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Hepatoprotective effects of Yi Guan Jian, an herbal medicine, in rats with dimethylnitrosamine-induced liver fibrosis

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ABSTRACT

Aims of the study: Yi Guan Jian (YGJ) has long been employed clinically to treat liver fibrosis in traditional Chinese Medicine but the mechanism underlying the regulation has not been clarified in detail. The present investigation was designed to assess the involvement of the fibrosis pathway in dimethylnitrosamine (DMN)-induced liver fibrosis in rats.
Materials and methods: Liver fibrosis was induced by DMN injection (10 mg/kg, i.p., given three consecutive days each week) following 4 weeks. YGJ was oral administered (1.8 g/kg daily via gastrogavage for two weeks). Liver sample were subjected to histological and western blot studies. For evaluation of hepatic fibrosis-related factors, *collagen α1-I*, *tissue inhibitor of metalloproteinase-1* (*TIMP-1*), and *α-smooth muscle actin* (*α-SMA*) mRNA and protein levels were analyzed.
Results: YGJ remarkably prevented body weight loss and DMN damage in the liver, and it inhibited the elevation of serum glutamate oxaloacetate transaminase (GOT), and glutamic pyruvic transaminase (GPT). Oral administration of YGJ extract significantly reduced the accumulation of *collagen α1-I*, *TIMP-1*, and *α-SMA* in liver tissues.
Conclusions: Taken together, these findings indicate that the YGJ Chinese herb showed hepatoprotective and anti-fibrogenic effects against DMN-induced hepatic injury. Our data suggest that the YGJ may be useful in reversing the development of hepatic fibrosis.

1. Introduction

Liver fibrosis is a result of severe liver damage that occurs in the presence of viral infections (especially hepatitis B or C), cholestasis, metabolic diseases, persistent alcohol abuse, autoimmune liver diseases or as a result of the administration of some medicines. Pathological liver fibrosis is mainly due to a loss of homeostasis that results in fibrogenesis and a disturbance of matrix degradation. The disease attributes include disruption of the architecture of the hepatic extracellular matrix (ECM) and an impairment of liver function (Iredale, 2003). It has been demonstrated that activated hepatic stellate cells (HSCs) are the primary cells responsible for the development of liver fibrosis (Tsukada et al., 2006). During liver injury, HSCs change from a quiescent to an activated state and undergo transformation into myofibroblast-like cells (Tsukada et al., 2006) that produce excessive ECM proteins, such as collagen α1-I and α1-III, glycoprotein, and other fibrosis-associated proteins, including tissue inhibitor of metalloproteinase-1 (TIMP-1) and matrix metalloproteinase-2 (MMP-2) (Friedman, 2000). Therefore, the inactivation of HSCs is the primary approach for blocking the progression of liver fibrosis (Bataller and Brenner, 2005).

Traditional Chinese herbal medicines (TCM) are multi-ingredient extracts with low adverse effects in the treatment of chronic liver diseases and are effective in preventing fibrogenesis and other chronic liver injuries (Yang et al., 2008; Mu et al., 2009; Lou et al., 2010). Yi Guan Jian (YGJ) is a complex prescription of Chinese herbal medicine consisting of 9 medical herbs (*Radix Rehmanniae*, *Radix Glehniae*, *Radix Angelicae sinensis*, *Fructus Lycii*, *Radix Ophiopogomis*, *Fructus Mediae*, *Radix Astragalus membranaceus*, *Trionyx sinensis*, and *Eupolyphaga sinensis*) (Mu et al.,

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2009). YGJ has been used to treat human liver fibrosis induced by hepatitis and has shown apparent efficacy in the reversal of liver fibrosis. The major active components of YGJ extract, ferulic acid and catalpol, significantly inhibit the progression of hepatic fibrosis induced by carbon tetrachloride (CCL_4) in animal model (Mu et al., 2009). In addition, a more clinical representation of liver fibrosis model showed that the livers in dimethylnitrosamine (DMN)-treated animals were shrunken with dark discoloration because of congestion (Nishikawa et al., 2009). However, the therapeutic effect of YGJ extract in the DMN-induced liver fibrosis has not been fully elucidated.

The HSCs produce the extracellular matrix and play important roles in injury repair and fibrosis in the liver. α-Smooth muscle actin (α-SMA) is a reliable marker for activated HSCs in human and rat liver. Strategies to prevent the proliferation of activated HSCs include prevention of the stimulation of growth factor receptors and/or to blockade of intracellular pathways required for proliferation (Friedman, 2008; Ikeda et al., 1999). Recent studies showed that the inhibition of HSC activation and the stimulation of HSC apoptosis enhance the resolution of liver fibrosis (Friedman, 2003; Elsharkawy et al., 2005). Furthermore, HSC apoptosis may also play a central role in the spontaneous recovery from biliary fibrosis (Issa et al., 2001). Therefore, it is important to discover the apoptosis mechanisms of HSC treated by traditional Chinese herbal medicines that might resolve progressive HSC proliferation and matrix synthesis (Lin et al., 2011).

The aim of this study was to investigate the therapeutic effects of YGJ extract, a traditional Chinese medicine (Mu et al., 2009) in a rat model of DMN-induced liver fibrosis (Jezequel et al., 1987). The therapeutic efficacy of YGJ extract for liver fibrosis was extensively evaluated on the basis of several HSC functions, histological examination, biochemical values, and fibrotic gene regulations.

2. Materials and methods

2.1. Herbal medicine

Herbal medicinal plants were purchased from Koda Pharmaceutics Ltd. (Taoyuan, Taiwan) for preparation of the YGJ extract. YGJ extract is a mixture of 9 crude plant ingredients: *Rehmannia glutinosa*, *Glehnia littoralis*, *Angelica sinensis*, *Lycium barbarum*, *Ophiopogon japonicus*, *Melia toosendan*, *Astragalus membranaceus*, *Trionyx sinensis* and *Eupolyphaga sinensis*. These 9 plants were extracted with boiling double-distilled water and allowed to infuse. The extract was decanted, filtered under vacuum, concentrated in a rotary evaporator, and then lyophilized. The concentration for final stock solution of YGJ extract was 175 mg/ml (Lin et al., 2011).

2.2. Chromatographic analysis of YGJ extract

The YGJ extract was dissolved in pyrogen-free isotonic saline (YF Chemical, Taipei, Taiwan) and filtered through a 0.45 μm filter (Microgen, Laguna Hills, CA, USA) before high performance liquid chromatography (HPLC) analyses. HPLC analyses were performed using a Mightysil RP-18 column (250 mm × 4.6 mm) at room temperature. Pure standards of ferulic acid and catalpol, purchased from Sigma (St. Louis, MO, USA), were used as external standards in the HPLC analyses. For ferulic acid, the mobile phase was acetonitrile and water in gradient mode as follows: 10:90–50:50 in 45 min. The effluent was monitored at 320 nm and flow rate was set at 1.0 ml/min (Lin et al., 1998). Measurement of catalpol was performed with a Cosmosil 5NH_2–MS Waters (250 mm × 4.6 mm) at room temperature. The mobile phase was acetonitrile and water in gradient mode as follows: 92:8–50:50 in 75 min. The effluent was monitored at 203 nm and flow rate was set at 1.0 ml/min (Liu et al., 1992). Identification of HPLC peak fractions was carried out by comparing retention times and UV spectra with those of standard samples. YGJ extract mainly contained a constant volume of ferulic acid (0.069 mg/g) and catalpol (0.499 mg/g) for quality control (Fig. 1).

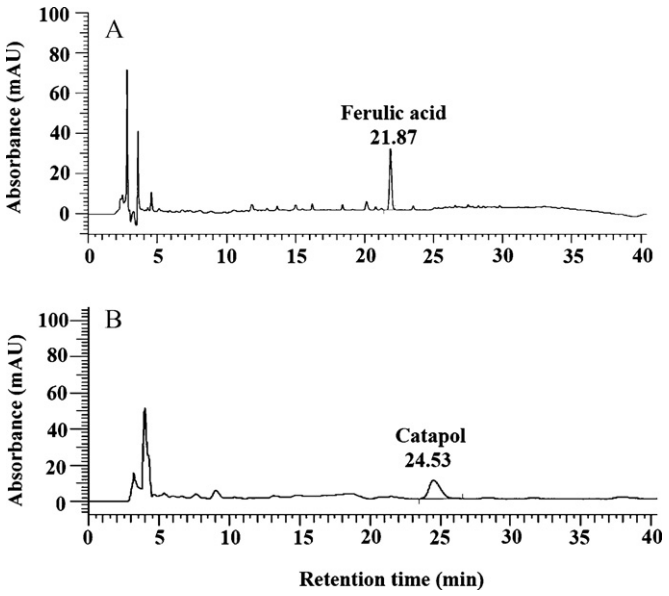


Fig. 1. Results of HPLC analyses of the YGJ extract. (A) Column: Mightysil RP18, GP 250 × 4.6 mm (5 (m); mobile phase: acetonitrile–H_3PO_4 (acetonitrile: 10–50% in 45 min); flow rate: 1.0 ml/min; detection wavelength: UV 320 nm. Ferulic acid was identified from YGJ extract at a retention time of 21.87 min. (B) Column: Cosmosil 5NH_2–MS Waters, GP; mobile phase: acetonitrile–H_3PO_4 (92-50% in 75 min); flow rate: 1.0 ml/min; detection wavelength: UV 203 nm. Catalpol was identified from YGJ extract at a retention time of 24.53 min.

2.3. Animals

Male Sprague–Dawley (SD) rats (aged 8–9 weeks and weighing 280–320 g) were purchased from BioLASCO Taiwan Co. Ltd. (Taipei, Taiwan) and fed a standard laboratory diet and tap water *ad libitum*. Animals were housed in an air-conditioned room at 23 ± 2 °C, 55–60% relative humidity and a 12 h light/dark cycle (Wu et al., 2007). Animals were acclimatized for 1 week and 6 rats were randomly assigned to each group. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University.

2.4. Experimental design

2.4.1. Hepatic fibrosis of rats

The hepatic fibrosis model in rats was produced by the administration of toxic DMN. After acclimatization of 1 week, DMN (Wako, Osaka, Japan) (10 mg/kg per day for 3 consecutive days of each week) was injected i.p. for 4 weeks to induce rat hepatic fibrosis as described previously (Jezequel et al., 1987).

2.4.2. Animal treatment groups

After treatment with DMN for 4 weeks, animals were administered 1.8 g/kg YGJ herbal extract orally ($n = 6$), or the same volume of saline as a placebo ($n = 6$), every day for the following two weeks (Fig. 2). A control group ($n = 6$), without DMN treatment, but also administered the same volume of saline as the normal control. All of the animal experiments were repeated at least three times. At the end of the experiment, all animals were sacrificed for tissue sampling and blood was collected from the tail artery. Blood samples were allowed to coagulate

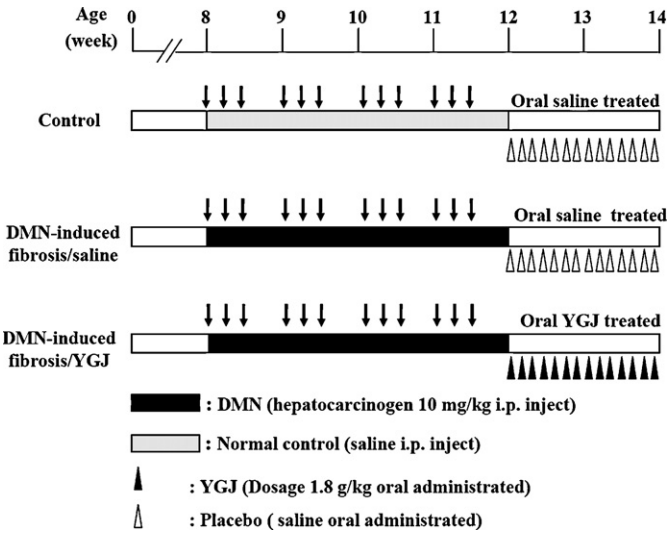


Fig. 2. The schedules for DMN-induction of liver fibrosis and for YGJ extract therapy. DMN was given i.p. for 3 consecutive days per week for a continuous 4 weeks (black box). In the placebo saline-treated group, saline alone was administered daily for 2 weeks (white arrows) after cessation of DMN-treatment. In the YGJ extract-treated group, YGJ extract was administered daily for 2 weeks (black arrows) after cessation of DMN-treatment.

at 4 °C for 30 min. Serum was then separated by centrifugation at 4 °C, 3000 rpm for 10 min (Hung et al., 2009; Yen et al., 2009).

2.5. Tissue hydroxyproline and blood biochemical assays

The liver index (liver wet weight/body weight) and spleen index (spleen wet weight/body weight) were calculated. Concentrations of serum GOT and GPT were determined using a JSCC kit (Roche, USA) (Liu et al., 2010). The hydroxyproline content in the liver tissue was measured as described previously (Jamall et al., 1981). Briefly, the liver tissues were dehydrated in 95% alcohol for 5–6 h and defatted with acetone for 2 days. The defatted tissue was dried in an oven at 65 °C for 2 days. HCl (6 M) was added to the tube (according to the ratio 1 ml HCl: 100 mg dried liver powder) and hydrolyzed at 121 °C for 1 h. NaOH (6 N) was added to hydrolysates that were neutralized and filtered to pH 6–6.5. A volume of 450 μl chloramine T was placed in a new tube, left for 25 min at room temperature followed by the addition of 500 μl Ehrlich's reagent and reacted for 20 min at 60 °C. Finally, the test tube was placed on ice for 5 min to stop the reaction. The absorbance was measured at 550 nm against a reagent blank using a spectrophotometer (Beckman DU650, USA).

2.6. Histopathological observations

Liver specimens were fixed with 10% neutralized formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E) (Hung et al., 2010). In addition, the Masson-Trichrome stain method was used to specifically stain fibrous tissue components. The criteria used for scoring fibrosis severity were described previously (Chevallier et al., 1994). Four main sites of fibrotic deposition were analyzed: the centrilobular vein (CLV), perisinusoidal space (PS) (Ou et al., 2003), portal tract (PT), and the septa of width (WS) and number (NS) (Tsukada et al., 2006). The histological score was defined as follows: (a) CLV score (Grade 0: normal vein or absent in the case of cirrhosis; 1: thickened wall with stellate aspect of the outer part of the vein wall; and 2: marked thickening of the wall with fibrous extensions between adjacent hepatocytes); (b) PT score (Grade 0: absence of fibrosis; 1: enlarged without septa; 2: enlarged with septa; and 3: cirrhosis); (c) PS score (Grade 0: absence of fibrosis; 1: localized fibrosis; and 2: diffused fibrosis); (d) NS score (Grade 0: absent septum; 1: 6 or fewer septa per 10 mm; 2: more than 6 septa per 10 mm; and 3: cirrhosis); and (e) WS score (Grade 0: thin, incomplete or both; 1: thick with loose connective matrix; 2: very thick with a dense collagenous organization; and 3: > 2/3 biopsy area). The total score is expressed as $SSS = CLV + PT + PS + 2(WS \times NS)$.

Table 1
Primer information for semi-quantitative RT-PCR used in this study.

mRNA	Primer sequence	T_m (°C)	Length (bp)
α-SMA	(+) 5′-TGGCTATTCCTTCGTGACTAC TG-3′ (−) 5′-AAAGATGGCTGGAAGAGAGTCAC-3′	59	230
Col Iα-1	(+) 5′-GTTCGTGACCGTGACCTTGA-3′ (−) 5′-TTGGGGTTCGGGCTGATGTA-3′	57	292
TIMP-1	(+) 5′-CCCCAACCCACCCACAGACAGC-3′ (−) 5′-CGCTGCGGTTCTGGGACTTGT-3′	67	228
GADPH	(+) 5′-ATCCCCAGAGCGTCATTCG-3′ (−) 5′-GAGAGAGCCCTGCCTGCC-3′	61	251

2.7. Detection of fibrogenesis-related mRNA by semi-quantitative RT-PCR

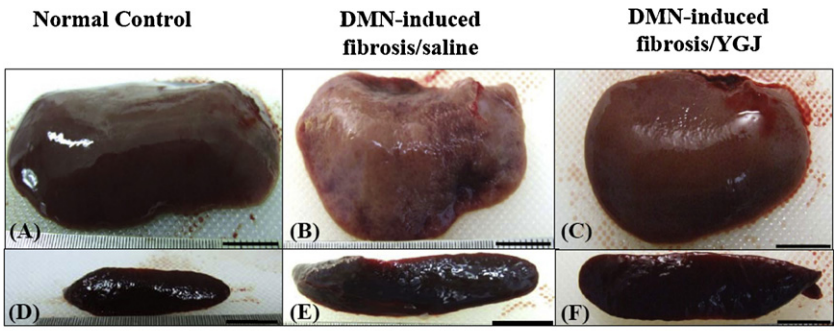
The mRNA expression of the fibrogenesis genes, *α-SMA*, *TIMP-1* and *collagen α1-I*, was determined by semi-quantitative RT-PCR. The housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GADPH*) was used as an internal control (Chen et al., 2008b). Total RNA was isolated from liver tissues using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was subsequently treated with deoxyribonuclease I (MBI Fermentas Inc., Lithuania, Germany) to remove any genomic DNA contamination (Chen et al., 2008a). Approximately 900 ng of total RNA was reverse-transcribed with MuLV Reverse Transcriptase using the GeneAmp RNA PCR Kit (Applied Biosystems, Foster, CA) and oligo d(T)16 primers. Aliquots of the reverse transcriptase mix were used for PCR amplification of *α-SMA*, *collagen α1-I*, *TIMP-1* and *GAPDH*. The primer sequences for these genes are listed in Table 1. The amplified RT-PCR products were subjected to electrophoresis at 100 V in a 2% agarose gel (Invitrogen, Carlsbad, CA) for 30 min. The transcription levels of each gene were normalized against those of *GAPDH* mRNA using a semi-quantitative method (Chen et al., 2006; Liu et al., 2008).

2.8. Protein extraction and Western blot

Liver tissues were washed with ice-cold PBS and lysed with cell lysis buffer (Cell Signal Technology Inc., MA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The samples were sonicated briefly and centrifuged. The supernatant was collected to obtain the whole-cell protein. Protein samples were subjected to Western blotting as described previously (Chen et al., 2004, 2008c). Primary antibodies against *α-SMA* (DAKO, Denmark) and β-actin (Santa Cruz Biotechnology, Inc., CA) as well as the respective horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, UK) were used for protein detection.

2.9. Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Experimental data were analyzed using the General Linear Model (GLM) procedure in SAS as described previously (Chen et al., 2003). The differences between the herbal medicinal group and



(G)

	Normal control	DMN-induced fibrosis/saline	DMN-induced fibrosis/YGJ
Body weight (g)	463.33 ± 42.85	345.67 ± 40.37###	358.33 ± 44.57###
Liver index (%)	4.10 ± 0.25	3.63 ± 0.71	4.51 ± 0.17**
Spleen index (%)	0.15 ± 0.02	0.45 ± 0.07###	0.37 ± 0.06###
Hydroxyproline (μg/g) liver	210.72 ±19.52	1277.06 ±220.08###	196.88 ±15.76***

Fig. 3. Effects of YGJ extract on body weight, liver and spleen index, morphology, hydroxyproline changes in DMN-induced fibrotic rats. Gross observations of liver and spleen morphologies were performed in normal control rats (A, D), DMN-treated rats (B, E), and YGJ-administered rats (C, F). (G) Effects of YGJ herb treatment on body weight, liver and spleen index and hydroxyproline of DMN-induced liver fibrosis in rats. Each group consisted of 6 rats; data are presented as mean ± SD. ###$p < 0.001$ compared with normal control group. **$p < 0.01$ compared with DMN treatment group. ***$p < 0.001$ compared with DMN treatment group.

the control group were evaluated via least mean squares comparison. Statements of statistical significance were based on $P < 0.05$(*).

3. Results

3.1. Effects of YGJ extract on rats with DMN-induced liver fibrosis

Rats liver fibrosis was evaluated based on several parameters of body weight, liver index and spleen index as shown in Fig. 3. Body weight in the DMN-treated group was decreased about 26% compared with the normal control group ($P < 0.001$). The weight of the YGJ-treated group was slightly heavier than that of the DMN-treated group but this difference was not statistically significant. Liver atrophy was induced by DMN treatment. The liver index in the DMN-treated group (Fig. 3B) was decreased but significantly recovered after YGJ medicine treatment (Fig. 3C) ($P < 0.01$). Marked splenomegaly was caused by DMN treatment. The spleen index in DMN-treated rats (Fig. 3E) was increased by approximately 300% compared with the control group (Fig. 3D). The abnormal spleen index induced by DMN treatment was effectively modulated by YGJ extract administration (Fig. 3F and G). As shown in Fig. 3, no ascites occurred in the control group, whereas 80% of DMN-treated rats displayed ascites. The percentage of rats showing ascites after DMN treatment was markedly reduced by YGJ extract administration.

A main constituent amino acid of collagen is proline, which is converted to hydroxyproline. To evaluate ECM production, the hydroxyproline content was measured. Hepatic hydroxyproline contents in the DMN-treated group were increased sevenfold over the untreated control group ($P < 0.01$). However, the hydroxyproline concentration was significantly decreased in DMN-injured rats after YGJ extract was administered (Fig. 3G) ($P < 0.001$).

3.2. Effects of YGJ extract on serum GOT and GPT

Circulating levels of liver function enzymes, i.e., serum GOT and GPT, were used as biochemical markers for the monitoring of hepatic injury after DMN treatment and YGJ herbal therapy. As shown in Fig. 4, both GOT (Fig. 4A) and GPT (Fig. 4B) were significantly increased after DMN treatment for 4 weeks compared with untreated normal 12-week-old rats ($P < 0.01$). After hepatic injury, the administration of YGJ extract for a continuous 2 weeks statistically decreased serum GOT and GPT levels compared to the saline-treated group ($P < 0.01$).

3.3. Effects of YGT extract on liver histological changes

Liver histopathological analyses consisted of tissue section staining with H&E and Masson's trichrome dye as shown in Fig. 5. Liver tissue from DMN-treated rats had more steatosis, fibrosis (FB), necrotic hepatocytes (NH) and degenerative hepatocytes (DH) (Fig. 5B) than that of normal control rats (Fig. 5A). DMN-injured rat livers showed a distinct histological pattern of fibrotic septa and incorporation of degenerated hepatocytes into pseudolobules as well as associated higher collagen content (Fig. 5E) compared with normal livers (Fig. 5D). After hepatic injury, YGJ extract treatment markedly alleviated the degree of liver fibrosis and significantly reduced the collagen deposition (Fig. 5C and F).

To quantitatively evaluate the therapeutic efficacy of YGJ extract on hepatic fibrosis induced by DMN, Masson's stained sections were graded from 24 (cirrhosis) to 0 (normal) on the basis of the extent

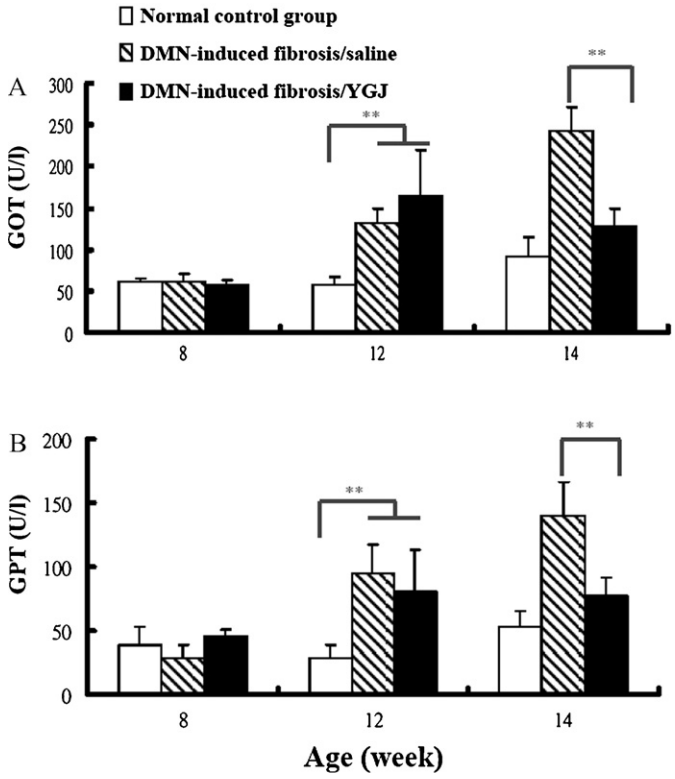


Fig. 4. YGJ extract prevents DMN-induced liver fibrosis as determined by serum GOT and GPT levels. Serum GOT (A) and GPT (B) were measured in 8-week, 12-week and 14-week-old rats in different groups (control group, white bars; DMN-induced and saline-treated group, filled bars; DMN-induced and YGJ-treated group, black bars). Data are expressed as mean ± SD ($n = 6$); $^{**}p < 0.01$ compared with DMN treatment group.

of collagen deposition. The fibrosis scoring system combined the localization of fibrotic deposits and their semi-quantitative evaluation. During fibrogenesis, three main sites, i.e., the lobule-CLV, PT and Disse's space could be involved depending on the etiological agent. The septa contained the four items evaluated by our score and these topographic data are important for correctly describing the distribution of fibrosis. The pathologic grade was significantly decreased in YGJ extract-treated rats (Table 2). Livers from injured rats treated with YGJ displayed marked improvements in terms of the fibrosis pathology score (3.83 compared with 12.17 for DMN-treated livers).

Table 2
The pathological score of DMN-induced liver fibrosis in rats.

Group	Pathological scores in different liver tissues[a]					
	CLV	PS	PT	WS	NS	SSS[b]
Control	0	0	0	0	0	0
DMN-induced fibrosis	1.67	1.67	1.83	2.17	1.50	12.17
YGJ-herb treated	1.00	1.50	1.00	0.17	1.00	3.83**

[a] LV, centrilobular vein; PS, perisinusoidal space; PT, portal tract; WS, septa of width; and NS, septa of number.
[b] SS is given by CLV + PS + PT + 2(WS × NS).
** $p < 0.01$ compared with DMN treatment group.

3.4. Effect of YGJ on gene expression of collagen α1-I, α-SMA and TIMP-1

The extent of liver fibrosis depends on rates of hepatic collagen synthesis and degradation. Gene expression for *TIMP-1*, *collagen α1-I* and *α-SMA*, which represent hepatic fibrosis factors, was analyzed using RT-PCR (Fig. 6A). Expression of *TIMP-1* and *α-SMA* was lower in normal tissue compared with that of activated HSCs and myofibroblasts in injured livers. Activated HSCs exhibited a marked difference in their gene expression profiles. Increases in various pro-fibrogenic genes, including *collagen α1-I* and *α1-III* as well as fibronectin, are frequently observed. High expression of the *α-SMA* gene was induced in the DMN-treated group compared with the normal group. The relative abundance of *collagen α1-I* (Fig. 6C) transcripts in the DMN-treated group was also increased compared with the control group. The YGJ extract treatment significantly reduced the *α-SMA* (Fig. 6B) ($P < 0.001$) and *TIMP-1* (Fig. 6D) ($P < 0.05$) mRNA levels by 200% and 51%, respectively.

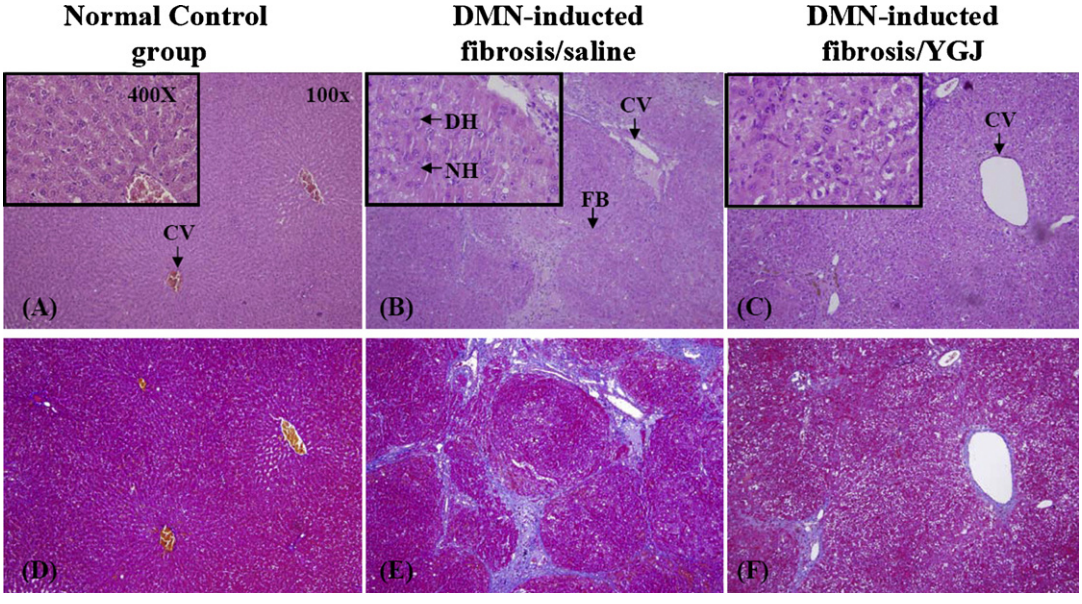


Fig. 5. Pathological features for effects of the YGJ extract on DMN-induced liver fibrosis. (A, D) The normal lobular architecture with central veins and radiating hepatic cords was observed in normal control rats. (B, E) Fatty degeneration, necrosis, infiltration of inflammatory cells and apparent formation of fibrotic septa were present in the DMN-treated group. (C, F) The degree of liver fibrosis was significantly reduced in the YGJ-treated group. Liver fibrosis is shown by cell necrosis and mitosis. (A–C) Tissues stained with H&E, 100× and 400×. (D–F) Tissues stained with Masson-trichrome, 100×. CV, central vein; NH, necrotic hepatocytes; FB, fibrosis; DH, degenerative hepatocytes. See the text for details.

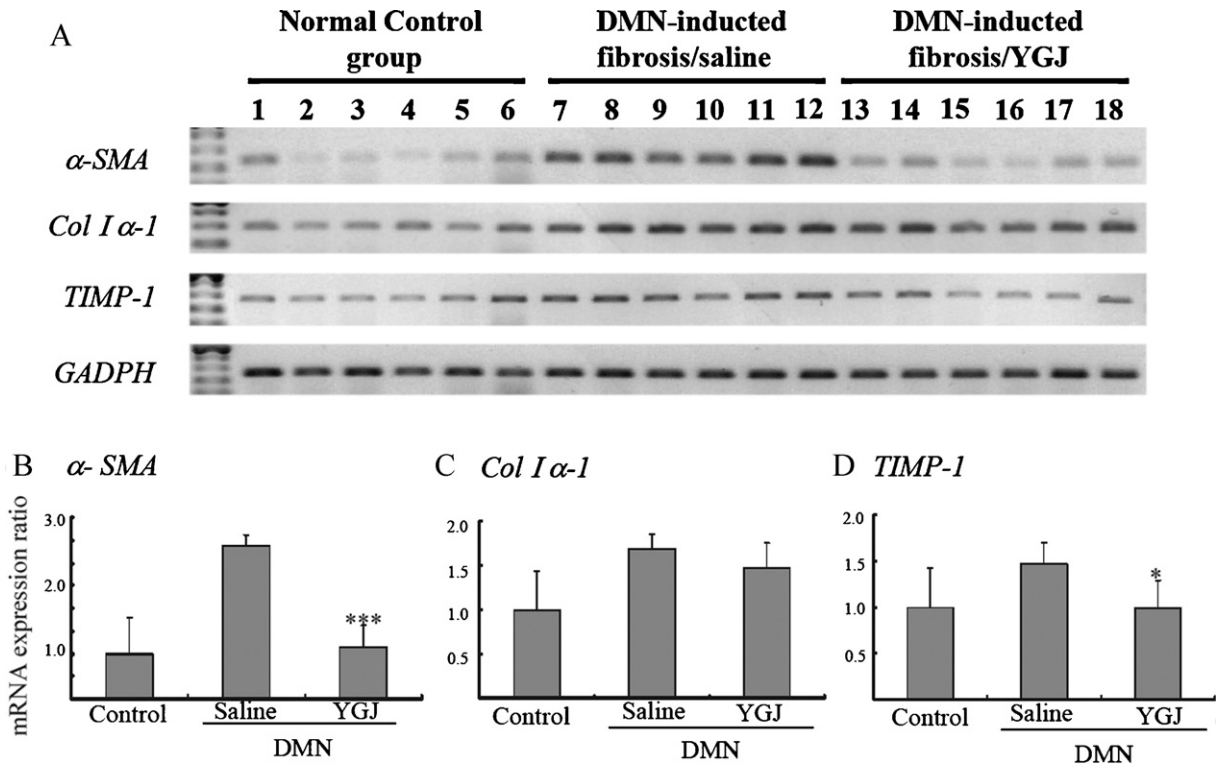


Fig. 6. Changes of mRNA expression levels of *α-SMA*, *Collα-1* and *TIMP-1* in DMN-induced fibrosis and YGJ herb-treated groups. (A) Gene expression patterns were amplified by RT-PCR. The expression levels of *α-SMA* (B), *Collα-1* (C) and *TIMP-1* (D) were quantified using densitometric measurement of RT-PCR bands. Values were normalized against those for *GAPDH* mRNA expression. $^{*}p < 0.05$ compared with DMN treatment group. $^{***}p < 0.001$ compared with DMN treatment group.

3.5. Effect of YGJ on α-SMA fibrotic protein expression

α-SMA is a cytoskeletal protein that is absent from resting fibroblastic cells in most human tissues but can be detected in quiescent HSCs during liver injury. To evaluate hepatic stellate cell activation, we performed Western blotting for α-SMA. α-SMA protein content was dramatically increased in liver tissue from DMN-treated rats (Fig. 7B). YGJ extract therapy significantly reduced α-SMA protein levels to 75% (Fig. 7C) of that observed in DMN-induced liver injury (Fig. 7D) ($P < 0.001$).

4. Discussion

Hepatic fibrosis is a wound healing response to chronic insults. The most characteristic feature of liver fibrosis is the excessive deposition of ECM, especially collagen α1-I, due to the activation of HSCs (Friedman, 1993, 2000; Tsukada et al., 2006). Increased collagen α1-I and TIMP-1 expression can stimulate HSCs to produce ECM and pro-fibrogenic factors in liver fibrosis (Kinnman et al., 2003; Bonner, 2004; Czochra et al., 2006). The mechanisms of liver injury involved in DMN-induced collagen α1-I and TIMP-1 expression of HSCs can be used to measure the fibrotic status in human and rats.

DMN was reported in the early 1950s to have hepatocarcinogenic effects (Magee and Barnes, 1956). DMN and other nitrosamine derivatives exhibit carcinogenesis and may be activated into DNA binding intermediates by cytochrome P450-dependent formation of unstable nitrosamine radicals, leading to hepatocarcinogenesis by free radical damage and DNA alkylation (Bartsch et al., 1989). The free radical scavenger activities of antioxidant-containing herbal drugs like silymarin and Sho-saiko-to might be hepatoprotective, as they reverse established fibrosis (Sakaida et al., 1998). Furthermore, some traditional Chinese herbal drugs are effective for preventing fibrogenesis and other chronic liver injuries, which may help in controlling liver fibrosis and cirrhosis (Shimizu et al., 1999). For centuries YGJ has been used in Chinese traditional herbal medicine for the improvement of liver function (Mu et al., 2009). One of the active compounds of YGJ extract is catalpol, a natural component of *Rehmannia glutionsa* that stimulates the secretion of insulin and reduces the glycogen content in the livers of normal mice (Mu et al., 2009). The other active compound, ferulic acid, is a natural component of *Angelica sinensis* that is well-known as an agent in traditional medicine to enrich blood and promote blood circulation. Ferulic acid is often used in anti-diabetic prescriptions or as a therapy for diabetic complications (Li et al., 2004).

The YGJ extract is a mixture of 9 crude plant ingredients. These 9 plants have some synergistic effects for hepatic injury therapy, for examples, *Astragalus membranaceus* delays the formation of liver fibrosis (Liu et al., 2009), *Lycium barbarum* and *Rehmannia glutinosa* inhibit proliferation and induce apoptosis of hepatocellular carcinoma cells (Chao et al., 2006). One of the components in YGJ extract, *Trionyx sinensis*, can induce hepatic stellate cell apoptosis or inhibit the proliferation of HSCs and soften the liver (Hu et al., 2008). To study the ability of YGJ to reverse liver fibrosis, rat body weight, liver index and spleen index were monitored in this study. A significant difference was found in rats exposed to DMN treatment compared with normal rats or CCl_4-damaged rats in previously reports (Hu et al., 2008; Mu et al., 2009). The DMN-damaged livers were shrunken with dark discoloration, grossly and shrunken because of congestion (Fig. 3B), which is more representative in clinical hepatic patients. The DMN-treated animal model has been shown to damage endothelial cells concurrently with cell morphological changes in adjacent hepatocytes (Fig. 5B). YGJ extract administration significantly relieved the DMN-induced histopathological deterioration of liver tissue ($P < 0.01$), but did not alter spleen tissue or body weight (Fig. 3).

During fibrosis progression, the main ECM-producing cells are activated HSCs, which secrete ECM proteins including collagen types I and III (Bataller and Brenner, 2001). Cross-remodeling of

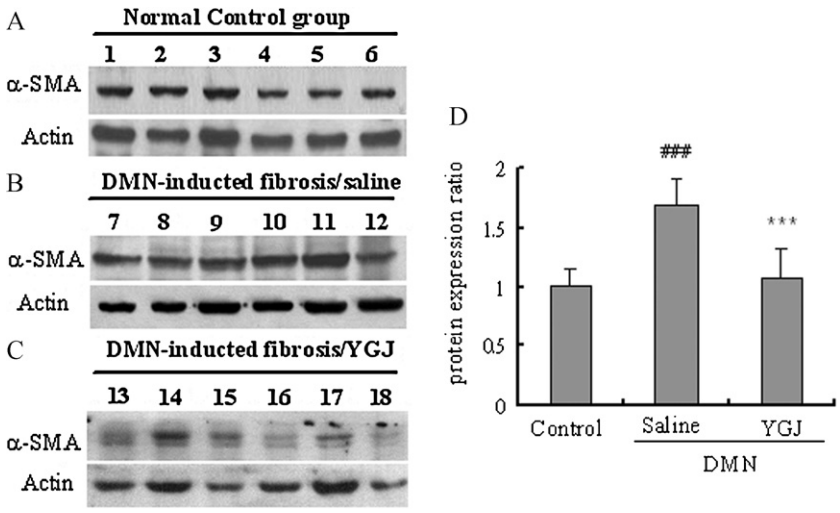


Fig. 7. Changes of α-SMA protein expression in DMN-treated rats. (A) Normal group; (B) DMN-treated group; (C) YGJ-treated group; (D) protein expression levels were quantified using immunoblots. Equal loading was assessed with an anti-actin antibody. $^{***}p < 0.001$ compared with DMN treatment group. $^{###}p < 0.001$ compared with normal control group.

the ECM in the fibrotic liver is likely to be regulated by the synthesis and enzymatic degradation of the ECM. Matrix degradation is catalyzed by the activity of matrix metalloproteinases (MMPs). The activities of MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) (Yoshiji et al., 2000). Our *in vivo* study showed the ability of YGJ extract to promote recovery from liver fibrosis not only by the removal of collagen-producing HSCs (Fig. 5), but also by the reduction of hepatic TIMP1 expression and the promotion of the collagenolytic activity of the liver (Fig. 6). Hydroxyproline measurement also demonstrated that YGJ extract treatment significantly decreased the accumulation of collagen compared with the DMN-treated and saline-administrated groups (Fig. 3). The pathological changes of liver tissue obviously improved with YGJ extract treatment (Fig. 5), indicating the inhibitory effect of the YGJ extract on hepatic fibrosis.

HSCs play a central role in the pathogenesis of liver fibrosis and cirrhosis. HSCs are quiescent in the normal liver, but when liver injury is present, HSCs undergo activation and transdifferentiation to myofibroblast-like cells (Schmitt-Graff et al., 1991). Myofibroblast-like cells differ from resting HSCs by their increased size and more pronounced immunostaining with α-SMA (Rockey et al., 1993; Levy et al., 1999). *TIMP-1* and *collagen α1-I* expression was increased concurrently with increased *α-SMA* expression in the liver tissue (Fig. 6). The YGJ extract drastically down-regulated gene expression of *α-SMA* in HSCs. Protein expression of α-SMA was decreased by 69% after administration of YGJ extract compared with the saline-treated group (Fig. 7). This result indicates that the inhibition of HSC activation might be an important mechanism of the YGJ extract against liver fibrosis.

In conclusion, the results from the present study demonstrated that the YGJ extract was effective in the treatment of DMN-induced liver fibrosis in rats. The primary mechanism of this therapeutic effect could be protection against hepatic injury via reduced serum levels of GOT and GPT as well as reduction of the activated HSCs via down-regulation of α-SMA mRNA and protein expressions. Therefore, this study provides scientific evidence for the clinical use of YGJ extract in treating fibrosis and cirrhosis.

Conflict of interest

None of the authors have any conflicts of interest.

Acknowledgments

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