Biomedicine & Pharmacotherapy 106 (2018) 426–433

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Flavonoid-rich Scabiosa comosa inflorescence extract attenuates CCl_4-induced hepatic fibrosis by modulating TGF-β-induced $Smad_3$ phosphorylation

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ARTICLE INFO

Keywords:
Scabiosa comosa
Flavonoids
Liver fibrosis
TGF-β1
Smad3

ABSTRACT

Scabiosa comosa inflorescence is a traditional Mongolian medicine in the treatment of liver diseases. In the study, we investigated the anti-fibrotic efficacy of flavonoid-rich Scabiosa comosa inflorescence extract (TF-SC) in a rat model of CCl_4-induced hepatic fibrosis and explored its underlying mechanism in vitro and in vivo. Rats (Wistar, Male, weight 200–250 g) were injected intraperitoneally with CCl_4 (1:1v/v in peanut oil, 2 mL/kg body weight) to induce liver fibrosis, followed by treatment with TF-SC or vehicle. In addition, transforming growth factor-β1 (TGF-β1)-activated hepatic stellate cells (HSCs) were used for measuring Smad3 phosphorylation. We found decrease in liver function and liver fibrosis markers in serums. Also, TF-SC decreased hydroxyproline content and collagen deposition in liver tissues. TF-SC also decreased the expression of α-SMA, collagen I and fibronectin in CCl4-induced hepatic fibrosis rats. Mechanistically, TF-SC attenuated liver fibrosis by selectively inhibiting Smad3 phosphorylation. In TGF-β1-stimulated HSCs, TF-SC blocked the interaction between Smad3 and TGF-β type I receptor (TβRI), suppressed subsequent phosphorylation and nuclear translocation of Smad3, and down-regulated the transcription of fibrotic genes. In conclusion, the study demonstrated that TF-SC was an effective therapeutic agent for treatment of hepatic fibrosis, and provided a molecular basis through which TF-SC exerts its anti-fibrotic effects.

1. Introduction

Liver fibrosis, characterized by excessive deposition of the extracellular matrix (ECM), is the essential pathophysiologic consequence of chronic hepatic injury [1,2]. Advanced liver fibrosis can lead to cirrhosis, resulting in a high-risk of portal hypertension, liver failure and hepatocellular carcinoma [3,4]. Thus, there is an urgent need to develop effective anti-fibrotic strategies.

Activated hepatic stellate cells, which express alpha-smooth muscle actin (α-SMA) and profibrogenic genes, are the main source of collagen during liver fibrosis [5], and are considered as a therapeutic target for treatment of hepatic fibrosis [6]. Numerous studies have established the crucial role of TGF-β1 in mediating inflammation, HSCs activation, and ECM accumulation [7–9]. HSCs change from a non-proliferating cell type into a proliferating activated phenotype after TGF-β1 stimulation, which in turn lead to liver fibrosis [10,11]. TGF-β1 signaling is transduced by transmembrane serine/threonine kinase receptors type I (TβRI) and type II (TβRII) and intracellular mediators known as Smads [12,13]. Upon TGF-β1 stimulation, Smad2 and Smad3 are phosphorylated by TβRI. Phosphorylated Smad2/3 heteroligomerizes with Smad4 and then translocate into the nucleus, leading to the expression of EMT-related genes [14]. Thus, TGF-β1/Smads signaling might be a therapy against for the intervention of liver fibrosis.

Based on particular efficacy, flavonoids have been considered as

Abbreviations: TF-SC, total flavonoids of *scabiosa comosa* inflorescences; TGF-β1, transforming growth factor-β1; HSCs, hepatic stellate cells; TβRI, TGF-β type I receptor; TβRII, TGF-β type II receptor; ECM, extracellular matrix; α-SMA, alpha-smooth muscle actin; Hyp, hydroxyproline

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https://doi.org/10.1016/j.biopha.2018.06.118
Received 8 February 2018; Received in revised form 21 June 2018; Accepted 22 June 2018

new therapies for liver diseases. The inflorescence of *Scabiosa comosa* (SC) is an important traditional medicine for treatment of liver diseases. Herbs in the genus Scabiosa contain diverse phyto-compounds including flavonoids, triterpenes and coumarins [15,16]. Previously, SC were demonstrated the excellent radical scavenging activity and anti-HCV activity [17]. However, the protective effects of SC on liver fibrosis have not been reported. Thus, the aim of the present study was to investigate the anti-fibrotic activities of the total flavonoids of SC on CCl_4-induced hepatic fibrosis and explore its underlying mechanism.

2. Materials and methods

2.1. Reagents

Reagents used in the study included: TGF-β1, ELISA kit (Rattus) for alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) (Sigma, St. Louis, MO). ELISA kit for hyaluronic acid (HA), laminin (LN), amino-terminal propeptide of type III procollagen (PIIINP), and collagen IV (CIV) (Bluegene Biotech, Shanghai, China). Antibodies to α-SMA, collagen I, fibronectin and β-actin (Santa Cruz Biotechnology, CA, USA). Antibodies to Smad3, p-Smad3, Smad2, p-Smad2, Smad4 and Smad7 (Cell Signaling Technology, Danvers, MA). Antibodies to TβRI and TβRII (Abcam, Cambridge, MA). HRP-conjugated secondary antibodies (Vazyme, Shanghai, China).

2.2. Isolation of TF-SC

Scabiosa comosa inflorescences were purchased from Chinese Medicinal Material Markets (Anguo, Hebei province) and the total flavonoids were extracted as previously described [18,19]. The *Scabiosa comosa* inflorescences were authenticated by professors in Inner Mongolia medical university and were identified as herbaria of dipsacaceae species *Scabiosa tschiliensis Grunning*. Briefly, dried *scabiosa comosa* inflorescences were crushed with a grinder and boiled with water for 2 h twice. The solution was then filtered, vacuum concentrated and redissolved with 95% ethanol for 8 h. Finally, TF-SC was obtained by preparative HPLC. The content (50 g) of total flavonoids was determined by colorimetric analysis.

2.3. Isolation and culture of HSCs

Primary mouse HSCs were isolated from adult male Wistar rats by collagenase perfusion and purified by density gradient in Nicodenz (AXIS-SHIELD PoC, Scotland). Cells were cultured in DMEM supplemented with 10% FBS (Life Technologies, Grand Island, USA) at 37 °C with 5% CO_2.

2.4. Safety assay

HSCs were cultured on a 96-well plate. After various concentrations (0–400 μg/ml) of TF-SC treatments, cells were incubated with MTT solution at 37 °C for 4 h. Then the medium was removed and the formazan crystals were dissolved in DMSO. The absorbance at 570 nm was measured with a microplate reader.

For in vivo toxicity, Wistar rats (body weight 200–250 g, obtained from the Experimental Animal Center of Inner Mongolia Medical University, China) were administrated by gavage with various doses (0–800 mg/kg) of TF-SC for 7 consecutive days.

2.5. Animal work and experimental protocols

Male Wistar rats (body weight 200–250 g) were housed under controlled conditions (25 ± 2 °C, 70% humidity and 12-light-dark periods) and fed on regular sterile chow diet and water ad libitum. Hepatic fibrosis model was induced by intraperitoneal injection of CCl_4 (1:1v/v in peanut oil, 2 mL/kg body weight) two times per week for 8 weeks. TF-SC was administrated by gavage alone or 15 min before CCl_4 injection. All rats were sacrificed after 8 weeks. Blood was collected by cardiac puncture, and the liver was rapidly removed and properly stored at −80 °C for further analysis.

2.6. Measurement of serum biochemical parameters and hydroxyproline content

Serum was obtained by centrifuging blood samples (3000 rpm at 4 °C for 10 min). The levels of ALT, AST, ALP, HA, LN, PIIINP and CIV were detected by ELISA kit according to the manufacturer's instructions. Hydroxyproline (Hyp) content (mg/g wet weight) in liver tissue was measured as previous described [20].

2.7. Histological analysis

Liver sections fixed in 4% buffered paraformaldehyde were embedded in paraffin and cut to a thickness of 4 μm. Masson's trichrome was performed according to standard procedure [21]. Sections were visualized by a microscope and the ratio of collagen deposition (blue color area) over the whole field area was quantified by ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

For Immunohistochemistry, sections were incubated with p-Smad3 antibody overnight at 4 °C, followed by incubation with fluorophore-conjugated secondary antibody for 1 h. The expression of p-Smad3 was quantified by ImageJ software.

2.8. Western blot analysis

Proteins were extracted from cells or tissues using RIPA lysis buffer (1 × PBS, pH 7.4, 0.5% Sodium deoxycholate, 1% Triton X-100, 0.1% SDS). Samples were heated at 95 °C and separated by 10% SDS-PAGE gel and then transferred into PVDF membranes. After blocking with 5% non-fat milk solution at room temperature for 1 h, the membranes were incubated with respective primary antibodies overnight at 4 °C. After extensive washing, the membranes were incubated with corresponding secondary antibodies. The targeted bands were visualized using enhanced chemolum.

2.9. Immunofluorescence assay

HSCs were cultured on coverslips and treated with TGFβ and TF-SC as indicated for 4 h. The cells were incubated with p-Samd3 antibody and subsequently Alexa Fluor 488 fluorescein conjugated secondary antibody (Abcam, USA). Finally, the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and visualized by a confocal microscope.

2.10. Immunoprecipitation

Immunoprecipitation was performed essentially as described before [22]. Briefly, cell lysates were prepared in RIPA buffer with protease inhibitor cocktail. Immunoprecipitates were obtained by incubating with indicated antibodies or normal IgG (as controls) overnight at 4 °C. And then, the immune complexes were separated by SDS-PAGE and analyzed by Western blot.

2.11. Statistical analysis

All results were presented as mean ± standard deviation (SD). Statistical analysis was performed with SPSS software (version 19). The statistical significance between groups was analyzed using one way ANOVA. The difference was considered significant at $P < 0.05$.