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Relationship between anti-fibrotic effect of Panax notoginseng saponins and serum cytokines in rat hepatic fibrosis

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ABSTRACT

The aim of this study was to investigate the relationship between anti-fibrotic effect of Panax notoginseng saponins (PNS) and serum cytokines in rat hepatic fibrosis. Hepatic fibrosis induced by carbon tetrachloride (CCl_4) was studied in animal models using SD rats. Liver index, serum alanine amino transferase (ALT), aspartate amino transferase (AST), transforming growth factor-β1 (TGF-β1), tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) were measured, respectively. Liver index and the degree of liver fibrosis were also determined. Our results showed that the levels of ALT, AST and liver index in PNS-treated group were markedly lower than those in model group. PNS therapy also significantly attenuated the degree of hepatic fibrosis, collagen area and collagen area percent in liver tissue. Furthermore, the levels of serum TGF-β1, TNF-α and IL-6 were strikingly reduced in PNS-treated group compared with model group while the production of IL-10 was up-regulated. These findings demonstrate that PNS has certain therapeutic effects on hepatic fibrosis probably by immunoregulating the imbalance between pro-fibrotic and anti-fibrotic cytokines.

Introduction

Hepatic fibrosis is a common pathological process of chronic liver injuries, regardless of etiology, and its progression leads to cirrhosis and liver cancer [1]. Despite extensive efforts, its etiology and pathogenesis remain unclear and effective therapies with limited side effects are still deficient [2]. PNS is the major active constituent of the isolated root of Panax notoginseng, a well known traditional Chinese medicine. We have reported that PNS has significant scavenging effects on oxygen free radicals and protective effects on liver injuries induced by CCl_4 [3,4]; however, its exact mechanisms remain unclear. Therefore, it is necessary to be further elucidated.

CCl_4-induced hepatic fibrosis is a well-established animal model to study the pathogenesis and therapy of chronic liver injury diseases. Zhang et al. have reported that several pro-fibrotic cytokines including TGF-β1, TNF-α and IL-6 play an important role in the initiation and perpetuation of CCl_4-induced liver fibrosis while IL-10 play an antifibrogenic role by counterbalancing their effects [5]. This study aimed to further investigate the effect of PNS on hepatic fibrosis induced by CCl_4 and its relationship with the expression of TGF-β1, TNF-α, IL-6 and IL-10.

Materials and methods

Reagents and animals. PNS was provided by Kunming Zhongxing Pharmaceutical Co., Ltd (purity > 98%, Yunnan, China). Colchicine was purchased from Xiamen Sanland Chemical Co., Ltd (purity > 99%, Fujian, China). CCl_4 was obtained from Chongqing Chemical Reagent Co., Ltd (Chongqing, China). Male SD rats weighing 150–180 g were purchased from the Experimental Animal Center of Third Military Medical University. All studies involving animals were approved by the Institutional Animal Care and Use Committee.

Induction of liver fibrosis and PNS treatment. Forty male SD rats were randomly divided into four groups: normal control group ($n = 9$), model group ($n = 11$), PNS-treated group ($n = 10$), colchicine-treated group ($n = 10$). Except normal control group, all rats were treated with subcutaneous injection of 40% CCl_4 (0.3 ml/kg, but 0.5 ml/kg for the first injection), mixed with vegetable oil, twice a week for 8 weeks. For the last two groups, PNS (130 mg/kg, dissolved in sterile normal saline, intraperitoneal injection, once daily) or colchicine treatment (50 μg/kg, dissolved in sterile normal saline, intraperitoneal injection, once daily as positive group) was initiated at the same day as CCl_4 administration and

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Table 1
Levels of liver index and serum AST, ALT in different treatment groups (mean ± SD).

Group	*n*	Liver index	ALT (U/L)	AST (U/L)
Normal	9	0.026 ± 0.004	114.50 ± 8.16	183.09 ± 26.70
Model	7	0.049 ± 0.009[b]	193.58 ± 24.35[b]	404.37 ± 68.29[b]
PNS	8	0.038 ± 0.005[b,d]	163.36 ± 19.91[b,c]	321.70 ± 50.94[b,c]
Colchicine	9	0.031 ± 0.004[a,d]	167.60 ± 21.66[b,c]	325.61 ± 52.83[b,c]

Significantly different: [a]$P < 0.05$, [b]$P < 0.01$ vs. normal control group; [c]$P < 0.05$, [d]$P < 0.01$ vs. model group.

continued till the end. The two drug doses were selected based on the previous study [4]. Simultaneously, normal control and model groups were intraperitoneally administered with the same volume of vehicle (sterile saline water) once daily. At the end of the 8-week experimental period, all animals were anesthetized with 3% chloral hydrate and dissected. Blood and liver were obtained for further analysis.

Measurement of serum AST and ALT. Serum AST and ALT levels were measured using on an automated analyzer of biochemistry (Hitachi 7170, Tokyo, Japan) according to the manufacturer's instructions.

Liver index calculation. Liver index was measured according to the formula: (rat liver weight/rat weight) × 100% [6].

Histopathology. Samples were obtained from the same liver lobe in all animals and fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin–eosin (HE) or Van Gieson (VG) solutions.

The degree of liver fibrosis was evaluated on HE stained sections according to the previous report [7]. The collagen content of the sections was also determined on VG stained sections by a computer image analysis system (CM2000B, Beijing University of Aeronautics & Astronautics, china). Five random fields were chosen in each section and the amount of total collagen was detected in the area stained by VG and expressed as percentage relative to the total area [8].

ELISA of TGF-β_1, TNF-α, IL-6 and IL-10. Cytokine levels in the serum samples were measured by a commercially available ELISA kit (Biosources, San Jose, CA, USA) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed with the SPSS software system (SPSS for Windows, version 13.0; SPSS Inc, Chicago, IL). Parametric data were statistically analyzed by one-way ANOVA followed by post hoc tests when appropriate. Degree of hepatic fibrosis was analyzed by Kruskal–Wallis nonparametric test. Data were expressed as the means plus or minus SD. A significant difference was defined as $p < 0.05$.

Results

Animals

Irritability, aggression, and weight loss was present predominantly in rats of the model group. At the end of 8-week experimental period, No death was found in normal control group. All rats' death in other groups was as follows: 4 rats died in model group, 1 in colchicine-treated group, 2 in PNS-treated group.

Liver index and serum aminotransferases

Liver index in the normal control group was 0.026 ± 0.004. However, 8 weeks after the CCl_4 injection, the level of Liver index increased markedly. The increase was significantly attenuated by PNS or colchicine treatment ($P < 0.01$; Table 1).

We then measured serum aminotransferase activities in different experimental groups. The levels of serum AST, ALT were significantly increased in model group compared with those in normal control group. In contrast, PNS or colchicine treatment significantly suppressed up-regulations of these parameters induced by CCl_4 ($P < 0.05$; Table 1).

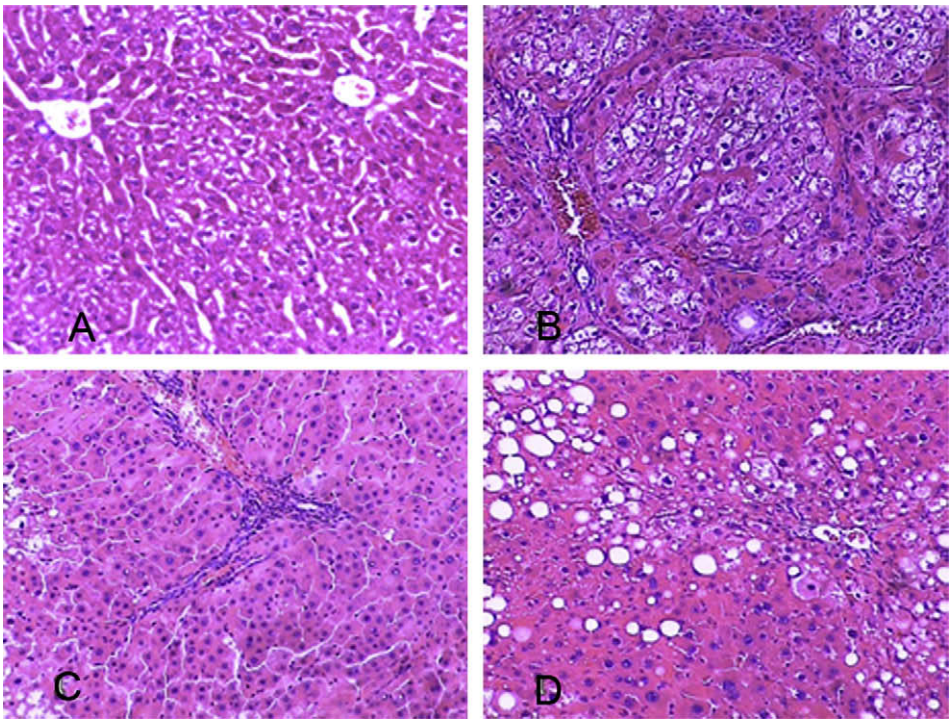

Fig. 1. The representative pathological changes of liver section taken from four experimental groups (A: normal; B: model; C: PNS; D: colchicine), HE stain, original magnification, ×100.

Table 2
Degree of liver fibrosis in different treatment groups.

Group	*n*	Degree of hepatic fibrosis					Average
		0	I	II	III	IV	
Normal	9	9	0	0	0	0	0
Model	7	0	0	0	4	3	3.43^{b}
PNS	8	0	0	3	5	0	$2.63^{b,c}$
Colchicine	9	0	3	5	1	0	$1.78^{b,d}$

Significantly different: $^{a}P < 0.05$, $^{b}P < 0.01$ vs. normal control group; $^{c}P < 0.05$, $^{d}P < 0.01$ vs. model group.

Table 3
Comparison of collagen area and collagen area percent in liver tissue of rats from different treatment groups.

Group	*n*	Collagen area (um^2)	Collagen area percent (%)
Normal	9	993.54 ± 145.31	1.97 ± 0.30
Model	7	2599.99 ± 488.32^{b}	10.15 ± 2.87^{b}
PNS	8	$1900.74 \pm 430.65^{b,c}$	$7.05 \pm 2.40^{b,c}$
Colchicine	9	$1396.00 \pm 276.07^{b,d}$	$4.65 \pm 2.11^{b,d}$

Significantly different: $^{a}P < 0.05$, $^{b}P < 0.01$ vs. normal control group; $^{c}P < 0.05$, $^{d}P < 0.01$ vs. model group.

Histopathology

Using HE staining, we observed that the liver tissue in normal control rats showed normal lobular architecture with central veins and radiating hepatic cords. However, liver sections taken from rats of model group exhibited more inflammatory infiltration, steatosis, hepatocyte coagulative necrosis and fibrous septa compared with normal control rats after 8 weeks of CCl_4 injection. In contrast, PNS or colchicine treatment markedly ameliorated these histopathological changes (Fig. 1).The results were further supported by a significantly decreased staging score of hepatic fibrosis after PNS or colchicine therapy ($P < 0.05$ or $P < 0.01$; Table 2).

We then examined the collagen levels in the liver tissue from different treatment groups by VG staining. Compared with normal control group, both collagen area and collagen area percent were significantly increased in model group. The increases were relieved by PNS or colchicine treatment, similar to changes of hepatic histological examination ($P < 0.05$ or $P < 0.01$; Fig. 2, Table 3). Therefore, the above findings shows that PNS can effectively prevent CCl_4-induced hepatic fibrosis in rats.

Effect of PNS on serum TGF-β1, TNF-α, IL-6, IL-10 production

As shown in Table 4, the levels of serum TGF-β1, TNF-α and IL-6 in model group were significantly higher than those in normal control group ($P < 0.01$). The up-regulations were markedly inhibited by treatment with PNS at the dose of 130 mg/kg ($P < 0.05$). On the other hand, IL-10 production in model group was sharply decreased compared with that in the normal control group (55% reduction, $P < 0.01$). However, PNS therapy significantly recovered the decrease induced by CCl_4 ($P < 0.05$).

Discussion

In the present study, PNS significantly lowered the levels of liver index, serum ALT and AST, reduced histological changes of liver fibrosis, suppressed the production of serum TGF-β1, TNF-α, IL-6 and increased significantly the level of IL-10. Furthermore, our previous study also show that the increases of several fibrosis indices including serum hyaluronic acid (HA), type IV collagen (IV-C) and hepatic hydroxyproline (Hyp) content after the CCl_4 injection can be notablely inhibited by PNS treatment [4]. The above findings demonstrated that PNS can effectively prevent CCl_4-induced hepatic fibrosis in rats and regulate the production of cytokines correlated with fibrosis.

Carbon tetrachloride (CCl_4), a known hepatotoxin, can cause liver necrosis, fibrosis and cirrhosis when administered sequentially. Hepatotoxicity is thought to involve two phases [9,10]. The initial phase involves bioactivation by a microsomal cytochrome P450 – dependent monooxygenase system, resulting in the formation of free radicals and lipid peroxidation [11]. The second step involves

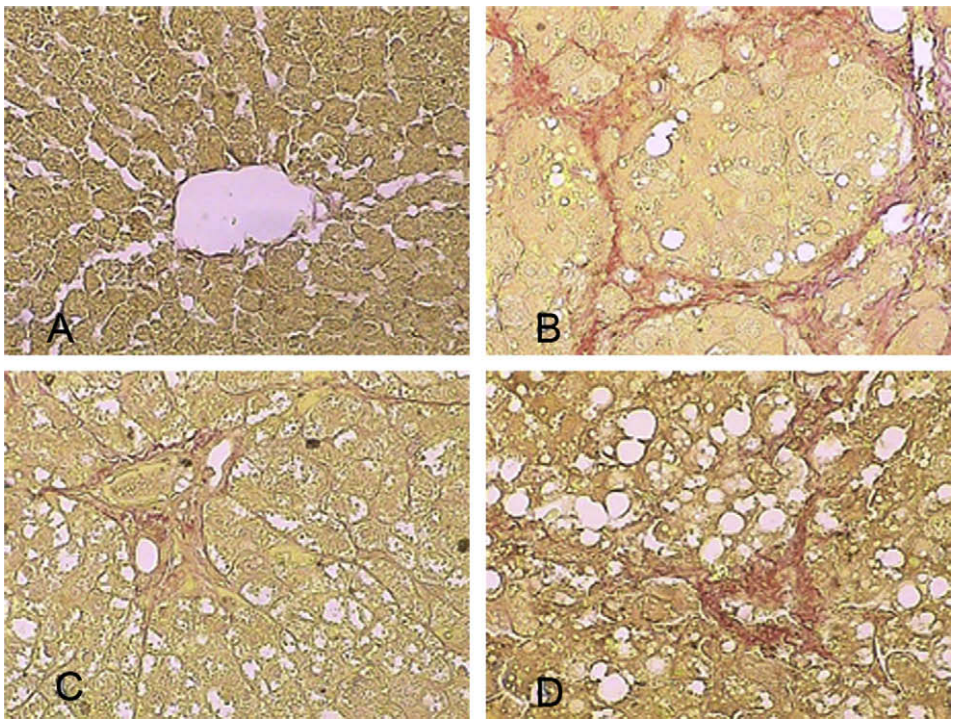

Fig. 2. The representative pathological changes of liver section taken from four experimental groups (A: normal; B: model; C: PNS; D: colchicine), VG stain, original magnification, ×200.

Table 4
Levels of serum TGF-β1, TNF-α, IL-6 and IL-10 in different treatment groups (mean ± SD).

Group	*n*	TGF-β1 (pg/ml)	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
Normal	7	199.78 ± 18.92	157.62 ± 11.77	187.98 ± 51.97	700.52 ± 138.63
Model	7	375.49 ± 57.47[b]	260.04 ± 37.70[b]	606.47 ± 130.73[b]	316.95 ± 62.74[b]
PNS	7	315.40 ± 40.22[b,c]	219.91 ± 18.20[b,c]	469.71 ± 92.06[b,c]	415.20 ± 82.20[b,c]

Significantly different: [a]$P < 0.05$, [b]$P < 0.01$ vs. normal control group; [c]$P < 0.05$, [d]$P < 0.01$ vs. model group.

the activation of Kupffer cells, which is accompanied by the production of pro-fibrotic mediators such as TGF-β1, TNF-α, IL-6 [12]. These pro-fibrotic factors act on hepatic stellate cells, leading to the formation of fibrous tissue [13]. In the study, we observed that PNS significantly reduced the increase of pro-fibrotic cytokines such as TGF-β1, TNF-α and IL-6 induced by CCl_4. The reduction of pro-fibrotic cytokines may be closely correlated with the previous reports that PNS has good radical scavenging action and also can significantly inhibit the activation and proliferation of Hepatic stellate cell (HSC) and enhance HSC apoptosis [3,4]. IL-10 is a pluripotent cytokine produced by many activated immune cell types, including T-helper (Th2) cells, B cells, macrophages, monocytes, and keratinocytes [14]. Recent studies indicate that IL-10 might play an important role in anti-fibrogenesis during CCl_4-induced hepatic fibrogenesis [15–17]. Our study showed that the level of circulating IL-10 in model group was lower than normal control group, which was consisted with the previous study [5]. In contrast, PNS significantly restored the decrease of IL-10 content induced by CCl_4, probably contributing to the anti-fibrotic effect of PNS.

In conclusion, PNS has significant anti-fibrogenesis effects on CCl_4-induced liver fibrosis in rats. In addition to the inhibition on the HSC activation and lipid peroxidation production as we previously reported, immunoregulation of the unbalance between pro-fibrotic and anti-fibrotic cytokines is one of the most important causes concerning with the preventive effect of PNS on CCl_4-induced liver fibrosis. The exact molecular mechanisms remain to be explored.

Conflict of interest

The authors state no conflict of interest.

References

[1] R. Alcolado, M.J. Arthur, J.P. Iredale, Pathogenesis of liver fibrosis, Clin. Sci. (Lond.) 92 (1997) 103–112.
[2] S. Lotersztajn, B. Julien, F. Teixeira-Clerc, P. Grenard, A. Mallat, Hepatic fibrosis: molecular mechanisms and drug targets, Annu. Rev. Pharmacol. Toxicol. 45 (2005) 605–628.
[3] W.B. Wang, L.L. Dai, Y.Y. Zheng, Apoptosis of rat hepatic stellate cells induced by Panax notoginseng saponins, Zhonghua Gan Zang Bing Za Zhi 13 (2005) 156–157.
[4] X. Li, X.D. Peng, W.L. Zhang, L.L. Dai, Inhibiting effects of denshensu, baicalin, astragalus and Panax notoginseng saponins on hepatic fibrosis and their possible mechanisms, Zhonghua Gan Zang Bing Za Zhi 16 (2008) 193–197.
[5] L.J. Zhang, J.P. Yu, D. Li, Y.H. Huang, Z.X. Chen, X.Z. Wang, Effects of cytokines on carbon tetrachloride-induced hepatic fibrogenesis in rats, World J. Gastroenterol. 10 (2004) 77–81.
[6] Q. Yang, R.J. Xie, X.H. Luo, B. Han, T. Yang, L. Fang, M.L. Cheng, Expression of PKC in rat hepatic fibrosis and the effect of Dan-shao-hua-xian Capsule on its expression pattern, Zhonghua Gan Zang Bing Za Zhi 13 (2005) 707–708.
[7] E.M. Brunt, Grading and staging the histopathological lesions of chronic hepatitis: the Knodell histology activity index and beyond, Hepatology 31 (2000) 241–246.
[8] P.Y. Cheung, Q. Zhang, Y.O. Zhang, G.R. Bai, M.C. Lin, B. Chan, C.C. Fong, L. Shi, Y.F. Shi, J. Chun, H.F. Kung, M. Yang, Effect of WeiJia on carbon tetrachloride induced chronic liver injury, World J. Gastroenterol. 12 (2006) 1912–1917.
[9] N. Kamalakkannan, R. Rukkumani, P.S. Varma, P. Viswanathan, K.N. Rajasekharan, V.P. Menon, Comparative effects of curcumin and an analogue of curcumin in carbon tetrachloride-induced hepatotoxicity in rats, Basic Clin. Pharmacol. Toxicol. 97 (2005) 15–21.
[10] J. Armendariz-Borunda, J.M. Seyer, A.H. Kang, R. Raghow, Regulation of TGF beta gene expression in rat liver intoxicated with carbon tetrachloride, FASEB J. 4 (1990) 215–221.
[11] R.R. Koch, E.A. Glende Jr., R.O. Recknagel, Hepatotoxicity of bromotrichloromethane-bond dissociation energy and lipoperoxidation, Biochem. Pharmacol. 23 (1974) 2907–2915.
[12] M.J. Edwards, B.J. Keller, F.C. Kauffman, R.G. Thurman, The involvement of Kupffer cells in carbon tetrachloride toxicity, Toxicol. Appl. Pharmacol. 119 (1993) 275–279.
[13] A.M. Gressner, Cytokines and cellular crosstalk involved in the activation of fat-storing cells, J. Hepatol. 22 (1995) 28–36.
[14] M. Goldman, T. Velu, Interleukin-10 and its implications for immunopathology, Adv. Nephrol. Necker Hosp. 24 (1995) 79–90.
[15] K. Thompson, J. Maltby, J. Fallowfield, M. McAulay, H. Millward-Sadler, N. Sheron, Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis, Hepatology 28 (1998) 1597–1606.
[16] H. Louis, J.L. Van Laethem, W. Wu, E. Quertinmont, C. Degraef, K. Van den Berg, A. Demols, M. Goldman, O. Le Moine, A. Geerts, J. Deviere, Interleukin-10 controls neutrophilic infiltration, hepatocyte proliferation, and liver fibrosis induced by carbon tetrachloride in mice, Hepatology 28 (1998) 1607–1615.
[17] D.R. Nelson, G.Y. Lauwers, J.Y. Lau, G.L. Davis, Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon nonresponders, Gastroenterology 118 (2000) 655–660.