JPP Journal of Pharmacy and Pharmacology

JPP 2010, 62: 935–942

Received October 5, 2009
Accepted February 26, 2010
DOI 10.1211/jpp.62.07.0016
ISSN 0022-3573

Research Paper

Preventive effects of a fractioned polysaccharide from a traditional Chinese herbal medical formula (Yu Ping Feng San) on carbon tetrachloride-induced hepatic fibrosis

Jia-jia Wang, Jun Li, Lei Shi, Xiong-wen Lv, Wen-ming Cheng and Ying-ying Chen

Anhui Medical University, China

Abstract

Objectives The study was to investigate the prevention effects and possible mechanism of Yu Ping Feng San fractioned polysaccharide (YPF-P) on CCl_4-induced liver fibrosis in rats.
Methods YPF-P was prepared from root of *Astragalus membranaceus*, rhizome of *Atractylodes macrocephaia* and root of *Raidix saposhnikoviae*, and compared with polysaccharide from root of *Astragalus membranaceus* (AP). Hepatic fibrosis was induced by subcutaneous injection with carbon tetrachloride twice weekly for 12 weeks in Sprague–Dawley rats. YPF-P, AP and colchicine were administered intragastrically daily to carbon tetrachloride-treated rats. Histopathological changes of the liver and hepatic stellate cells were evaluated by Masson staining and transmission electron microscopy, respectively. Markers of fibrosis were determined by radioimmunoassay, biochemistry assay and ELISA. The mRNA expressions of tissue inhibitor of metalloproteinase-1 (TIMP-1), matrix metalloproteinase-13 (MMP-13), procollagen I and collagen III were detected by RT-PCR.
Key findings YPF-P dose-dependently alleviated the degree of liver fibrosis and inhibited hepatic stellate cell transformation into myofibroblast-like cells, markedly reduced the elevated levels of hyaluronic acid, laminin, type IV collagen, type III procollagen, hydroxyproline and transforming growth factor beta-1, suppressed procollagen I, collagen III and TIMP-1 expression, and improved the TIMP-1/MMP-13 ratio. MMP-13 expression was only promoted moderately by YPF-P. Compared with AP, YPF-P showed more potency on most markers except laminin, type IV collagen and MMP-13 mRNA.
Conclusions YPF-P prevented the progress of rat liver fibrosis induced by carbon tetrachloride and had a more potent preventative effect. The preventative effect may be associated with the ability of YPF-P to inhibit the synthesis of matrix collagen and balance the TIMP/MMP system.
Keywords liver fibrosis; mechanism; polysaccharide; prevention; Yu Ping Feng San

Introduction

Hepatic fibrosis, a dynamic process caused by various chronic liver injuries, is predominantly characterised by excessive accumulation of extracellular matrix (ECM) caused by both an increased synthesis and decreased or unbalanced degradation of this material.[1] Interrupting or reversing the progress of hepatic fibrosis may be a new approach for preventing its progression to hepatic cirrhosis or hepatocellular carcinoma. However, the therapy is not yet well established.[2] Recently, Chinese herbal medicine has become of interest in hepatic fibrosis therapy from laboratory discovery to clinical evaluation,[3,4] but the active ingredients in most of these medications have not yet been determined.

Yu Ping Feng San (YPF, Jade Windscreen Powder in English, *Gyokuheifusan* in Japanese) is an officially approved herbal formula in China, and consists of the root of *Astragalus membranaceus (Fisch.) Bge. var. monghlicus (Bge.) Hsiao*, rhizome of Compositae *Atractylodis macrocephalae* Koidz. and root of *Saposhnikovia divaricata* (Turez.) Schischk. Its earliest use was recorded in Danxi's *Experiential Therapy* in 1281 AD. YPF is commonly used to treat biomedically defined disorders such as upper respiratory tract infection, exacerbation of chronic bronchitis, mild bronchitis, hyperthyroid condition, autonomic dystonia, allergic rhinitis, etc.,[5] and its immunomodulating activities, including enhancement of innate immunity, stimulation of the cellular immune response and the raising of humoral and

Correspondence: Jun Li, School of Pharmacy, Anhui Medical University, Hefei, 230032, China. E-mail: ljedu@163.com

cellular immune functions, have been reported.[6] However, to our knowledge, little is known about the effects of its active components on hepatic fibrosis.

We have previously separated several polysaccharide fractions from YPF and have confirmed that one YPF fractioned polysaccharide (YPF-P) is the most active fraction for immunomodulating effects in cyclophosphamide-treated mice.[7] Furthermore, YPF-P has a hepatoprotective effect on acute liver injury induced by carbon tetrachloride (CCl_4), D-galactosamine or Bacillus Calmette Guerin-lipopolysaccharide by inhibition of lipid peroxidation, as we have previously reported.[8] In the present study, we aimed to investigate the preventive effects of YPF-P on rat liver fibrosis induced by CCl_4 as well as its possible mechanisms.

In traditional Chinese medicine theory, the three essential herbs in YPF play different roles in the pharmacodynamic actions: root of *Astragalus membranaceus (Fisch.) Bge. var. monghlicus (Bge.) Hsiao* is the principal drug, rhizome of Compositae *Atractylodis Macrocephalae* Koidz. is the ministerial drug and root of *Saposhnikovia divaricata* (Turez.) Schischk. is the adjuvant. This essential combination emphasises the fact that the synergistic effects of the active components of the formula often exceed those of any one of its active ingredients used individually. Based on this theory, we evaluated YPF-P compared with its counterpart polysaccharide (AP) prepared in the same manner from the principal herb in order to demonstrate the superiority of the mixture over the individual components in preventing liver fibrosis.

Materials and Methods

Plant materials and reagents

Root of *Astragalus membranaceus (Fisch.) Bge. var. monghlicus (Bge.) Hsiao*, rhizome of Compositae *Atractylodis Macrocephalae* Koidz. and root of *Saposhnikovia divaricata* (Turez.) Schischk. were purchased from Heyitang Co. Ltd (Hefei, China) and identified by Dr Wenming Cheng at the School of Pharmacy, Anhui Medical University, China. CCl_4 was purchased from Shanghai Changjiang Chemical Plant and mixed with peanut oil in a ratio of 1 : 1 under aseptic conditions. Colchicine was obtained from Kunming Pharmaceutical Group, China. Primers produced by Shanghai Shenergy Biotechnology Co. Ltd and agarose from Sigma were used in the reverse transcription–polymerase chain reaction (RT-PCR) experiment. The other chemicals used were reagent grade from commercial sources.

Preparation of YPF-P

The formula YPF is composed of root of *Astragalus membranaceus (Fisch.) Bge. var. monghlicus (Bge.) Hsiao*, rhizome of Compositae *Atractylodis Macrocephalae* Koidz. and root of *Saposhnikovia divaricata* (Turez.) Schischk. in proportions of 3 : 1 : 1 by weight. The dried YPF (6.0 kg) was defatted with petroleum ether (30 l) for 2 h to remove liposoluble constituents and then extracted twice with hot water at 75°C (60 l) for 2 h. The supernatant was combined, filtered and concentrated six-fold under reduced pressure. Based on the distinct solubilities of the polysaccharides, the water-soluble total polysaccharide was fractioned into two parts by adding the concentrated extract with ethanol step by step until the final concentrations of ethanol reached 30 and 60%. The latter fraction was centrifuged at 1400*g* at room temperature for 20 min, washed successively with absolute ethanol and acetone, and then dried in a vacuum, yielding the fractioned polysaccharide powder (yield: 5.67% of YPF weight). Afterwards, the Sevag method was applied five times to remove protein.[9] The deproteinated and dried brown product, YPF-P, was obtained with a yield of 2.85% of YPF weight. The sugar and protein contents of YPF-P were determined by the modified phenol–sulfuric acid assay[10] and the Lowry method,[11] with respective values of 79 and 5.37% of the YPF-P dry weight.

The fractioned polysaccharide, AP, was prepared in a similar manner from the same batch number of *Astragalus membranaceus (Fisch.) Bge. var. monghlicus (Bge.) Hsiao* root (2.5 kg), acheiving 82% sugar content and 4.77% protein content of AP dry weight. Recently, the HPLC-GPC method has been used to further analyse the ingredients of YPF-P, indicating that YPF-P mainly consists of three homogeneous sugars, YPF-P1, YPF-P2 and YPF-P3, with molecular weights of 488.5, 2232 and 1863 kDa, respectively. Figure 1 shows the HPLC-GPC chromatograms of YPF-P1, YPF-P2 and YPF-P3.

The structural features of YPF-P1 were further investigated by monosaccharide composition analysis, periodic acid oxidation, infrared spectrophotometry and ^{1}H- and ^{13}C-NMR spectroscopy. It is suggested that YPF-P1 is a highly branched heteroglycan, mainly composed of glucose, galactose, fructose and arabinose in a molar ratio of 3 : 2.5 : 1.75 : 1. The main chain is composed of 1,6-linked α-D-glucopyranoside and 1,6-linked β-D-galactopyranose in a molar ratio of 6 : 5.

Animal model and drug treatment

Male Sprague–Dawley rats weighing 130–150 g were supplied by the Experimental Animal Center of Anhui Medical University. All rats were maintained under controlled conditions (20–22°C, 50% humidity and alternating 12 h dark/light cycles) and provided with rodent chow and water *ad libitum*. The rats were randomly divided into seven groups: the control group ($n = 10$), the model group ($n = 15$), YPF-P groups (30, 60 and 120 mg/kg, $n = 12$ in each group), the AP group (60 mg/kg, $n = 12$) and the colchicine group (0.1 mg/kg, $n = 12$) as positive control. In order to produce liver fibrosis, all groups were treated by subcutaneous injection of 50% (v/v) CCl_4 diluted

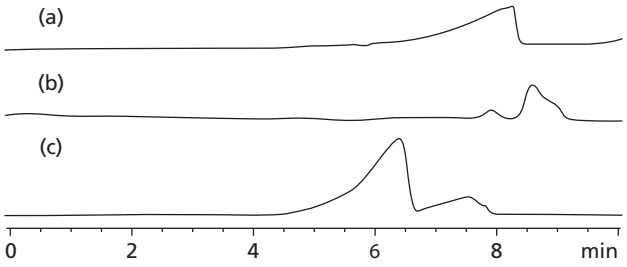

Figure 1 HPLC-GPC chromatograms of YPF-P1, YPF-P2 and YPF-P3. (a) YPF-P1, RT: 8.241 min; (b) YPF-P2, RT: 8.564 min; (c) YPF-P3, RT: 6.354 min.

in peanut oil, at a dose of 1 ml/kg, twice weekly for 12 weeks, with the exception of the control group, which was treated with peanut oil only, as described by Zhang *et al*. but with some modifications.[12] In the treatment groups, YPF-P and AP dissolved in normal saline were given once a day via gavage starting from the injection of CCl_4. An equal volume of normal saline was given to the model and the control groups. All rats were weighed and the dosage was adjusted once a week. At the end of the 12-week experimental period, 68 rats remained, there having been one death in the control group, five in the model group and eleven in the treated groups. The rats were sacrificed when fasted and blood samples were centrifuged at 1000*g* for 15 min at room temperature. The blood serum obtained was stored at −80°C. Left hepatic lobes were promptly collected for pathological examination as described later. Partial liver tissues were stored in liquid nitrogen for hydroxyproline (Hyp) and messenger RNA examinations.

All animal experiments were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals Guidelines, using protocols approved by the Institutional Animal Ethics Committee.

Histopathological examination

Left hepatic lobe tissues were fixed in 10% neutral formalin followed by routine histological procedures, embedded in paraffin, cut into 3-μm sections and mounted on a slide. Tissue sections were stained with Masson collagen kit (Guangzhou Huajun Medicine Equipment Co., China). Specimens were examined and scored by two pathologists who were blinded to the treatment protocol. The criteria used for scoring fibrosis severity was as follows:[13] 0, normal; 1+, fibrosis present (collagen fibres extended from the portal triad or central vein to peripheral regions); 2+, mild fibrosis (collagen fibres present with extension without compartment formation); 3+, moderate fibrosis (collagen fibres present with some pseudolobe formation); 4+, severe fibrosis (collagen fibres present with thickening of the partial compartments and frequent pseudlobe formation). Specimens were observed with a Nikon Eclipse 80i fluorescence microscope (Nanjing Xinfeida Optical Instrument Co., China). Semi-quantitative histological analysis was performed with an Image Pro Plus (version 6.0). The ratio of collagen area to hepatic tissue area was calculated and the result was determined as the mean of 10 different fields on each slide.

Transmission electron microscope

Morphological changes of hepatic stellate cells (HSC) were examined by transmission electron microscope (TEM). Liver tissue specimens were sliced into small pieces, fixed with 2.5% glutaraldehyde and 1% osmic acid, dehydrated, substituted by propylene, embedded in epoxy resin, and cut into ultrathin sections as previously described.[14] Ultrastructure was observed under a JEM-1230 transmission electron microscope (Jeol Ltd, Japan).

Measurement of serum fibrotic markers and hepatic hydroxyproline

Serum concentrations of hyaluronic acid (HA), laminin (LN), type IV collagen (CIV) and type III procollagen (PCIII) were assayed with radioimmunoassay using commercial kits (Peking Northern Biotech Institute, China). The measurement was performed with a GC-911 γ automatic radioimmunity computer (USTC Chuangxin Co., Ltd, Zonkia Branch, Hefei, China). Hepatic Hyp content was determined according to the procedure of the kits (Nanjing Jiancheng Biological Company, Nanjing, China).

Serum transforming growth factor-beta 1 (TGF-β1)

Serum TGF-β1 level was detected using a rat enzyme-linked immunosorbent assay (ELISA) kit obtained from Sigma Chemical Co. (St Louis, MO, USA) with a Muliskan MK3 ELISA Reader (Thermo Labsystems Oy, Finland).

Reverse transcription-polymerase chain reaction for mRNA detection

RT-PCR was performed as previously described[15] with some modifications. Briefly, total RNA was extracted by Trizol reagent (Life Technologies Inc., Carlsbad, CA, USA). RNA concentrations were determined by ultraviolet spectrophotometric measurement at wavelengths of 260 and 280 nm. Total RNA (5 μl) was reversely transcribed into cDNA using the Reverse Transcription System (Promega Co., Sunnyvale, CA, USA). The cDNA was amplified by Mastercycle Epgradient (Eppendorf, Hamburg, Germany) using recombinant Taq DNA polymerase (Fermentas Co.) with the primers listed in Table 1. The total reaction mixure volume was 25 μl. The thermal profile consisted of denaturation at 94°C for 1 min, annealing respectively at 56, 58, 58, 51 and 54°C for 40 s, and extension at 72°C for 1 min by 35 cycles for β-actin, tissue inhibitor of metalloproteinase-1 (TIMP-1), matrix metalloproteinase-13 (MMP-13), procollagen I and collagen III. The β-actin was used as an internal calibrant. The PCR products were electrophoresed on 1.5% (w/v) agarose gel. Relative quantification of gene expression was analysed using a BIO-RAD GS-700 densitometer.

Statistical analysis

Quantitative data were expressed as mean ± SD, and subjected to one-way analysis of variance (ANOVA) followed by

Table 1 Primer pairs used for RT-PCR in this study

Primer	Forward	Reverse	Size (bp)
β-Actin	5′-CAGTAACAGTCCGCCTAGAA-3′	5′-GATTACTGCTCTGGCTCCTA-3′	195
TIMP-1	5′-TCCCTTGCAAACTGGAGAGT-3′	5′-GTCATCGAGACCCCAAGGTA-3′	148
MMP-13	5′-TGACTATGCGTGGCTGGAA-3′	5′-AAGCTGAAATCTTGCCTTGGA-3′	355
Procollagen I	5′-TACAGCACGCTTGTGGATG-3′	5′-TTGAGTTTGGGTTGTTGGTC-3′	259
Collagen III	5′-AGATCATGTCTTCACTCAAGTC-3′	5′-TTTACATTGCCATTGGCCTGA-3′	493

Bartlett's test to determine the homology of variance. Ordinal data were analysed by Ridit analysis.[16] Differences were considered to be significant at a level of $P < 0.05$.

Results

Histopathological analysis

Representative photographs of the liver morphology with Masson staining are shown in Figure 2. The rat livers in the control group showed normal lobular architecture, with central veins, radiating hepatic cords and no regenerating collagen fibre. Compared with the control group, subcutaneous injection of CCl_4 for 12 weeks significantly aggravated the severity of hepatic fibrosis: lobular structure was destroyed, hepatic plates were in disorder, there were fibrous connective tissues with many inflammatory cells regenerated in the portals, collagen fibres expanded into the hepatic parenchyma, and fibrous septa surrounding and separating the normal lobules formed (Figure 2b). These changes occurred primarily around the central and portal veins, as described in previous reports.[13] However, administration of 60 mg/kg YPF-P apparently attenuated the degree of liver fibrosis with no evidence of connective tissue septa and a reduced deposition of collagen (Figure 2d). The liver histology in the YPF-P 120 mg/kg group appeared to be closer to normal histology, with few fibrotic septa. In the AP (60 mg/kg) group, continuous fibrotic septa were reduced between the central and portal veins, but much more fatty degeneration was observed compared with theYPF-P (60 mg/kg) group. In both the YPF-P 30 mg/kg group and the colchicine group, reduced numbers of collagen fibres were observed compared with the model group, but the deposition of collagen was still considerable.

The pathologic grading in CCl_4-induced hepatic fibrosis rats by semi-quantitative histological analysis is shown in Table 2. The results show that the hepatic fibrosis staging score was significantly raised, to 3.71 ± 0.38 in the model group ($P < 0.01$ vs the control group). Administration of YPF-P (60, 120 mg/kg) or AP (60 mg/kg) greatly reduced the scores for liver fibrosis and collagen deposition ($P < 0.01$).

To study the effects of YPF-P and AP on fibrogenesis inhibition, we compared the YPF-P 60 mg/kg group with the

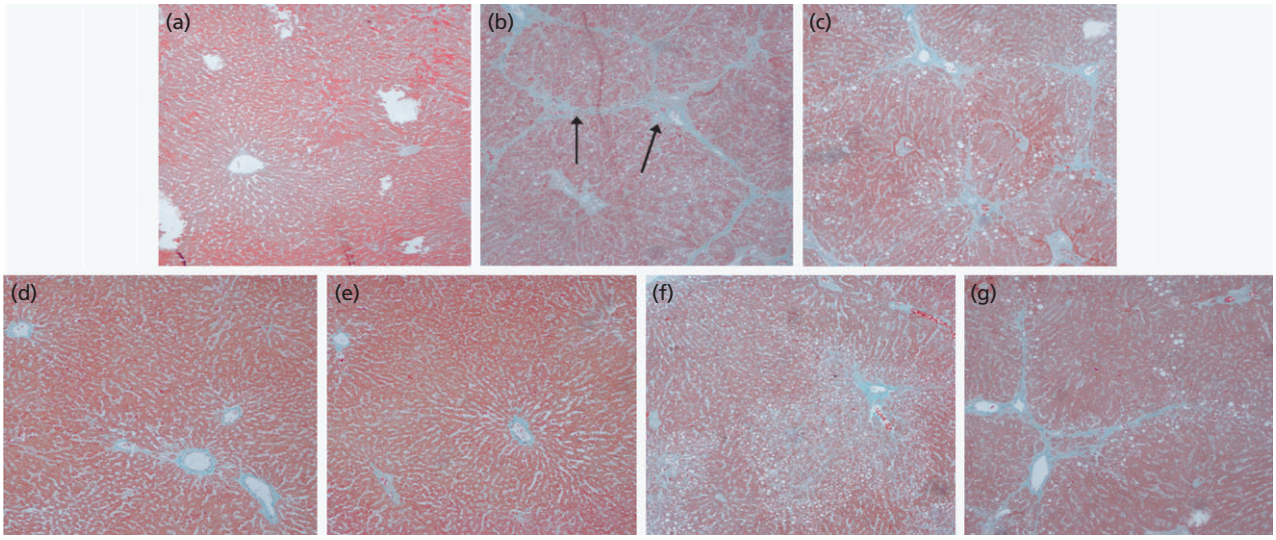

Figure 2 Representative microphotographs of liver morphology with Masson staining. (a) Control group; (b) model group; (c) YPF-P at 30 mg/kg; (d) YPF-P at 60 mg/kg; (e) YPF-P at 120 mg/kg; (f) AP at 60 mg/kg; (g) colchicine at 0.1 mg/kg. The Masson stain colours collagen green and hepatocytes red. The arrows indicate collagen deposition. (Original magnification: ×100).

Table 2 Effect of YPF-P on pathologic grading in carbon tetrachloride-induced hepatic fibrosis rats

Group		Staging score of hepatic fibrosis					*P*-value	Average ratio[a]
	n	0	1	2	3	4		
Control	9	9	0	0	0	0		0.0 ± 0.0
Model	10	0	0	1	5	4	#	3.71 ± 0.38#
YPF-P 30 mg/kg	9	0	0	2	6	1		3.53 ± 0.21
YPF-P 60 mg/kg	11	0	6	4	1	0	**▲	1.98 ± 0.45**▲
YPF-P 120 mg/kg	10	1	7	2	0	0	**	1.43 ± 0.38**
AP	10	0	2	5	3	0	**	2.30 ± 0.47**
Colchicine	9	0	1	4	3	1	*	3.20 ± 0.42*

#$P < 0.01$ vs the control group, *$P < 0.05$, **$P < 0.01$ vs the model group, ▲$P < 0.05$ vs the AP group. [a]The individual average ratio is expressed as mean ± SD. Results are determined as the mean of 10 randomly-selected fields on each slide.

AP group, due to their comparability based on the same dosage. Compared with the AP group, YPF-P 60 mg/kg treatment significantly improved liver morphological changes and fibrosis ($P < 0.05$). However, no remarkable liver fibrosis resolution was found in the YPF-P 30 mg/kg group (Table 2).

Ultrastructure of hepatic stellate cells

Hepatic stellate cells (HSC), known as fat-storing, perisinusoidal or Ito cells, have now been clearly identified as the primary cellular source involved in the pathogenesis of liver fibrosis.[17] We therefore examined their ultrastructure using TEM. TEM revealed that HSC of the model rats displayed the typical fibrotic morphology in Disse's spaces, i.e. few fat droplets, hypertrophied rough endoplasmic reticulum, and the so-called myofibroblast-like cell (or activated stellate cell) with excessive collagen deposition (Figure 3a,b). The HSC of rats treated with 120 mg/kg YPF-P for 12 weeks showed major fat droplets and unremarkable extension of the rough endoplasmic reticulum, similar to the stellate cell in the resting state (Figure 3c).[18,19]

Effects of YPF-P on serum hepatic fibrosis markers and hepatic hydroxyproline

Serum HA, LN, PCIII and CIV have been reported to reflect the extent of liver fibrosis as the surrogate markers of liver fibrogenesis, and Hyp is crucial in quantifying the extent of fibrosis.[20] Table 3 shows the effects of YPF-P on those fibrosis markers. In model rats, the levels of serum liver fibrosis markers and hepatic Hyp showed clear increases. YPF-P administration (60 or 120 mg/kg) greatly decreased all the serum markers and Hyp levels ($P < 0.01$), while the 30 mg/kg administration caused a decrease in PCIII, CIV and Hyp levels ($P < 0.05$). It is therefore concluded that concurrent administration of YPF-P significantly reduced the levels of the four elevated markers in a dose-dependent manner. The administration of AP also caused decreases in the four markers ($P < 0.01$) as well as the colchicine group ($P < 0.05$). However, compared with AP, the YPF-P 60 mg/kg group showed further decreases in HA and PCIII, by 36% ($P < 0.05$) and 38% ($P < 0.01$), respectively, with no apparent differences in serum LN, CIV and hepatic Hyp levels.

Effects of YPF-P on serum TGF-β1

As shown in Figure 4, a significantly elevated concentration of serum TGF-β1 (3.88 ± 0.75 μg/l) was observed in the model group compared with 1.25 ± 0.34 μg/l in the control group ($P < 0.01$). With increasing administration of YPF-P, serum TGF-β1 levels were significantly and dose-dependently decreased to 1.80 ± 0.41 and 1.51 ± 0.45 μg/l ($P < 0.01$) in parallel with the reduction of the serum fibrotic markers. The AP group also showed a significant decrease in serum TGF-β1, but the YPF-P group with an identical dose showed lower levels of reduction ($P < 0.05$).

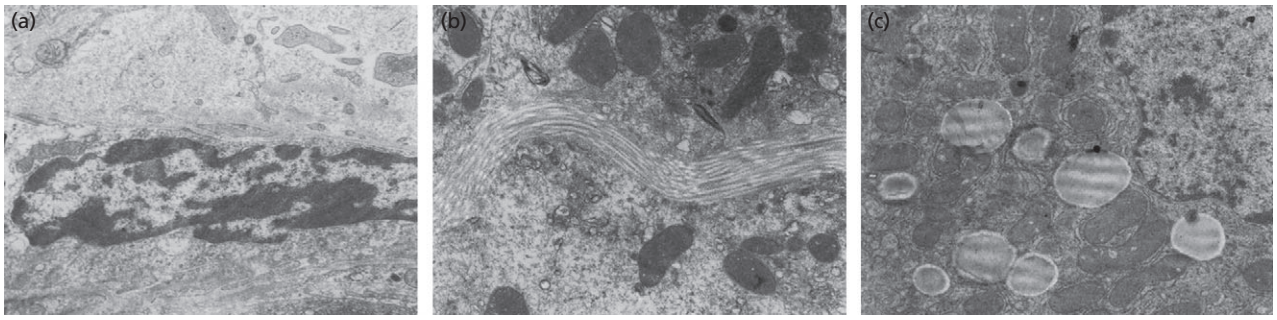

Figure 3 Electron micrographs of representative hepatic stellate cells of CCl_4-induced liver fibrosis rats. (a) Fat droplets are rarely seen in fibrosis rats and the rough endoplasmic reticulum is markedly extended; (b) extension of excessively deposited collagen is clearly seen in fibrosis rats; (c) hepatic stellate cells in Disse's spaces of rats treated with 120 mg/kg YPF-P for 12 weeks – fat droplets remain, and extension of the rough endoplasmic reticulum is not as marked as in (a). (Magnification: (a), (b) × 8000; (c) × 15 000).

Table 3 Effect of YPF-P on serum levels of fibrosis markers and hepatic hydroxyproline content in carbon tetrachloride-induced hepatic fibrosis rats

Group	Doses (mg/kg)	HA (μg/l)	LN (μg/l)	PCIII (μg/l)	CIV (μg/l)	Hyp (μg/g)
Control	–	102.87 ± 31.47	81.88 ± 22.05	71.12 ± 25.55	59.28 ± 24.51	254.01 ± 13.85
Model	–	377.75 ± 68.09$^{\#}$	316.64 ± 59.66$^{\#}$	293.52 ± 33.23$^{\#}$	253.48 ± 40.28$^{\#}$	430.27 ± 43.04$^{\#}$
YPF-P	30	309.11 ± 48.84	283.86 ± 40.33	226.45 ± 25.05*	196.94 ± 22.37*	384.64 ± 28.50*
	60	153.13 ± 58.07**$^{\blacktriangle}$	172.13 ± 28.59**	107.97 ± 16.28**$^{\blacktriangle\blacktriangle}$	142.68 ± 31.61**	316.58 ± 20.56**
	120	148.75 ± 26.88**	115.79 ± 25.15**	89.24 ± 22.35**	94.22 ± 17.33**	282.32 ± 23.06**
AP	60	239.46 ± 33.55**	187.11 ± 32.34**	175.13 ± 31.51**	156.09 ± 27.91**	309.88 ± 10.40**
Colchicine	0.1	282.74 ± 57.33*	244.69 ± 25.72*	229.62 ± 32.59*	189.36 ± 30.98*	325.48 ± 12.46**

HA, hyaluronic acid; Hyp, hydroxyproline; LA, laminin; CIV, type IV collagen; PCIII, type III procollagen. $^{\#}P < 0.01$ vs the control group, *$P < 0.05$,**$P < 0.01$ vs the model group, $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ vs AP group. All values are expressed as mean ± SD.

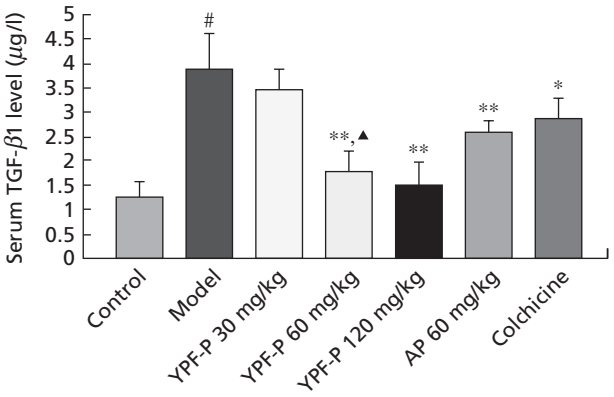

Figure 4 Effect of YPF-P on serum TGF-β1 levels in rats of hepatic fibrosis induced by CCl_4. Mean ± SD. $^{\#}P < 0.01$ vs the control group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs the model group, $^{\blacktriangle}P < 0.05$ vs AP group.

Effects of YPF-P on TIMP-1, MMP-13, procollagen I and collagen III gene expressions

In an attempt to evaluate the mechanism of YPF-P on hepatic fibrosis rats, we measured gene expressions of the TIMP/MMP enzyme system and fibrin-forming collagens, which play important roles in the balance of synthesis and enzymatic degradation of ECM. As shown in Figure 5a and b, the expression levels of TIMP-1 were significantly higher in the model group than in the control group, and they were significantly decreased in the YPF-P and AP groups. In addition, YPF-P 60 mg/kg group showed lower levels than the AP group ($P < 0.05$). We also found that the rat interstitial collagenase MMP-13 mRNA expression in the current study was relatively unchanged in each experimental group, except in the YPF-P 120 mg/kg group, which was distinct from the control group ($P < 0.05$). Notably, MMP-13 mRNA expression in the model group was quite close to the control value, in accordance with previous reports.[21,22] In addition, the ratio of TIMP-1/MMP-13, which has been reported to play an important role in regulating ECM remodelling,[23–25] corresponded statistically with the TIMP-1 result (Figure 5b). The expressions of procollagen I and collagen III were significantly decreased in the YPF-P groups, in a dose-dependent manner, as well as in the AP group. Also, the decrease was more marked in the YPF-P 60 mg/kg group than in the AP group ($P < 0.05$) (Figure 5a,c).

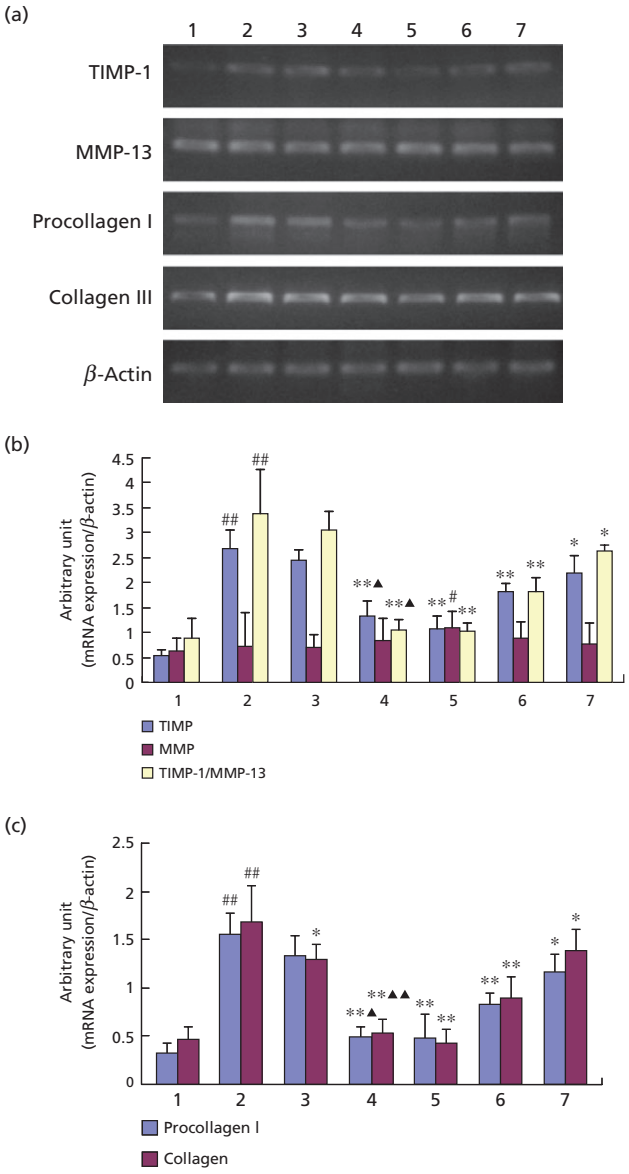

Figure 5 Effect of YPF-P on mRNA expression of TIMP-1, MMP-13, procollagen I and collagen III in liver fibrosis rats induced by CCl_4. (a) Representative electrophoretograms of three individuals in each group; (b) TIMP-1, MMP-13 mRNA expression and TIMP-1/MMP-13 ratio in graphic representation of Figure 5a; (c) procollagen I and collagen III mRNA expression in graphic representation of Figure 5a. Columns 1 to 7 indicate the control group, the model group, YPF-P 30, 60, 120 mg/kg groups, the AP group and the colchicine group in order. The results of the densitometric analysis after normalisation against β-actin optical density value are shown as mean ± SD of three independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the control group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs the model group, $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ vs the AP group.

Discussion

Evidence has been accumulating that even advanced fibrosis is reversible[26] and the key to treatment is to inhibit or reverse the occurrence and progression of fibrosis.[27] Recently, herbal medicine formulations as well as their active components have shown conspicuous advantages in the antifibrosis field, such as the results of the famous Sho-saiko-to studies.[28,29] The present study demonstrates the preventive effects and antifibrotic mechanisms of YPF-P, the active polysaccharide fraction of the Chinese herbal formulation YPF, on liver fibrosis in rats.

Chronic CCl_4 exposure was used in rats to produce the experimental hepatic fibrosis model. Histological analysis showed that typical hepatic fibrosis was successfully established in the model rats. However, YPF-P therapy effectively ameliorated the aggravation of liver histomorphology in a dose-dependent manner.Moreover, HSC underwent activation, characterised by morphological transformation into myofibroblast-like cells and synthesis of excessive extracellular matrix

components.[29] The electron microscopy findings suggest an inhibition effect of YPF-P on the transformation process. However, the effect was only shown at the 120 mg/kg concentration, indicating that YPF-P at higher doses may be a potent inhibitor of stellate cell activation. Further studies are required on this issue. Additionally, serum HA, LN, PCIII, CIV and hepatic Hyp, the commonly used biomarkers of liver fibrosis, were examined. The results revealed that treatment with YPF-P reduced the increase of these markers in a dose-dependent manner, and the effects were moderate or marked in the liver.

As reported previously, TGF-β1 is one of the most powerful and widely distributed profibrogenic mediators in the progress of hepatic fibrosis. It is produced by a number of non-parenchymal cells, such as activated HSC, Kupffer cells and endothelial cells.[30] Notably, TGF-β1 not only causes an increase in the production of type I, III and IV collagen, but also alters the balance of matrix degradation and accumulation by inhibiting production of interstitial collagenase, stromelysin and promoting the production of TIMP-1.[31] According to our investigation, it is presumed that the inhibitory effects of YPF-P are related to its reducing TGF-β1, which it does in a dose-dependent manner.

Matrix metalloproteinases (MMPs) are a family of extracellular zinc- and calcium-dependent proteases that degrade the ECM and other extracellular proteins.[32] MMP-13, the interstitial collagenase of rodents, is a highly specific protease capable of degrading insoluble fibrillar collagens, especially type I collagen.[33] The protease inhibitor TIMP-1 inhibits activity of MMPs, including MMP-13.[34] The ratio between MMP and TIMP expression has been shown to play an important role in ECM remodelling.[25] In addition, type I and type III collagen are the most prevalent ECM proteins deposited in liver fibrosis.[35]

In our study, TIMP-1 expression was significantly increased, whereas MMP-13 levels remained relatively unchanged during the chronic course of fibrosis in the model rats. This result is similar to previous reports,[36–38] although overexpression of the interstitial collagenase has been observed in the early progression phase of liver fibrosis in mice.[39] The results show that YPF-P administration greatly suppresses TIMP-1 expression and moderately promotes MMP-13 expression. As Kuo has reported, the suppressed TGF-β1 expression is correlated with up-regulation of MMP-13,[40] and it is presumed that the moderate up-regulation of MMP-13 expression after 12 weeks of treatment with YPF-P at high doses is correlated with the suppressed TGF-β1. Meanwhile, the down-regulation of TIMP-1 expression suggests the restoration of MMP-13 activity.

The ratio between TIMP-1 and MMP-13 is improved by YPF-P, which can be attributed to a decreased synthesis of TIMP-1. Otherwise, the effectiveness of YPF-P is also strongly reflected in the marked decrease of procollagen I and collagen III gene expression, with even YPF-P administered at 30 mg/kg producing a significant decrease in collagen III, which is consistent with the reduced production of PC III.

It has been reported that both the extract from the root of *Astragalus membranaceus (Fisch.) Bge. var. monghlicus (Bge.) Hsiao* as well as its active polysaccharides extract have inhibitory effects on hepatic fibrosis.[41,42] In this study we verified the effectiveness of active polysaccharide fractions from the root of *Astragalus membranaceus (Fisch.) Bge. var. monghlicus (Bge.) Hsiao* on CCl_4-induced hepatic fibrosis in rats. However, to investigate the antifibroplastic advantages of YPF-P and AP, the two active polysaccharides from the formula YPF and its principal drug, we compared YPF-P (60 mg/kg) with AP at the same dosage. From the results, it was clear that YPF-P is more potent than AP, based on morphologic observation, serum fibrosis marker evaluation (with the exception of serum LN and CIV) and ECM-related gene expression. Although the comparison of the comprehensive compatability studies is relatively imperfect, with incomplete compatibility between the two drugs and uncertain component comparison, the advantages of YPF-P on fibrosis seem clear. This suggests that the combination of active polysaccharides in this YPF formulation may possess synergistic or additive effects on antifibrosis, and it is thus more potent than that in its constituent herbs.

Conclusions

YPF-P dose-dependently alleviated the degree of liver fibrosis, inhibited HSC transformation into myofibroblast-like cells, markedly reduced the elevated levels of serum HA, LN, CIV, PCIII and TGF-β1, suppressed procollagen I, collagen III and TIMP-1 expression, and improved the TIMP-1/MMP-13 ratio. MMP-13 expression was only promoted moderately by YPF-P (120 mg/kg). Although AP showed the greatest effect on these markers, with the exception of MMP-13 expression, YPF-P showed more potency on PC III and collagen III mRNA ($P < 0.01$) than AP used individually, as well as on HA, TGF-β1, TIMP-1 mRNA, the TIMP-1/MMP-13 ratio and procollagen I mRNA ($P < 0.05$), but with no apparent differences in MMP-13 mRNA and serum LN and CIV. These findings suggest that YPF-P possesses preventive effects in CCl_4-induced hepatic fibrosis in rats and presents superior compatibility by balancing the TIMP-1/MMP-13 system and inhibiting the synthesis of matrix collagen. YPF-P may be a potent inhibitor of liver fibrosis.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work was supported by the National Natural Science Foundation of China (No. 30973917) and the Foundation of Provincial Key Laboratory of Anhui Medical University (SBSYS-0803). The authors acknowledge the help of the staff members of School of Pharmacy, Anhui Medical University.

References

1. Beljaars L *et al*. Targeting hepatic stellate cells for cell-specific treatment of liver fibrosis. *Front Biosci* 2002; 7: e214–e222.
2. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; 115: 209–218.

3. Luk JM *et al*. Traditional Chinese herbal medicines for treatment of liver fibrosis and cancer: from laboratory discovery to clinical evaluation. *Liver Int* 2007; 27: 879–890.
4. Fowell AJ, Iredale JP. Emerging therapies for liver fibrosis. *Dig Dis* 2006; 24: 174–183.
5. Bensky D, Barolet R. *Chinese Herbal Medicine Formulas & Strategies*. Seattle: Eastland Press, 1990.
6. Xin H *et al*. Progress in the immunopharmacological study of yupingfeng powder. *Zhongguo Zhong Yao Za Zhi* 1998; 23: 505–507 [in Chinese].
7. Chen XT *et al*. Immunomodulating effects of fractioned polysaccharides isolated from Yu-Ping-Feng-Powder in cyclophosphamide-treated mice. *Am J Chin Med* 2006; 34: 631–641.
8. Chen FJ *et al*. Protective effect of polysaccharides from yupingfeng powder on immunological liver injury in mice. *Anhui Med Pharm J* 2008; 12: 302–304 [in Chinese].
9. Wang X *et al*. Deproteinization of gellan gum produced by Sphingomonas paucimobilis ATCC 31461. *J Biotechnol* 2007; 128: 403–407.
10. Dong Q *et al*. Modified phenol-sulfuric acid method for determination of the content of oligo- and polysaccharides. *Chin Pharm J* 1996; 31: 550–553.
11. Lowry OH *et al*. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265–275.
12. Zhang JT. *Modern Experimental Methods in Pharmacology (Xian Dai Yao Li Shi Yan Fang Fa Xue)*. Beijing: Beijing Medical University Press, 1998: 1398.
13. Tsai JH *et al*. Effects of silymarin on the resolution of liver fibrosis induced by carbon tetrachloride in rats. *J Viral Hepat* 2008; 15: 508–514.
14. Sakaida I *et al*. The prolyl 4-hydroxylase inhibitor HOE 077 prevents activation of Ito cells, reducing procollagen gene expression in rat liver fibrosis induced by choline-deficient Lamino acid-defined diet. *Hepatology* 1996; 23: 755–763.
15. Okuno M *et al*. Prevention of rat hepatic fibrosis by the protease inhibitor, camostat mesilate, via reduced generation of active TGF-beta. *Gastroenterology* 2001; 120: 1784–1800.
16. Liu R, Bai RH. Ridit analysis with SPSS. *Chin J Health Stat* 2004; 21: 236–238 [in Chinese].
17. Friedman SL. Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. *N Engl J Med* 1993; 328: 1828–1835.
18. Horn T *et al*. Early alcoholic liver injury: activation of lipocytes in acinar zone 3 and correlation to degree of collagen formation in the Disse space. *J Hepatol* 1986; 3: 333–340.
19. Chen W *et al*. Isolation, culture and main characteristics of mouse fat-storing cells: interaction with viruses. *Hepatology* 1989; 9: 352–362.
20. Liu J *et al*. Serum fibrosis markers in diagnosing liver fibrosis. *Zhonghua Nei Ke Za Zhi* 2006; 45: 475–477 [in Chinese].
21. Lee HS *et al*. Expression of matrix metalloproteinases in spontaneous regression of liver fibrosis. *Hepatogastroenterology* 2001; 48: 1114–1117.
22. Yoshiji H *et al*. Tissue inhibitor of metalloproteinases-1 attenuates spontaneous liver fibrosis resolution in the transgenic mouse. *Hepatology* 2002; 36: 850–860.
23. Olaso E, Friedman SL. Molecular regulation of hepatic fibrogenesis. *J Hepatol* 1998; 29: 836–847.
24. Arthur MJ. Degradation of matrix proteins in liver fibrosis. *Pathol Res Pract* 1994; 190: 825–833.
25. Woessner JF Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J* 1991; 5: 2145–2154.
26. Hammel P *et al*. Regression of liver fibrosis after biliary drainage in patients with chronic pancreatitis and stenosis of the common bile duct. *N Engl J Med* 2001; 344: 418–423.
27. Nieto N *et al*. Ethanol and arachidonic acid increase alpha 2(I) collagen expression in rat hepatic stellate cells overexpressing cytochrome P450 2E1. Role of H2O2 and cyclooxygenase-2. *J Biol Chem* 2000; 275: 20136–20145.
28. Shimizu I *et al*. Effects of Sho-saiko-to, a Japanese Herbal Medicine, on Hepatic Fibrosis in Rats. *Hepatology* 1999; 29: 149–160.
29. Kayano K *et al*. Inhibitory effects of the herbal medicine Shosaiko- to (TJ-9) on cell proliferation and procollagen gene expressions in cultured rat hepatic stellate cells. *J Hepatol* 1998; 29: 642–649.
30. Wu J, Zern MA. Hepatic stellate cells: a target for the treatment of liver fibrosis. *J Gastroenterol* 2000; 35: 665–672.
31. Liu X *et al*. Therapeutic strategies against TGF-b signaling pathway in hepatic fibrosis. *Liver Int* 2006; 26: 8–22.
32. Nagase H, Woessner JF Jr. Matrix metalloproteinases. *J Biol Chem* 1999; 274: 21491–21494.
33. Uchinami H *et al*. Loss of MMP 13 attenuates murine hepatic injury and fibrosis during cholestasis. *Hepatology* 2006; 44: 420–429.
34. Brocks B *et al*. Generation and optimization of human antagonistic antibodies against TIMP-1 as potential therapeutic agents in fibrotic diseases. *Human Antibodies* 2006; 15: 115–124.
35. Brenner DA *et al*. New aspects of hepatic fibrosis. *J Hepatol* 2000; 32: 32–38.
36. Iredale JP *et al*. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J Clin Invest* 1998; 102: 538–549.
37. Iredale JP *et al*. Tissue inhibitor of metalloproteinase-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis. *Hepatology* 1996; 24: 176–184.
38. Watanabe T *et al*. Gene expression of interstitial collagenase in both progressive and recovery phase of rat liver fibrosis induced by carbon tetrachloride. *J Hepatol* 2000; 33: 224–235.
39. Hagiwara S *et al*. Overexpression of NK2 promotes liver fibrosis in carbon tetrachloride-induced chronic liver injury. *Liver Int* 2007; 28: 126–131.
40. Kuo YR *et al*. Suppressed TGF-beta1 expression is correlated with up-regulation of matrix metalloproteinase-13 in keloid regression after flashlamp pulsed-dye laser treatment. *Lasers Surg Med* 2005; 36: 38–42.
41. Sun WY *et al*. Effects and mechanisms of extract from Paeonia lactiflora and Astragalus membranaceus on liver fibrosis induced by carbon tetrachloride in rats. *J Ethnopharmacol* 2007; 112: 514–523.
42. Li SP *et al*. Astragalus polysaccharides and astragalosides regulate cytokines ecretion in LX-2 cellline. *Journal of Zhe-Jiang University (Medical Sciences)* 2007; 36: 543–548 [in Chinese].