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Protective effects of total flavonoids of *Bidens bipinnata* L. against carbon tetrachloride-induced liver fibrosis in rats

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Abstract

Bidens bipinnata L. is well known in China as a traditional Chinese medicine and has been used to treat hepatitis in clinics for many years. In a previous study we found that total flavonoids of *Bidens bipinnata* L. (TFB) had a protective effect against carbon tetrachloride (CCl_4)-induced acute liver injury in mice. Now this study was designed to investigate its therapeutic effect against CCl_4-induced liver fibrosis in rats and to determine, in part, its mechanism of action. The liver fibrosis model was established by subcutaneous injection of 50% CCl_4 twice a week for 18 weeks. TFB (40, 80 and 160 mg kg^{-1}) was administered by gastrogavage daily from the 9th week. The results showed that TFB (80 and 160 mg kg^{-1}) treatment for 10 weeks significantly reduced the elevated liver index (liver weight/body weight) and spleen index (spleen weight/body weight), elevated levels of serum transaminases (alanine aminotransferase and aspartate aminotransferase), hyaluronic acid, type III procollagen and hepatic hydroxyproline. In addition, TFB markedly inhibited CCl_4-induced lipid peroxidation and enhanced the activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase. Moreover, TFB (80 and 160 mg kg^{-1}) treatment improved the morphologic changes of hepatic fibrosis induced by CCl_4 and suppressed nuclear factor (NF)-κB, α-smooth muscle actin (SMA) protein expression and transforming growth factor (TGF)-β1 gene expression in the liver of liver fibrosis of rats. In conclusion, TFB was able to ameliorate liver injury and protect rats from CCl_4-induced liver fibrosis by suppressing oxidative stress. This process may be related to inhibiting the induction of NF-κB on hepatic stellate cell activation and the expression of TGF-β1.

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Introduction

Liver fibrosis is a common sequel to diverse liver injuries (Okazaki et al 2001) and is characterized by the excessive deposition of extracellular matrix (ECM) (Okazaki et al 2000). Without effective treatment, reversible liver fibrosis at an early stage leads to irreversible cirrhosis. Now it is well known that hepatic stellate cells (HSCs) play an important role during hepatic fibrogenesis (Friedman 2000). In normal liver HSCs are the principal storage sites for retinoids. Upon activation, HSCs changed their phenotype from retinoid-storing quiescent cells to ECM-producing myofibroblast (Friedman et al 1985). The activation of HSCs could be initiated by the stimulation of cytokines, oxidative stress (Kim et al 2000; Aboutwerat et al 2003; Marotta et al 2007) and deposition of degraded ECM. The activated HSCs then increase the production of ECM and different cytokines, such as transforming growth factor (TGF)-β1, which further stimulates the activation of HSCs and the production of ECM (Barcellos-Hoff & Dix 1996).

Cellular oxidative stress is one of the main trigger factors in the development of liver fibrosis (Kim et al 2000; Aboutwerat et al 2003; Mahmood et al 2004; Marotta et al 2007). Reducing oxidative stress by antioxidants, such as α-tocopherol and butylated hydroxytoluene, blocks HSC activation and suppresses the expression of collagen genes in HSC in-vitro (Lee et al 1995) as well as preventing fibrosis in iron-overloaded rat liver in-vivo (Pietrangelo et al 1995). Experimental results suggest that reducing oxidative stress by antioxidants could be a potential and effective therapeutic strategy for treatment and prevention of liver fibrosis. However, the therapeutic efficacy of currently well-known

antioxidants, such as superoxide dismutase and vitamin E, in the treatment of human liver fibrosis is generally unimpressive.

The *Bidens* genus has about 230 types of weeds all over the world. In China there are 9 kinds of weeds of *Bidens* genus, such as *Bidens bipinnata* L., *B. pisola* L., *B. aurea* Sherff, etc. *Bidens bipinnata*, commonly known as po-po-z-hen, is the most widely distributed in China, and has been used as a traditional Chinese folk medicine for a long time. Studies show that *Bidens bipinnata* L. has anti-inflammatory activity, antimicrobial activity, cardiovascular activity and antileukaemia activity. It has been applied in the treatment of jaundice, rheumatism, laryngitis, headache and digestive disorders. Our previous study (Zhong et al 2007) demonstrated that the total extracted flavonoids from *Bidens bipinnata* L. (TFB) had a hepatoprotective effect on acute liver injury in mice. Now, this study was designed to evaluate the therapeutic effect of TFB on CCl_4-induced liver fibrosis in the rat and the possible mechanism of its action.

Materials and Methods

Plant material

Bidens bipinnata L. was purchased from a crude drug market in Bozhou, Anhui Province, China, in October 2005. It was classified by Dr De-qun Wang (Department of Pharmacy, Anhui College of Traditional Chinese Medicine, China) and a voucher specimen (No. AH20051012) was deposited in the herbarium of the College of Pharmacy, Anhui Medical University, China.

Drugs and chemicals

CCl_4 was purchased from Shanghai Changjiang Chemistry Plant (Shanghai, China) and dissolved in olive oil to a final concentration of 50% before use. Kits for determining serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), hepatic thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) levels and hepatic hydroxyproline (Hyp) content were purchased from Jiancheng Institute of Biotechnology (Nanjing, China). Test kits for evaluating serum hyaluronic acid (HA) and type III procollagen (PCIII) were purchased from Jiancheng Institute of Biotechnology (Nanjing, China). Other biochemicals and reagents used in these experiments were of analytical grade from commercial sources.

Preparation of TFB

The crude extract was obtained from the dried leaves (500 g) of *Bidens bipinnata* L. by reflux extraction using 80% ethanol (3.5 L, three times). The extracts were combined and concentrated in-vacuo to syrup, then the concentrated crude extract was dissolved again with distilled water (1:20 w/v); undissolved impurities were filtered, and the clarified liquid was collected. The clarified liquid was passed through HPD100 macroporous adsorptive resin columns (Cangzhou Bon Chemical Co. Ltd, Hebei Province, China). An ordered elution was performed using distilled water, 30% ethanol, 50% ethanol and 95% ethanol. Only the 30% ethanol-eluted solution was collected. The 30% ethanol eluate was concentrated in-vacuo and dried by lyophilization to yield a brown powder that reacted intensely with magnesium hydrochloric acid. This powder was referred to as TFB. Forty grams of TFB could be obtained from 100 g of the crude extracts in a single operation. The preparation of the individual flavonoids was carried out on an RP-HPLC with a Symmetry Prep C18 column, and the mobile phase was water–acetonitrile at a flow rate of 5.0 mL min^{-1}. The compounds were identified with ultra violet absorbance (UV), infrared (IR), nuclear magnetic resonance (NMR) and mass spectrometry (MS). They were hyperoside (15 mg), rutin (18 mg), maritimetin (10 mg), quercetin (15 mg), okanin (11 mg), iso-okanin (15 mg), 7-*O*-(4″,6″-diacetyl)-*b*-D-glucopyranoside (11 mg), (*Z*)-6-*O*-(3,6-di-*O*acetyl-D-glucopyranosyl)-6, 7,3′,4′-tetrahydroxyaurone (17 mg) and 2′,4′,6′-trimethoxy-4-*O*-D-glucopyranosyl-dihydrochalcone (12 mg). The total flavonoid content, measured using a colorimetric assay developed by Zhishen et al (1999), was 66.2% (w/w) and this was suspended in physiological saline and administered orally.

Animals

Forty male Sprague–Dawley rats, 200–250 g, were obtained from the Animal Center of Anhui Medical University and were bred under controlled temperature (21–22°C), humidity (50%), and light conditions (12-h light–dark cycle). Rats were fed a commercially available chow, and water was freely available. The rats were divided randomly into 5 groups: control group (normal rats), CCl_4 group, TFB (40, 80 and 160 mg kg^{-1}) + CCl_4 groups. All rats received humane care in compliance with the Guidelines of the Animal Care and Use of Laboratory Animals as set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Experimental model of liver fibrosis and TFB treatment

All rats were administered with carbon tetrachloride (CCl_4, 0.1 mL/100 g of rat body weight, dissolved in an equal volume of peanut oil), injected subcutaneously twice weekly (18 weeks in total), to induce liver fibrosis, except for the rats in the control group which received injection of peanut oil. From the 9^{th} week, rats receiving different doses of TFB (40, 80 and 160 mg kg^{-1}) were given TFB via gastric gavage together with CCl_4 injection throughout the remaining 10 weeks. All rats were sacrificed 24 h after the last CCl_4 injection. Whole blood (2–3 mL) was harvested and centrifuged to determine the levels of the aforementioned biochemical markers. The liver and spleen were immediately removed and weighed to calculate the liver and spleen indexes. The left lobe of the liver was sampled for histopathology assessment and α-smooth muscle actin (α-SMA), nuclear factor (NF)-κB and TGF-β1 immunohistochemistry analysis, a part of the right lobe for TBARS, SOD and GSH-Px assays and another part of the right lobe for Hyp assay.

Biochemical variables

Serum ALT and AST activity was determined according to the procedure of kits (Nanjing Jiancheng Biological Company, Nanjing, China). Serum HA and PCIII contents were assessed using radioimmunoassay methods (Beijing biotechnology company, Beijing, China). Hepatic Hyp and TBARS content and SOD and GSH-px activity were determined according to the procedure of kits (Nanjing Jiancheng Biological Company, Nanjing, China).

Histopathological examination and grading of liver tissue in liver fibrosis rats

Hepatic tissues excised from each rat were fixed in 10% formalin and embedded in paraffin wax. Sections, 3–4 μm thin, from blocks were stained with haematoxylin–eosin (H&E). Areas in sections stained for collagens by Masson trichrome were quantified by image analysis using a Universal Imaging Image-1/AT image acquisition and analysis system (West Chester, PA, USA) incorporating an Axioskop 50 microscope (Carl Zeiss, Thornwood, NY, USA) and ×4 objective lens. For each rat, the area of stained collagens tissue in five randomly selected fields was measured, and the average was expressed as collagen per unit area of liver tissue (termed the FI) as described by MacIntosh et al (1992). At least five fields contained a central vein of each specimen, and the microscopic examination was performed independently by two pathologists who had no prior knowledge of their source.

Immunohistochemistry

NF-κB

Sections of formalin-fixed, paraffin-embedded tissue were cut onto silanized glass slides and stained by means of an SP kit (Zymed, South San Francisco, CA, USA). Rabbit anti-human monoclonal NF-κB (p65) IgG (Santa Cruz, CA, USA) was used as the primary antibody. As immunohistochemistry controls, some sections were incubated in the same way but with normal rabbit serum or with phosphate-buffered saline (0.01 mol L^{-1}, pH 7.4) alone instead of the primary antibody. After being immunostained, the sections were counterstained with haematoxylin.

α-SMA

To evaluate whether activated HSCs were present in CCl_4-induced liver fibrosis rats, liver sections were subjected to immunohistochemistry for α-SMA. The liver sections were deparaffinized in xylol and rehydrated by gradient alcohol before exposure to 3% hydrogen peroxide in water to quench endogenous peroxidases. They were then incubated with a mouse monoclonal anti-α-SMA primary antibody (Sigma Chemical Co., St Louis, MO, USA) and stained by means of a SP kit (Zymed, South San Francisco, CA, USA). The negative control used was normal rabbit serum. Sections were counterstained with eosin.

RNA preparation

Frozen liver tissue was mechanically pulverized and total hepatic RNA was isolated for the analysis effect of TFB on TGF-β1 mRNA expression. Total RNA was isolated by the guanidinium isothiocyanate–phenol–chloroform method using RNAzol (Introgen Life Technologies Company). In brief, tissue samples were homogenized with RNAzol and 0.2 mL chloroform was added to 2 mL of homogenate. The samples were shaken vigorously for 15 s and incubated for 5 min on ice, and then centrifuged at 12 000 *g* for 15 min. The aqueous phase was then transferred to a fresh tube, mixed with an equal volume of isopropanol and placed for 15 min on ice. After centrifugation at 12 000 *g* for 15 min, the RNA pellet was washed once with ice-cold 75% ethanol with overtaxing, centrifuged at 7500 *g* for 5 min, dried for 10 min and dissolved in diethylpyrocarbonate (Sigma)-treated RNase-free solution. The total RNA content of the samples was estimated spectrophotometrically by absorbance at 260 nm. Purity of RNA was determined from the 260/280 absorbance ratio.

RT-PCR analysis

Total RNA (1 μg) was reverse transcribed into cDNA by using AMV reverse transcriptase (Fermentas Company) and the cDNAs were amplified by PCR. The primers were designed and synthesized by Shanghai Biology Engineering Corporation. TGF-β1 sense primer: 5-GGACTCTCCACCTGCAAGAC-3, antisense primer: 5-CTCTGCAGGCGCAGC TCTG-3 (product length 392 bp); β-actin sense primer: 5-ACCA-CAGCTGAGAGGG AAATCG-3, antisense primer: 5-AGAGGTCTTTA CGGATGT CAACG-3 (product length 277 bp). Briefly, 1 μg of total RNA was denatured by heating for 10 min at 70°C and added to a mixture containing 2 μL of oligo-dT (0.5 mg mL^{-1}) (Fermentas Company), 6 μL of 5-fold concentrated reverse transcriptase buffer, 2 μL of DTT (Fermentas Company; 0.1 M), 1 μL of dNTP (Fermentas Company; 10 mM) and 1 μL of reverse transcriptase. Each cDNA synthesis was performed for 1 h at 42°C and stopped by incubation for 10 min at 98°C. PCR was performed in a volume of 50 μL containing 400 pM of each primer, 2 μg of cDNA, Tris-HCl (10 mM, pH 8.3), KCl (50 mM), $MgCl_2$ (1.5 mM), dNTP (0.2 mM) and 2 U of Taq DNA polymerase (Boehringer). Thermal cycle conditions were 94°C for 45 s for denaturing, 55°C for 45 s for annealing and 72°C for 2 min for extension. The number of amplification cycles was 35 for each set of primers. For each set of primers, dilutions of cDNA were amplified for 20, 25, 30, 35 and 38 cycles to define optimal conditions for linearity and to permit semiquantitative analysis of signal strength. After the last cycle of amplification, the samples were incubated for 7 min at 72°C. The PCR products were electrophoresed on 1.2% agarose gels and stained with ethidium bromide. To ensure that contaminating DNA had not been amplified, PCR without reverse transcriptase was simultaneously demonstrated. Quantitation of TGF-β1 mRNA was performed by competitive PCR using the PCR Mimic Protocol (Clontech). TGF-β1 competitor primers yielding product sizes of 392 bp were used in each reaction.

Statistical analysis

Numerical data were presented as mean ± standard deviation. Statistical analyses were performed using SPSS 10.0 software.

The significance of the difference between the groups was assessed by the Kruskal–Wallis test, in conjunction with Dunn's post-hoc test. $P < 0.05$ was considered significant.

Results

Effect of TFB on liver and spleen weights and serum ALT and AST content in CCl_4-induced liver fibrosis rats

The relative liver and spleen weights were significantly increased after treatment with CCl_4 alone compared with the control group. In contrast, treatment with TFB (80 and 160 mg kg^{-1}) significantly reduced the liver and spleen weights compared with the rats that received CCl_4 treatment alone (Table 1). Similarly, the CCl_4-treated rats had elevated serum ALT and AST levels, demonstrating marked liver damage. Administration of TFB (80 and 160 mg kg^{-1}) attenuated the CCl_4-induced increase in ALT and AST activity ($P < 0.05$) (Table 1).

Effect of TFB on the levels of serum HA, PCIII, hepatic Hyp and hepatic TBARS, SOD and GSH-Px in liver fibrosis rats

The levels of serum HA, PCIII and hepatic Hyp were significantly increased in rats treated with CCl_4 alone compared with the control group ($P < 0.01$). The elevation of those markers of liver fibrosis were significantly lower in TFB (80 and 160 mg kg^{-1})-treated rats (Table 2). Moreover, the content of hepatic TBARS in the control group was increased and hepatic SOD and GSH-Px activity were decreased. The abnormal changes of these indexes in TFB (80 and 160 mg kg^{-1}) groups were markedly ameliorated (Table 2).

Effect of TFB on the pathological changes in hepatic fibrosis rats

Liver tissues were collected to assess the effect of TFB on liver pathological changes. The morphological analysis showed that more fibrous tissues were formed, extending into the hepatic lobules to separate them completely, in the rats treated with CCl_4 alone (Figure 2B). In addition a large number of inflammatory cells infiltrated into the intralobular and interlobular regions. The liver structure was disordered and there were more necrotic and fatty degenerated liver cells (Figure 1B). In the rats treated with TFB for 10 weeks the hepatocyte degeneration, necrosis and infiltration of inflammatory cells were all apparently ameliorated (Figure 1C) and collagen deposition was also markedly reduced (Figure 2C). However no evidence of inflammatory cells (Figure 1A) or accumulation of collagen were observed in the normal group (Figure 2A). Image analysis revealed that Masson trichrome

Table 1 Effect of total flavonoid of *Bidens bipinnata* L. (TFB) on the liver and spleen indexes and the activity of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in CCl_4-induced liver fibrosis rats

Group	Dose (mg kg^{-1})	Index of liver	Index of spleen	ALT(U L^{-1})	AST(U L^{-1})
Control	—	2.36 ± 0.25	0.32 ± 0.07	50.01 ± 19.18	57.24 ± 15.08
CCl_4	—	3.49 ± 0.74##	0.45 ± 0.09##	81.64 ± 15.05##	78.14 ± 10.50##
TFB + CCl_4	160	2.85 ± 0.47*	0.36 ± 0.06*	58.74 ± 19.26*	62.06 ± 9.29**
	80	2.75 ± 0.42*	0.38 ± 0.04*	55.51 ± 19.98*	62.02 ± 5.77**
	40	3.79 ± 0.40	0.40 ± 0.08	75.27 ± 13.46	74.15 ± 6.74

Rats were treated by gastrogavage with TFB daily from the 9th week. Liver and spleen were removed and weighed 24 h after the last CCl_4 injection at the 18th week. Meanwhile blood was collected and measured. Data are expressed as mean ± s.d., n = 8 per group. ##$P < 0.01$ compared with the control group; **$P < 0.01$, *$P < 0.05$ compared with the CCl_4 group.

Table 2 Effect of total flavonoid of *Bidens bipinnata* L. (TFB) on the content of serum hyaluronic acid (HA), type III procollagen (PCIII), hepatic hydroxyproline (Hyp) and thiobarbituric acid reactive substances (TBARS) formation, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in CCl_4-induced liver fibrosis rats

Group	Dose (mg kg^{-1})	HA (ng mL^{-1})	PCIII (ng mL^{-1})	Hyp (ug (g liver)$^{-1}$)	TBARS (nmol (mg protein)$^{-1}$)	GSH-Px (U (mg protein)$^{-1}$)	SOD (U (mg protein)$^{-1}$)
Control	—	274.87 ± 31.47	2.91 ± 1.68	219.72 ± 18.70	3.80 ± 0.96	24.65 ± 4.79	18.56 ± 4.12
CCl_4	—	827.75 ± 334.09##	11.78 ± 4.73##	289.42 ± 40.75##	9.36 ± 2.87##	13.28 ± 3.43##	8.32 ± 2.45##
TFB + CCl_4	160	529.8 ± 174.39*	6.78 ± 2.95*	248.33 ± 36.34*	6.66 ± 1.52*	18.44 ± 3.37**	15.94 ± 2.11**
	80	488.75 ± 120.45*	6.25 ± 1.49**	235.01 ± 33.21*	6.66 ± 0.53*	18.56 ± 2.90**	12.87 ± 2.55*
	40	737.34 ± 299.46	8.92 ± 2.40	269.92 ± 44.62	8.99 ± 1.88	12.19 ± 1.80	10.25 ± 3.46

Serum HA, PCIII content and hepatic Hyp content were measured in liver collected 24 h after the last CCl_4 injection at week 18. Hepatic TBARS, SOD and GSH-Px were measured in liver homogenates 24 h after the last CCl_4 injection at week 18. Data are expressed as mean ± s.d, n = 8 per group. ##$P < 0.01$ compared with the control group; **$P < 0.01$, *$P < 0.05$ compared with the CCl_4 group.

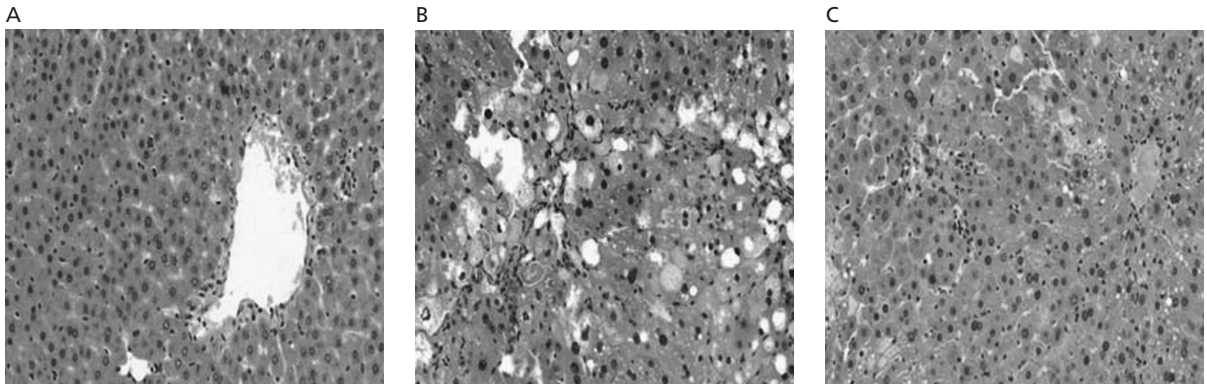

Figure 1 Histological examination performed under a light microscope (magnification ×100) with haematoxylin and eosin on liver specimens. Livers were harvested 24 h after the last CCl_4 injection at week 18, and haematoxylin and eosin staining was performed to evaluate pathological changes. Shown are representative images: liver section from normal rats treated with saline and olive oil (control group) showing normal liver architecture (A); liver section from CCl_4-treated rats showing severe hepatocellular necrosis, fatty degeneration and disordered liver structure (B); liver section from rats treated with 160 mg kg^{-1} total flavonoid of *Bidens bipinnata* L. (TFB) + CCl_4 showing a marked reduction in the severity of hepatocellular necrosis and fatty degeneration (C).

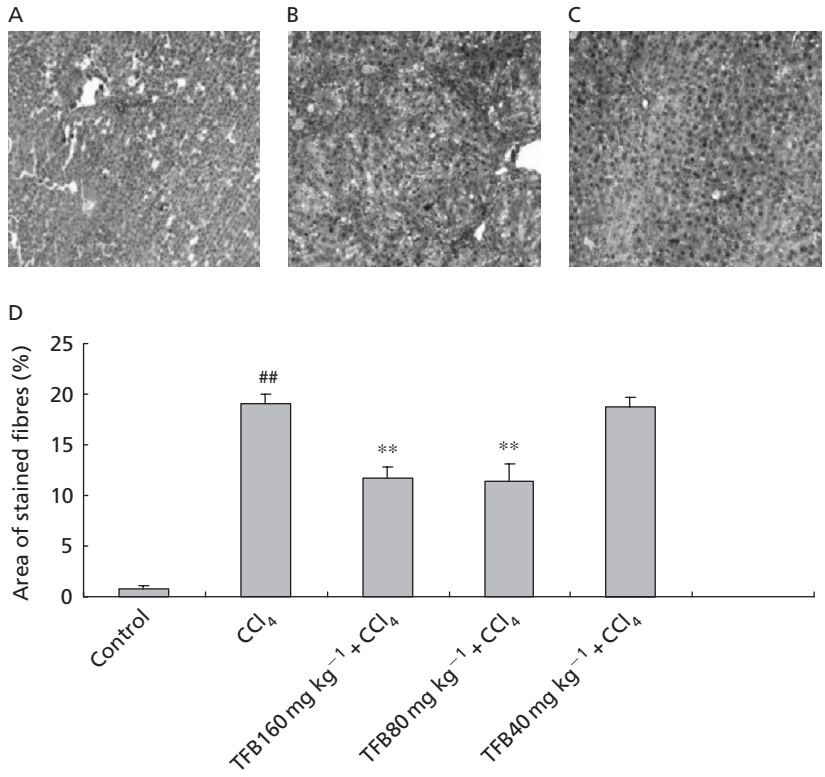

Figure 2 Collagen examination performed under a light microscope (magnification ×100) with Masson on liver specimens. Collagen fibres were stained with Masson as described in Materials and Methods. Shown here are: liver section from normal rats treated with saline and olive oil (control group) showing no evident accumulation of collagen fibres (A); liver section from CCl_4-treated rats showing more collagen fibres stained as blue (B); liver section from rats treated with 160 mg kg^{-1} total flavonoid of *Bidens bipinnata* L. (TFB) + CCl_4 showing a marked reduction in collagen fibres stained as blue (C). The percentage area of stained collagens was further characterized by using image analysis (D). Data are expressed as mean ± s.d., n = 8 per group. ##$P < 0.01$ compared with the control group; **$P < 0.01$ compared with the CCl_4 group.

stained an area of 0.81% of liver sections from the normal control rats. Masson trichrome staining increased to 19.05% after treatment with CCl_4 alone for 18 weeks ($P < 0.05$). Treatment with TFB (160, 80 mg kg^{-1}) suppressed this increase in Masson trichrome staining to 11.77% in the 160 mg kg^{-1} group and 11.47% in the 80 mg kg^{-1} group of the measured areas (Figure 2D, $P < 0.05$ compared with the CCl_4-treated rats alone).

Effect of TFB on NF-κB and α-SMA expression of hepatic fibrosis rats

Immunohistochemical analysis of liver tissue sections revealed that NF-κB expression, as brown or yellow granulae in the cytoplasm and nucleus were apparently increased in response to 18 weeks of subcutaneous injection of CCl_4 (Figure 3B) while no, or rare, NF-κB expression was observed in normal rats (Figure 3A). TFB significantly reduced NF-κB expression in the same section (Figure 3C). The image analysis of the positive area of NF-κB confirmed the effect of TFB in reducing NF-κB protein expression (Figure 3D).

Activated stellate cells are the major source of matrix proteins in diseased liver. Accordingly, we evaluated α-SMA, an indicator of stellate cell activation, by immunohistochemical staining after CCl_4 injection for 18 weeks. In the normal rats, occasional stellate cells were positive for α-SMA protein (Figure 4A). After treatment with CCl_4, α-SMA expression was significantly increased, predominantly in the smooth muscle and endothelium of blood vessels in the liver (Figure 4B). Rats treated with TFB showed a marked reduction in the number of α-SMA-positive stellate cells (Figure 4C). The image analysis of the number of cells showing positive staining for α-SMA confirmed the effect of TFB in reducing α-SMA protein expression, and thus inhibiting the activation of stellate cells (Figure 4D).

Effect of TFB on TGF-β1 gene expression in liver

To determine whether TGF-β1 contributes to the hepatic fibrosis, we analysed the mRNA expression level of TGF-β1, which was reported to affect tissue fibrogenesis. Using a semiquantitative RT-PCR technique, the mRNA expression of TGF-β1 in liver tissue in the control group was up-regulated after 18 weeks of CCl_4 injection. Treatment with TFB (40, 80 and 160 mg kg^{-1}) inhibited the up-regulation of TGF-β1 mRNA significantly (Figure 5).

Discussion

CCl_4 is one of the most widely used hepatic toxins for experimental induction of hepatic fibrosis in laboratory animals. CCl_4-induced fibrosis in experimental animals resembles human cirrhosis in some aspects of morphology and pathophysiology (Basu 2003). For example, in both cases regeneration of hepatocytes occurs after necrosis, and fibrotic

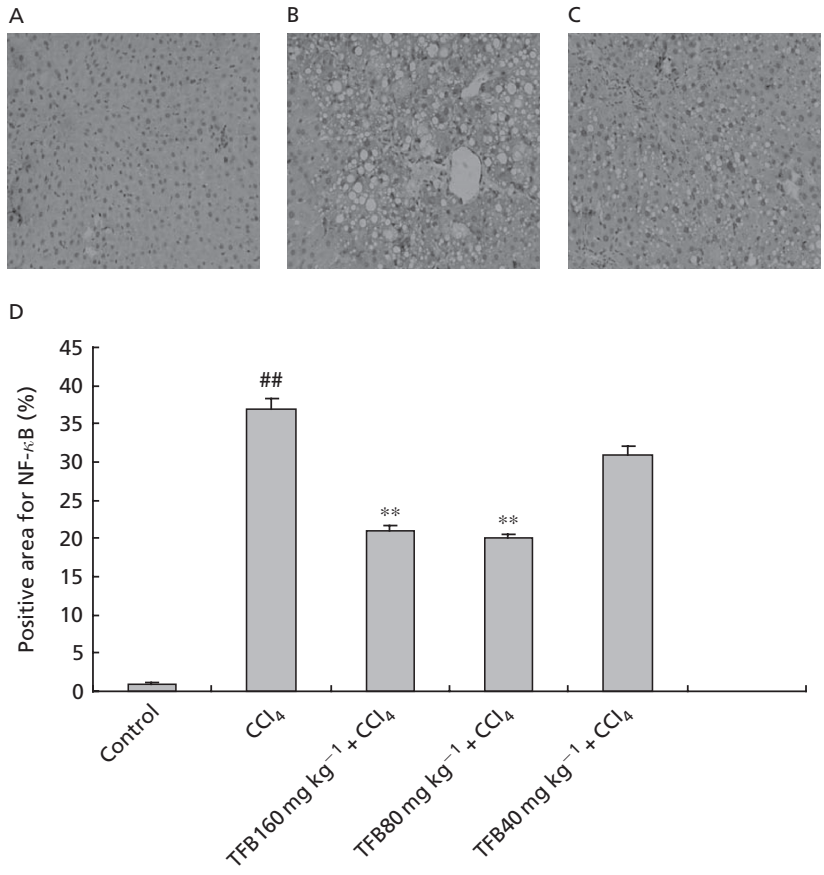

Figure 3 Effect of total flavonoid of *Bidens bipinnata* L. (TFB) on nuclear factor-κB p65 in liver (magnification ×400). Liver specimens were obtained from rats treated with saline and olive oil (control group, A), rats treated with carbon tetrachloride (CCl_4) alone (B) and rats treated with 160 mg kg^{-1} TFB + CCl_4 (C). The presence of NF-κB was further characterized by image analysis (D). Data are expressed as mean ± s.d., n = 8 per group. ##$P < 0.01$ compared with the normal control group; **$P < 0.01$ compared with the CCl_4 group.

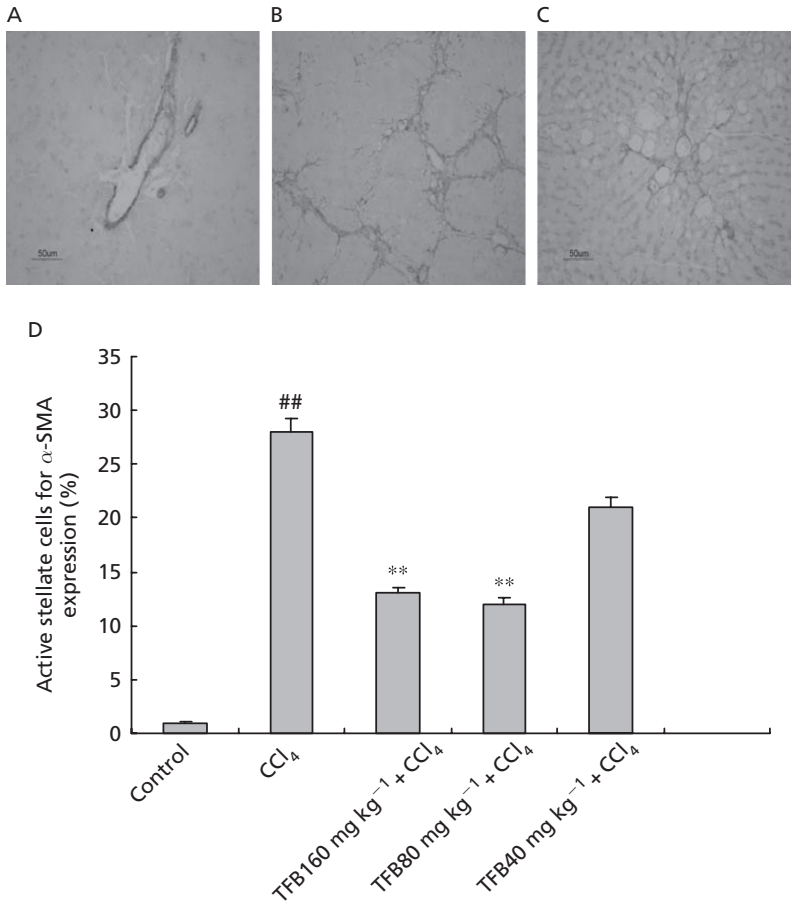

Figure 4 Effect of total flavonoid of *Bidens bipinnata* L. (TFB) on α-SMA (α-smooth muscle actin) expression in liver (magnification ×100). Liver specimens were obtained from rats treated with saline and olive oil (control group, A), rats treated with carbon tetrachloride (CCl_4) alone (B) and rats treated with 160 mg kg^{-1} TFB + CCl_4 (C). The presence of α-SMA was further characterized by image analysis (D). Data are expressed as mean ± s.e., n = 8 per group. ##$P < 0.01$ compared with the normal control group; **$P < 0.01$ compared with the CCl_4 group.

infiltration is almost irreversible in the advanced stage of cirrhosis. Thus, CCl_4-induced hepatic fibrosis has also been used to assess the efficacy of anti-fibrotic reagents and to verify correlation between pathophysiologic features of the liver and serum markers of fibrosis (Wu & Norton 1996).

In this article, hepatic fibrosis was successfully induced by subcutaneous injection with sterile CCl_4 0.1 mL/100 g body weight in a ratio of 1:1 with olive oil twice weekly for a total of 18 weeks. On this basis, treatment with TFB reduced the elevated liver and spleen weights, serum levels of ALT, AST, HA, PCIII and hepatic Hyp content in liver fibrosis rats. Histological examination showed the degree of histological changes in liver injury and liver fibrosis were also remarkably ameliorated after TFB treatment. Therefore administration of TFB had an apparently inhibitory effect on hepatic fibrosis induced by CCl_4 in rats.

Now there is more and more evidence that the pivotal cellular event underlying liver fibrosis is HSC activation towards a myofibroblast-like phenotype (Gutiérrez-Ruiz & Gómez-Quiroz 2007). Evidence indicates that oxidative stress plays a critical role in the activation of HSC during liver fibrosis (Lee et al 2001; Urtasun & Nieto 2007). Oxidative stress has been detected in almost all the clinical and experimental conditions of chronic liver diseases with different aetiology and fibrosis progression in rats, often in association with decreased antioxidant defences (Poli 2000). It has been shown that TBARS, an index of lipid peroxidation and oxidative stress, damages cells and tissues (Halliwell & Whiteman 2004). SOD is responsible for neutralizing the most common free radical known as superoxide. It also aids the body's utilization of the minerals copper, zinc and manganese. GSH-Px, along with SOD, is one of the body's endogenous antioxidants, and is known to protect liver cells against oxidative damage through chemical or enzymatic reactions. In this study, the level of hepatic TBARS in CCl_4-induced liver fibrosis rats was significantly increased as compared with that in the control rats, which may result from a strong oxidative stress and enhanced reactive oxygen species (ROS) formation. Moreover, the activity of both GSH-Px and SOD was down-regulated in the CCl_4 group. The lower activity of SOD and GSH-Px in the CCl_4 group could be a consequence of depleting effect due to excess ROS generation. All these observations agree with the view that oxidative stresses likely contribute to the onset and progression of liver fibrosis (Parola & Robino 2001). Our results showed that TFB reduced the elevated contents of liver TBARS and increased the

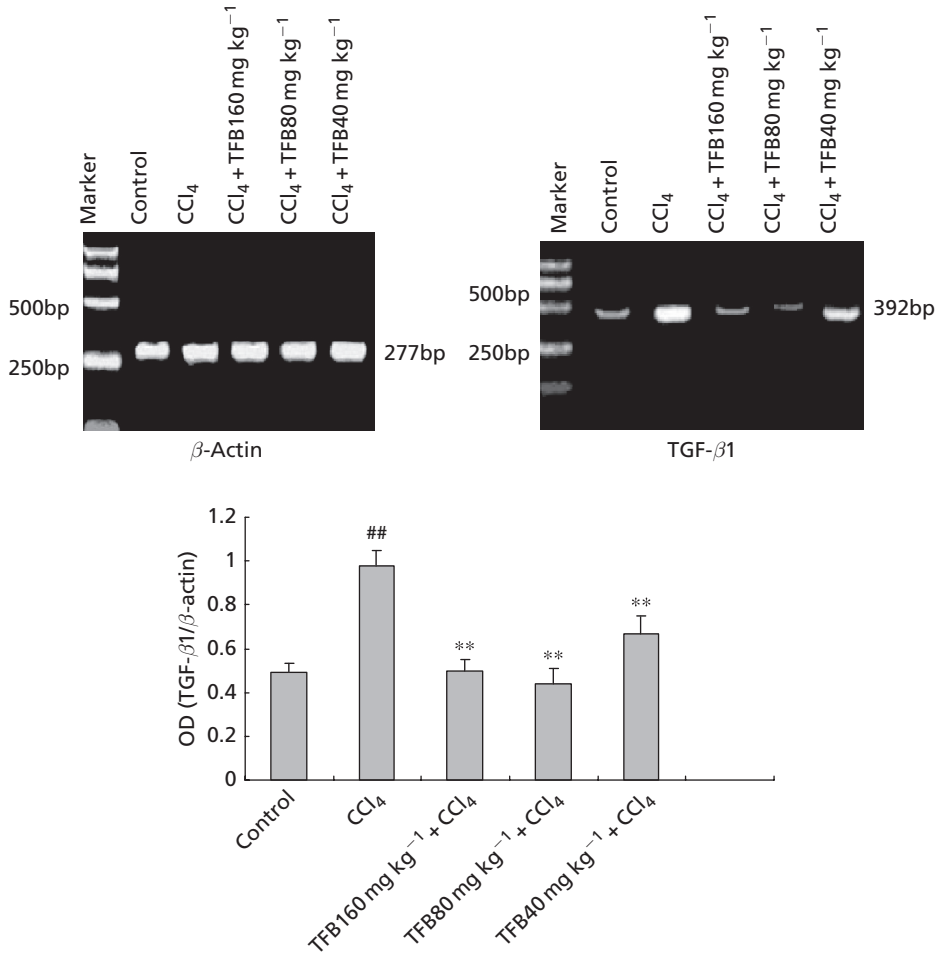

Figure 5 Effect of total flavonoid of *Bidens bipinnata* L. (TFB) on TGF-β1 mRNA in liver of liver fibrosis rats. TFB inhibited increased TGF-β1 mRNA expression after CCl_4 injection for 18 weeks. Livers were harvested 24 h after the last injection of CCl_4, perfused with normal saline and frozen in liquid nitrogen. Values are means ± s.d., n = 8 per group. ##$P < 0.01$ compared with the control group; **$P < 0.01$ compared with the CCl_4 group.

diminished SOD and GSH-Px activity in liver fibrosis rats, suggesting that the therapeutic effect of TFB might be associated with its anti-oxidative activity.

It has been reported that hepatocytes which are undergoing oxidative stress release ROS, which stimulate HSC proliferation and transformation into α-SMA-positive myofibroblast-like cells (Svegliati et al 1998). The key to this process may be due to the activation of NF-κB, a redox-sensitive transcription factor that transactivates promoters of many types of inflammation, infection and stress genes, including cytokines (Hellerbrand et al 1998; Lang et al 2000; Schwabe et al 2001). It was reported that bile-duct ligation increased 4-hydroxynonenal, a product of lipid peroxidation, activated NF-κB and increased synthesis of TNF-α and TGF-β; these effects were also blunted significantly by Ad-Mn-SOD (Saile et al 2001). Much research has been devoted to identification of upstream signalling for activation of NF-κB (Vasiliou et al 2000), but the precise mechanism by which oxidant stress participates in this signalling is yet to be determined. Clues to this key question may be attained through studies on the mechanisms of sustained or accentuated NF-κB activation in chronic liver diseases. In this experiment, the expression of NF-κB p65 and α-SMA were significantly increased in the CCl_4 group, synchronously. Activated NF-κB induced gene expression only in activated, but not in quiescent, HSCs (Buck et al 2000). Mechanisms involved in this process have not been elucidated completely. In-vivo study showed that HSC activation was associated with the expression of C-myb and NF-κB, which bind to the specific regulating sequence of the α-SMA gene (Mann & Smart 2002). Therefore, NF-κB and C-myb may play an essential role in the activation of HSC. On the other hand, NF-κB has the capacity of restraining apoptosis in many cells, including HSC (Nieto et al 2002). This may be another mechanism of liver fibrosis. Furthermore, a series of studies have outlined a close relationship between oxidative stress, redox-sensitive transcription factor NF-κB and activation of HSCs (Castilla et al 1991). As shown in this study, in contrast with the CCl_4 group, the expression of NF-κB P65 and α-SMA were reduced in the TFB-treated groups consistently. Oxidative stress may play an essential role through the induction of NF-κB on HSC activation (Hellerbrand et al 1998) and this process can be inhibited by TFB.

TGF-β1 is a major fibrogenic cytokine, regulating the production, degradation and accumulation of ECM proteins in liver fibrogenesis. This cytokine induces its own expression in activated HSCs, thereby creating a self-perpetuating cycle of events referred to as an autocrine loop. TGF-β1 gene expression correlates with the extent of liver fibrosis (Shek & Benyon 2004) and an increased production of ROS such as H_2O_2 in fibrotic livers is associated with the up-regulation of TGF-β1. Data also demonstrate a direct connection between TGF-β1-mediated accumulation of H_2O_2 and the up-regulation of collagen I in HSCs (García-Trevijano et al 1999). Secreted as a latent precursor, TGF-β1 is activated at sites of injury. Active TGF-β1 binds to specific, high-affinity receptors present on most cells, initiating a signalling cascade that results in biological effects. The findings of this study suggest that treatment with TFB leads to a significant decrease in ROS generation, TBARS production and TGF-β1 mRNA expression, causing the potent inhibition of activation of NF-κB and HSCs with a significant decrease in both α-SMA expression and collagen fibre production, compared with the CCl_4 group. TGF-β1 may act as surviving factors for activated rat HSCs through up-regulating the anti-apoptotic factor NF-κB. TFB may protect rats from liver fibrosis through suppressing this pathway.

In summary, TFB has a strong antioxidative capacity. This antioxidant is able to ameliorate liver injury and prevent CCl_4-induced liver fibrosis by suppressing oxidative stress. HSCs may be activated via the activation of NF-κB, which is induced by oxidative stress and may be related to TGF-β1 — this process can be inhibited by TFB. This may be the main mechanism of TFB protection against liver fibrosis. However, as a therapeutic agent, the effect of TFB on restraining liver fibrosis still needs to be investigated. Further studies are necessary to investigate the upstream and downstream pathways of NF-κB and TGF-β1 to elucidate the underlying molecular mechanisms. Of course, other mechanisms may also be involved in this process.

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