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Full paper

Chlorogenic acid protects against cholestatic liver injury in rats

Duohu Wu, Chuanyu Bao, Longhe Li, Mingliang Fu, Desen Wang, Jinyuan Xie, Xiaoguang Gong*

Department of Emergency, Hainan General Hospital, No. 19, Xiuhua St, Xiuying Dist., Haikou, Hainan 570311, PR China

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ABSTRACT

The aim of this study was to investigate the protective effect of chlorogenic acid (CA) on liver injury caused by bile duct ligation (BDL), as well as the potential mechanism. Permanent bile duct ligation induced liver injury was evaluated by liver index, liver function and pathological observation. Oral administration of CA for 3 weeks markedly attenuated liver swelling and fibrosis. Blood biochemistry results revealed that CA decreased alanine transaminase, aspartate aminotransferase, alkaline phosphatase, total bilirubin, direct bilirubin and total bile acid. PCR analysis indicated that collagen I, collagen III, transforming growth factor and vascular endothelial growth factor mRNA were increased markedly by BDL treatment but these increases were suppressed by CA. Additionally, CA effectively alleviated the expression of α-smooth muscle actin induced by BDL. Taken together, our data indicate that CA can efficiently inhibit BDL-induced liver injury in rats, which is a candidate drug for preventing liver injury against cholestasis.

1. Introduction

Cholestasis is a clinical syndrome including obstructive and metabolic type. Obstructive cholestasis is a mechanical blockage in the duct system that can occur from a gallstone or malignancy, and metabolic types of cholestasis which are disturbances in bile formation that can occur because of genetic defects or acquired as a side effect of many medications. Intrahepatic accumulation of cytotoxicbile acids can directly induce hepatocytes apoptosis (1), which may eventually lead to fibrosis and cirrhosis (2–4). The pathogenesis of liver fibrosis is characterized by excessive extracellular matrix deposition and is based on complex interactions between an abundance of liver-resident cells and matrix-producing hepatic stellate cells (HSCs). The activated hepatic stellate cells can up-regulate pro-fibrogenic cytokines such as TGF-β1 and VEGF. The mechanism of hepatocyte apoptosis triggers HSCs activation is not clear, either directly by phagocytosis of the apoptotic bodies (5), or indirectly by damage-associated molecular patterns inducing the migration or activation of HSCs (6). Thus effective treatment approaches for liver fibrosis may include drugs that target HSCs activation, hepatocyte apoptosis, or both.

Chlorogenic acid (CA), an ester of quinic acid and caffeic acid, is a major active ingredient found in many traditional Chinese herbs and herbal medicine (7). It exerts a broad spectrum of pharmacological activities including anti-hypertension (8), anti-inflammation (9,10), antioxidantion (11), antitumor (12) as well as inhibiting histamine production in the epidermis (13). Increasing studies show that chlorogenic acid presents remarkable hepatoprotective effects on different liver injury models. For example, chlorogenic acid can efficiently inhibit CCl_4-induced liver fibrosis in rats (14) and protect against acute hepatotoxicity induced by lipopolysaccharide in mice (15). A recent study showed that chlorogenic acid exerts therapeutic detoxification against acetaminophen-induced liver injury in mice (16). Surgical bile duct ligation is a well-established model that can induce obstructive jaundice (17). Moreover, it is an acceptable model to evaluate the potential hepatoprotective effect of some candidate drugs (18). In this study, we investigated the potential protective effects of chlorogenic acid and anti-fibrotic activity on hepatic damage rats induced by common bile duct ligation.

2. Materials and methods

2.1. Drugs

Chlorogenic acid (purity >95% tested by titration) was purchased from Sigma–Aldrich (St Louis, MO, USA). CA was dissolved in distilled water and administrated i.g. at a volume of 10 ml/kg.

* Corresponding author. Tel./fax: +86 898 68661664.
E-mail address: wdhhx@126.com (X. Gong).
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2.2. Animals

Adult male Sprague–Dawley rats, weighing 220–250 g, were purchased from Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China). The rats were maintained in a controlled environment at 22 ± 2 °C and 55 ± 5% relative humidity under a 12-h dark/light cycle and were acclimatized for at least 1 week prior to use. All experimental procedures were approved by the Animal Care and Use Committee of Hainan General Hospital (Haikou, China).

2.3. Surgical procedure

Rats were fasted for 12 h with water *ad libitum* before the operation. Each rat was weighed and anaesthetized with choral hydrate (350 mg/kg, i.p.). Following a midline incision, the common bile duct was exposed and a double-ligature with 5–0 silk suture was performed and the bile duct was sectioned between the ligatures. In the control-operated animals, the common bile duct was freed from surrounding soft tissue without ligation. A two-layer running suture was used for abdominal closure with 4–0 dexon and 2–0 nylon. The operation was performed on a heating pad to maintain body temperature at 37.5 ± 0.5 °C, and the animal was kept on the pad until recovery from anesthesia.

2.4. Experimental treatment

Rats subjected to bile duct ligation were randomly divided into 5 groups containing 12 rats each (1): Rats in Group 1 were treated with distilled water (2); Rats in Group 2 were treated with CA 37.5 mg/kg (3); Rats in Group 3 were treated with CA 75 mg/kg (4); Rats in Group 4 were treated with CA 150 mg/kg (5); Rats in Group 5 were treated with Silibinin 150 mg/kg; The control-operated rats treated with distilled water were served as Group 6. Daily oral administration of CA, Silibinin or vehicle (distilled water) started on day 1 post-surgery, and lasted for the termination of the experiment on day 21.

2.5. Sample collection

The animals were anaesthetized and a second laparotomy was performed. After blood samples were drawn from the inferior vena cava, the liver was carefully dissected from its attachment and totally excised. The blood samples were kept at 4 °C for biochemical analyses of alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), direct bilirubin (DBIL), and total bile acid (TBA). The left lobe of the liver was excised and flushed with physiological saline and then cut into two pieces, of which one piece was immediately frozen in liquid nitrogen and stored at −80 °C for western blotting and PCR, and another was fixed in 40 g/L paraformaldehyde for histological analyses.

2.6. Blood biochemistry

After clotting, blood samples were centrifuged at 800 g for 5 min at 4 °C. The top, clear layer was centrifuged again under the same conditions to prepare serum. Alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), direct bilirubin (DBIL), and total bile acid (TBA) were measured using TBA-40FR automatic biochemistry analyzer (TOSHIBA, Japan). The detailed procedures of measurements followed the manufacture instruction in different reagent kits (Nanjing Jiancheng Institute of Biological Engineering, China).

2.7. Histopathological examination

A portion of liver in each group was fixed in 10% formalin, processed by routine histological procedures, embedded in paraffin, and cut into 5-μm sections. The sections were stained with hematoxylin and eosin (H&E) for histopathological examination and with Masson's trichrome for assessment of fibrosis. Sections were examined in a blinded manner under a microscope (Leica TCS SP, German). Each sample was observed at 100× magnification.

Fibrosis was graded according to the criteria (19) as follows: grade 0, normal liver; grade 1, increase in collagen without septa formation (small stellate expansions of the portal fields); grade 2, formation of incomplete septa from the portal tract to the central vein (septa that do not interconnect with each other); grade 3, complete but thin septa interconnecting with each other so as to divide parenchyma into separate fragments; and grade 4, the same as grade 3, except for the presence of thick septa (complete cirrhosis).

2.8. RNA isolation, cDNA synthesis, and reverse transcription-polymerase chain reaction (PCR)

Samples (50 mg) of the liver tissue were first cleaned with cold physiological saline and then dried with absorbent paper. Total RNA for RT-PCR was isolated by lysing the tissue in 1 ml of Trizol (Gibco, NY, USA). The RNA was treated with 200 μL chloroform, centrifuged (12,000 rpm, 15 min, 4 °C), and precipitated with ethanol. Total RNA (1 μg) from each liver sample was subjected to reverse transcription in a reaction volume of 10 μL using a Revert Aid™ First Strand cDNA Synthesis Kit. Aliquots of the first strand cDNA were used to amplify fragments specific to collagen I, collagen III, VEGF, TGF-β1 and GAPDH by PCR using the primer pairs listed in Table 1. The identities of the resulting PCR products were confirmed by sequence analysis. PCR products were run on a 2% agarose gel stained with ethidium bromide recorded on polaroid film, and the bands quantified by densitometry. The expression levels of all the transcripts were normalized to that of GAPDH mRNA in the same tissue samples.

2.9. Western blot analysis

Liver tissues stored at −80 °C were homogenized on ice in five volumes of the lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM sodium chloride, 1% NP-40, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM EDTA, 1 mM EGTA, 1 mg/ml leupeptin and 1 mM PMSF. The lysate was centrifuged at 15,000 g for 10 min at 4 °C and the supernatant was used for Western blot analysis of α-SMA. Briefly, samples were separated by a SDS-polyacrylamide gel electrophoresis and then transferred onto PVDF membranes. The membranes were blocked with 3% BSA in Tris-buffer saline. Then

Table 1
Primer sequences for PCR amplification.

mRNA	Primer sequence
Collagen I	FP: 5′-CAGAGTGGAAGAGCGATTA-3′ RP: 5′-CAAGGACAGTGTAGGTGAA-3′
Collagen III	FP: 5′-GTCCACAGCCTTCTACAC-3′ RP: 5′-TTCCTGACTCTCCATCCTT-3′
TGFβ1	FP: 5′-GCAACAACGCCATCTATG-3′ RP: 5′-CAAGGTAACGCCAGGAAT-3′
VEGF	FP: 5′-CTGCTGTACCTCCACCAT-3′ RP: 5′-ACAGGACGGCTTGAAGAT-3′
GAPDH	FP: 5′-TCTCCTGCGACTTCAACA -3′ RP: 5′-TGTAGCCGTATTCATTGTCA-3′

the membranes were incubated at 4 °C overnight with α-SMA antibody (1:1000) and β-actin antibody (1:1000), respectively (Santa Cruz Biotechnologies, Santa Cruz, CA). After washing, the membranes were incubated with a horseradish peroxidase conjugated secondary antibody (mouse anti-rabbit IgG, 1:5000; rabbit anti-goat IgG, 1:5000) (Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h at room temperature and processed for visualization by enzyme-linked chemiluminescence. Relative intensities of the bands were quantified by densitometric analysis.

2.10. Statistical analysis

Histopathological scoring data were analyzed by the Kruskal–Wallis test to determine the possible statistically significant differences among the groups and the Mann–Whitney U-test was used to test the differences between two groups. The other data were analyzed using by one-way analysis of variance with the Student–Newman–Keuls test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. General observation and liver index

Bile duct ligation results in severe liver damage and deaths were observed in all groups except control group. During the course of experiment, 3 rats died in model group and 1–3 rats died in other treated group. There was no statistical difference in survival between treated groups. Bile duct ligated animals in model group gradually lost body weight after BDL. CA or Sil treatment significantly reduced this weight loss. As shown in Fig. 1, the liver index in the model group increased highly compared to control group (2.1 ± 0.09 *vs.* 4.5 ± 0.23, $P < 0.001$), which suggested that BDL caused liver swelling. Compared to the model group, rats treated with the dose 75 mg/kg and 150 mg/kg of CA significantly deceased the liver index (3.1 ± 0.16, 3.0 ± 0.11, $P < 0.01$, $P < 0.01$), which shown that CA can ameliorate the swelling induced by BDL.

3.2. Blood biochemistry

Ligation of the common bile duct caused substantial hepatocellular injury, as indicated by a more than 31.5-fold, 4.7-fold and 6.1-fold increase in ALT, AST and ALP, respectively (Fig. 2A,B,F). Also, BDL significantly elevated TBIL, DBIL and TBA levels compared with sham-treated animals, suggesting that significant cholestasis was induced in this model (Fig. 2C,D,E). As shown in Fig. 2, administration of different dose of CA, especially 150 mg/kg, considerably suppressed the release of ALT, AST and ALP from the liver by 84.1%, 76.4% and 51.6%, respectively, compared with model group. TBIL, DBIL and TBA levels in rats treated with CA (75 mg/kg and 150 mg/kg) after BDL were significantly decreased than those in model animals.

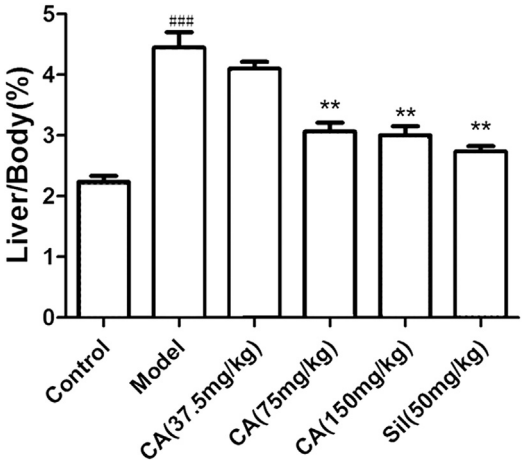

Fig. 1. Effect of chlorogenic acid (CA) on liver index of BDL-rats. Data were expressed as mean ± SD (n = 9). Liver index: liver weight (g)/body weight (g) ×100%. ###P < 0.001 compared with control group, **P < 0.01 compared with model group.

3.3. Effect of CA on liver histopathologic changes

The liver histological changes were observed by HE staining as shown in Fig. 3. The structure of the hepatic lobules in model group was in disarray with the infiltration of many periportal inflammatory cells. The hepatic plate was dissociated and hepatocytes were swelling or lytic necrosis. Massive or sub massive necrosis of the regenerated nodules was observed in some areas. However, liver histology was significantly improved in rats of CA treatment groups, especially those in CA 75 mg/kg and 150 mg/kg. There was an obvious decrease in inflammatory cell infiltration, and well-arranged hepatic cords and the alleviation of hepatocyte swelling was observed.

3.4. Effect of CA on liver fibrosis

BDL-induced liver fibrosis was evidenced by fiber extension and collagen accumulation, as visualized by increased staining intensity with Masson's trichrome. As shown in Fig. 4, the development of hepatic fibrosis in the CA-treated groups as compared with the model group was inhibited in the pericentral region, and occurred mainly in the periportal region. There was a small degree of bridging fibrosis in the CA-treated groups. As shown in Table 2, there were differences among the groups in terms of histopathological scoring according to the Kruskal–Wallis test ($H_C = 28.8$, $P < 0.005$). Meanwhile, the liver fibrosis scoring of CA (150 mg/kg)-treated rats were significantly lower than those in the model group ($P < 0.05$).

3.5. Inhibitory effect of CA on collagen synthesis and cytokine expression

Total RNA for RT-PCR was isolated from rats livers of each group used as a template for PCR amplification. As shown in Fig. 5, the levels of collagen mRNA in BDL-treated rats was significantly higher than those in the control group, and the collagen mRNA levels were decreased in the rats treated with 75 mg/kg or 150 mg/kg CA. Meanwhile, the expression of VEGF and TGF-β1 mRNA was increased in the model group as compared with the control group. The increased synthesis of VEGF and TGF-β1 mRNA in response to BDL was suppressed markedly by 75 mg/kg or 150 mg/kg CA.

3.6. Analysis of α-SMA expression by western blotting

To explore the mechanism underlying the protective effects of CA against bile duct ligation induced liver injury, we examined α-SMA expression. As shown in Fig. 6, the level of α-SMA in the model group, as determined by western blotting, was increased significantly compared to that in the control group, but it was reduced significantly after the treatment with CA (75 mg/kg and 150 mg/kg).

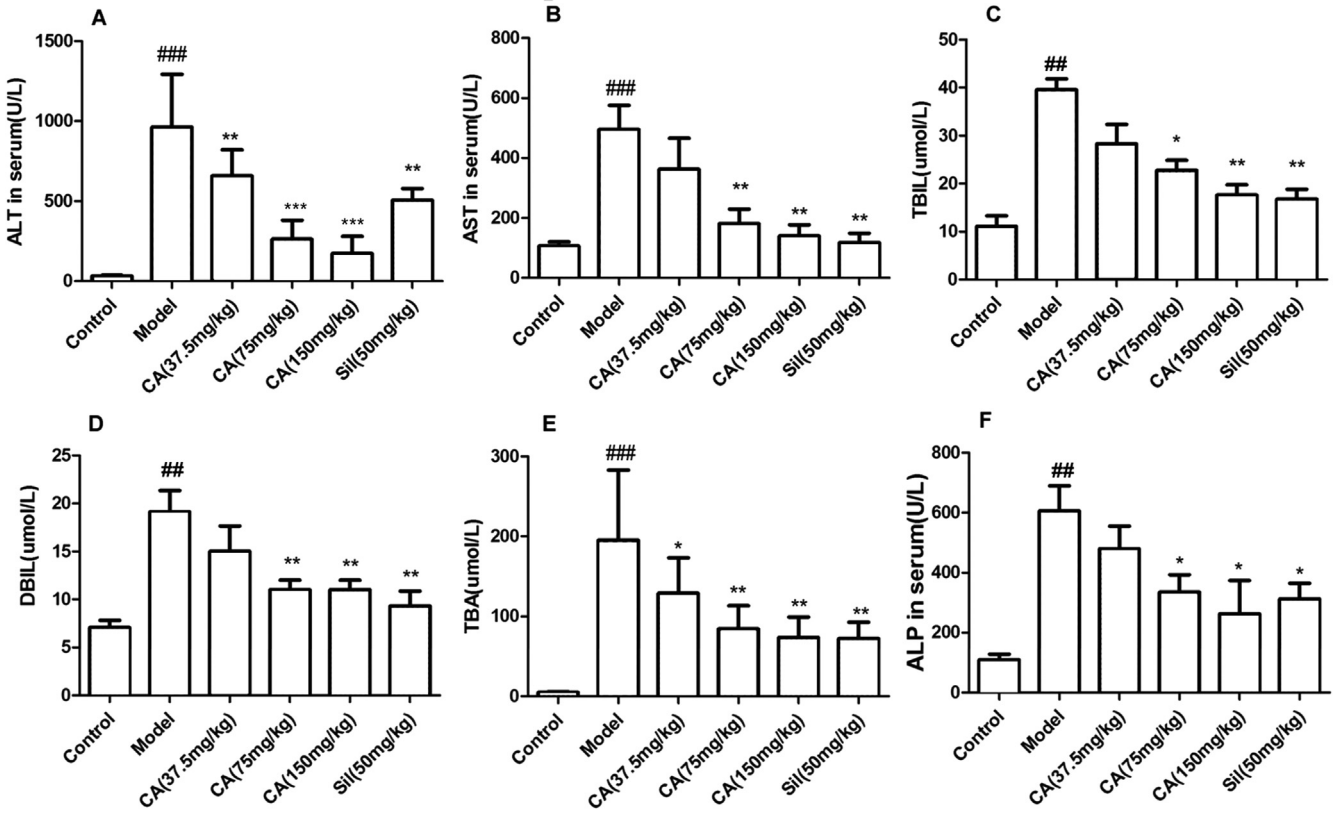

Fig. 2. Effect of chlorogenic acid (CA) on blood biochemistry. The serum levels of (A) ALT, (B) AST, (C) TBIL, (D) DBIL, (E) TBA, and (F) ALP were determined. Data were expressed as mean ± SD (n = 9). ##P < 0.01, ###P < 0.001 compared with control group, *P < 0.05, **P < 0.01, ***P < 0.001 compared with model group.

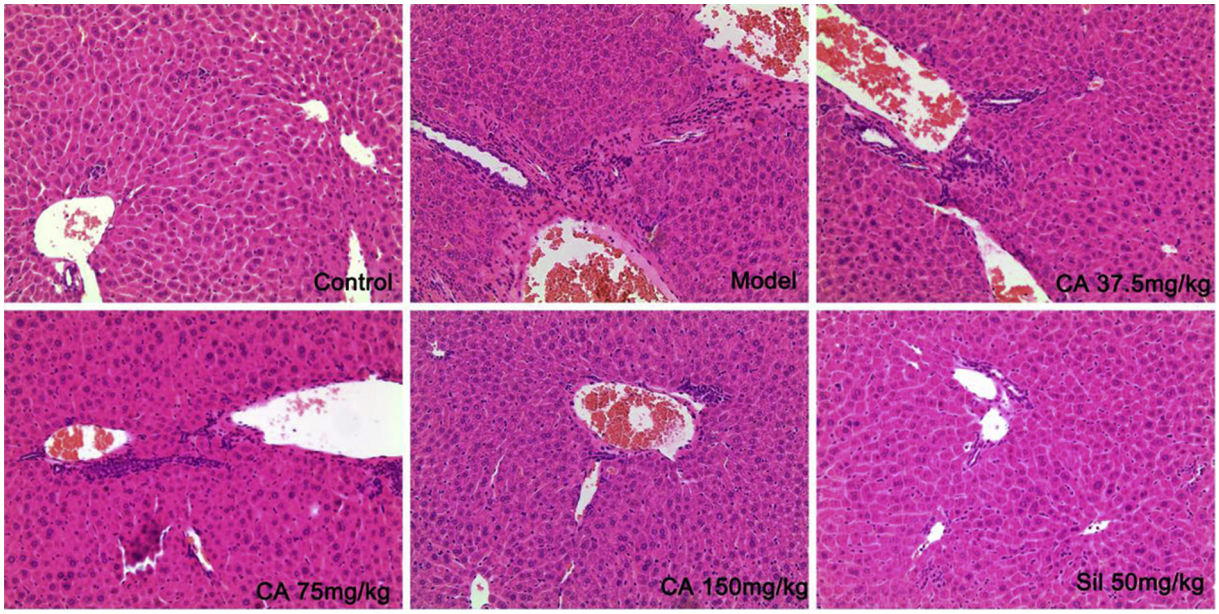

Fig. 3. Histological images of rat livers stained with hematoxylin and eosin staining. Representative micrographs: 100× magnification.

4. Discussion

The current study showed that CA could attenuate BDL-induced liver injury, and suggested a beneficial effect to protect liver from fibrosis. CA treatment improved liver function in term of serum ALT, AST, ALP, TBIL, DBIL and TBA on BDL-induced liver injury rats. Moreover, CA may inhibit the expression of α-SMA, collagen I and collagen III, and decrease the secretion of TGF-β1 and VEGF. Taken together, our results suggested that CA may have protective effect against liver injury in rat model.

Rats subjected to bile duct ligation cause the retention of biliary constituents and high biliary pressure caused hepatocellular injury

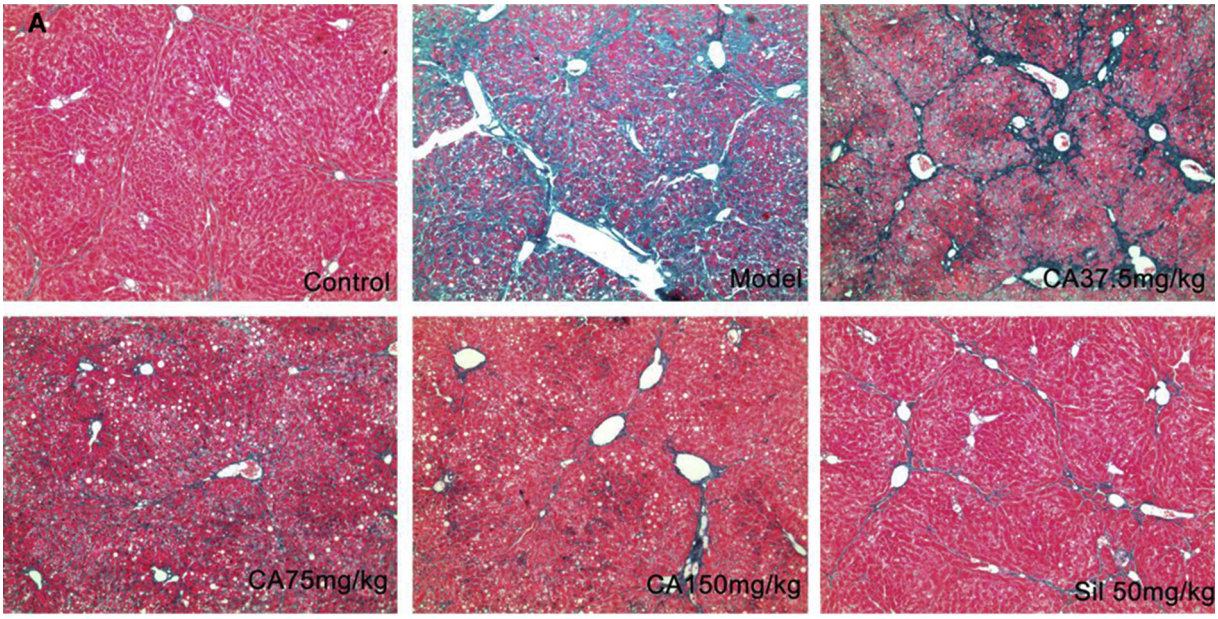

Fig. 4. Histological images of rat livers stained with Masson's trichrome. Representative micrographs: 100× magnification.

Table 2
Scoring of histopathological changes of liver.

Group	Dose (mg/kg)	Score of hepatic fibrosis					Mean rank
		0	1	2	3	4	
Control	–	9	0	0	0	0	5
Model	–	0	0	2	3	4	38.6[a]
CA-L	37.5	0	0	2	4	3	37.1
CA-M	75	0	0	3	5	1	32.3
CA-H	150	0	2	3	2	2	25.2[b]
Sil	50	0	1	4	2	2	26.9[b]

Each group consists of 9 rats and figures represent number of rat per grade.
[a] $P < 0.001$ compared with control group.
[b] $P < 0.05$ compared with model group.

(20). In this study, serum bilirubin, bile acid and liver enzymes such as ALT, AST and ALP were all increased in BDL treated rats. The liver pathological changes observed by HE staining indicated that hepatic lobules, hepatic plates and hepatocytes were abnormal in BDL rats. However, the serum factors in CA treated rats were decreased, especially in 75 and 150 mg/kg. Meanwhile, there was an obvious decrease in inflammatory cell infiltration, and well-arranged hepatic cords and the alleviation of hepatocyte swelling in CA treated rats. These observations suggested that CA exerts anti-inflammatory effect, which was consistent with previous study results that CA reduces liver inflammation in CCl_4-induced liver injury in rats (21).

Hepatic fibrosis, characterized by an increased production and deposition of extracellular matrix component accompanies most chronic liver disorders (22). In our study, collagen accumulation was observed by Masson's trichrome in BDL rats after 3 weeks of operation. Activated HSCs undergo phenotypic transformation, which includes increased proliferation and contractility together with the appearance of α-SMA filaments (23) and the accumulation of ECM including type I and III collagen (24). In the current study, as compared to the control group, the level of α-SMA was increased in BDL rats, but the increasing expression level was decreased by CA treatment. The levels of collagen I and collagen III mRNA in the BDL group was higher than those in the CA-treated group. This might suggest that the inhibition of HSCs activation contributes to the

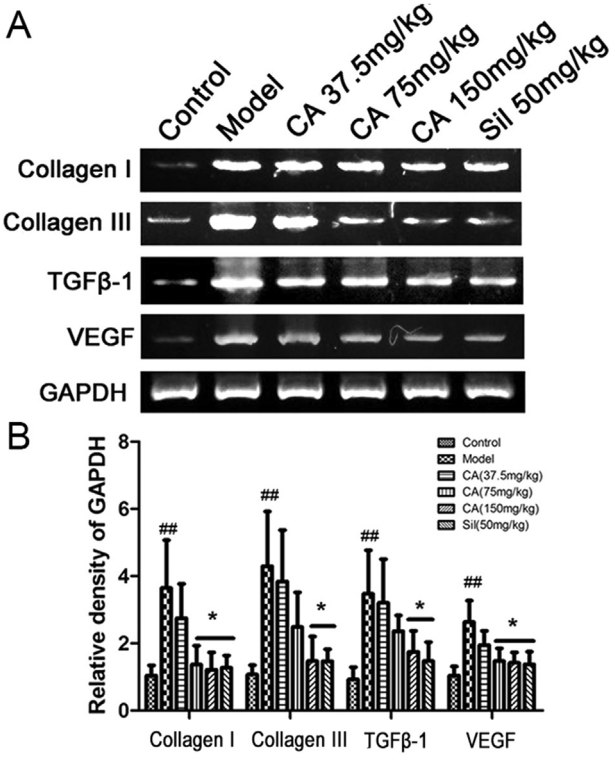

Fig. 5. Effects of chlorogenic acid (CA) on collagen I, collagen III, TGF-β1, and VEGF mRNA in the liver of BDL rats. (A) Representative RT-PCR of collagen I, collagen III, TGF-β1 and VEGF. (B) Quantification of the intensities of collagen I, collagen III, TGF-β1 and VEGF. ##$P < 0.01$ compared with the control group; *$P < 0.05$ compared with the model group.

decreased production of collagen in CA treated rats. Previous studies showed that TGF-β1 is involved in the excessive production of ECM components and hepatic fibrosis (25), and VEGF may contribute to the development of liver fibrosis by inducing the proliferation of HSCs (26). In this study, we found that both of TGF-

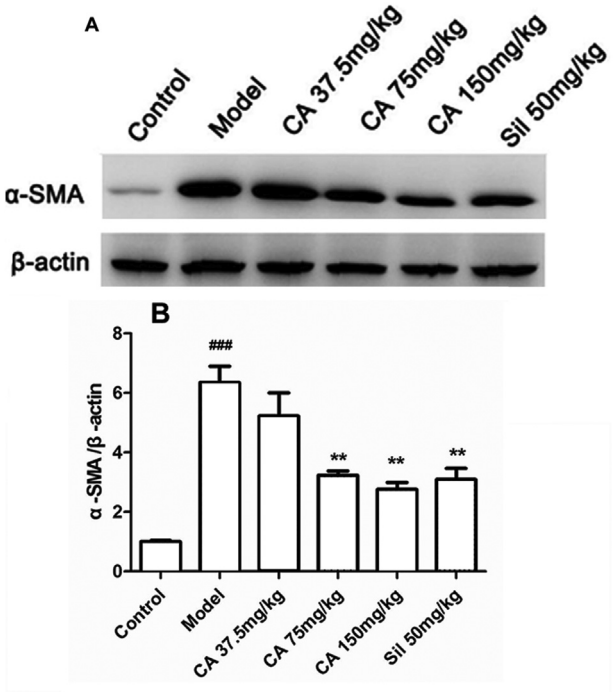

Fig. 6. Effects of chlorogenic acid (CA) on α-SMA protein in the liver of BDL rats. (A) Representative western blots of α-SMA (B) Quantification of the intensities of α-SMA. ###P < 0.001 compared with control group; **P < 0.01 compared with model group.

β1 and VEGF mRNA were decreased in rats treated with CA. Therefore, it is likely that inhibition of the expression of TGF-β1 and VEGF by CA plays a major role in the down-regulation of collagen production.

In summary, all these results suggested that CA may protect against liver injury caused by BDL. Although the mechanism was not entirely clear, the present study revealed that the inhibitory effect of CA on liver injury was associated with its ability to inhibit the production of TGF-β1, VEGF and α-SMA. Therefore, our study suggested that CA may be a potential drug for treating cholestasis-related liver injury.

Conflict of interest statement

The authors have no conflict of interest to declare.

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