EXPERIMENTAL RESEARCH

Effects of Danggui Buxue Decoction (当归补血汤) on Lipid Peroxidation and MMP-2/9 Activities of Fibrotic Liver in Rats*

CHEN Yuan (陈 园)[1], CHEN Qian (陈 倩)[1], LU Jing (吕 靖)[1], LI Feng-hua (李凤华)[2], TAO Yan-yan (陶艳艳)[1], and LIU Cheng-hai (刘成海)[1,3]

ABSTRACT **Objective:** To explore the mechanism of Danggui Buxue Decoction (当归补血汤, DBD) on the liver fibrosis related to hepatic lipid peroxidation and matrix metalloproteinases (MMP) -2/9 activities. **Methods:** The liver fibrosis in 28 rats was induced by an injection of carbon tetrachloride (CCl_4) and fed with high lipid and low protein diet for 6 weeks, the model rats were randomly divided into the model group and DBD treated group, 14 in each group, and another 10 rats as the normal group were observed as well. Rats in the DBD group were administered with DBD at the dose of 6 g/kg body weight for 6 weeks since CCl_4 intoxication. The hepatic inflammation and fibrosis were examined with HE and Sirius red stain. The liver function including serum alanine aminotransamine (ALT), aspartate transamine (AST), albumin (Alb) and total bilirubin (TBIL), liver triglyceride (TG) and malondialdehyde (MDA) contents, superoxide dismutase (SOD) activity were assayed. Hepatic hydroxyproline (Hyp) content was detected with Jamall's method. The α-SMA expression was analyzed by immunohistochemistry and the Western blot. Liver MMP-2 mRNA was analyzed with Real-time PCR, and MMP-2/9 activities were measured with gelatin zymography and in situ zymography. **Results:** Compared with the normal group, the levels of ALT, AST and TBIL, the content of Hyp, TG and MDA were remarkably increased, the Alb content and SOD activity were significantly decreased in the model group ($P<0.05$), and higher levels of MMP-2 mRNA and MMP-2/9 activities ($P<0.01$), the hepatic fatty degeneration and collagen accumulation and fibrosis at liver were observed. Compared with the model control, DBD group showed slighter hepatic fatty degeneration and collagen deposition, and had lower levels of ALT, AST and TBIL activities, lower contents of MDA, TG and Hyp, but higher SOD level and Alb content ($P<0.05$), and DBD also down-regulated MMP-2 mRNA expression and decreased MMP-2/9 activities in the fibrotic livers ($P<0.01$). **Conclusion:** The action of DBD against liver fibrosis is related to prevent lipid peroxidation and inhibit MMP-2/9 activities in the fibrotic livers.

KEY WORDS Danggui Buxue Decoction, liver fibrosis, hepatic lipid peroxidation, matrix metalloproteinases-2/9

Liver fibrosis, characterized by over-production and deposition of extracellular matrix (ECM) in liver tissue, is the common pathological process in most chronic liver diseases and its progression leads to cirrhosis regardless of etiologies(1). At present there is no effective biochemical and chemical drug for liver fibrosis in clinic yet(2), therefore antifibrotic treatment is crucial for chronic liver diseases, and much effort has been made to find the anti-fibrotic remedies, such as malotilate, colchicine, S-adenosylmethionine and interferon, etc.

Chinese medicine has been used by over one-fifth of the world population for thousands years, and the valuable knowledge has been accumulated on liver diseases treatment, which is a rich resource for developing anti-fibrotic remedies. Danggui Buxue Decoction (当归补血汤, DBD), an ancient classic decoction composed of *Radix Astragali* and *Radix Angelicae Sinensis* in a weight ratio of 5:1, has wide pharmacological actions such as regulating

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1. Shuguang Hospital, Liver Disease Institute, Shanghai University of Traditional Chinese Medicine, Shanghai (201203), China; 2. The Experimental Center of Science and Technology, Shanghai University of Traditional Chinese Medicine, Shanghai (201203), China; 3. Shanghai Traditional Chinese Medicine Internal Medicine E-Institute, Key Lab of Liver & Kidney Diseases, Shanghai University of Traditional Chinese Medicine, Minister of Education, Shanghai (201203), China
Correspondence to: Prof. LIU Cheng-hai, Tel: 86-21-5132888 Ext. 8111, Fax: 86-21-51328500, E-mail: chenghai_liu@yahoo.com.cn

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immune ability and protecting liver injuries and so on[3]. Recently, it was reported that DBD could be effective in decreasing portal pressure cirrhosis in animal models, and we also found DBD had potential effect on preventing liver fibrosis induced by carbon tetrachloride (CCl_4) in rats in the ratio of 5:1 (*Radix Astragali:Radix Angelicae Sinensis*) better than that in the ratio of 1:1 or 1:5[4]. However, oxidative stress and abnormal collagen metabolism mainly regulated by matrix metalloproteinases (MMP) are closely related to liver fibrosis pathogenesis but the mechanism of DBD against liver fibrosis is still unclear. In this study, we aim to further elucidate the mechanism of DBD against the liver fibrosis in rats induced by CCl_4 related to hepatic lipid peroxidation and MMP activity.

METHODS

Materials and Methods

CCl_4 and olive oil were purchased from Shanghai National Chemicals Co.,Ltd., China. The serum liver function kits including alanine aminotransamine (ALT), aspartate transamine (AST), albumin (Alb) and totalbilirubin (TBIL), triglyceride (TG), malondialdehyde (MDA), and superoxide dismutase (SOD) kits were purchased from Nanjing Jiangcheng Institute of Biological Products, China. Mouse-anti-human α-SMA monocolonal antibody was from Sigma, USA. Nitrocellulose membrane (Hybond-C, optimized for protein transfer) was purchased from Amersham Biosciences UK Ltd., UK. Horseradish peroxidate labeling goat-anti-mouse antibody and goat-anti- rabbit antibody were obtained from Santa Cruz Biotechnology, USA. SuperSignal chemiluminescent substrate was obtained from Pierce, USA. Fluorescence gelatinase assay kit (E12055) was purchased from Molecular Probes Company, USA. Trizol was obtained from Invitrogen, USA. First strand cDNA synthesis kit (K1622) was purchased from Fermentas, Germany. SYBR Green Real-time PCR Kit (DRR041A) was from TaKaRa Biotechnology Co., Ltd., China.

DBD is composed of *Radix Astragali* and *Radix Angelica sinensis* in the ratio of 5:1, the herbs were produced in Gansu province and purchased from Shanghai Hua Yu Chinese Herbs Co., Ltd., and they were accredited by the pharmacognosist as well. The herbs were mainly prepared with water boiling and alcoholic extraction, then the extracts were sprayed to dry to obtain the powder and used for the experiments by dissolving in pure water at required concentrations.

Animal Model of Liver Fibrosis and Treatment

Thirty-eight male Wistar rats were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (certificate No. 0001452), the rats were fed with free access to food and drinking water. The model rats with liver fibrosis were induced by injection of CCl_4 subcutaneously and administration of high lipid and lower protein food[5]. The rats were randomly divided into three groups:14 in the model group, and 14 in the DBD treated group and 10 in the normal control group. At the beginning, the rats were administrated with 100% CCl_4 subcutaneous injection at the dose of 5 mL/kg body weight, once time, then 3 mL/kg body weight of 40% CCl_4 was dissolved by olive oil twice every week for 6 weeks, and they were fed with high lipid and lower protein diet at the first 2 weeks, then with pure corn until the end of experiment. Those in the normal group took normal diet, and all animals were free to drink water during the whole experiment.

The rats in the DBD treated group were administrated with DBD by gastrogavage at the dose of 6 g /kg body weight raw herbs for 6 weeks through the experimental period, the dose was equivalent to 10 times of the 60 kg adult dose. While the rats in the normal and model groups received the same dose of normal saline.

Pathological Examination

Liver specimens were preserved in 4% paraformaldehyde and dehydrated in a graded alcohol series, and embedded in paraffin blocks, cut into 5 μm-thick slices and placed on glass slides. And then the slices were stained with hematoxylin/eosin (HE) and Sirius red.

Assessment of Serum Parameters of Liver Function

The serum was obtained from the rat blood, and then centrifuged at 4 700 r/min for 15 min at 4 ℃. The serum ALT and AST activities, TBIL and Alb contents were assayed with the kits according to manufacturer's instructions.

Measurement of TG in Liver Tissues

The hepatic sample for measurement TG was extracted from liver tissue with chloroform/methanol

mixed solution (1:1, v/v), the prepared sample was then centrifuged at 1 200× *g* for 10 min, the supernatant was collected for TG measurement with colorimetric method.

Measurement of SOD, MDA and Glutathione in Liver

Liver homogenates were prepared and centrifuged at 3 000 r/min for 10 min at 4 ℃, and the supernatants were collected and immediately assayed for enzyme activities. For total glutathione (GSH) 50 μg of liver was homogenized in 5% trichloroacetic acid in a ratio of 1:10 (w:v) and centrifuged for 5 min at 8 000 r/min and 4 ℃. Then, GSH level, total SOD activities, MDA level were determined. All theses parameters were expressed by gram of liver protein, which was assayed with BCA kit.

Hepatic Hydroxyproline Content Determination

Hepatic hydroxyproline (Hyp) content was measured according to the modified method of Jamall, et al[6]. The concentration of Hyp in each sample was determined from a standard curve, which was generated from a serial of known quantities of Hyp from 0.2 to 1.6 μg hyp (Peptide Co., Japan). Hyp content was expressed as μg/g of liver wet weight.

Immunohistochemistry

Immunohistochemistry was performed with two-step staining method. After deparaffinization and dehydration, microwave antigen retrieval was performed for 5 min prior to peroxidase quenching with 3% H_2O_2 in PBS for 15 min. Consequently, the slices were preblocked with 5% bovine serum albumin for 30 min. Slices were incubated at 4 ℃ with anti-α-SMA antibody (Sigma, A2547, 1:400, USA) overnight, then with biotinylated secondary antibodies for 45 min. After that, they were developed with DAB (AR1022) for 3 min and finally counterstained with hematoxylin. For the negative control the primary antibody was replaced with PBS.

Western Blotting Analysis of Protein Expression

The liver tissue was homogenized in RIPA buffer, the homogenate was centrifuged at 12 000 x *g* for 30 min, and the protein concentrations of supernatant were determined by BCA protein assay kit (Pierce, USA). Each solubilized tissue sample with 30 μg protein was subjected to 10% SDS-PAGE electrophoresis under the reducing and denaturing condition, and transformed onto the nitrocellulose membrane. The membranes were then blocked in 5% nonfat milk and in Tris-buffered saline containing 0.1% Tween for 1 h at room temperature, followed by incubation with primary antibodies as follows: mouse anti-α-SMA (1:400 dilution) and GAPDH overnight at 4 ℃. The blots were washed with TBS-T for 3 times, followed by incubation with the second antibody (anti-mouse, or anti-rabbit horseradish peroxidase-conjugated antibody) for 1 h at room temperature. After washed, the blots were developed with Supersignal west pico chemiluminescenct substrate and exposed to X-ray films (Kodak, USA) according to the manufacturer's protocol. The density of immunoreactive bands exposed on the films was quantified and corrected for GAPDH signal with SmartView analysis software (Smartview, China).

Gelatinase Activity Assay

Liver tissue gelatinase activity was assayed by tissue gelatin zymography and in situ fluorescent zymography of liver section respectively. The total liver tissue gelatin zymography was performed as described by Kleiner, et al[7], the liver tissue was homogenized and the homogenate was centrifuged at 12 000 x *g* for 30 min. And the supernatant was aliquoted according to protein concentrations determined as the same procedures mentioned above. Aliquots (30 μg protein/lane) of liver tissue were prepared by dilution in the zymogram sample buffer (5×) consisting of 0.4 mol/L Tris, pH 6.8, 5% SDS, 20% glycerol and 0.03 % bromphenol blue, then were separated with electrophoresis in 10% SDS-PAGE containing 1 mg/mL gelatine as a substrate under non-reducing conditions, then the gel was rinsed with 50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$ and 2.5% Triton-X 100 (pH 7.5) to remove SDS, and then incubated at 30 ℃ for 18 h with the same buffer without Triton-X100. After the incubation, the gel was stained with 0.1 % Coomassie brilliant blue G-250 in 30% methanol/20% acetic acid, and destained with 30% methanol/10% acetic acid. Areas of digestion were visualized as non-staining regions of the gel, which represents the gelatinase activity. The gel was scanned and gelatinolytic activity was analyzed with the Furi Gel Image software.

In situ fluorescent zymography of liver section was performed according to Wielockx, et al[8] with

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modification. Briefly, 1 mg/mL fluorescein-conjugated gelatine (Molecular Probes, USA) solution was diluted in 1:10 in the agarose-containing solution. Liver sections were mounted to the glass slide with the gelatin agarose mixture and incubated with 40 μg/mL in 0.5 mol/L Tris-HCl (pH 7.6), 50 mmol/L $CaCl_2$ and 1.5 mol/L NaCl for 6 h at 37 ℃. The sections were washed 3 times with water, and subsequently the nuclei were counterstained by adding Hoechst (Beyotime, China). Gelatinase activity in situ was visualized with fluorescent microscopy.

RNA Extraction and Quantitative Real-time PCR

The total RNA was isolated from the liver tissues with Trizol according to the manufacturer's instructions. The RNA quantity was determined spectrophotometrically and its integrity checked with agarose gel electrophoresis. The first strand cDNA was synthesized with reverse transcribing 4 μg of total RNA in a final reaction volume of 20 μL with the first strand cDNA synthesis kit according to the manufacturer's instructions. Primer oligonucleotide sequences specific for the Real-time PCR were shown in Table 1, which were designed and synthesized by Sangon Biotech Inc. (China). PCR mixtures contained 1 μL cDNA, 10 μL SYBR® Premix Ex Tq 2×, 0.25 μmol/L forward and reverse primers in a final volume of 20 μL. The triplicate reactions were performed with a Rcorbett 6.0 system (Rotor-Gene 3000) starting with a polymerase activation step for 10 s at 95 ℃, followed by 40 cycles of 5 s at 95 ℃ and 20 s at 60 ℃. Fluorescence data were acquired after each cycle. The absence of primer dimers and unspecific products were verified after every run by melting curve analysis (72 ℃ to 95 ℃) and agarose gel electrophoresis.

Table 1. Primers Used for Real-time PCR

Gene	Primer sequence	Gene bank accession number	Length (bp)
MMP-2	5'-CCAAGAACTTCC GACTATCCAATGA -3'	NM-031054	107
	5'- CAGTGTAGGCG TGGGTCCAGTA -3'		
β -actin	5'- TGA CGA GGC CCA GAG CAA GA -3'	DQ237887	331
	5'- ATG GGC ACA GTG TGG GTG AC -3'		

Statistical Analysis

The statistical tests were performed with SPSS software version 11.5. The differences between two groups were analyzed with two-tailed Student's *t*-test. *P* values lower than 0.05 were considered statistically significant.

RESULTS

Effect of DBD on Liver Pathohistology

The model rats showed obvious liver inflammation including gross hepatocyte fatty degeneration and necrosis, broad infiltration of lymphocytes around the central vein, also marked collagen deposition, which formed fibrous septa and cirrhotic nodules or pseudolobules. The rats treated with DBD showed much less liver inflammation and collagen accumulation in the liver as compared with the model control (Figure 1).

Effect of DBD on Liver Function

CCl_4 intoxication resulted in significant increase

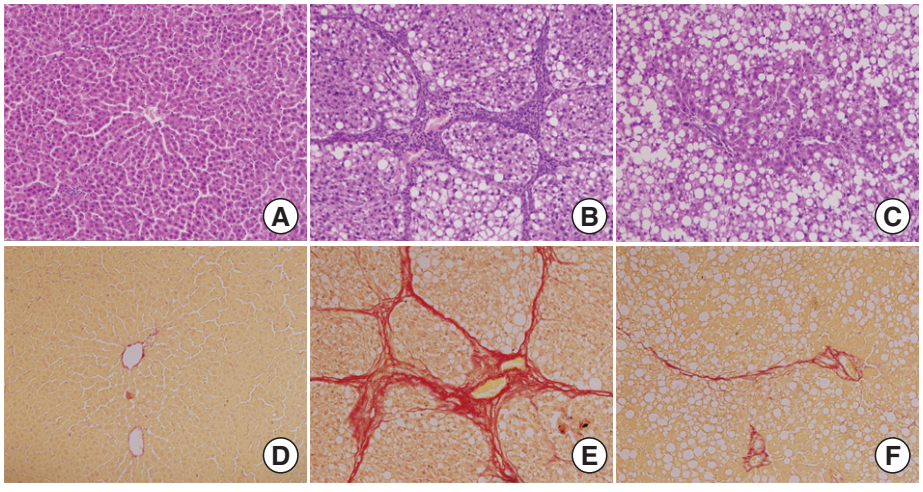

Figure 1. Effect of DBD on Liver Inflammation and Collagen Deposition in CCl_4-induced Liver Fibrotic Rats (× 200)

Notes: A, D: normal group; B, E: model group; C,F: DBD group; HE (3 photos in the up line) and Sirius red (3 photos in the down line) staining

of serum ALT, AST activities and TBIL contents, but lower Alb content in the model group than those in the normal group. DBD significantly reduced the increase of ALT, AST activities and TBIL contents, and improved the Alb level (Table 2).

Table 2. Effect of DBD on Liver Function ($\bar{x} \pm s$)

Group	*n*	ALT (U/L)	AST (U/L)	TBIl (μmol/L)	Alb (g/L)
Normal	10	37.7±13.2	101.4±15.0	11.3±2.7	31.1±1.1
Model	14	182.1±67.5*	190.1±55.0*	18.9±6.1*	26.4±2.0*
DBD	14	143.1±32.3△	152.5±59.4△	14.9±4.1△	32.1±3.2△

Notes: *P<0.05, compared with the normal group; △P<0.05, compared with the model group

Effect of DBD on Liver Lipid Peroxidation and Hyp Content

Compared with the normal rats, the model rats showed significant decrease in SOD activity, but remarkable increase in TG, MDA and Hyp respectively. While DBD increased liver SOD activity, significantly decreased the hepatic levels of TG, MDA and Hyp respectively (Table 3).

Table 3. Effect of DBD on Liver Lipid Peroxidation and Hyp Content ($\bar{x} \pm s$)

Group	*n*	SOD (NU/g)	Hyp (μg/g)	TG (μg/g)	MDA (μmol/g)
Normal	10	852.5±58.5	239.7±39.9	6.1±0.8	5.7±1.4
Model	14	716.7±158.5*	408.1±63.2*	15.1±2.6*	21.5±5.7*
DBD	14	1051.3±135.1△	298.7±57.6△	10.8±2.4△	10.3±2.8△

Notes: *P<0.05, compared with the normal group; △P<0.05, compared with the model group

Effect of DBD on Hepatic Stellate Cell Activation

The α-SMA expression, a marker of hepatic stellate cells (HSCs) activation, was determined by immunohistochemical stain and Western blotting with an antibody against α-SMA. As shown in Figure 2, model rats resulted in a significantly increased expression of α-SMA-positive HSCs around damaged hepatocytes and fibrotic bands as compared with the normal group. The numbers of α-SMA-positive HSCs in livers was markedly reduced after treated with DBD, the α-SMA expression level was almost 3 times higher in the model group than that in the normal group confirmed by Western blotting, however DBD resulted in a more remarkable decrease than that in the model group (Figure 3).

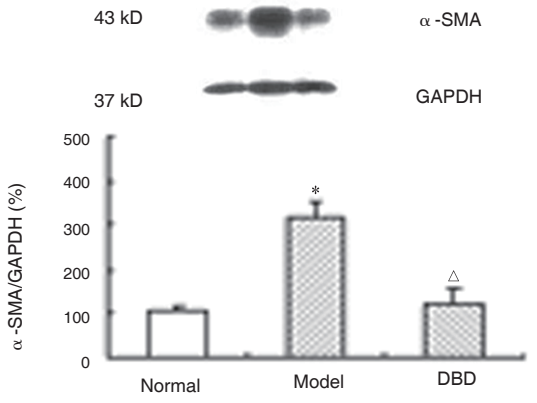

Figure 3. Effect of DBD on α-SMA Protein Expression in CCl_4-induced Liver Fibrotic Rat

Notes: *P<0.01, compared with the normal group; △P<0.01, compared with the model group

Effect of DBD on MMP-2 mRNA and MMP-2/9 Activities

Real-time PCR analysis showed that the expression of MMP-2 mRNA in the liver of the model group was 5 times more than that in the normal group, it was significantly decreased in the DBD treated group when compared with the model ones (Table 4). MMP-2/9 activities were assessed using gelatin zymography and in situ zymography. As shown in Figures 4 and 5, the normal rats expressed little active MMP-2/9, model rats had much higher MMP-2/9 activities in liver tissue, in particular around hepatic sinusoidal area and fibrous septa checked by in situ zymography as compared with the normal group. DBD significantly reduced the activities of MMP-2/9, especially of active MMP-2 confirmed by gelatin zymography, and decreased MMP2/9 activities in the hepatic sinusoidal area.

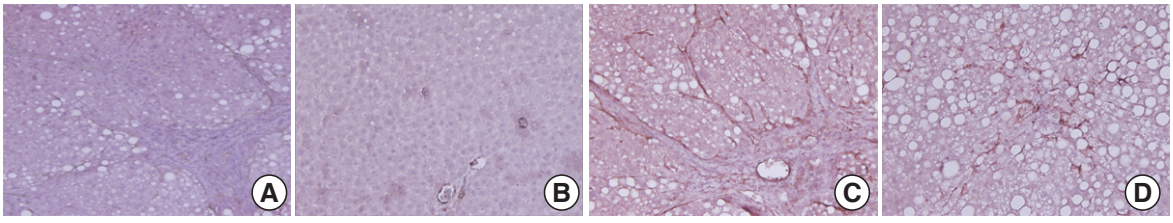

Figure 2. Effect of DBD on α-SMA Expression in Liver Tissue of CCl_4-induced Liver Fibrotic Rats (DAB, × 200)

Notes: A: negative control; B: normal group; C: model group; D: DBD group

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Table 4. Effect of DBD on MMP-2 mRNA ($\bar{x} \pm s$)

Group	*n*	MMP-2 mRNA (folds)
Normal	3	0.87 ± 0.16
Model	3	$4.37 \pm 1.02^{*}$
DBD	3	$1.61 \pm 0.27^{\triangle}$

Notes: $^{*}P<0.01$, compared with the normal group; $^{\triangle}P<0.01$, compared with the model group

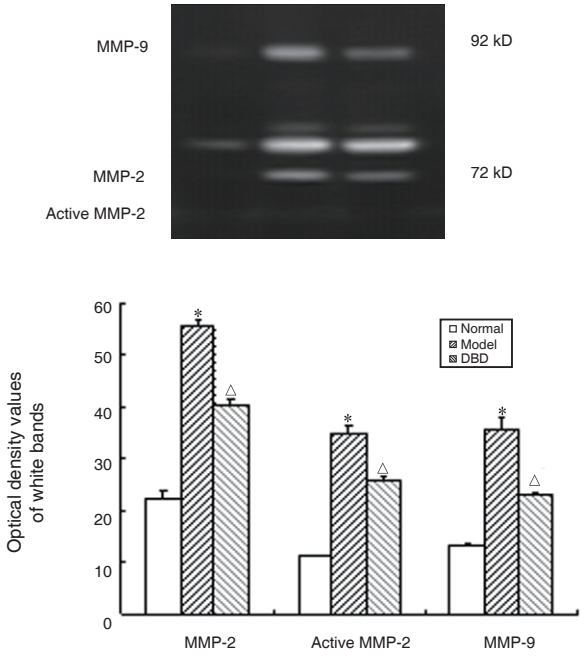

Figure 4. Effect of DBD on Liver MMP-2/9 Activities in CCl_4-induced Liver Fibrotic Rats

Notes: $^{*}P<0.01$, compared with the normal group; $^{\triangle}P<0.01$, compared with the model group

DISCUSSION

Many etiologies including virus, toxins, alcohol and cholestasis etc. can cause liver fibrosis. It is widely recognized that HSC activation is a key cellular basis of liver fibrosis, while oxidative stress plays a pivotal role in the initiation and perpetuation of fibrogenesis[9]. Oxidative stress is a cellular damage process, which is mainly caused by reactive oxygen species (ROS), and can lead to lipid peroxidation. It is clearly demonstrated that both ROS and lipid peroxidation can directly activate HSC and improve collagen gene expression, and indirectly activate HSC through triggering Kupffer cell activation, Kupffer cells secrete fibrogenic factors such as ransforming growth factor-β[10]. Recently, it is reported that oxidative stress can increase MMP-2 expression and activity through extracellular signal-regulated kinase (ERK) and phosphatidyl inositol 3-kinase (PI3K) pathways, and promote HSC proliferation and invasiveness[11]. While antioxidants, such as α-tocopherol, polye nyphosphatidycholine, flavonoids silymarin and N-acetylceiteine etc., could effectively prevent or reduce liver fibrosis[9].

CCl_4 is a chemical reagent for free radical damage, which is converted to $CCl_3^{\cdot}$ by cytochrome P450 and then causes lipid peroxidation. The repeated damages of CCl_4 and lipid peroxidation will lead to steatohepatitis and liver fibrosis or cirrhosis at last. In this study, we found that there was obvious lipid degeneration and fibrous septa in liver, and hepatic lipid peroxidation manifested by decreased SOD activity and increased MDA level. Not only did we find liver MMP-2 mRNA and activity increase in fibrotic liver, but as far as we known, it was the first time found that MMP-2/9 activity mainly expressed and increased around sinusoidal area in CCl_4 induced fibrotic liver. It confirmed that CCl_4 fibrotic model has a close relation with lipid peroxidation and increased MMP-2/9 activity caused by oxidative stress.

Chinese medicine theory regards that liver fibrosis is caused by dual deficiency of qi-yin and blood stasis[12]. DBD is often used in menopausal disorders for its good effect on nourishing qi and blood, and activating blood stasis[3]. Now there is no report about the effect of DBD against liver fibrosis, but its ingredients—*Radix Astragali* and

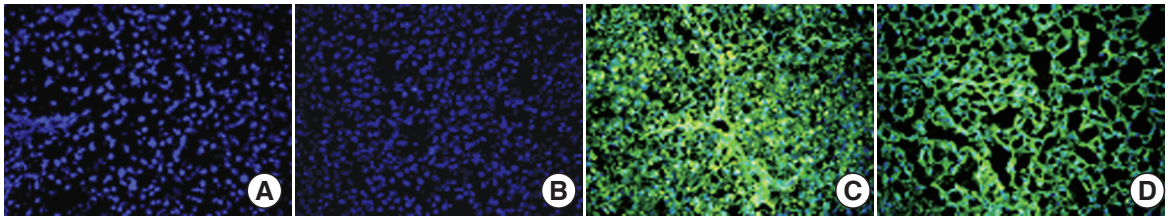

Figure 5. Effect of DBD on Liver Tissue MMP-2/9 Activities in CCl_4-induced Liver Fibrotic Rat in Situ Zymography ($\times$ 200)

Notes: A: negative control; B: normal group; C: model group; D: DBD group

Radix Angelica Sinensis individually or combined with other herbs reportedly have good actions on chronic liver diseases, and *Radix Astragali* and *Radix Angelica Sinensis* decoction can efficiently treat chronic kidney diseases patients with renal fibrosis[13]. Our pervious study first found that DBD had good action against liver fibrosis[3], in this study, we found that DBD could attenuate hepatic inflammation and fibrosis, improve liver function, reduce hepatic TG and MDA contents and enhance SOD activity in fibrotic liver, diminish α-SMA expression confirmed with immunohistochemical stain and Western blotting. Since α-SMA is the marker of HSC activation, it indicated that DBD could prevent hepatic lipid peroxidation, and this action may be one of mechanisms against liver fibrosis.

In addition, DBD decreased MMP-2 gene expression, MMP-2/9 activity, in particular MMP-2/9 in the hepatic sinusoidal area with in situ gelatin zymography. Normally, the sub-endothelial Disse space contains basement membrane including type Ⅳ collagen, laminin and fibronectin, etc., which are essential to preserve the differentiated functions of hepatocytes, hepatic stellate cells and endothelial cells, etc. These basement membrane matrixes can be degraded by MMP-2/9. It is known that MMP-2 expression was markedly increased during liver fibrogenesis[14], and will lead to ECM turn over and remodeling. This remodeling alters the liver normal microenvironment, simulates HSC activation, replacement of sub-endothelial matrix with intensive interstitial collagens and hepatic sinusoidal capillarization, all these contribute to liver fibrogenesis.

In the study, DBD inhibited MMP-2/9 expression and activities, while MMP-2 was implicated to be closely associated with fibrogenesis, and it suggested that DBD exerts the action against liver fibrosis through inhibiting hepatic oxidative stress and MMP-2/9 activities and protecting the hepatic microenvironment.

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