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Original Article

7,8-dihydroxycoumarin has a dual mechanism of action in hepatic ischemia reperfusion injury

Hai-Yang Zhu, Hong-Wei Gao, Shu-Feng Zhang

Department of Gastroenterology, The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou 450000, Henan, China

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Abstract: The present study was designed to investigate the protective effect of 7,8-dihydroxycoumarin on hepatic ischemia/reperfusion (I/R) injury in the rats. The rats were divided in three groups of 10 each; normal control, untreated and the 7,8-dihydroxycoumarin treatment groups. The rats in the treatment group received 7,8-dihydroxycoumarin at doses of 15 mg/kg body weight 1 h prior to ischemia and then daily for 2 days. The animals were sacrificed after 1, 12, 24, 36, and 48 h of reperfusion. The results revealed that 7,8-dihydroxycoumarin protected the liver against I/R injury via inhibition of inflammatory response at the early stage (0-24 h). However, in 7,8-dihydroxycoumarin treatment group autophagy was inhibited resulting in intensified I/R injury following 36 h of reperfusion. 7,8-dihydroxycoumarin treatment caused reduction in the level of serum aminotransferase, liver inflammatory cytokines and showed minor liver histopathologic alterations. However, after 36 h of reperfusion treatment group showed similar I/R injury as that of untreated group. It was observed that 7,8-dihydroxycoumarin enhanced the activation of mitogen-activated protein kinase, decreased nuclear release of high-mobility group box 1 and production of inflammatory cytokines. After 36 h 7,8-dihydroxycoumarin promoted hepatic injury through suppression of autophagy and induction of hepatic apoptosis. Therefore, 7,8-dihydroxycoumarin exhibits inhibitory effect on hepatic ischemia during 0-24 h but causes its promotion after 36 h.

Keywords: 7,8-dihydroxycoumarin, ischemic injury, reperfusion, apoptosis, cytokines

Introduction

Liver ischemia/reperfusion (I/R) injury is the leading cause behind failure of liver function and is associated with liver surgery, especially after hepatectomy and hepatic transplantation. The common causes for the I/R injury involve post ischemia blood supply disruption and deficient oxygen supply to the hepatic tissues. The liver blood supply disruption is induced by the factors which include hemorrhagic shock, severe trauma and disruption stage sepsis [1]. Ischemia reperfusion injury induces damage to various body organs like kidneys, heart, brain, and liver [2, 3]. Different mechanisms including response to hypoxia, reaction involving inflammation and free radical induced injury are responsible for the hepatocyte damage during liver ischemia after reperfusion [4]. For hepatocyte damage various signaling pathways involving interaction between multiple inflammatory pathways are responsible [5-7]. During reperfusion higher expression of pro-inflammatory cytokines like tumor necrosis factor (TNF)-α, high-mobility group box 1 (HMGB1) and interleukin (IL)-1b in the rat serum and hepatic tissues is induced. The pro-inflammatory cytokines play a vital role in the liver I/R injury pathology [8-11]. Currently, liver I/R injury is a challenge to the clinicians all over the world and its prevention can be of great importance for the successful transplantation of liver and hence for the treatment of liver diseases.

7,8-dihydroxycoumarin, an isolate from thymelaeceae family, exhibits a promising role in the protection of cardiovascular system and has free access to the blood-brain barrier [12]. It is reported that 7,8-dihydroxycoumarin induces expression of GAP-43 during early stage of liver ischemia/reperfusion and also removes necrotic substances from the neurons. It improves water and electrolyte balance, promotes neurotransmission and metabolism in addition to

increase in the secretion of neurotrophic factors [12-14]. The present study was performed to investigate the role of 7,8-dihydroxycoumarin in the prevention of hepatocyte damage induced during I/R.

Materials and methods

Treatment strategy

The animals were divided into 3 groups; control, untreated and treatment groups. The animals in the treatment group were given 7,8-dihydroxycoumarin (15 mg/kg) 2 h before ischemia and daily for 2 days post ischemia. The rats were sacrificed after 1, 12, 36 and 48 h reperfusion to investigate the effect of 7,8-dihydroxycoumarin in liver injury, level of cytokines, expression of hepatic HMGB1 released, activation of MAP kinase, autophagy and apoptosis.

Animals

Male inbred Lewis rats (250-320 g) were purchased from Charles River Laboratories (Sulzfeld, Germany). The animals were acclimatized to the laboratory atmosphere 1 week before the start of experiment under standard animal care conditions and had free access to water and food. The study was approved and performed according to the German Animal Welfare Legislation.

7,8-dihydroxycoumarin administration

7,8-dihydroxycoumarin was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in normal saline solution. Dimethyl sulphoxide (DMSO) and other common chemicals were obtained from Merck and Fluka chemical companies.

Assay for analysis of liver cell damage

For the investigation of hepatocyte damage the levels of AST and ALT in the rat serum was measured by using Automated Chemical Analyzer (Bayer Advia; Leverkusen, Germany).

Histopathology: The animals were sacrificed to extract liver which was fixed in paraformaldehyde and then embedded in paraffin. The tissues were then cut into thin sections of around 2 μm, deparaffinized using xylene followed by rehydration through a series of ethanol concentrations. The sections were stained with hematoxylin-eosin stain. The semiquantitative method of scoring system was used for the histological evaluation of the tissue sections using light microscopy.

Immunohistochemistry for HMGB1

For the purpose of immunohistochemisty the tissue sections after deparaffinization were rehydrated and citrate buffer (pH 6.0) was used for antigen retrieval. The hydrogen peroxide solution was used to block the endo-genous peroxidase. The slides after blocking with 10% normal horse serum were incubated with HMGB1 antibody (Cambridge, UK) at 4°C overnight. The slides after washing were incubated for 45 min with the biotinylated secondary antibody and then ABC reagent (Vector Labs) and diaminobenzidine. The slides after hematoxylin counterstaining were dehydrated using gradient ethanol and xylene, mounted and then analyzed. PowerVision goat-anti-Rabbit-AP (ImmunoLogic, DPVR-110AP, Duiven, the Netherlands) and Fast-red were used for the detection.

Gel electrophoresis and western blotting

The protein samples (30 μg) after electrophoresis on a 10% SDS-PAGE gel under reducing conditions were electroblotted onto nitrocellulose membrane. The 5% non-fat milk was used to block the membrane followed by incubation at 4°C with primary antibodies overnight. Primary antibodies used were against phosphorylated or total ERK (Cell Signaling Technology), JNK (Cell Signaling Technology) and p38 (Cell Signaling Technology,); and anti-caspase-3 (Cell Signaling Technology), caspase-7 (Cell Signaling Technology), LC3 (Abcam), SQSTM1/p62 (Cell Signaling Technology), as well as anti-glyceraldehyde-3-phosphate dehydrogenase (Sigma-Aldrich). The blots were washed and then incubated with secondary antibody for 1 h. For the detection of signals ECL substrate was used where as GAPDH was used as internal control. Densitometric analysis was used for quantification of the intensity of bands.

Enzyme-linked immunosorbent assay

The ELISA assay (Shino-Test, ST51011, Kanagawa, Japan) was used to measure the level of HMGB1 in the rat serum according to the manufacturer's instructions.

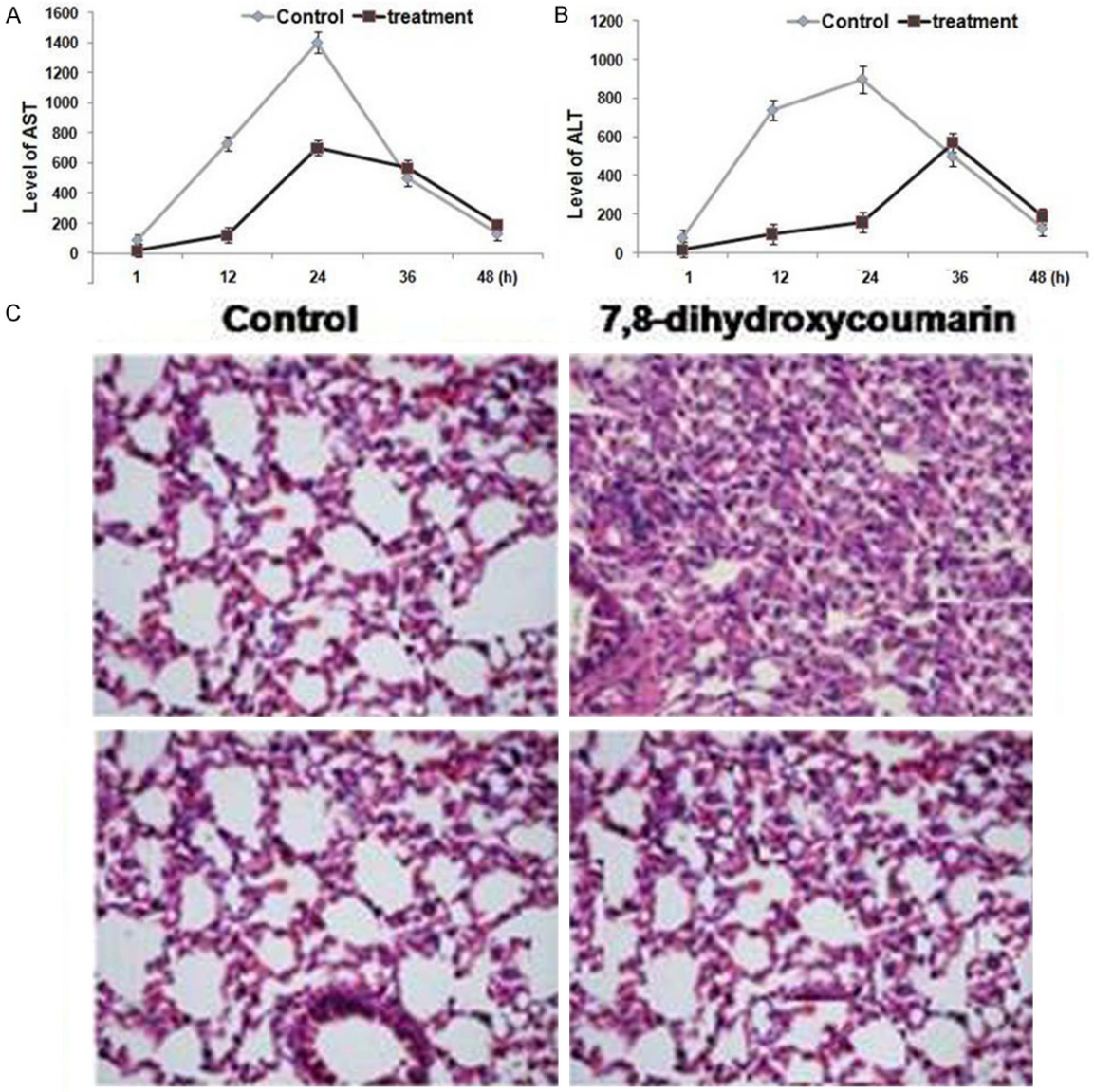

Figure 1. Effect of 7,8-dihydroxycoumarin treatment in the prevention of liver I/R injury at early phase and was intensified after 36 h of I/R injury.

Quantitative polymerase chain reaction (PCR)

From the liver tissues total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The RNA samples were subjected to DNase I (Promega Corporation) treatment according to the manufacturer's instructions. From the 2 μg total RNA, cDNA was synthesized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA amplification was performed using Power SYBR-Green Master Mix (Applied Biosystems) in the Applied Biosystems 7500 Fast Real Time PCR system and analyzed with 7500 software v.2.0.4. The primers were mixed with Brilliant probe-based QPCR Master Mix (Santa Clara, CA, USA) and then diluted with distilled deionized H_2O up to 20 ml. PCR amplification was performed on an M × 3000P QPCR System (Stratagene, La Jolla, CA, USA).

Statistical analysis

All the data presented are the mean ± S.D. One-way ANOVA was used for the determination of differences among the groups. SigmaStat v3.5 (Systat-Software, Erkrath, Germany)

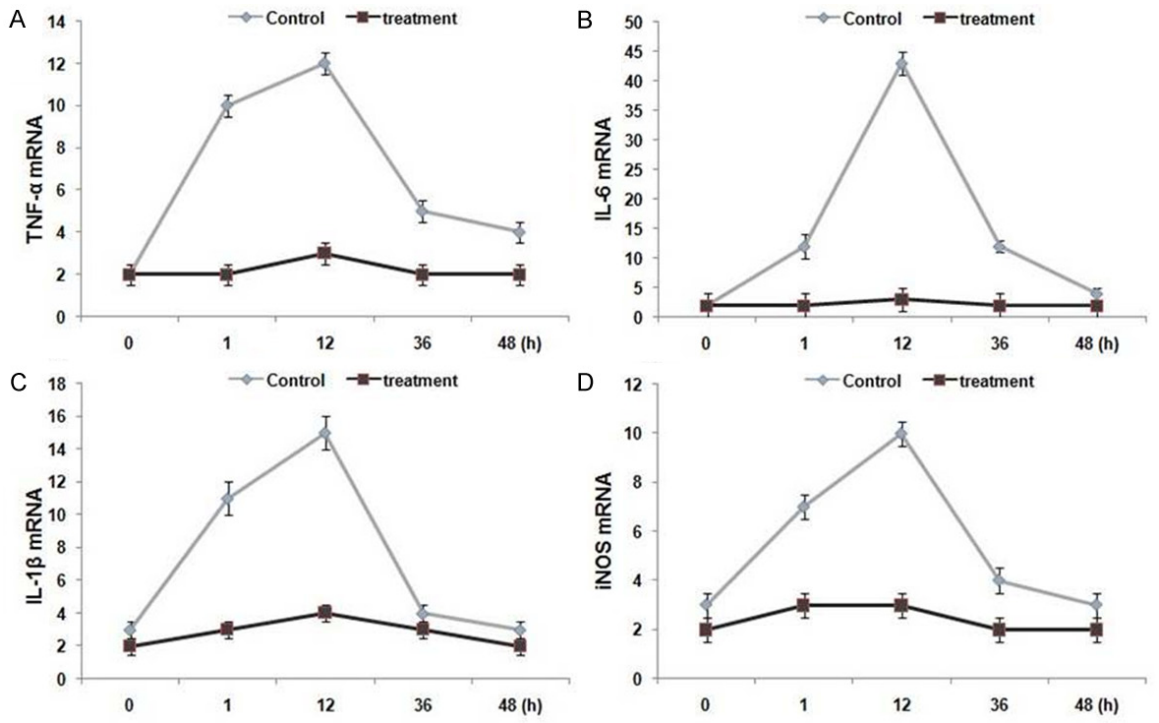

Figure 2. 7,8-dihydroxycoumarin induced decrease in the expression of inflammatory cytokines during early stage of I/R injury.

to perform all the tests. The differences were considered significant statistically at *P* values below 0.05.

Results

Effect of 7,8-dihydroxycoumarin on liver injury

To investigate the effect of 7,8-dihydroxycoumarin on the liver injury, warm ischemia was induced in the rats. The rats with liver warm I/R showed markedly higher levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) in the serum compared to the control group. The rats in the treatment group received 7,8-dihydroxycoumarin injections 2 h prior to the induction of warm ischemia and daily after induction for 2 days. Examination of the rats showed that 7,8-dihydroxycoumarin treatment caused a significant reduction in the I/R injury after 1 and 12 h which was evident from decreased secretion of enzyme in liver (**Figure 1A**) after 60 min of warm ischemia. However, after 36 and 48 h of the reperfusion the levels of ALT and AST in the serum increased significantly compared to the control group of rats ($P < 0.05$).

The above findings were further confirmed by the treatment of rats with 7,8-dihydroxycoumarin after 90 min of warm I/R. The results revealed that 7,8-dihydroxycoumarin treatment led to the similar dual effects as observed above after the rats were exposed to longer warm ischemic injury (**Figure 1B**).

The histopathological investigation of the liver tissues in the control group showed the presence of necrotic cells, cytoplasmic vacuoles and dilation of sinusoids after 6 h of reperfusion. However, 7,8-dihydroxycoumarin treatment in the rats inhibited the cell necrosis, caused disappearance of cytoplasmic vacuoles and dilation of sinusoids after 12 h of reperfusion. But the presence of cytoplasmic vacuoles and cell necrosis were clearly visible in the 7,8-dihydroxycoumarin treatment group after 36 h compared to control group (**Figure 1C**).

Effect of 7,8-dihydroxycoumarin on gene expression of hepatic inflammatory cytokines

We used quantitative PCR to investigate the hepatic mRNA for TNF-a, IL-6, IL-1b and inducible nitric oxide synthase (iNOS) expression. After 60 min of warm ischemia, the rats in the

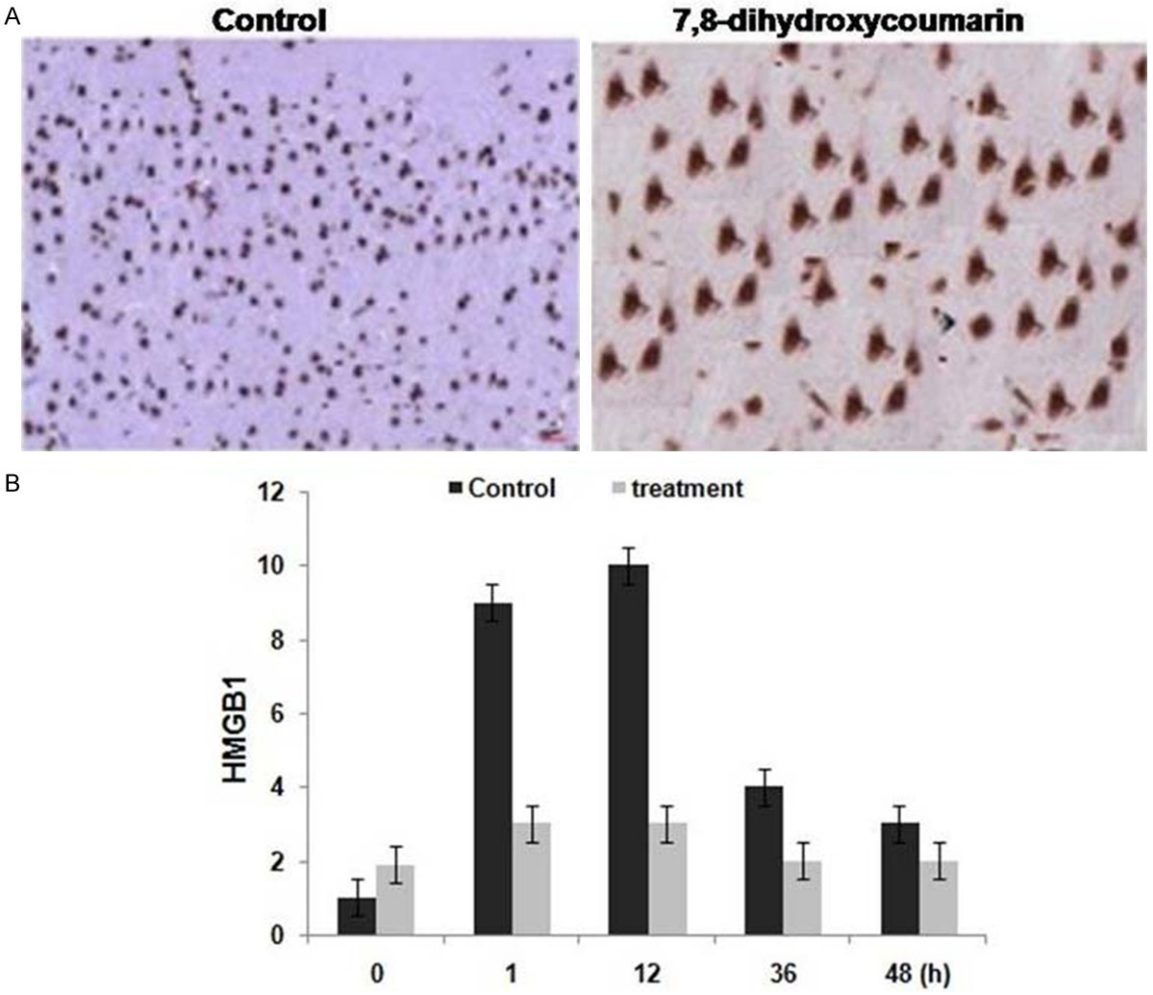

Figure 3. 7,8-dihydroxycoumarin induced reduction in the release of HMGB1 at early stage of I/R injury.

untreated group showed significantly higher levels of TNF-a, IL-6, IL-1b and iNOS expression compared to the 7,8-dihydroxycoumarin treatment group at 1 and 12 h after reperfusion. The levels of TNF-a, IL-6, IL-1b and iNOS mRNA in the untreated group were 7-, 28-, 14- and 8-fold, respectively higher after 24 h compared to the 7,8-dihydroxycoumarin treatment group (**Figure 2**).

Chloroquine treatment decreases HMGB1 release at early phase: It is reported that hepatic ischemia is accompanied by the increase in the expression of cytokine, HMGB1 [10, 11]. In the untreated group, a marked increase in the nuclear HMGB1 staining of hepatocytes was observed after 1 h of the reperfusion (**Figure 3**). However, 7,8-dihydroxycoumarin treatment inhibited the nuclear release of HMGB1 in the hepatocytes.

Investigation of the HMGB1 level in the rat serum after I/R injury was performed using enzyme-linked immunosorbent assay (ELISA). The results revealed that the serum levels of HMGB1 was significantly higher in the untreated group compared to the 7,8-dihydroxycoumarin treatment group. In the treatment group, the levels of HMGB1 in serum were respectively, 0.67 ± 0.12 and 0.47 ± 0.14 ng/ml after 1 and 12 h of reperfusion compared to 2.03 ± 0.56 in untreated and 0.47 ± 0.24 ng/ml in the normal group following 12 h of reperfusion.

Effect of 7,8-dihydroxycoumarin on I/R-induced inflammatory signaling pathways

Activation of mitogen-activated protein (MAP) kinases is most commonly observed during I/R induction [15]. Examination of the untreated rats showed a significant increase in the p38

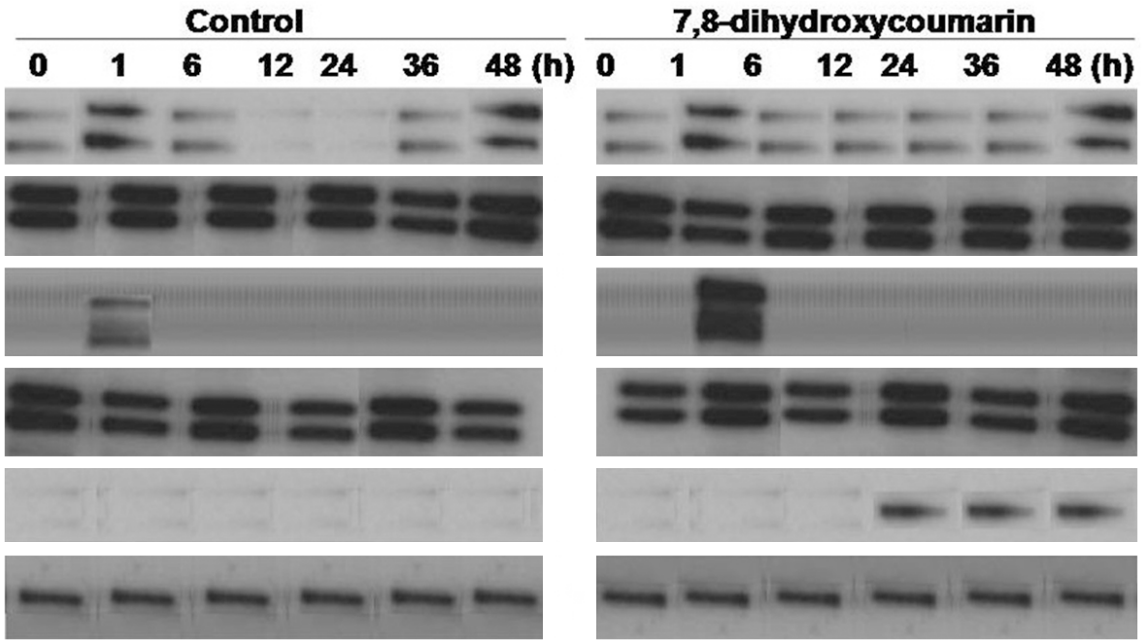

Figure 4. 7,8-dihydroxycoumarin induced modulation in the MAP kinase activation.

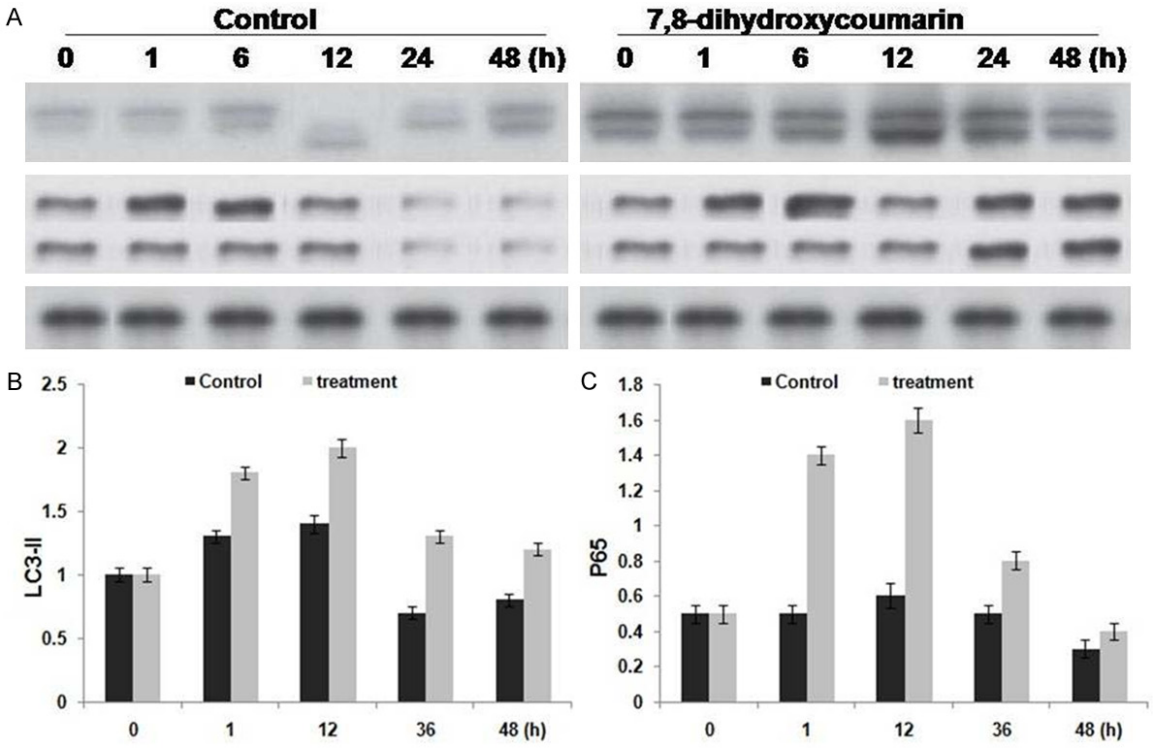

Figure 5. Effect of 7,8-dihydroxycoumarin treatment on the reduction in liver autophagy after I/R injury.

and JNK phosphorylation following I/R (**Figure 4**). However, treatment of the rats with 7,8-dihydroxycoumarin led to a marked inhibition of JNK and promotion of p38 phosphorylation. ERK phosphorylation in the untreated group was enhanced significantly after 12 h of the I/R, however, 7,8-dihydroxycoumarin treatment prevented dephosphorylation of ERK.

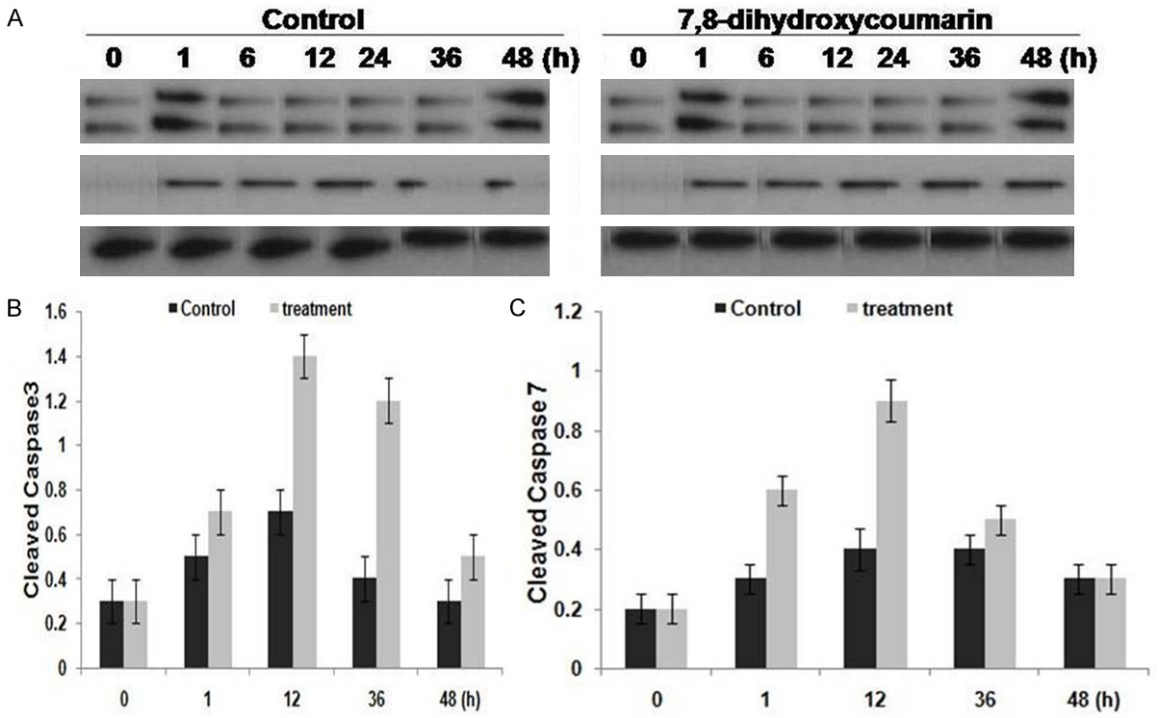

Figure 6. Role of 7,8-dihydroxycoumarin treatment in enhancing liver apoptosis after I/R injury.

7,8-dihydroxycoumarin treatment decreases autophagy

The process is autophagy involves recycling of the non-functional and damaged tissues [15, 16]. The results from western blot analysis revealed that 7,8-dihydroxycoumarin treatment inhibits hepatic autophagy after I/R. In the rats treated with 7,8-dihydroxycoumarin, LC3-II and p62 expression was significantly increased compared to the untreated rats after 12 h of the liver I/R (**Figure 5**). Therefore, 7,8-dihydroxycoumarin treatment leads to the suppression of autophagy after hepatic ischemia.

To investigate the effect of 7,8-dihydroxycoumarin on apoptosis in the in the rats, the expression of caspase-3 and caspase-7 proteins was investigated. The results showed that liver I/R caused increase in the expression of cleaved caspase-3 compared to the control rats (**Figure 6**). The expression of cleaved caspase-3 was further increased in the rats on treatment with 7,8-dihydroxycoumarin. The similar results were observed for the expression of caspase-7 levels. Therefore, 7,8-dihydroxycoumarin treatment enhances the I/R-induced apoptosis.

Discussion

The present study was aimed to examine the effects of 7,8-dihydroxycoumarin in hepatic I/R injury. It was observed that 7,8-dihydroxycoumarin significantly prevented liver injury at the early stage of the reperfusion (0-12 h) where as in the late stage (24-48 h) I/R was intensified. During early stage, 7,8-dihydroxycoumarin treatment increased expression of MAP kinase, suppressed nuclear translocation of HMGB1 and decreased the expression of inflammatory cytokines. On the other hand, after 24 h of 7,8-dihydroxycoumarin treatment liver injury was intensified through the suppression of autophagy and apoptosis induction.

Various studies have shown that cytokines exhibit an important role in the pathology of liver I/R injury and induce inflammatory reactions [8, 9, 18]. The results from the present study revealed that 7,8-dihydroxycoumarin treatment inhibited the expression of cytokines including TNF-a, IL-6, IL-1b and iNOS genes during early stage of reperfusion.

Liver I/R injury induces translocation of HMGB1 from the hepatocyte nucleus into the extracellular region where it leads to the synthesis of

pro-inflammatory cytokines and induces liver damage [10, 11, 19, 20]. The results from our study showed that 7,8-dihydroxycoumarin treatment decreased serum levels and translocation of liver HMGB1 during the early stage of the reperfusion. During the liver injury MAP kinase pathway is activated which plays crucial role in damaging the liver tissues [14]. 7,8-dihydroxycoumarin led to a marked reduction in the p38 and ERK phosphorylation. Phosphorylation of p38, JNK and ERK was induced during I/R injury. However, 7,8-dihydroxycoumarin suppressed phosphorylation of JNK and promoted phosphorylation of ERK during the early stage of reperfusion.

Autophagy regulates the process of degradation of the organelles and their recycling via lysosomal degradation pathway using autophagy-related gene (Atg) proteins [16, 17, 21]. Conjugation of LC3-1 with phosphatidyl ethanolamine forms LC3-II which is present in the autophagic membranes. In the present study 7,8-dihydroxycoumarin treatment significantly inhibited autophagy and intensified hepatic injury in the late stage of the reperfusion. Autophagy is essential for the organ damage evolution and its suppression plays an important role in the induction of cell apoptosis [22]. The results from the current study showed that 7,8-dihydroxycoumarin treatment enhanced apoptotic cell death which was evident by increase in the cleavage of caspase proteins.

Therefore, the present study demonstrates that 7,8-dihydroxycoumarin treatment has potential to prevent the hepatic ischemic injury induced damage during the early stage through increased expression of MAP kinase, suppressed nuclear translocation of HMGB1 and decreased the expression of inflammatory cytokines. However, after 24 h of 7,8-dihydroxycoumarin treatment liver injury was intensified through the suppression of autophagy and apoptosis induction.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hai-Yang Zhu, Department of Gastroenterology, The Fifth Affiliated Hospital of Zhengzhou University, 3 Kangfu Street, Eeqi District, Zhengzhou 450000, Henan, P. R. China. Tel: 0086-371-66902543; Fax: 0086-371-66902543; E-mail: zhuhaiyang09@hotmail.com

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