Tropical Journal of Pharmaceutical Research September 2017; 16 (9): 2107-2112
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v16i9.9

Original Research Article

Mitigation of TGF-β/Smad signaling pathway-associated liver fibrosis by paeoniflorin

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Sent for review: 13 April 2017 *Revised accepted: 24 August 2017*

Abstract

Purpose: *To evaluate paeoniflorin (PF) as a possible protective agent against liver fibrosis and its likely mechanisms of action.*
Methods: *A rat model of liver fibrosis was induced by carbon tetrachloride (CCl_4) injection. Liver damage was determined by serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. The hydroxyproline content of proteins was measured as an indirect way of assessing collagen deposition. TGF-β1 levels and TGF-β/Smad signaling pathway-related genes and proteins were analyzed by quantitative polymerase chain reaction (qPCR) assay and Western blot assay.*
Results: *After PF administration, serum ALT and AST levels were significantly ($p < 0.01$) reduced by 34.9 and 37.6 %, respectively. Collagen deposition was also significantly ($p < 0.01$) reduced by 31.0 %. Hepatic stellate cell activation was significantly inhibited, as evidenced by suppressed α-SMA expression. PF inhibited phospo-Smad 2/3 by elevating Smad 7 level.*
Conclusion: *PF alleviates CCl_4-induced liver fibrosis in a dose-dependent manner probably by restoring the balance between activated Smad 2/3 and Smad7. Thus, PF might be a source of a novel anti-fibrotic agent.*

Keywords: *Paeoniflorin, Fibrosis, Liver, TGF-β, Hepatic stellate cell, Smad, Hydroxyproline*

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INTRODUCTION

Hepatic fibrosis is a major disease condition with severe morbidity. Hepatitis B and C virus infections, together with helminthic infection and biliary obstruction are the dominant causes of liver fibrosis [1]. Hepatic stellate cells (HSCs) play a pivotal role in the development of liver fibrosis. When activated, HSCs assume myofibroblast phenotype, resulting in rapid proliferation and massive collagen synthesis with attendant excessive extracellular matrix deposition. The collagen deposition could be degraded, thereby reversing the processes leading to the development of fibrosis, as evidenced in recent studies [2]. However, there is as at now no available therapeutic modality for preventing and/or effectively managing this condition. Therefore, there is urgent need to identify anti-fibrotic agents and to develop novel therapeutic approaches in the field of liver fibrosis management.

Cortex moutan is commonly used in Traditional Chinese Medicine for treating hepatic disorders. Paeoniflorin (PF), isolated from C*orte moutan*, is a major bioactive compound with anti-inflammatory and immune-regulatory effects

[3,4]. The anti-fibrotic effect of PF has been studied on both pulmonary [5,6] and renal interstitial fibrosis [7]. Studies of the effect of PF'on *Schistosomiasis japonica*-induced liver fibrosis have also been carried out [8,9].

The present study was aimed at examining the possible anti-fibrotic effect of PF in the liver and its underlying mechanisms of action.

EXPERIMENTAL

Chemicals and regents

Paeoniflorin (PF) ($C_{23}H_{28}O_{11}$, MW: 480.47, purity ≥ 98 %) was purchased from Nanjing Ze-lang Biotechnology Inc. (Nanjing, China). Other chemicals were products of Sigma-Aldrich (St. Louis, USA).

Animals and CCl_4-induced liver fibrosis model

Fifty male Sprague-Dawley rats (weighing 180-220 g) obtained from the Animal Center of Nantong University (Nantong, China), were used for this study. They were maintained on rat chow and water *ad libitum* while being acclimatized. After acclimatization for 7 days, rats were divided randomly into five experimental groups of 10 rats each: group1, the normal control; group 2, the negative control exposed to CCL_4 only; group 3, CCL_4 + 50 mg PF/kg /day; group 4, CCL_4 + 100mg PF/kg/day and group 5, CCL_4 + 200 mg PF/kg/day. Liver fibrosis was induced as previously described, with CCl_4 (30 % *v/v* diluted with corn oil) injection, twice a week for eight consecutive weeks [10]. Rats in the normal control group received equivalent volume of corn oil (CCL_4 vehicle), at the same frequency in which CCL_4 was administered to the test rats. The study protocol was approved by the local Ethical Committee of Changzhi Medical College (CZMC 2015/23) and the rats were treated in line with "National Research Council for Animal Care" [11].

Liver function assay

The rats were sacrificed, and blood samples were collected and centrifuged after clotting (3000 g, 4 °C). Serum AST and ALT were determined by automated analyzer (Roche, Indianapolis, USA).

Quantitative polymerase chain reaction (qPCR) assay

qPCR was performed with SsoFast EvaGreen Supermix (Bio-Rad, Hercules, USA). Primers were purchased from Sangon Biotechnology Inc. (Shanghai, China). These were: *Col1α1* (Forward: 5'-ACTCAGCCGTCTGTGCCTCA-3'; Reverse: 5'-GGAGGCCTCGGTGGACATTA-3'); *α-SMA* (Forward: 5'-CGAGAGGACGTTGTTAG CATAGAG-3'; Reverse: 5'-GGGCATCCACGAA ACCA-3') and *GAPDH* (Forward: 5'-AGTTCAACGGCACAGTCAAG-3'; Reverse: 5'-TACTCAGCACCAGCATCACC-3'). The fold change in target gene expression was analyzed by $2^{-\Delta\Delta Ct}$ method.

Hydroxyproline assay

Rats were sacrificed and, the livers excised and hydrolyzed with the hydrolyzing solution (1 mL) at 95 °C for 20 min in water. The content of hydroxyproline in the supernatant was analyzed by specific kit (Nanjing Jian-cheng, Nanjing, China), and was expressed as μg hydroxyproline /g liver.

Western blot assay

Western blot was conducted as described previously [12] with primary antibodies specific for α-smooth muscle actin (SMA), Smad 2/3, Smad 7, phospho-Smad 2/3 and GAPDH (Santa Cruz Biotechnology, Santa Cruz,USA).

Enzyme-linked immunosorbent assay (ELISA)

Rats were sacrificed and blood samples were collected, allowed to clot and centrifuged (3000 g, 4 °C). Serum TGF-β1 level in the supernatant was determined by specific ELISA kit (R&D, Minneapolis, USA).

Statistical analysis

All the studies were performed in triplicate and data are presented as mean ± S.D. One-way ANOVA followed by Dunnett's test was performed using SPSS version 19. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of PF on CCl_4-induced liver damage

The results of the effect of PF on CCL4- induced liver injury are shown in Figure 1. The serum of rats treated with CCl_4 only (the negative control group) had elevated ALT and AST activities compared to the normal control (3.15- and 2.96-fold, respectively). PF (100 and 200 mg/kg) treatment significantly reduced serum levels of both enzymes relative to the CCL_4 only group of rats.

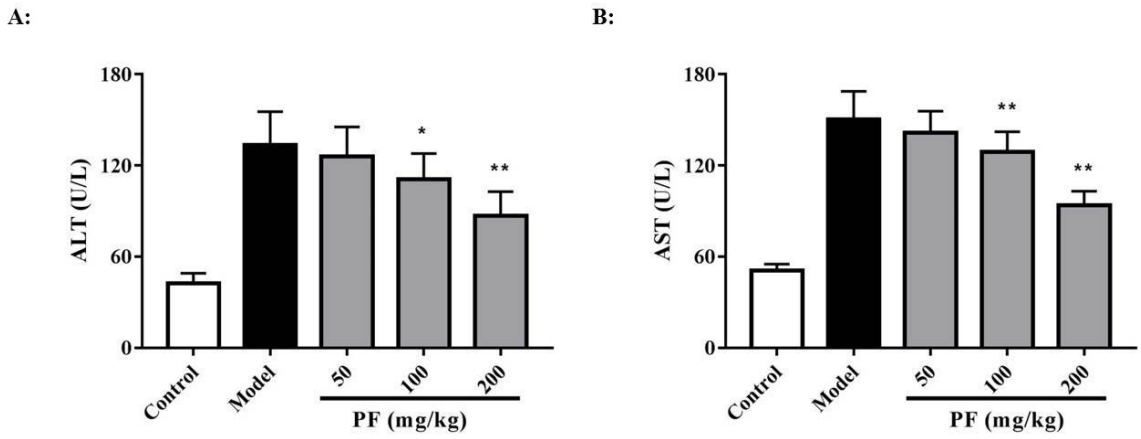

Figure 1: Serum levels of ALT (A) and AST (B). $^{**}p < 0.01$, $^{*}p < 0.05$

Effect of PF on collagen type I expression

Collagen type I is dominantly expressed as the extracellular matrix in fibrotic liver. The results of the analysis of the effect of PF on its biosynthesis are presented in Figure 2. The production of the mRNA of *Col1a1*, which is translated to give the α chain of collagen type I, was elevated 2.62-folds compared to the negative control group. PF (100 and 200 mg/kg/d) treatment significantly inhibited the *Col1a1* expression ($p < 0.05$ and $p < 0.01$, respectively).

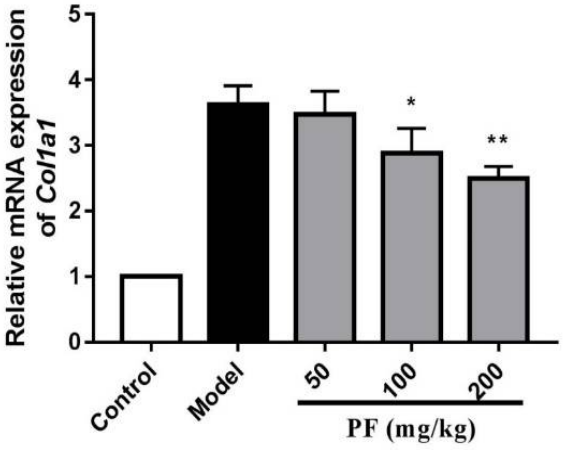

Figure 2: Expression of the mRNA of *Col1a1*
$^{**}p < 0.01$, $^{*}p < 0.05$

Effect of PF on collagen deposition

Hydroxyproline content is a common biomarker for assessing the severity of collagen deposition and hence is a test for liver fibrosis. As shown in Figure 3, PF (100 and 200 mg/kg) significantly reduced CCl_4-induced liver fibrosis as evidenced by the low level of hydroxyproline (reduced by 13.2 and 31.0% respectively).

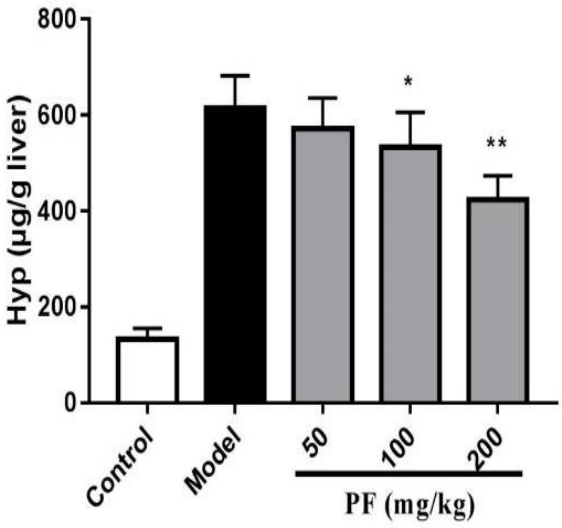

Figure 3: Rat liver hydroxyproline level
$^{**}p < 0.01$, $^{*}p < 0.05$

Effect of PF on HSCs activation

α-SMA is a typical marker for activated myofibroblast-like HSCs. As the results presented in Figure 4 show, α-SMA expressions were significantly decreased in a dose-dependent manner due to PF treatment, when compared to the negative control. Thus HSCs activation was partially suppressed.

Effect of PF on TGF-β1 expression

TGF-β1 plays a central role in activation of HSCs which culminates in liver fibrosis. As shown in Figure 5, TGF-β1 level was significantly elevated in rats treated only with CCl_4. PF (100 and 200 mg/kg/d) significantly decreased TGF-β1 expression by 19.5 and 25.6 %, respectively, relative to fibrotic rats (group 2).

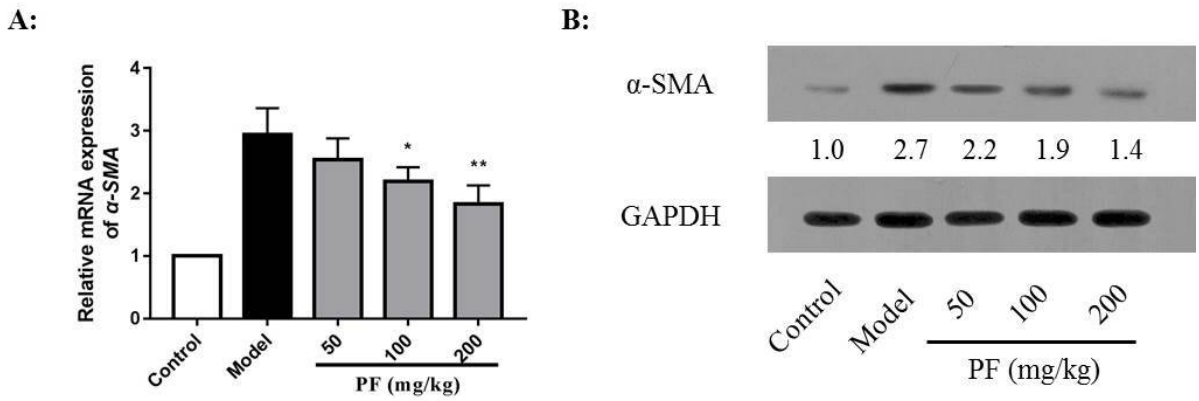

Figure 4: Expression of α-SMA mRNA (A) and its protein levels (B)
***p* < 0.01, **p* < 0.05

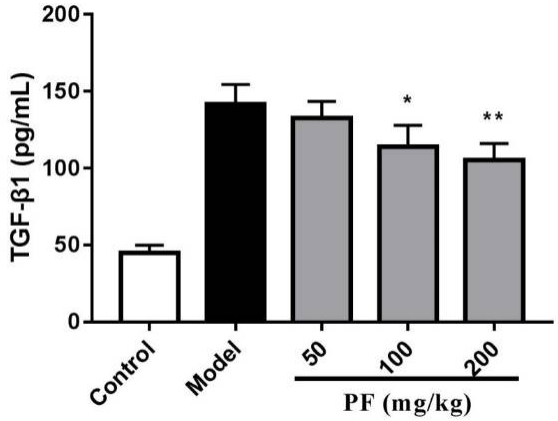

Figure 5: Effect of PF on serum TGF-β1 level
***p* < 0.01, **p* < 0.05

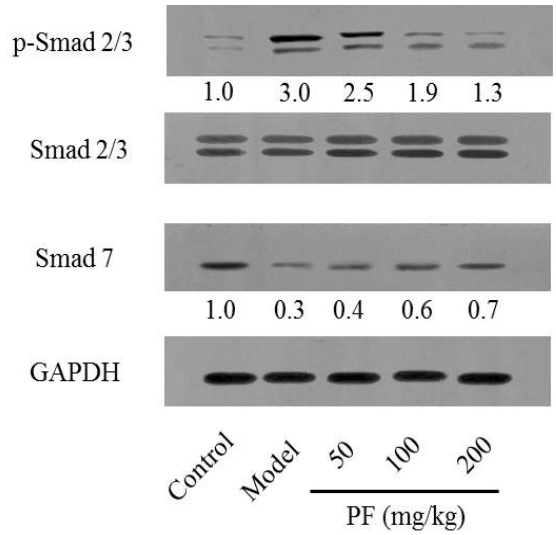

Figure 6: Western blots of TGF-β/Smad signaling pathway-associated proteins

Effect of PF on TGF-β/Smad signaling pathway

As shown in Figure 6, phosphor.-Smad 2/3 level was markedly increased in the group of rats treated with only CCl_4 when compared with the normal control value. In the group of rats exposed to carbon tetrachloride and the higher doses of PF, there were remarkable reductions in phosphor-Smad 2/3 levels. In the CCl_4-only group, there was corresponding reduction in inhibitory Smad 7 level which was partially reversed after PF treatment. This observation suggests that reversal of liver level of inhibitory Smad 7 is likely to be the underlying mechanism of the anti-fibrotic effect of PF.

DISCUSSION

It is currently believed that liver fibrosis could be reversed by collagen degradation. However, there is as at now no therapeutic agent(s) for such reversal. This study has revealed that PF isolated from *Cortex moutan*, could alleviate CCl_4-induced hepatic fibrosis, as evidenced by lower ALT and AST levels and hydroxyproline content. The expression of α-SMA both in form of mRNA and protein product was also suppressed by PF administration in a dose-dependent manner. This indicates that the HSCs were activated. Investigation of TGF-β/Smad signaling pathway and the findings appear to suggest that PF inhibited HSCs activation by up -regulating Smad 7 expression, and by so doing mitigated CCl_4-induced liver fibrosis.

The anti-fibrotic effects of *Paeonia lactiflora* and *Astragalus membranaceus* extract on CCl_4-induced rat liver fibrosis has been reported previously [13]. However, the bioactive agent(s) in the extracts with anti-fibrotic effect has remained unclear. It does appear, based on the findings in this study that PF might be the major phytochemical entity in *Paeonia lactiflora* and *Astragalus membranaceus* extracts responsible for the anti-fibrotic bioactivity. This views is supported by the fact that it had similar effects on

both pulmonary [5,6] and renal interstitial fibrosis [7].

The activation of fibroblast HSCs is one of the central events in the fibrotic process. Activated HSCs exhibit a myofibroblast-like phenotype with positive α-SMA expression and excessive collagen secretion [2]. Among the central events, TGF-β1 is a key mediator that has been given considerable attention by a sizable number of research scientists. The chronic chemical assaults in the liver cause the mobilization and activation of macrophages. Activated macrophage is the primary cell type of TGF-β1 secretion. The autocrine of activated HSCs is another source of TGF-β1 with positive feedback control of the TGF-β/Smad signaling pathway [14]. In the current study, TGF-β1 was down-regulated in a dose-dependent way after PF administration. The findings in this study could be attributed to the inhibition of both the macrophages and the autocrine of HSCs, as well as the anti-inflammatory effect of PF, which have been widely reported [3,15].

The *in vitro* effect of PF on TGF-β/Smad pathway has been reported previously, based on the induction of *S. japonicum* egg antigen which has the ability to induce liver fibrosis also [16]. Chronic assaults on the liver, whether from the egg antigen of *S. japonicum* or hepatitis B virus infection, contribute to the activation of TGF-β/Smad pathway. Bound by TGF-β1, the TGF-β receptors can phosphorylate Smad 2/3. Phosphor-Smad 2/3 binds to Smad 4 and translocates to specific promoter region in the nucleus to induce collagen expression [17]. As an inhibitory molecule, the deletion of Smad7 gene product accentuates CCl_4-induced liver fibrosis [18]. In addition, overexpression of Smad7 attenuated TGF-β/Smad pathway in CCl_4-induced liver fibrosis model [19]. Although the underlying mechanisms are well elucidated, the development of anti-fibrotic agents, which target TGF-β/Smad remains unexplored. In the current work, marked elevation of phosphor-Smad 2/3 accompanied by down -regulation of Smad 7 as observed following exposure to CCl_4, indicate that there was an imbalance between activated Smad 2/3 and Smad7. However, PF reversed the imbalance in a dose-dependent manner, and thus suppressed TGF-β/Smad pathway activation and further downstream events.

CONCLUSION

The findings of the present work demonstrate that PF is a potential anti-fibrotic agent. Smad7 induction and the consequent restoration of the balance between activated Smad 2/3 and Smad 7, is the probable underlying mechanism by which PF alleviates liver fibrosis. PF could therefore be a possible therapeutic agent for liver fibrosis management.

DECLARATIONS

Acknowledgement

None.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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