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Original Article

Effects of swertiamarin on TGF-β1/Smad signaling pathway in rats with carbon tetrachloride-induced liver fibrosis

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Abstract: *Background & aims:* Swertiamarin (SM) is a typical iridoid isolated from *Swertia mussotii* Franch (Gentianaceae) exerting broad pharmacological activities including hepatoprotective effect. SM was evaluated for anti-fibrotic effect and mechanism in rats with carbon tetrachloride-induced liver fibrosis in this study. Methods: Adult male Sprague-Dawley rats were randomly divided into control group, SM 200 mg/kg group, CCl_4 group, CCl_4+ SM 100 mg/kg group and CCl_4+ SM 200 mg/kg group. Rats in experimental groups were subcutaneously injected with 40% CCl_4 twice weekly for eight weeks to develop liver fibrosis. SM (100 and 200 mg/kg per day) was orally given to experimental rats by gavage for eight consecutive weeks. The histopathology of the liver tissue was evaluated by use of hematoxylin-eosin (H&E) staining. Immunohistochemical examination was carried out to detect the protein expressions of collagen I, collagen III and TGF-β1 in the liver, and the mRNA expressions of these genes were determined in the liver by real time PCR. The protein expression of p-Smad2 and p-Smad3 was determined in the liver by Western blot analysis. Results: SM ameliorated histological changes and significantly suppressed collagen deposition. Immunohistochemical staining revealed that collagen I, collagen III and TGF-β1 expression were decreased with SM treatment. SM significantly reduced the mRNA expression of collagen I, collagen III and TGF-β1 in the liver. Western blot assay showed p-Smad2 and p-Smad3 expression was significantly decreased after SM treatment. Conclusion: SM is able to attenuate CCl_4 induced liver fibrosis in rat at least partly through its inhibition of TGF-β1/Smad signaling pathway.

Keywords: Swertiamarin, liver fibrosis, collagen, TGF-β1, Smad

Introduction

Swertiamarin (SM) (**Figure 1**) is a typical iridoid isolated from *Swertia mussotii* Franch (Gentianaceae), one of the original plants of Tibetan medicine "Zangyinchen" [1]. SM exerts broad pharmacological activities, particularly hepatoprotective effects [2-4]. SM has been reported to alleviate dimethylnitrosamine (DMN)-induced rat hepatic fibrosis previously [5]. However, the effect and underlying mechanism of SM on carbon tetrachloride-induced liver fibrosis remains unclear.

Liver fibrosis remains a major health problem arise from chronic liver injury caused by a variety of pathogenic factors including viruses, alcohol, metabolic syndrome and autoimmune diseases [6]. Chronic liver injury, as triggered by different etiologies, induces repetitive tissue damage, resulting in impaired regenerative capacity marked by an altered inflammatory infiltrate and a chronic wound healing response [7]. Although initially beneficial, the wound healing process becomes pathogenic if it progressively replaces parenchyma with scar tissue and distorts the liver vascular architecture. The fibrogenic response, as characterized by scar formation due to increased production and deposition of extracellular matrix (ECM) proteins, is the essential step that culminates in major changes in liver architecture. The activated hepatic stellate cell (HSC) is the key fibrogenic effector cell type in the liver and the major producer of scar ECM [8]. Multiple cytokines participate in the process of liver fibrosis in auto-

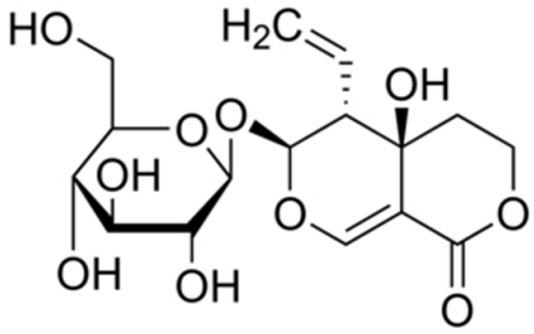

Figure 1. Chemical structure of swertiamarin (SM).

crine and paracrine dependent manners [9]. TGF-β1 has been identified as the most profibrotic cytokine, and can elevate the expression of collagen I in HSC, promote their transition to a myofibroblast-like phenotype, and modulate key elements of ECM in the homeostasis [10, 11]. Although lots of preclinical researches have led to considerable improvements in the understanding of liver fibrosis pathogenesis, specific biomarkers to measure fibrosis progression and effective antifibrotic approaches are still lacking [12]. Some herbal medicines have been demonstrated to improve the experimental liver fibrosis effectively, indicating the therapeutic potential of natural drugs [13-15].

Carbon tetrachloride (CCl_4) is an extensively used hepatotoxic agent to induce liver fibrosis in rodents. Repeated CCl_4 exposure leads to repeated rounds of wound-healing, HSC activation, imbalance between ECM production and degradation, thus inducing hepatic fibrosis [16]. Since the pathological lesions developed in CCl_4-treated rats and mice closely resemble the symptoms of liver cirrhosis in human, this agent serves as an excellent model for determining mechanisms of fibrosis and for evaluating the efficacies of novel hepatoprotectants [17, 18].

In this study, we investigated the ability of SM to protect against CCl_4-induced liver fibrosis in rats, and the impact of SM on TGF-β/Smad signal pathway.

Materials and methods

Chemicals and reagents

Swertiamarin (purity >98%, dissolved in 1% Tween-20 saline) was provided by Xian Jiatian Biotechnology Co., Ltd (Xian, China). Carbon tetrachloride was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Rabbit anti-TGF-β1 and mouse anti-collagen I antibodies were purchased from Santa Cruz Biotechnology Inc (California, USA). Rabbit anti-collagen III antibody was purchased from BOSTER Biotechnology., Ltd (Wuhan, China). Rabbit anti-p-Smad2 and rabbit anti-p-Smad3 antibodies were purchased from Cell Signaling Technology (MA, USA). Rabbit anti-GAPDH was purchased from Beijing Biosynthesis Biotechnology., Ltd (Beijing, China).

Animals and groups

This study was carried out in strict accordance with the guideline of the Council on Animal Care of Academia Sinica. The protocol was approved by the Ethical Committee on Animal Ex--perimentation of Puai Hospital, Huazhong Un iversity of Science and Technology, China. Sixty male Sprague-Dawley rats weighing 250-280 g were obtained from the Center of Experimental Animal of Hubei Province (Wuhan, China). All animals were kept under the same laboratory conditions of temperature (25 ± 2°C) and lighting (12:12 h light: dark cycle), and were given free access to standard laboratory chow and tap water. All rats were allowed to acclimatize for one week before experiment. The animals were randomly assigned to five experimental groups, namely:

Control group, rats were given 1% Tween-20 saline by gavage once per day for consecutive eight weeks with co-administration of vehicle (peanut oil, solvent of Carbon tetrachloride, 0.3 mL/100 g, s. c. twice a week).

SM 200 mg/kg group, rats were given SM dissolved in 1% Tween-20 saline (200 mg/kg B.W.) by gavage once per day for consecutive eight weeks with co-administration of vehicle (peanut oil, solvent of Carbon tetrachloride, 0.3 mL/100 g, s. c. twice a week).

CCl_4 group, rats were given 1% Tween-20 saline by gavage once per day for consecutive eight weeks with co-administration of 40% CCl_4 mixed peanut oil solution (0.3 mL/100 g, s. c. twice a week).

CCl_4+SM 100 mg/kg group, rats were treated with SM dissolved in 1% Tween-20 saline (100 mg/kg B.W.) by gavage once per day for consecutive eight weeks with co-administration

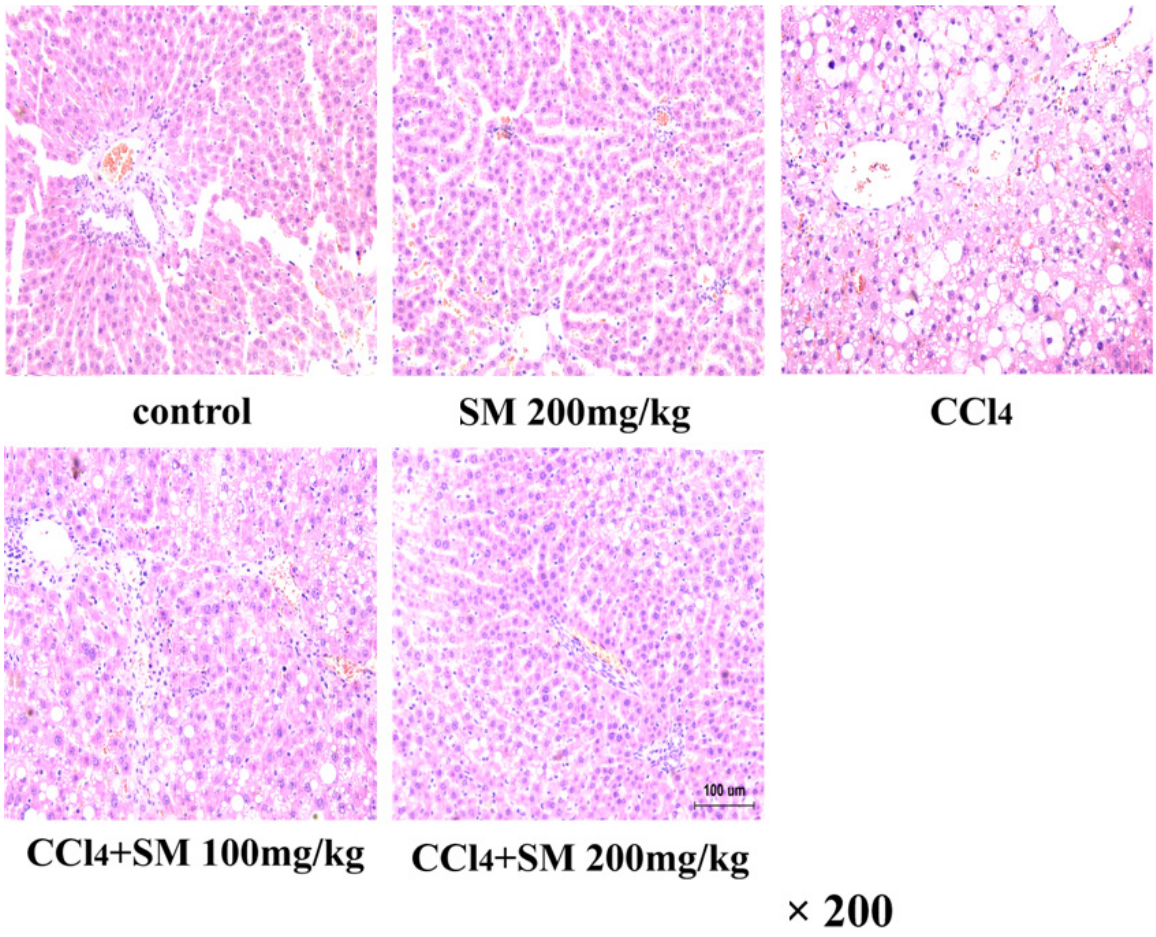

Figure 2. Histological images of liver samples. Control and SM 200 mg/kg: normal liver histology without fibroplasia and inflammatory cell infiltration; CCl_4: The normal structure of hepatic lobules was damaged with many inflammatory cells infiltration; hepatic fibrosis, hepatocytes degeneration/necrosis, fatty changes and infiltration of lymphocytes were observed; CCl_4+SM 100 mg/kg: comparatively low degree of fibrotic changes with SM treatment (100 mg/kg B.W.); CCl_4+SM 200 mg/kg: relieved liver histopathology with SM treatment (200 mg/kg B.W.). (H-E, original magnification × 200).

of 40% CCl_4 mixed peanut oil solution (0.3 mL/100 g, s. c. twice a week).

CCl_4+SM 200 mg/kg group, rats were treated with SM dissolved in 1% Tween-20 saline (200 mg/kg B.W.) by gavage once per day for consecutive eight weeks with co-administration of 40% CCl_4 mixed peanut oil solution (0.3 mL/100 g, s. c. twice a week).

Rats were fasted for 12 h after the last dose of agents before they were anaesthetized with urethane (1 g/kg B.W., intraperitoneally). The livers were removed and used for histological assessment and the measurements of protein expression. The livers were stored at -80°C until use.

Histological examination

Liver samples were derived from the central part of the right large lobe of the rats. For hematoxylin-eosin (H&E) staining, the liver tissues were fixed with 10% formalin for 24 h, and then washed with tap water, dehydrated and embedded in paraffin. At last, each slide was subjected to histological assessment successively.

Measurement of hepatic hydroxyproline content

The intrahepatic content of hydroxyproline was determined using a commercially available kit (Jiancheng Institute of Biotechnology, Nanjing, China) following the manufacturer's instruc-

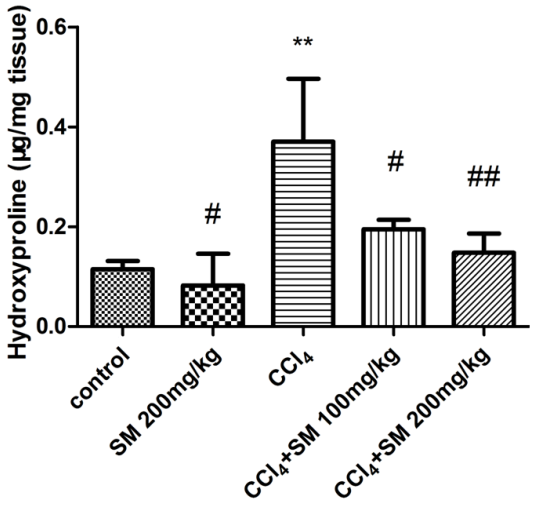

Figure 3. Effect of SM on hepatic hydroxyproline content. The hydroxyproline content of rats in CCl_4+SM 100 mg/kg and CCl_4+SM 200 mg/kg groups were significantly lower than that in CCl_4 group. Data are represented as means ± S.D. for 7-12 animals per group. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ versus CCl_4, $^{\#\#}P < 0.01$ versus CCl_4 by one-way ANOVA and LSD post hoc test.

tions. Final data are represented as μg/mg tissue.

RNA extraction and Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from liver samples using TRIzol (Invitrogen, Carlsbad, CA) according to standard protocol. cDNA was produced by using the SuperScript Preamplification System for first-standing cDNA synthesis. Real-time quantitative RT-PCR was performed on cDNA samples using the MiniOpticom RT-PCR System (Bio-Rad Laboratories, Hercules, CA, USA). Quantification of the expression rat genes was performed by RT-qPCR using the following sense and antisense primers: TGF-β1, 5'-ctttaggaaggacctgggttg-3' and 5'-ggttgtgttggtgtagaggg-3'; collagen I, 5'-caggttgcagccttggttagg-3' and 5'-agaggcataaagggtcatcgtg-3'; collagen III, 5'-cattgcgtccatcaaagcctc-3' and 5'-gtcggaggaatgggtggctat-3'; GAPDH, 5'-ttcctaccccaatgtatccg-3' and 5'-catgaggtccaccaccctgtt-3'. The amplified product size by each pair of primers was 140, 540, 322 and 281 bp for TGF-β1, collagen I, collagen III and GAPDH respectively. At the end of experiments, PCR products were removed from tubes and analyzed by gel electrophoresis to confirm the product of interest. GAPDH was used as an endogenous reference gene. Quantification of the target cDNAs in samples was normalized to the reference gene RNA (Ct_{target} - $Ct_{reference}$ genes = ΔCt) and the difference in expression for each target cDNA in the treated groups was expressed to the amount in the control group ($\Delta Ct_{treated}$ - $\Delta Ct_{control}$ = ΔΔCt). Fold changes in target gene expression were determined by taking 2 to the power of this number ($2^{-\Delta\Delta Ct}$).

Immunohistochemistry

Livers from rats were perfused with phosphate-buffered saline (PBS) and sliced and fixed with 4% parafoamaldehyde. The liver samples were incubated overnight in PBS with 6.8% sucrose, dehydrated with acetone and embedded. Before staining, semithin sections were incubated for 5 min at 37°C in 0.01% trypsin/0.1% $CaCl_2$ (PH 7.8). Sections were incubated for 5 h at 37°C with TGF-β1, collagen I and collagen III antibodies and then treated with corresponding secondary antibodies, followed by an incubation with peroxidase-antiperoxidase at a dilution of 1:100 for 1 h at 37°C. At last, the immunolabeling was visualized by incubation with 3,3-diaminobenzidine-H_2O_2 medium for 10 min at room temperature. Images were captured with an OLYMPUS photomicroscope with digital camera.

Western blot analysis

The whole liver lysate was prepared to evaluate the expression level of p-Smad2 and p-Smad3. The protein concentration was determined using the bicinchoninic acid (BCA) assay and samples were stored at -80°C. An equal amount of membrane protein (100 μg) per lane was separated with 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. After electrophoresis, the gels were transferred onto polyvinylidene difluoride membranes, which were blocked with Tris-buffered saline containing 5% nonfat milk at 4°C. Then, the membranes were incubated overnight at 4°C in solution containing 0.1% Tween 20, 5% nonfat milk and the following primary antibodies: p-Smad2 (1:400); p-Smad3 (1:400); GAPDH (1:20000). After three washes in Tris-buffered saline Tween 20, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature and subsequently processed for enhanced chemiluminescence (ECL) detection using potent ECL kit (MUL-

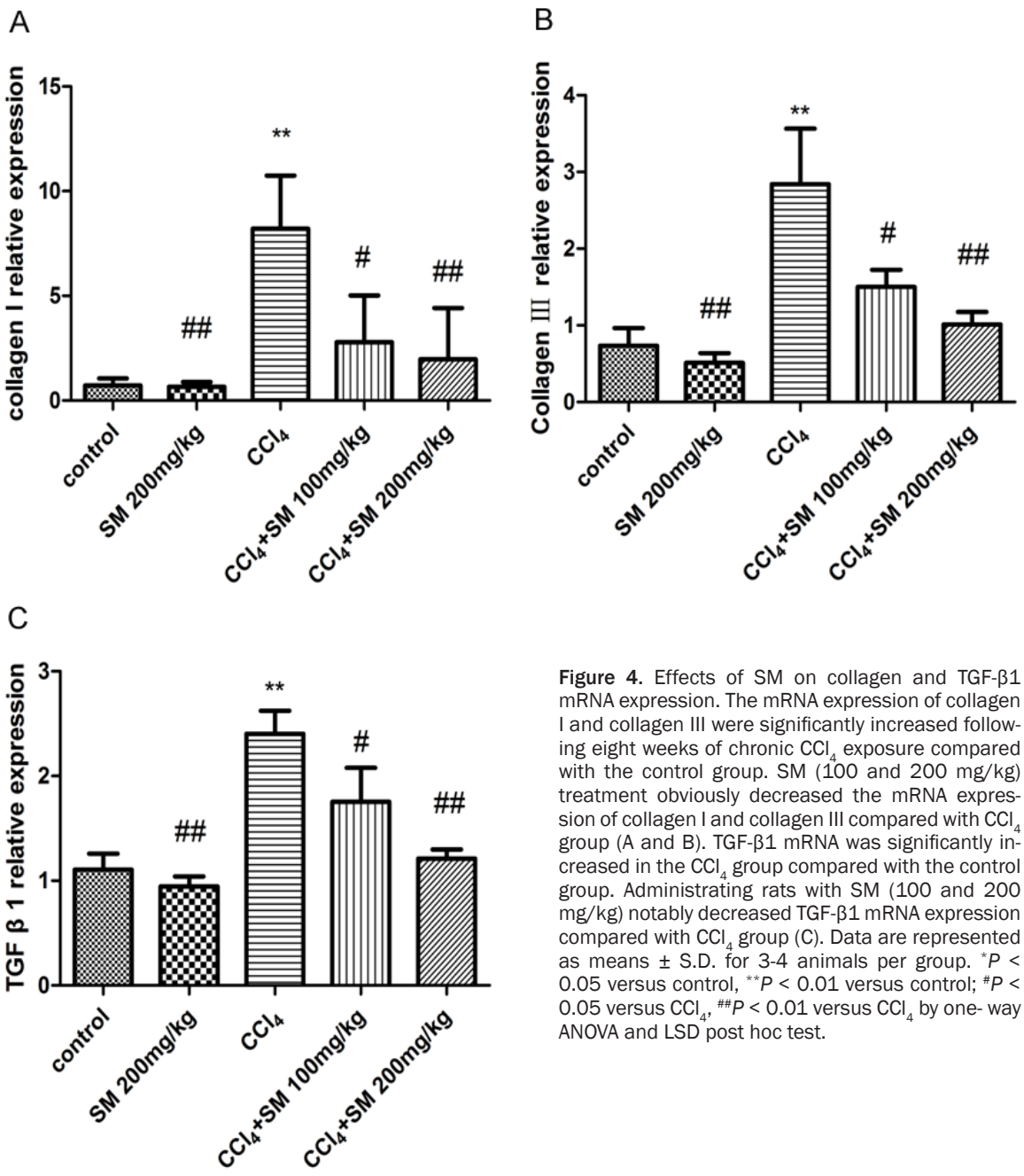

Figure 4. Effects of SM on collagen and TGF-β1 mRNA expression. The mRNA expression of collagen I and collagen III were significantly increased following eight weeks of chronic CCl_4 exposure compared with the control group. SM (100 and 200 mg/kg) treatment obviously decreased the mRNA expression of collagen I and collagen III compared with CCl_4 group (A and B). TGF-β1 mRNA was significantly increased in the CCl_4 group compared with the control group. Administrating rats with SM (100 and 200 mg/kg) notably decreased TGF-β1 mRNA expression compared with CCl_4 group (C). Data are represented as means ± S.D. for 3-4 animals per group. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ versus CCl_4, $^{\#\#}P < 0.01$ versus CCl_4 by one- way ANOVA and LSD post hoc test.

TISCIENCES, China) and a chemiluminescence detection system (IS4000MM Pro, Kodak, USA). GAPDH was used as an internal index.

Statistical analysis

Data were expressed as mean ± S.D. The significant differences between groups were assessed with SPSS version 13.0. The differences between group means were calculated by one-way ANOVA with LSD post hoc analysis. Difference was considered statistically significant when $P < 0.05$, and extremely significant when $P < 0.01$.

Results

Histological changes

The hepatic samples of the control and SM 200 mg/kg groups presented livers with normal architecture. However, liver tissues from rats treated with CCl_4 showed extensive histopathological changes, characterized by severe hepa-

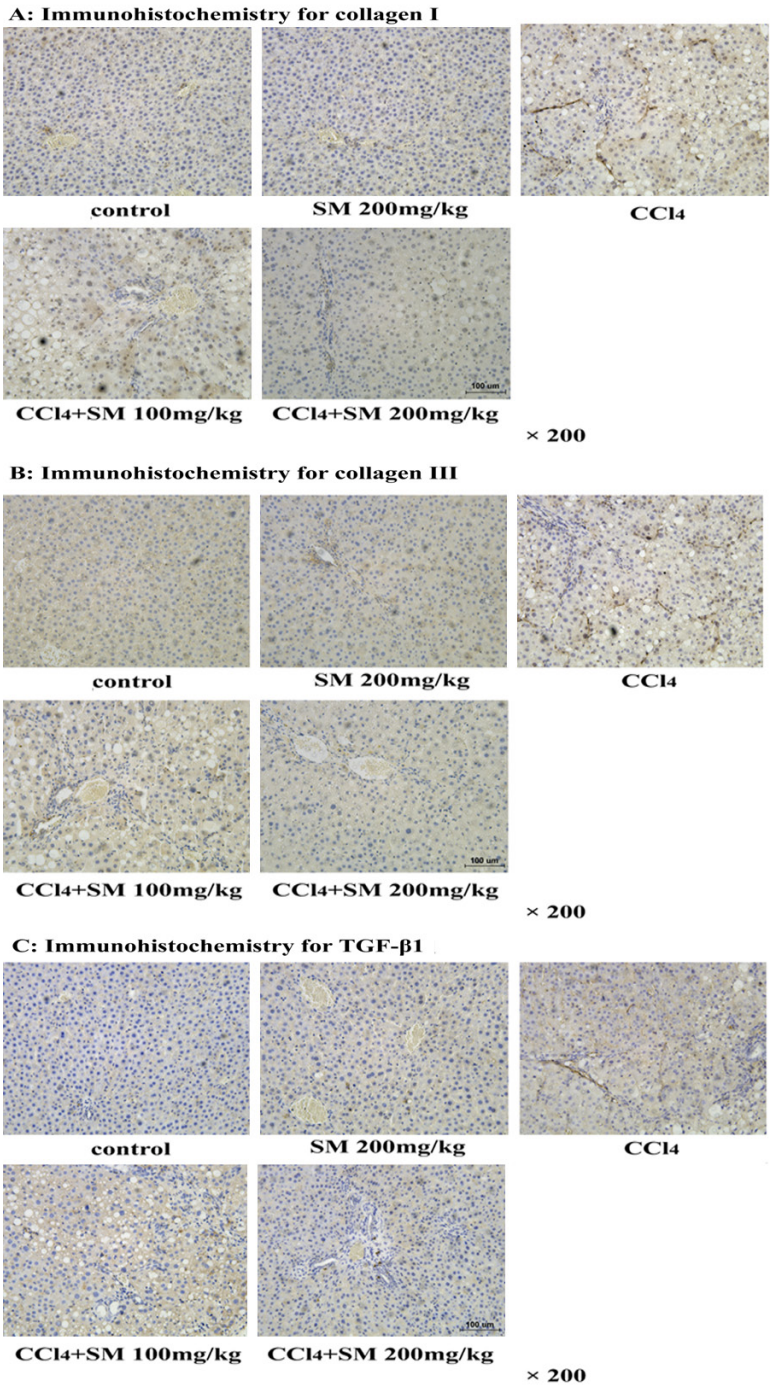

Figure 5. Immunohistochemical staining of collagen and TGF-β1 in the rat liver after SM treatment. Collagen fibers were observed comparably in rats after the long-term CCl_4 injection, whereas no fibrosis was observed in control and SM 200 mg/kg group. And the extent of fibrosis in CCl_4+SM 100 mg/kg and CCl_4+SM 200 mg/kg groups was significantly alleviated than CCl_4 group, which was confirmed by IHC for collagen I and collagen III (A and B). TGF-β1 was overexpressed in CCl_4 group, and mainly distributed in cells surrounding the portal area, sinusoidal endothelial cells, some hepatocytes and fiber septum. The TGF-β1 positive cells reduced significantly in CCl_4+SM groups when compared with CCl_4 group (C). Immunohistochemistry was performed to detect the protein expression of collagen I, collagen III (A-B) and TGF-β1 (C) in control group, SM 200 mg/kg group, CCl_4 group, CCl_4+SM 100 mg/kg and CCl_4+SM 200 mg/kg group. Positive cells had brown granules (Magnification × 200).

tocytes degeneration/necrosis, fatty changes, inflammatory cell infiltration and congestion. The incidence and severity of histopathological lesions in CCl_4+ SM groups were less than those in the CCl_4 group (**Figure 2**).

Measurement of hepatic hydroxyproline content

The content of hydroxyproline in the liver can display the content of collagen in the liver, and reflect the degree of hepatic fibrosis directly. The hydroxyproline content of rats in CCl_4+SM 100 mg/kg and CCl_4+SM 200 mg/kg groups (0.19 ± 0.02 μg/mg, 0.15 ± 0.04 μg/mg) were significantly lower than that in CCl_4 group (0.37 ± 0.13 μg/mg, $P < 0.05$), indicating the improvement of the degree of hepatic fibrosis with SM treatment (**Figure 3**).

Effects of SM on the expression of collagen and TGF-β1

The mRNA expression of the two prototypical profibrotic markers, collagen I and collagen III, in the hepatic tissues was significantly increased following eight weeks of chronic CCl_4 exposure compared with the control group. And SM (100 and 200 mg/kg) treatment obviously decreased the mRNA expression of collagen I and collagen III compared with the CCl_4 group (**Figure 4A** and **4B**).

TGF-β1 mRNA was significantly increased in the CCl_4 group compared with the control group. Administrating rats with SM (100 and 200 mg/kg) or SM alone notably decreased TGF-β1 mRNA expression compared with the CCl_4 group (**Figure 4C**).

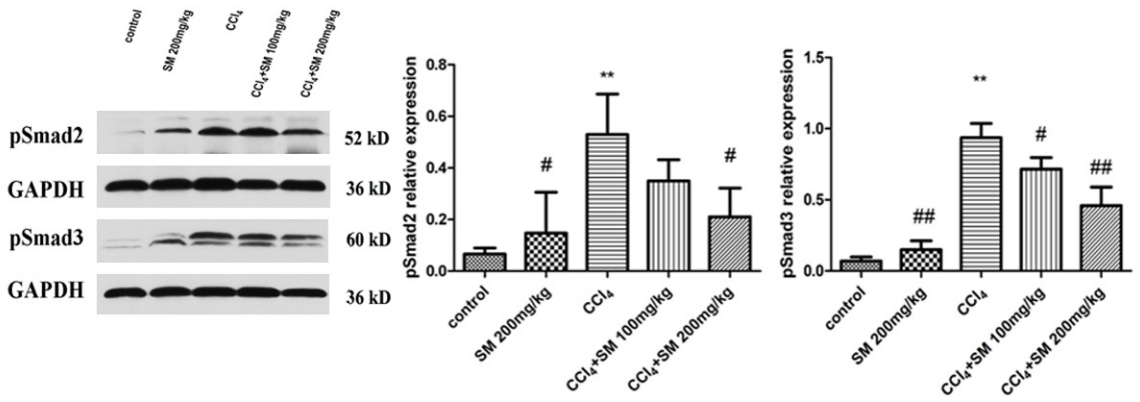

Figure 6. Western blot assay of pSmad2/3 expression in the rat liver after SM treatment. The expression of pSmad2, the activated form of Smad2, increased following CCl_4 treatment. The expression of pSmad2 was decreased in CCl_4+SM groups compared with CCl_4 group. And CCl_4 treatment also obviously elevated the expression of pSmad3 compared with the control group. In CCl_4+SM groups, pSmad3 expression was decreased significantly compared with the CCl_4 group. Data are represented as means ± S.D. for 3-4 animals per group. $^{*}P < 0.05$ versus control, $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ versus CCl_4, $^{\#\#}P < 0.01$ versus CCl_4 by one- way ANOVA and LSD post hoc test.

Detection of liver collagen deposition and TGF-β1

Liver fibrosis is characteristic of extra collagen deposition in extracellular matrix (predominantly type I and III fibrillar collagens) [19]. Collagen fibers were observed comparably in rats after the long-term CCl_4 injection, whereas no fibrosis was observed in control and sertiamarin 200 group. And the extent of fibrosis in CCl_4+SM 100 mg/kg and CCl_4+SM 200 mg/kg groups was significantly alleviated than CCl_4 group, which was confirmed by IHC for collagen I and collagen III (**Figure 5A** and **5B**).

In control group, TGF-β1 protein expression was only detectable in cells surrounding the portal vein and central veins of hepatic lobules with a small amount of TGF-β1 surrounding cells. In CCl_4 group, TGF-β1 was overexpressed, and mainly distributed in cells surrounding the portal area, sinusoidal endothelial cells, some hepatocytes and fiber septum. The TGF-β1 positive cells reduced significantly in CCl_4+SM groups when compared with CCl_4 group (**Figure 5C**).

Effects of swertiamarin on the expression of pSmad2/3

TGF-β1 acts through a very well described classical signaling pathway that involves the phosphorylation and activation of Smad2 and Smad3 [20]. Our results showed the expression of pSmad2, the activated form of Smad2, increased following CCl_4 treatment. The expression of pSmad2 was decreased in CCl_4+SM groups. And CCl_4 treatment also obviously elevated the expression of pSmad3 compared with the control group. In CCl_4+SM groups, pSmad3 expression was decreased significantly compared with the CCl_4 group (**Figure 6**).

Discussion

The main finding of this study was that swertiamarin (SM) was instrumental in attenuating liver injury in rats induced by a long-term CCl_4 exposure. In the present study, a chronic CCl_4 treatment resulted in a significant increase in hepatic hydroxyproline levels, a marker of fibrosis and the expression of other prototypical profibrotic markers such as collagen I and collagen III. Besides, severe liver lesion was also induced by CCl_4. All of the above-mentioned pro-fibrotic changes were significantly abrogated with SM treatment, suggesting that SM is able to attenuate CCl_4 induced liver fibrosis in rats.

Hepatic fibrosis refers to the liver dysfunction characterized by excess ECM deposits. The over-expression of ECM leads to liver fibrosis, in which other stress events enhance liver injury, such as immune cell infiltration [21]. The major producer of scar ECM in liver is the activated HSC, although other cells and processes can make significant contributions. HSC is characterized by the ability to store retinyl esters in intra cytoplasmic lipid droplets, and by ultrastructural features of vascular pericytes consistent with their role in regulating sinusoidal blood flow [22]. In the normal liver, HSC display

a quiescent phenotype, characterized by the expression of a large number of adipogenic genes and neural markers. Once HSC is activated, collagen or fibers would deposit in perisinusoidal spaces, progressively forming liver fibrosis [23]. Upon acute or chronic liver injury, a complex network of autocrine/paracrine fibrogenic signals promotes transdifferentiation of quiescent HSC to a myofibroblastic phenotype characterized by the expression of α-smooth muscle actin and a parallel loss of retinoids and lipid droplets [24]. The transition of HSC into myofibroblasts is regulated by the interaction with several cell types and the activation of specific signaling pathways. Besides injured hepatocytes, hepatic macrophages, endothelial cells, and lymphocytes drive HSC activation. The death of hepatocytes leads to the release of cellular contents (e.g. DNA and damage-associated molecular patterns known as DAMPs) and reactive oxygen species that activate resident macrophages (Kupffer cells) to release pro-inflammatory factors like TNFa, IL-1b, and IL-6, and pro-fibrogenic factors, especially TGFβ [25].

TGF-β1 is closely related to the progression of liver fibrosis. Castilla et al. found that TGF-β1 mRNA expression correlated closely with the mRNA expression of procollagen Type I and serum procollagen Type III peptide in 42 patients with chronic hepatitis and cirrhosis [26]. Many fibrotic diseases are associated with increased levels of TGF-β which initially recruit inflammatory cells and fibroblasts into an area of injury and then stimulate these cells to produce cytokines and extracellular matrix, respectively [27]. Animal model experiments have suggested an important role for TGF-β in the pathogenesis of fibrosis [28]. And the TGF-β1 expression was significantly elevated in rats with CCl_4-induced liver fibrosis [29]. TGF-β1 exerts its biological properties by binding to high-affinity receptors with intrinsic serine/threonine kinase activity and subsequently activates intracellular signaling intermediates called Smad proteins, which modulate the transcription of target genes. Members of the TGF-β superfamily elicit signaling through distinct combinations of transmembrane type I (TβRI) and type II receptors (TβRII) [30]. Upon TGF-β1 binding to the type II receptor, the type II receptor kinase phosphorylates the GS domain of type I receptor, leading to the activation of type I receptor [31]. Activated type I receptors then trigger the downstream Smad signaling pathway by phosphorylating Smad2 and Smad3 at two serine residues in the SSXS motif of their Cterminal [32]. Phosphorylated Smad2 and Smad3 form oligomeric complexes with Smad4, which then translocate into the nucleus and mediate the transcriptional regulation of target genes [33]. Liu et al. proposed that inhibiting TGFβ-induced Smad2/Smad3 phosphorylation and nuclear translocation in HSCs would attenuate hepatic fibrosis [34]. And application of Smad2/Smad3 antisense oligonucleotides or cDNA is also effective to block the biological functions of TGF-β [35]. Whereas TGF-β1 is the most potent stimulus to the synthesis of collagen I and other matrix constituents, inhibiting its actions remain a major focus of antifibrotic therapy in liver fibrosis [36]. In order to further elucidate the mechanism by which SM protects against liver fibrosis, the impact of SM on the downstream transcription factors pSmad2/3 was investigated. SM treatment significantly inhibited pSmad2 and pSmad3 expression, highlighting a potential anti-fibrotic mechanism.

In conclusion, SM effectively ameliorates the CCl_4-induced liver fibrosis in rats through inhibiting collagen I and III expression and inhibiting TGF-β/Smad signal pathway. Targeting a single pathway may be of limited efficacy, and further studies on the possible effect of SM on multiple cell signaling pathways involved in the pathogenesis of liver fibrosis are still ongoing.

Disclosure of conflict of interest

None.

Authors' contribution

Hongping Song as the principal director was responsible for the design of the study. Kang Chen, Tao Wu and Ruyi Zhang did the whole experiments of the study and wrote the manuscript. All authors participated in the preparation of, and have approved the final version of the manuscript.

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