macrophages, result in the activation of HSCs [12]. To manifest whether rhEPO inhibits activation of macrophages via inducing HO-1 expression, Raw264.7 cells were treated with LPS, rhEPO, and ZnPP, alone or in combination. This study subsequently detected the intracellular

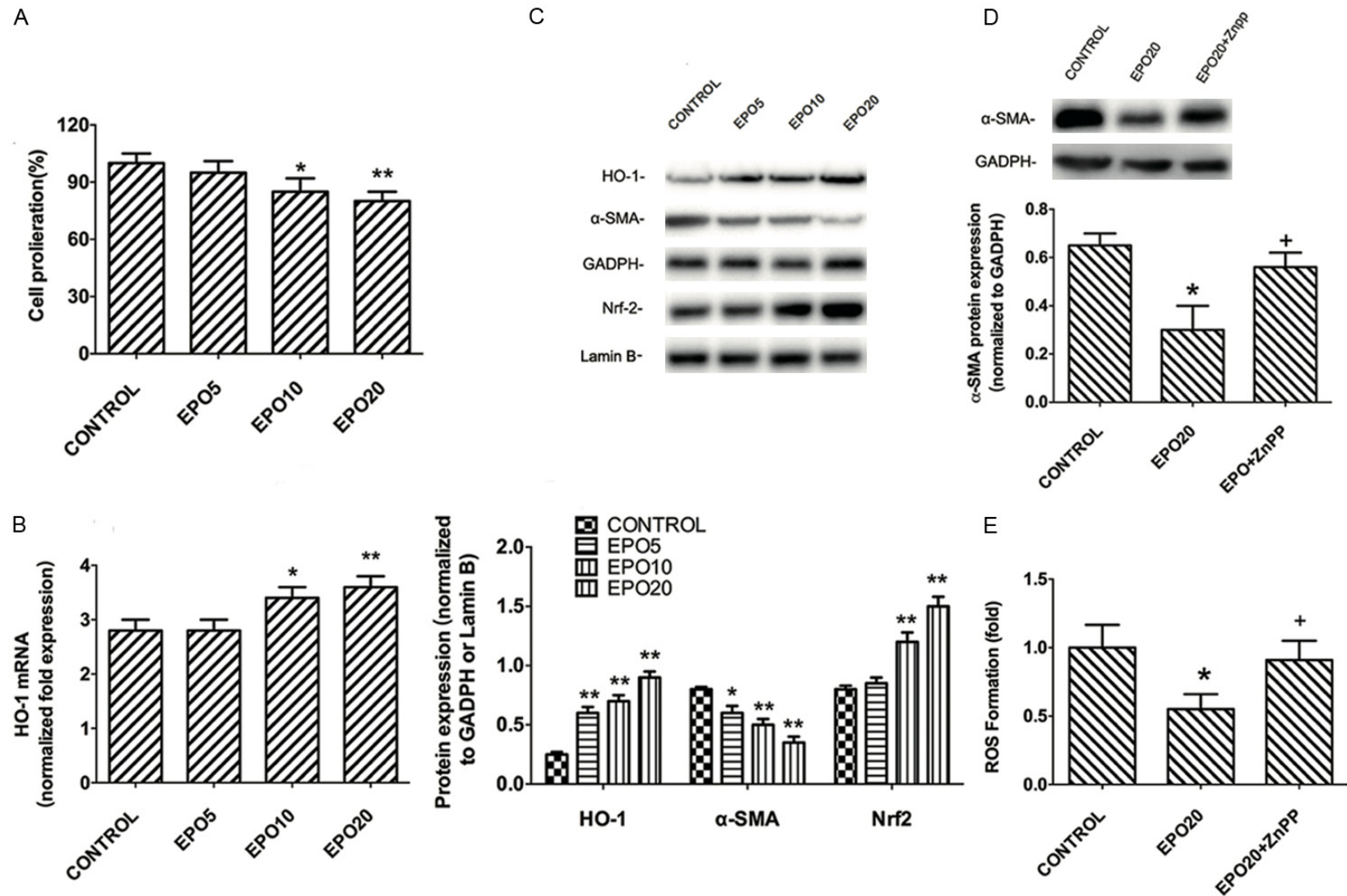


Figure 5. Roles of Nrf2/HO-1 signaling in rhEPO-induced effects on HSC-T6 cells. (A) Effects of rhEPO on cell proliferation, as determined by MTT. (B) Effects of rhEPO on HO-1 mRNA, as determined by Rt-PCR. (C) Effects of rhEPO on total HO-1, nuclear Nrf2, and α-SMA expression, as determined by Western blot analyses. (D) Effects of rhEPO on α-SMA expression and (E) levels of ROS in HSC-T6 cells which were treated with ZnPP and then exposed to 20 U/mL rhEPO for 24 hours. *P < 0.05, compared with control, **P < 0.01 compared with control. +P < 0.05 compared with CCl₄ group.

localization of NF- κ B p65 subunit by immunofluorescence staining. As shown in **Figure 6A**, HO-1 inhibitor ZnPP effectively abolished the inhibitory effects of rhEPO on LPS-induced nuclear translocation of NF- κ B p65 subunit. In addition, ZnPP diminished the effects of rhEPO against LPS-induced intracellular ROS levels and mRNA expression of inflammatory cytokines (**Figure 6B-F**).

Discussion

In the current study, the CCl₄-treated group exhibited significant hepatotoxicity and hepatic fibrosis, while rhEPO treatment significantly improved hepatic oxidative stress, inflammation, and hepatic fibrosis. According to *in vitro* models, rhEPO mitigates HSCs activation by inhibiting oxidative stress. This study found that EPO inhibits NF- κ B activation and induction of pro-inflammatory genes in LPS treated Raw-264.7 cells. However, inhibition of HO-1 can partially reverse the above beneficial effects.

Oxidative stress has been considered a major mechanism in the pathogenesis of hepatic fibrosis [12]. Free radicals generated under high oxidative stress can react with polyunsaturated fatty acids and induce the generation of MDA, which is known to increase collagen synthesis. Consistent with previously reported findings from studies of CCl₄-induced hepatic fibrosis [20], the current study found that MDA levels were elevated significantly in CCl₄-treated rats. There are also several endogenous antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase, that maintain a balance between ROS and the anti-oxidative defense system. Specifically, decreased GSH, CAT, and SOD levels in the liver tissue in CCl₄-induced hepatic fibrosis have been shown in this study and previous studies [20, 21]. EPO has antioxidative activities [22, 23]. In this study, rhEPO treatment significantly attenuated MDA levels and improved hepatic GSH, CAT, and SOD levels. Results of pathological detection and serum liver function testing were consistent with the above results.

Nrf2 activation promotes expression of antioxidant enzymes, such as HO-1, which play important roles in anti-inflammation and anti-oxidation, according to previous studies [5, 7, 9, 10]. Therefore, regulating the production of oxidative stress via Nrf2-dependent upregulation

of HO-1 would be effective for the prevention and treatment of a variety of oxidative injuries. Many studies have confirmed that HO-1 has protective effects on the liver under several pathological conditions, such as ischemia/reperfusion injury, chronic liver disease, and so forth [6, 8]. Several studies have indicated that EPO exhibits protective effects through HO-1 signaling pathways [11]. The current study found that rhEPO induced Nrf2 translocation and promoted the transcription of antioxidant gene, HO-1. These data support the hypothesis that rhEPO might mitigate liver fibrosis through Nrf2/HO-1 signaling pathways.

Hepatic stellate cells and Kupffer cells are key players in liver fibrosis [12, 13]. Their cross-talk is triggered and favored by a series of inflammatory and pro-fibrogenic cytokine mediators, such as TGF- β 1. Expression and synthesis of these mediators are mainly modulated through redox-sensitive reactions. To further confirm that HO-1 upregulation, induced by rhEPO, mediates suppressive effects on hepatic fibrosis, *in vitro* models of activation of HSCs and Raw264.7 cells were used [24, 25]. In this model of activation of HSCs, it was found that rhEPO treatment significantly increased the nuclear translocation of Nrf2 and HO-1 mRNA expression. Compared with control HSC-T6 cells, rhEPO could inhibit activation of HSCs and oxidative stress. An inhibitor of HO-1 can partially reverse the effects. It may, therefore, be postulated that rhEPO mitigates liver fibrosis through Nrf2/HO-1 signaling pathways by inhibiting oxidative stress-induced activation of HSCs.

Direct or indirect activation of Kupffer cells appears to be involved in the liver fibrosis process due to CCl₄ intoxication [12]. Cytokines from infiltrating inflammatory cells, especially macrophages, result in the activation of HSCs. Several transcription factors in particular nuclear factor κ B (NF- κ B) have been detected to be modulated by intracellular ROS [12]. A previous study showed that EPO inhibits the induction of pro-inflammatory in activated macrophages, which is mechanistically attributable to blockage of nuclear factor (NF)- κ B p65 activation by EPO [19]. This study subsequently found that HO-1 inhibitor ZnPP effectively reversed the inhibitory effects of rhEPO on LPS-induced nuclear translocation of NF- κ B p65 subunit in the model of Raw264.7 cells. In addi-

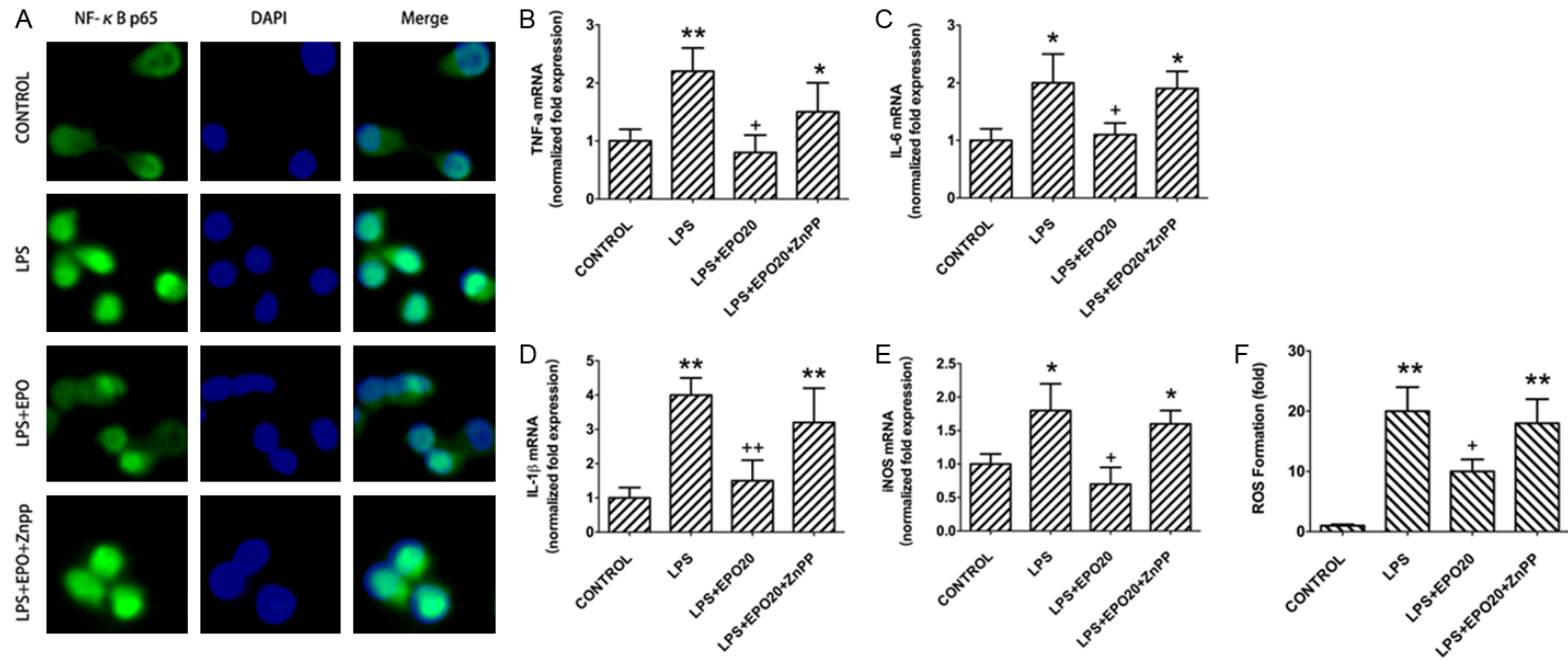


Figure 6. Roles of Nrf2/HO-1 signaling in rhEPO-induced effects on macrophage activation. A. Effects of rhEPO on NF-κB p65 nuclear translocation in LPS-stimulated Raw264.7 cells, as determined by immunofluorescence. The cell nuclei were stained with DAPI. Representative images were shown. B-E. Effects of rhEPO on cell TNF-α, IL-6, IL-1β, and iNOS mRNA, as determined by Rt-PCR. F. Effects of rhEPO on the levels of ROS, as determined by flow cytometry after DCFH-DA staining. *P < 0.05, compared with control, **P < 0.01, compared with control. +P < 0.05, compared with CCl₄ group, ++P < 0.01, compared with CCl₄ group.

tion, ZnPP diminished the activities of rhEPO against LPS-induced intracellular ROS levels and mRNA expression of inflammatory cytokines. These data suggest that rhEPO inhibits activation of macrophages via inducing HO-1 expression.

However, current studies on whether the immune-modulatory effects of EPO are beneficial are not always consistent. A few studies are available about rhEPO treatment concerning the clinical issue of infections. In critical patients diagnosed with pneumonia or sepsis, receiving appropriate antimicrobial therapy, the administration of EPO appears to be safe [1]. However, In systemic salmonella infection, treatment of mice with EPO results in reduced survival and impaired pathogen clearance because of diminished formation of antimicrobial effector molecules, such as TNF- α and NO [19]. In this respect, it is unlikely that EPO will have beneficial effects in infection induced inflammation without appropriate antimicrobial therapy. Thus, the clinical effects of EPO administration in sepsis or in chronic infections (decompensated liver cirrhosis) have to be carefully evaluated.

In summary, the present study demonstrated that rhEPO has a protective effect on CCl₄-induced oxidative stress, inflammation, and liver fibrosis in rats. Mechanisms of the protective effects of rhEPO may be dependent on inhibition of HSCs and macrophage activation via Nrf2/HO-1 induction.

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Disclosure of conflict of interest

None.

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