Protein extracts of *Crassostrea gigas* alleviate CCl_4-induced hepatic fibrosis in rats by reducing the expression of CTGF, TGF-β1 and NF-κB in liver tissues

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Abstract. Hepatic fibrosis may contribute to liver carcinoma and the mortality of patients with hepatic fibrosis is gradually increasing. However, no definitive treatment has been established for hepatic fibrosis. The hepatic fibrotic process is reversible and can be controlled; therefore, the creation of novel and effective therapeutic methods to prevent or reverse the disease is required. The aim of the present study was to identify whether protein extracts from Pacific oysters (PEPO) could alleviate the hepatic fibrosis induced by CCl_4 and to examine the mechanisms involved. A total of sixty rats were randomly divided into the following experimental groups: The normal control group; the hepatic fibrosis model group; the high-dose; medium-dose; and low-dose PEPO groups; and the colchicine group. The results indicated that compared with those of the model group, PEPO treatment significantly decreased the serum levels of alanine aminotransferase, aspartate aminotransferase, γ-glutamyltransferase, alkaline phosphatase, hyaluronic acid, laminin, collagen type IV and procollagen III in rats with hepatic fibrosis. The hematoxylin and eosin staining demonstrated that PEPO markedly alleviated hepatic fibrosis. The experiments using immunohistochemistry, western blotting and quantitative PCR indicated that protein and mRNA expression levels of connective tissue growth factor (CTGF), transforming growth factor β1 (TGFβ-1) and nuclear factor κB (NF-κB) in the liver tissues were significantly reduced by PEPO treatment. Therefore, it was concluded that PEPO successfully alleviated hepatic fibrosis induced by CCl_4 and reversed the effects of hepatotoxicity by regulating the serum levels of enzymes and decreasing the expression levels of CTGF, TGF-β1 and NF-κB in liver tissues. These findings may provide a novel treatment option for patients with hepatic fibrosis in the future.

Introduction

Hepatic fibrosis, a pathological condition characterized by impaired hepatic function and nodule formation, results from multiple types of liver injury, including drug intoxication, viral hepatitis, alcohol abuse, autoimmunity and non-alcoholic steatohepatitis (1-3). According to 2004 statistics, chronic hepatitis B virus (HBV) infection was the most common among all of the factors leading to hepatic fibrosis in China, and in the majority of the European countries and the USA, the main factors were hepatitis C virus infection, alcohol abuse, and non-alcoholic steatohepatitis (4-6). As hepatic fibrosis may contribute to liver carcinoma, the mortality of patients with hepatic fibrosis is gradually increasing (7). Certain medicines, including corticosteroids, penicillamine, methotrexate, silymarin and colchicines have been widely used in the treatment of hepatic fibrosis; however, no definitive treatment has been established (8-11). Consequently, novel and effective therapeutic methods for treating hepatic fibrosis are urgently required, as the fibrotic process is reversible and can be controlled (12).

The Pacific oyster (*Crassostrea gigas*) is one of the most economically important bivalves (13), and its global annual production reached 4.2 million tons in 2007 (14). The protein extracts of the Pacific oyster (PEPO) have been demonstrated to protect human epithelial cells against oxidative stress and to increase glutathione and the glutathione S-transferase activity levels in several organs of rats (15,16). However, to the best of out knowledge, no studies have examined the effects and underlying mechanisms of the alleviation of hepatic fibrosis by PEPO.

Connective tissue growth factor (CTGF) is used as a biomarker of hepatic fibrosis (17) as its levels have been

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Abbreviations: CTGF, connective tissue growth factor; TGF-β1, transforming growth factor β1; NF-κB, nuclear factor κB

Key words: Pacific oyster (*Crassostrea gigas*), hepatic fibrosis, CTGF, TGF-β1, NF-κB

associated with the development of hepatic fibrosis (18-20), and it can be used to evaluate the severity of cases of fibrosis (21). Transforming growth factor β (TGF-β), the key growth factor inducing the transcription of the CTGF gene (22), is important in the development hepatic fibrosis (23). Nuclear factor κB (NF-κB) can accelerate recovery from hepatic fibrosis (24) as it functions to protect hepatic stellate cells (HSCs) from apoptosis, and is key in the regulation of TGF-β1 levels (25,26).

The present study was designed to explore whether PEPO can alleviate the hepatic fibrosis induced by CCl_4 in rats, and the main focus was the differential expression levels of CTGF, TGF-β1 and NF-κB in liver tissues from rats exposed to CCl_4, with or without PEPO treatment.

Materials and methods

Materials. The Pacific oyster specimens were collected from the waters around Zhoushan, China. Once the fresh whole bodies were removed from the shells, they were stored at -20˚C. The shelled Pacific oysters (3.0 kg) were chopped and homogenized, which were then processed with hot water (75˚C) for 3.5 h. Once cooled to room temperature, the homogenized mixture was filtered with Celite powder (Linjiang Dahua Cellite Products Co., Ltd., Jilin, China) and filter paper (Shijazhuang Golden Link Science Laboratory Equiptment Co., Ltd., Shijiazhuang, China). The filtrate produced was used as the crude PEPO in the present study. Colchicine and CCl_4 were provided by Sigma-Aldrich, St. Louis, MO, USA.

Animals and groups. Sixty male Sprague-Dawley rats weighing 180-220 g were provided by the Experimental Animal Centre, School of Medicine, Zhejiang University (Hangzhou, China). All rats were housed in a temperature-controlled room with a 12-h light/dark cycle and maintained at a constant temperature of 25˚C and a humidity of 55%. They were fed with standard pellet food and tap water *ad libitum* for 1 week. The current study was performed according to The Care and Use of Laboratory Animals protocol of the National Research Council, and was approved by the Ethics Committee of The Third Clinical College of Zhejiang Chinese Medicine University. The hepatic fibrosis model rats were established through intragastric administration of CCl_4 (mixed 1:1 with olive oil) at 2 ml/kg body weight twice a day for 12 consecutive weeks. The normal control rats were administered the equivalent dosage of olive oil only (27-29). The rats were maintained for 1 week prior to further experiments to allow CCl_4 penetration. A randomization chart constructed in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) was used to assign rats into six groups (n=10 in each group). Each group received individual treatments that were orally administered for 12 weeks: Group A (normal control group), olive oil (2 ml/kg) twice a day; group B (model group), CCl_4 (2 ml/kg) twice a day; group C (high-dose PEPO group), CCl_4 (2 ml/kg) twice a day and PEPO (8 mg/kg) once a day; group D (medium-dose PEPO group), CCl_4 (2 ml/kg) twice a day and PEPO (4 mg/kg) once a day; group E (low-dose PEPO group), CCl_4 (2 ml/kg) twice a day and PEPO (2 mg/kg) once a day; and group F (colchicine group), colchicine (2 mg/kg) once a day. During the treatment period, the numbers of animal mortalities were 0, 3, 0, 1, 2 and 2 in groups A, B, C, D, E and F, respectively.

Sample collection and measurement. On the day following the 12-week treatments, after fasting for 12 h, the body weight of each rat was measured. The rats were then intraperitoneally anesthetized with urethane (1.2 g/kg). Blood samples were drawn from the abdominal aorta into heparinized injectors (Huayi Biotech, Co., Ltd., Shanghai, China) and then centrifuged at 550 xg at 4˚C for 10 min. The supernatant serum was then transferred to clean Eppendorf tubes and stored at -80˚C until required for the assay. Following the collection of blood samples, the animals were sacrificed using cervical dislocation, and the livers were immediately removed, washed with physiological saline and weighed. The left lateral lobe of the liver was sliced, and the tissue slices were fixed in 10% neutral-buffered formalin (Beijing Reagen Biotechnology Co. Ltd., Beijing, China) for 24 h in preparation for the histological examinations. The other parts of the livers were frozen and stored at -80˚C until required for the assay. Using commercial standard assay kits (Wuhan Boster Bio-Engineering Limited Company, Wuhan, China) the following enzymes were detected: Alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT) and alkaline phosphatase (ALP). The serum levels of hyaluronic acid (HA), laminin (LN), collagen type IV (IV-C) and procollagen III (PC III) were detected by radioimmunoassay kits (HaiYan Medical Biotechnology Center, Shanghai, China). All measurements were performed in duplicate and were conducted according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation were <10%. The liver index was calculated according to the following formula: (Liver weight/rat weight) x 100.

Histological examinations and immunohistochemistry. Once the fixed liver tissue slices were embedded in paraffin, sectioned, deparaffinized and rehydrated, they were cut into sections and mounted on slides. The slides were then stained with hematoxylin and eosin (HE) for histopathological examination. For immunohistochemical staining of CTGF, TGF-β1 and NF-κB, a number of the sections were incubated with the monoclonal CTGF antibody (rabbit-anti-rat; 1:200 dilution; Sigma, St Louis, MO, USA), monoclonal TGF-β1 (rabbit-anti-rat; 1:400 dilution; Sigma) and monoclonal NF-κB antibody (rabbit-anti-rat; 1:300 dilution; Sigma) at 4˚C overnight. Following washing of the slides with Tris-buffered saline (TBS) twice, biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin (Google Biotechnology Co., Ltd., Wuhan, China) were applied to the liver sections, and the expression was visualized by adding 3,3'-diaminobenzidine substrate (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) using an inversion fluorescence microscope (Olympus IX71S1F-3; Olympus Corporation, Tokyo, Japan).

Detection of CTGF, NF-κB and TGF-β1 mRNA expression in liver tissues with quantitative polymerase chain reaction (qPCR). Total RNA was isolated with RNAiso™ reagent (Takara Biotechnology, Dalian, China) according to the instructions of the manufacturer. The purity and concentration of the RNAs were detected with a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cDNA was prepared from 500 ng total RNA by reverse transcription (RT) with the PrimeScript™ RT

Table I. Body and liver weights.

Group	n	Body weight (g)		Liver weight (g)	Liver index (%)
		Initial	Final		
A	10	200.2±13.3	475.6±41.7	7.1±0.8	1.5±0.2
B	7	201.2±8.7	295.2±23.5[a]	13.2±1.6[a]	4.5±0.5[a]
C	10	197.8±10.9	423.4±30.7[a-c]	9.2±0.5[a-c]	2.2±0.2[a-c]
D	9	201.1±11.2	367.6±39.1[a,b]	10.4±0.9[a-c]	2.9±0.5[a,b]
E	8	203.0±9.9	322.6±22.5[a-c]	11.4±1.1[a,b]	3.6±0.5[a-c]
F	8	198.1±13.7	360.4±30.3[a,b]	11.2±0.5[a,b]	3.1±0.3[a,b]

Data are presented as the mean ± standard deviation. A, normal controls (2 ml/kg olive oil); B, models (2 ml/kg CCl_4); C, high-dose PEPO (8 mg/kg PEPO+2 ml/kg CCl_4); D, medium-dose PEPO (4 mg/kg PEPO+2 ml/kg CCl_4); E, low-dose PEPO (2 mg/kg PEPO+2 ml/kg CCl_4); F, colchicine (2 mg/kg colchicine+2 ml/kg CCl_4). P<0.05 was considered to indicate a statistically significant difference. [a]P<0.05 vs. group A; [b]P<0.05 vs. group B; [c]P<0.05 vs. group F (analysis of variance). PEPO, protein extracts of Pacific oyster.

Reagent kit (Perfect Real Time; Takara Biotechnology). The cDNA samples were then diluted in DNase- and RNase-free water at a proportion of 1:3 prior to further analysis. PCR was performed using the iCycler iQ Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA). The rat CTGF, TGF-β1 and NF-κB gene-specific primers were provided by Sangon Biological Engineering Technology (Shanghai, China). The sequences of the primers were as follows: CTGF forward, 5'-GGCCCTGTGAAGCTGACCTA-3' and reverse, 3'-CAGCCAGAAAGCTCAAACTTGAC-5'; NF-κB forward, 5'-AAAAACGCATCCCAAGGTGC-3' and reverse, 3'-AAGCTCAAGCCACCATACCC-5'; TGF-β1 forward, 5'-CACCGGAGAGCCCTGGATA-3' and reverse, 3'-TCCAACCCAGGTCCTTCCTA-5'; GAPDH forward, 5'-GCAAGTTCAACGGCACAG-3' and reverse, 3'-CGCCAGTAGACTCCACGAC-5'. PCR reactions were performed using 2 μl cDNA, 10 μM each primer, and 2X SYBR® Premix Ex Taq™ (Takara Biotechnology) in 20-μl reactions. Thermal cycling conditions were as follows: 95˚C for 10 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. A final melting curve was used to verify single-product formation. Gene starting quantity was based on the cycle threshold (Ct) method. Each value was normalized to GAPDH, a housekeeping gene, to control the amount of input cDNA. The Ct value for GAPDH mRNA was subtracted from that of the target gene, and the mRNA levels of the target gene were expressed as $2^{-\Delta Ct}$.

Detection of CTGF, NF-κB and TGF-β1 protein expression in liver tissues by western blotting. The protein lysates were prepared by homogenizing the frozen liver tissue. A Bicinchoninic Acid Protein Assay kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used to determine the protein concentration. Protein (40 μg) was incubated in the loading buffer at 95˚C for 5 min, cooled and then loaded into lanes. Gel electrophoresis was performed on Mini-Protean III gel apparatus (Bio-Rad) using 8% gel with 0.1% (w/v) SDS under a constant current of 26 mA prior to being transferred to nitrocellulose membranes (Dingguo Biotechnology, Beijing, China) for 2 h. The membranes were then blocked for 1.5 h at room temperature with 5% milk in TBS with Tween (TBST; 10 mM Tris pH 7.6, 150 mM NaCl and 0.05% Tween-20). Membranes were incubated with the following primary antibody (rabbit-anti-rat) dilution: CTGF antibody from Abcam, Cambridge, MA, USA, 1:2,000; NF-κB antibody (rabbit-anti-rat) from Cell Signaling Technology, Inc., Danvers, MA, USA, 1:1,500; TGF-β1 antibody (rabbit-anti-rat) from Abcam, 1:800; and β-actin antibody (rabbit-anti-rat) from Santa Cruz Biotechnology, Inc., 1:5,000) overnight at 4˚C. Once washed, the membranes were incubated with their corresponding secondary antibody (1:5,000) at room temperature for 2 h. The proteins were detected with Enhanced Chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ, USA). Densitometric intensity was measured with a GS-800 densitometer (Bio-Rad) and normalized against the internal control β-actin.

Statistical analysis. All data were analyzed with Statistical Package for Social Sciences (SPSS, version 13.0 for Windows; SPSS, Inc., Chicago, IL, USA). Analysis of variance was employed to analyze all data. Two-tailed tests were used for all hypothesis tests and P<0.05 was considered to indicate a statistically significant difference.

Results

Body and liver weights. No significant differences in the initial body weights were identified among the different groups (P>0.05; Table I). Following the 12-week treatment, the average final body weight of the normal control rats (group A) was significantly higher, while the liver weight and liver index were significantly lower than those of all other groups (P<0.05). Compared with the model group (group B), each of the groups C-F displayed a significantly greater average final body weight and significantly lower liver weight and liver index (P<0.05). The weight of the rats in group B decreased markedly over the treatment period and they displayed eminent irritability and aggression. PEPO treatment inhibited the reduction of body weight and the increase of liver weight and liver index in a dose-dependent manner. High-dose PEPO treatment was demonstrated to significantly inhibit the reduction of body weight and the increase in liver weight and

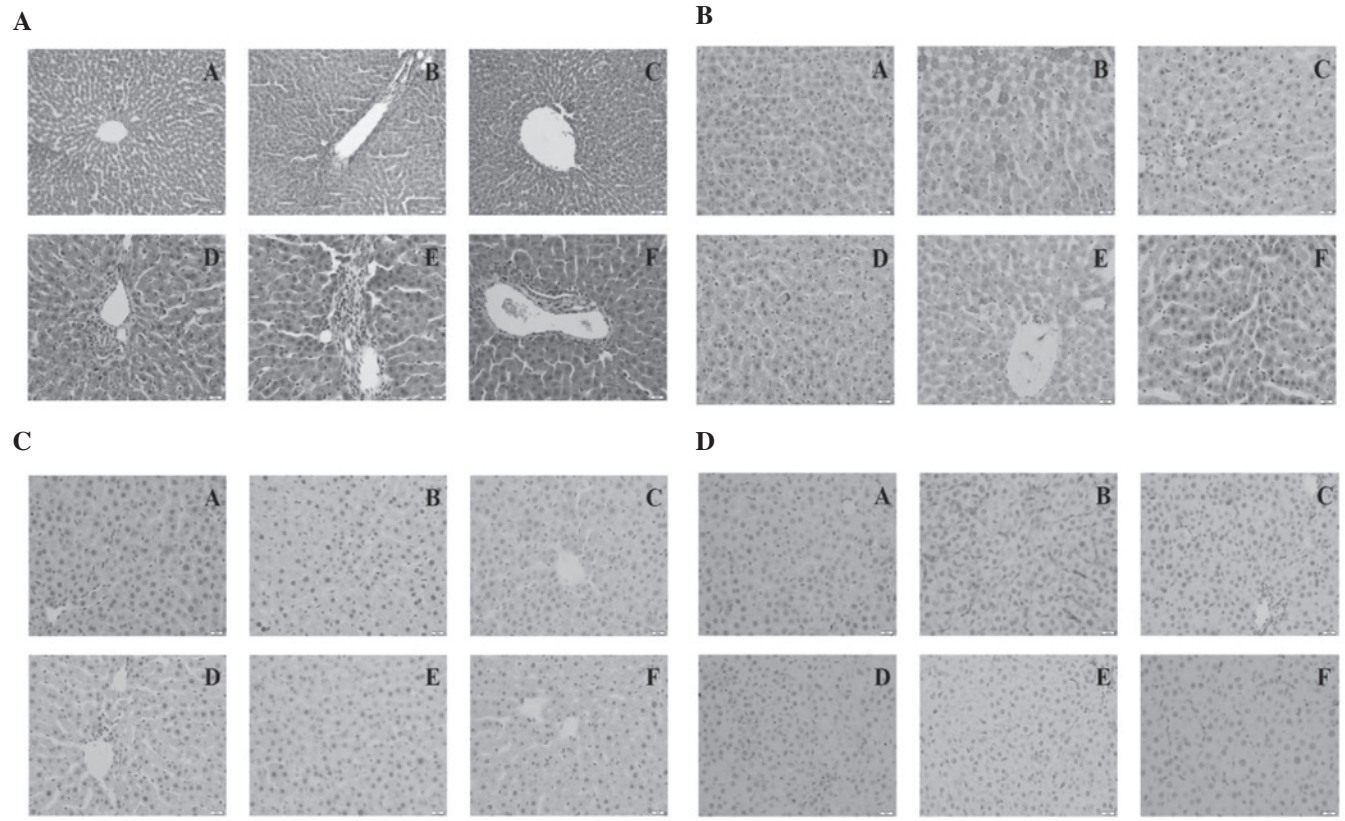

Figure 1. Representative photomicrographs of histopathological findings. (A) Rat liver tissues with HE stain. (B) CTGF immunohistochemistry. (C) NF-κB immunohistochemistry. (D) TGF-β1 immunohistochemistry. Magnification, x200. Group A, normal control group (2 ml/kg olive oil); group B, model group (2 ml/kg CCl_4); group C, high-dose PEPO group (8 mg/kg PEPO+2 ml/kg CCl_4); group D, medium-dose PEPO group (4 mg/kg PEPO+2 ml/kg CCl_4); group E, low-dose PEPO group (2 mg/kg PEPO+2 ml/kg CCl_4); group F, colchicine group (2 mg/kg colchicine+2 ml/kg CCl_4). HE, hematoxylin and eosin; CTGF, connective tissue growth factor; NF-κB, nuclear factor κB; TGF-β, transforming growth factor β; PEPO, protein extract of Pacific oyster.

liver index compared with the colchicine group (P<0.05). No significant differences in the final body weight and liver index were identified between the medium-dose PEPO treatment group and the colchicine treatment group (P>0.05); however, the rats in the medium-dose PEPO treatment group exhibited a significantly lower liver weight (P<0.05). Significant differences in the final body weight and liver index were identified between the low-dose PEPO treatment group and the colchicine treatment group (P<0.05); however, no difference was noted in their average liver weights (P>0.05).

Histopathological findings. Normal lobular architecture with central veins and radiating hepatic cords were observed in the normal control rats, whilst the hepatic fibrosis model rats displayed marked fat degeneration, portal inflammation and necrosis, evident collagen deposition, perihepatocyte fibrosis and hepatocyte loosening (Fig. 1A). All PEPO treatment and colchicine treatment groups demonstrated an amelioration in the severity of hepatic fibrosis; however, high-dose and medium-dose PEPO treatment were the most effective.

Immunohistochemistry. The results of the immunohistochemical staining demonstrated that the protein expression levels of CTGF, NF-κB and TGF-β1 were lowest in the normal control group and highest in the model group (Fig. 1B-D). All of the PEPO and colchicine treatment groups exhibited decreased expression levels of the three genes compared with those of the model group.

Serum levels of ALT, AST, GGT and ALP. The serum levels of ALT, AST, GGT and ALP in the normal control rats (group A) were significantly lower than those in all the other groups (P<0.05) (Fig 2A-D). Groups C-F exhibited significantly lower levels of ALT, AST, GGT and ALP in the serum compared with those of group B (P<0.05). The PEPO treatment decreased the serum levels of ALT, AST, GGT and ALP in a dose-dependent manner. High-dose PEPO treatment significantly reduced the serum levels of ALT, AST, GGT and ALP compared with those in the colchicine group (P<0.05). The medium-dose PEPO treatment group displayed significantly lower levels of AST and ALP than the colchicine treatment group (P<0.05); however, no significant differences in their ALT and GGT levels were detected (P>0.05). Significant differences in the serum levels of ALT, AST and GGT were detected between the low-dose PEPO and colchicine treatment groups (P<0.05); however, no difference was noted in their ALP levels (P>0.05).

Serum levels of HA, LN, IV-C and PC III. The serum levels of HA, LN, IV-C and PC III in the normal controls (group A) were significantly lower than those in all other groups (P<0.05) (Fig. 2E-H). Groups C-F displayed significantly lower levels

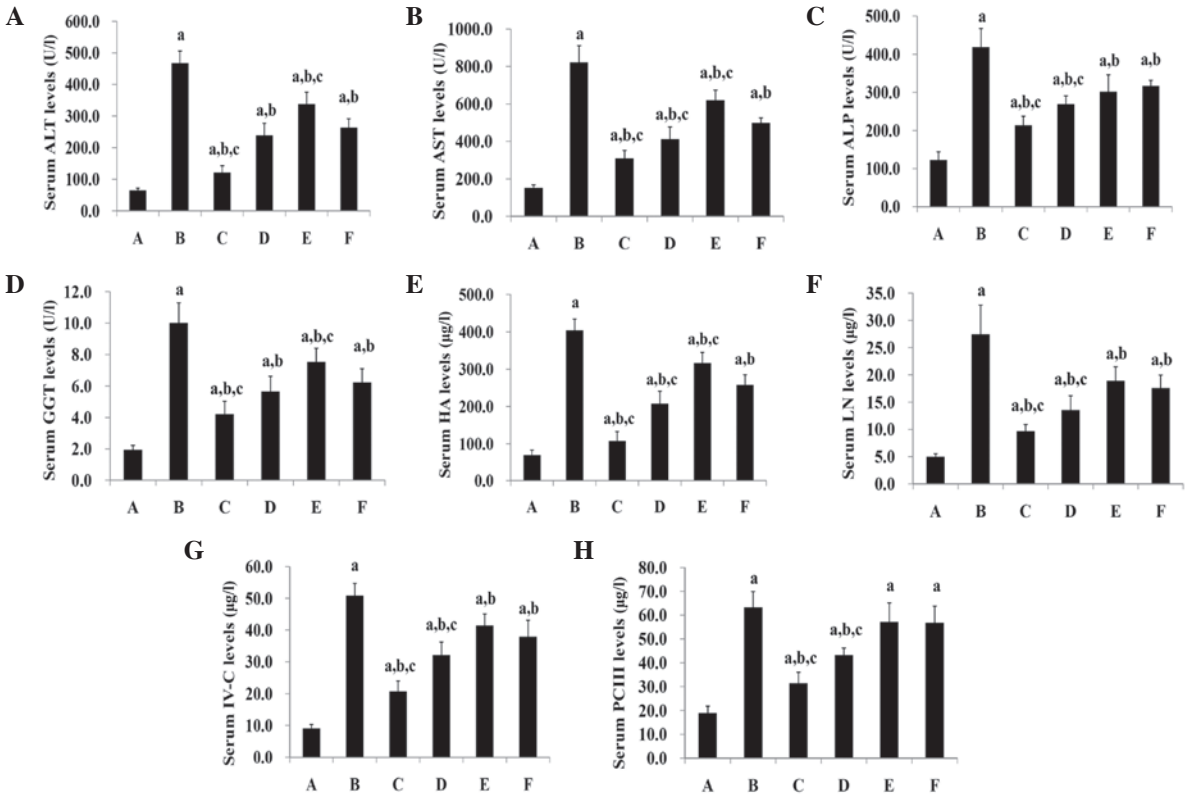

Figure 2. Serum levels of (A) ALT, (B) AST, (C) ALP, (D) GGT, (E) HA, (F) LN, (G) IV-C and (H) PC III. Group A, normal controls (2 ml/kg olive oil); group B, models (2 ml/kg CCl_4); group C, high-dose PEPO (8 mg/kg PEPO+2 ml/kg CCl_4); group D, medium-dose PEPO (4 mg/kg PEPO+2 ml/kg CCl_4); group E, low-dose PEPO (2 mg/kg PEPO+2 ml/kg CCl_4); group F, colchicine (2 mg/kg colchicine+2 ml/kg CCl_4). Data are presented as the mean ± standard deviation. (n=10, 7, 10, 9, 8 and 8 in groups A, B, C, D, E and F, respectively). $P<0.05$ was considered to indicate a statistically significant difference. [a]$P<0.05$ vs. group A; [b]$P<0.05$ vs. group B; [c]$P<0.05$ vs. group F (analysis of variance). ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyltransferase; HA, hyaluronic acid; LN, laminin; IV-C, collagen type IV; PC III, procollagen III; PEPO, protein extract of Pacific oyster.

of HA, LN, and IV-C in the serum compared with those of group B ($P<0.05$). High-dose and medium-dose PEPO treatment significantly decreased the serum PC III levels, compared with those of group B ($P<0.05$); however, no significant differences in the serum PC III levels were identified between group B and groups E and F ($P>0.05$). The PEPO treatment reduced the serum levels of HA, LN, IV-C and PC III in a dose-dependent manner. High- and medium-dose PEPO treatment significantly decreased the serum levels of HA, LN, IV-C and PC III compared with the colchicine group ($P<0.05$). A significant difference between the low-dose PEPO and colchicine treatment groups was identified in the serum levels of HA ($P<0.05$), but not in those of LN, IV-C and PC III ($P>0.05$).

CTGF, NF-κB and TGF-β1 protein expression in liver tissues. The CTGF, NF-κB and TGF-β1 protein expression levels in liver tissues of the normal control rats (group A) were demonstrated to be significantly lower than those in all other groups ($P<0.05$) and the levels in the model group (group B) were significantly higher than those in groups C-F ($P<0.05$) (Fig. 3A and B). The PEPO treatment decreased the protein expression of CTGF and NF-κB in liver tissues in a dose-dependent manner. High-dose PEPO treatment significantly reduced the expression levels of all three proteins in liver tissues compared with the levels in the colchicine treatment group ($P<0.05$). Medium-dose PEPO treatment significantly decreased the protein expression levels of CTGF and NF-κB in the liver tissues compared with those in the colchicine treatment group ($P<0.05$); but it did not significantly affect the TGF-β1 levels ($P>0.05$). A significant difference was noted in the CTGF protein expression levels between the low-dose PEPO and colchicine treatment groups ($P<0.05$); but not in those of NF-κB or TGF-β1 ($P>0.05$).

CTGF, NF-κB and TGF-β1 mRNA expression in liver tissues. The CTGF and NF-κB mRNA expression levels in liver tissues of the normal control group (group A) were significantly lower than those of all other groups ($P<0.05$), whilst the levels in the model group (group B) were significantly higher than those in all other groups ($P<0.05$) (Fig. 4A-C). The PEPO treatment reduced the mRNA expression levels of CTGF and NF-κB in the liver tissues in a dose-dependent manner. High-dose and medium-dose PEPO treatment significantly decreased the mRNA expression levels of these two genes in liver tissues compared with those of the colchicine treatment group ($P<0.05$). No significant differences in CTGF and NF-κB mRNA expression levels were identified between the low-dose PEPO and colchicine treatment groups ($P<0.05$). With regards to TGF-β1, the normal control rats (group A) displayed significantly lower mRNA expression levels than those in the other groups ($P<0.05$), with the exception of the high-dose PEPO treatment group

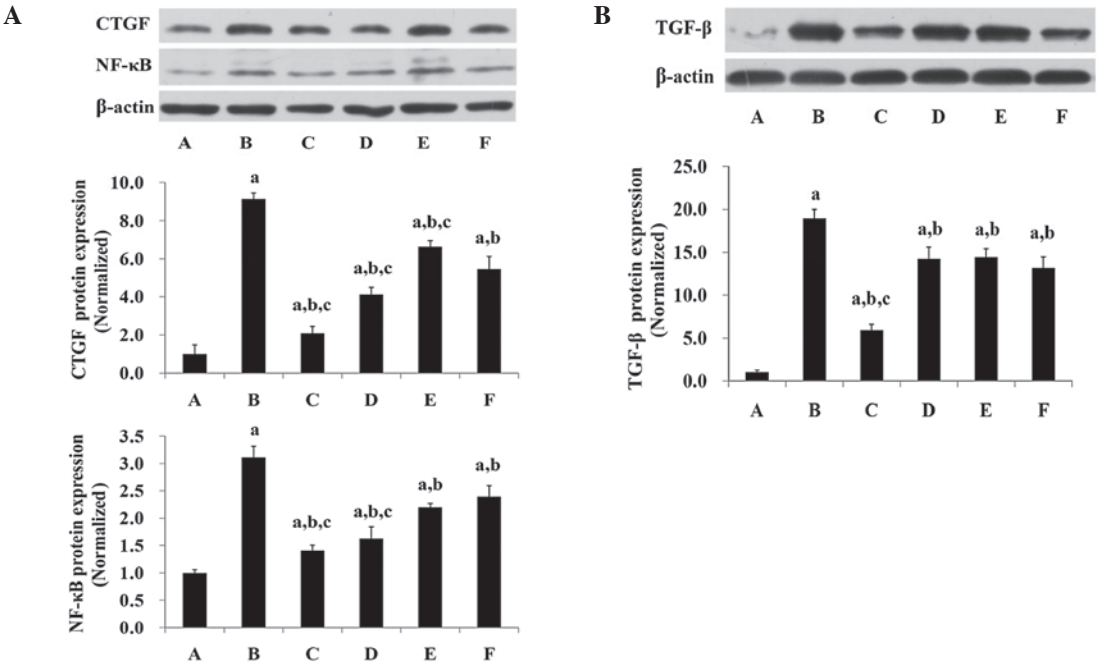

Figure 3. (A) CTGF and NF-κB; and (B) TGF-β1 protein expression in liver tissues. Group A, normal controls (2 ml/kg olive oil); group B, models (2 ml/kg CCl_4); group C, high-dose PEPO (8 mg/kg PEPO+2 ml/kg CCl_4); group D, medium-dose PEPO (4 mg/kg PEPO+2 ml/kg CCl_4); group E, low-dose PEPO (2 mg/kg PEPO+2 ml/kg CCl_4); group F, colchicine group (2 mg/kg colchicine+2 ml/kg CCl_4). Data are presented as the mean ± standard deviation. (n=10, 7, 10, 9, 8 and 8 in groups A, B, C, D, E and F, respectively). P<0.05 was considered to indicate a statistically significant difference. [a]P<0.05 vs. group A; [b]P<0.05 vs. group B; [c]P<0.05 vs. group F (analysis of variance). CTGF, connective tissue growth factor; NF-κB, nuclear factor κB; TGF-β, transforming growth factor β; PEPO, protein extract of Pacific oyster.

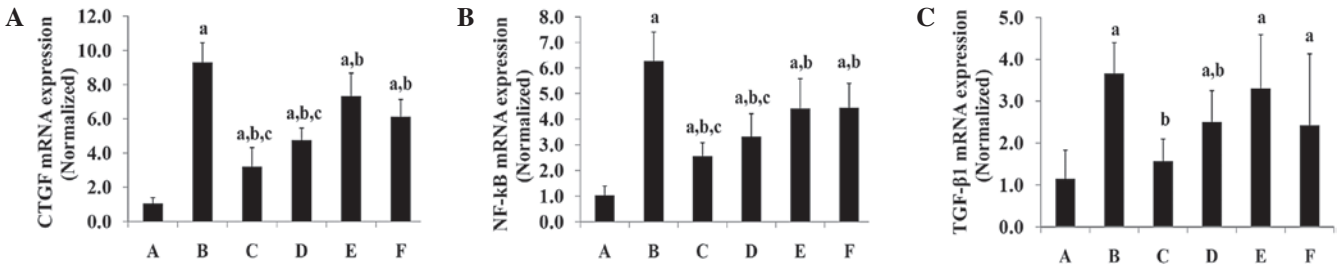

Figure 4. (A) CTGF; (B) NF-κB; and (C) TGF-β1 mRNA expression in liver tissues. Group A, normal controls (2 ml/kg olive oil); group B, models (2 ml/kg CCl_4); group C, high-dose PEPO (8 mg/kg PEPO+2 ml/kg CCl_4); group D, medium-dose PEPO (4 mg/kg PEPO+2 ml/kg CCl_4); group E, low-dose PEPO (2 mg/kg PEPO+2 ml/kg CCl_4); group F, colchicine (2 mg/kg colchicine+2 ml/kg CCl_4). Data are presented as the mean ± standard deviation. (n=10, 7, 10, 9, 8 and 8 in groups A, B, C, D, E and F, respectively). P<0.05 was considered to indicate a statistically significant difference. [a]P<0.05 vs. group A; [b]P<0.05 vs. group B; [c]P<0.05 vs. group F (analysis of variance). CTGF, connective tissue growth factor; NF-κB, nuclear factor κB; TGF-β, transforming growth factor β; PEPO, protein extract of Pacific oyster.

(group E) (P>0.05). TGF-β1 mRNA expression levels in the model group (group B) were indicated to be significantly higher than those in the high-dose and medium-dose PEPO treatment groups (P<0.05). No significant differences in TGF-β1 mRNA expression levels were identified between the three PEPO treatment groups and the colchicine treatment group (P>0.05).

Discussion

Corticosteroids, penicillamine, methotrexate, silymarin and colchicines have been widely applied to treat hepatic fibrosis clinically, but none of them have been recognized as a definitive intervention (8-11). In the current study, it was demonstrated that PEPO successfully alleviates the hepatic fibrosis induced by CCl_4 and reverses hepatotoxicity by regulating the expression of serum enzymes and decreasing the expression levels of CTGF, TGF-β1 and NF-κB in liver tissues. These findings may provide a novel treatment option for patients with hepatic fibrosis in the future.

In the present study, CCl_4 was used to establish model rats with hepatic fibrosis. This method has been widely used in previous studies (30,31). The hepatoprotective and anti-hepatofibrosis effects of PEPO were evaluated in the current study by measuring body and liver weights, examining histological changes, detecting serum levels of ALT, AST, GGT, ALP, HA, LN, IV-C and PC III, and determining alterations in the liver tissue protein and mRNA expression levels of CTGF, NF-κB and TGF-β1, which are overall indicators of hepatic function and hepatic fibrosis conditions. It was demonstrated that PEPO treatment significantly reduced the serum levels of ALT, AST,

GGT, ALP, HA, LN, IV-C and PC III in hepatic fibrosis model rats compared with those in untreated models. HE staining exhibited that PEPO treatment markedly alleviated the fibrosis.

CTGF is synthesized in hepatocytes, and has been demonstrated to alter cellular responses of growth factors (17,32). The expression of CTGF has been demonstrated to be eminently induced in fibrotic liver, and its levels are significantly elevated in fibrotic lesions (33-35). Serum levels of CTGF have been associated with the development of hepatic fibrosis (18-20). For patients with HBV-induced hepatic fibrosis, the serum CTGF levels can be used to distinguish between mild and severe cases of fibrosis (21).

TGF-β is a well-known profibrotic cytokine, which can stimulate the activation and proliferation of HSCs to induce their transition to myofibroblast-like cells (17,36-38). TGF-β1 may be the principal growth factor inducing the transcription of the CTGF gene (22). The key roles of TGF-β1 in the induction of fibrosis have been demonstrated by various experimental models (17,39-41). As the main HSC-transformation promoting cytokine in Kupffer cells and infiltrating mononuclear cells, TGF-β1 is key in the development of hepatic fibrosis (23). The secretion of CTGF depends on levels of TGF-β1 (17,42-44), and CTGF expression levels, which are linked to TGF-β pathways in fibro-proliferative diseases, are increased in fibrotic human liver (45). Tissue fibrosis has been indicated to be associated with increased TGF-β and CTGF production, and there exists a coordinate expression of TGF-β before CTGF in regenerating tissues (46). As an enhancer of profibrogenic TGF-β1, CTGF functions to mediate fibre-fibre, fibre-matrix and matrix-matrix interactions (44). NF-κB is important during the processes of hepatocyte survival/damage, stellate and inflammatory cell activation and inflammatory cytokine production (47,48). As NF-κB has been demonstrated to protect HSCs from apoptosis, it may be useful to accelerate recovery from hepatic fibrosis (24). The activation of HSCs and other liver-originated cells have been demonstrated to be closely correlated with activation of the transcription factors TGF-β/Smad and NF-κB (25,48,49). As NF-κB is key in regulating TGF-β1 levels, a potential cross-talk mechanism between NF-κB and TGF-β/Smad has been proposed previously (47), and potential NF-κB binding sites in the promoter of the CTGF gene have been located (25,26). In the present study, it was demonstrated that the protein and mRNA expression levels of CTGF, TGF-β1 and NF-κB in the liver tissues were significantly reduced by PEPO treatment. It was therefore concluded that PEPO successfully alleviated the hepatic fibrosis induced by CCl_4 and reversed hepatotoxicity through regulating the levels of serum enzymes and decreasing the expression levels of CTGF, TGF-β1 and NF-κB in the liver tissues. However, further *in vitro* experiments using HSCs are required to elucidate other mechanisms underlying PEPO alleviation of hepatic fibrosis induced by CCl_4.

In conclusion, in the present study, PEPO successfully alleviated the hepatic fibrosis induced by CCl_4 and reversed hepatotoxicity by regulating serum enzymes and reducing the expression of CTGF, TGF-β1 and NF-κB in liver tissues.

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