Scoparone Protects Against Pancreatic Fibrosis via TGF-β/Smad Signaling in Rats

Min Xuᵃ Jing Caiᵃ Hong Weiᵃ Meng Zhouᵃ Ping Xuᵃ Hongmei Huangᵃ Wanxin Pengᵇ Fengyi Duᵇ Aihua Gongᵇ Youli Zhangᵃ

ᵃDepartment of Gastroenterology, Affiliated Hospital of Jiangsu University, Jiangsu University, Zhenjiang, China
ᵇDepartment of Cell Biology, School of Medicine, Jiangsu University, Zhenjiang, China

Key Words
Scoparone • Oxidative stress • Pancreatic stellate cells • Fibrosis

Abstract
Background/Aims: This study was to investigate the influence of scoparone on pancreatic fibrosis in vitro and in vivo. Methods: Pancreatic stellate cells (PSCs) were isolated from pancreas tissue blocks, and cultured for 3-5 generations for the experiment. PSCs were treated with scoparone in different doses as experimental groups, salvianolic acid B as a positive control and PBS as a blank group. We measured the effects of scoparone on cellular proliferation, oxidative stress, epithelial-mesenchymal transition (EMT), and pancreatic fibrosis. Cellular oxidative stress was detected by using commercially available kits. The impact of scoparone on EMT and fibrosis was detected through immunofluorescence or western blotting. Results: Compared with the control group, scoparone significantly inhibited stellate cell proliferation, and reduced MDA, the expression of mesenchymal markers, and increased the levels of SOD and the expression of E-cadherin (P < 0.05). Western blot analysis showed that scoparone downregulated the expression of TGF-β and p-smad2/3, and upregulated the expression of smad7 (P < 0.05). Conclusion: Scoparone can reduce the levels of oxidative stress, repress pancreatic stellate cells activation, and alleviate fibrosis by regulating TGF-β/Smad pathway.

Introduction
Pancreatic stellate cells (PSCs) are emerging as key players in physiopathological processes underlying the occurrence and progress of pancreatic disease, including pancreatitis and cancer. The activation of PSCs is closely connected with chronic pancreatitis (CP) [1]. Oxidative stress can promote pancreatic fibrosis. In fact, multiple experiments in vivo and vitro have identified that antioxidants can inhibit pancreatic fibrosis effectively [2-4].

M. Xu and J. Cai contributed equally to this work.
Traditional Chinese herbs are multi-link and multi-point targeting. It has comprehensive advantages in dealing with complex diseases. In recent years, it has been proven that traditional Chinese herbs and their main active components both have a beneficial effect and prospects for drug development in anti-organ fibrosis [5-7]. Salvianolic acid B is one of the major water-soluble components from *Salvia miltiorrhiza* and is widely recognized as one clear anti-hepatic fibrotic agent of traditional Chinese herbal medicine. Furthermore, the involvement of functional salvianolic acid B in pancreatic fibrosis has been demonstrated [8]. Scoparone, which is the main component of *Artemisia capillaris*, is an antioxidant, anti-inflammatory compound, protects the liver among other benefits [9-11].

Presently, it is recognized that epithelial-to-mesenchymal transition (EMT) plays an important role in organ fibrosis. When inflammation is constantly present, EMT persists, excessive myofibroblastic-like cells appear and secrete extra cellular matrix (ECM), eventually leading to organ fibrosis [12]. The TGF-β/Smad pathway is a predominant promoter of EMT.

This study aimed to determine the influence and mechanism of scoparone on oxidation and pancreatic fibrosis in rats.

**Materials and Methods**

**Animals**

All experiments were performed on 250 - 300g male Sprague Dawley rats obtained from the Jiangsu University Animal Experimental Center. Rats were housed in rack-mounted cages under conditions of constant temperature and a 12:12-h light-dark cycle. All experiments were performed according to the guidelines of the Animals Committee.

**Chemicals**

Scoparone, Salvianolic acid B and Hoechst 33343 (nuclear counterstaining) were purchased from Sigma Chemical (Sigma-Aldrich, St. Louis, MO, USA). Anti-α-SMA, anti-collagen I, anti-desmin, and anti-GFAP were purchased from Abcam (Abcam, Cambridge, MA, USA), malondialdehyde (MDA), superoxide dismutase (SOD) were purchased from Jiancheng Bioengineering Institute (Nanjing, China), goat anti-rabbit IgG-Cy3 and goat anti-mouse IgG-Cy3 were from Com Win Biotech Co.Ltd. (Beijing, China).

**Isolation, Identification and Culture of PSCs**

We isolated PSCs according to a published method [13]. Briefly, the PSCs were outgrown from the tissue blocks 1–3 days later. Isolated pancreatic stellate cells from pancreas tissue blocks were then identified by immunofluorescence and cultured 3-5 generations for the experiment. PSCs were cultivated in DMEM/F12 (Invitrogen, USA) supplemented with 10% FBS and 1% penicillin–streptomycin at 37°C, 5% CO2. Incubations with the primary antibodies, anti-desmin (1:200), anti-GFAP(1:100) and anti-α-SMA(1:100), were performed at room temperature in a humid chamber for 2 hours. Goat anti-rabbit IgG-Cy3 and goat anti-mouse IgG-Cy3 were used for the specific secondary antibodies.

PSCs were allocated into the control group, the salvianolic acid B group (SA-B) 0.1 mmol/L and different concentrations of scoparone (low concentration (L-SC): 0.1 mmol/L; middle concentration (M-SC): 0.2 mmol/L; high concentration (H-SC): 0.4 mmol/L). After 24 h, the control group was cultured in DMEM/F12 containing 10% fetal bovine serum. Additionally, the SA-B and the respective concentrations of scoparone were added at 24 h.

**MTT Assay**

PSCs were plated into each well of a 96-well plate with 5 × 10³ cells. After incubation at 37°C in a CO₂ incubator for 24 and 48 h, the MTT assay was used to measure cell proliferation. In brief, 20 µL of 5 mg/ml MTT was added to each well and the cells were incubated at 37°C for 4h, MTT was then removed and replaced with 200 µL of dimethyl sulfoxide (DMSO) for 15 min at 37°C until the crystals were dissolved. The OD value of each well was measured using a microplate reader (Bio Tek Instruments, USA) at 490nm.
Detection levels of MDA and SOD in PSCs

PSCs were plated into each well of a 6-well plate with 2 × 10^4 cells. After 48 h, levels of MDA and SOD in PSCs of each group were detected by using commercially available detection kits (Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer’s instructions.

Immunofluorescence

PSCs were plated into each well of a 24-well plate with 1 × 10^4 cells. The primary antibodies were performed at working dilutions--mouse monoclonal antibodies: α-SMA (1:100), vimentin (1:100); rabbit polyclonal antibodies: Collagen I (1:100), E-cadherin (1:100). Nuclear counter staining was performed using Hoechst 33342 and staining was observed by confocal microscopy (Zeiss, LSM710).

Western blotting

Total proteins were prepared by standard procedures and quantified by the BCA method. Protein per sample was loaded onto a SDS-polyacrylamide gel. After electrophoresis and transfer, the membranes were incubated with a monoclonal mouse anti-α-SMA (1:300), polyclonal rabbit anti-collagen I (1:500), anti-E-cadherin (1:1000), anti-vimentin A (1:1000), anti-TGF-β (1:500), anti-Smad2/3 (1:1000), anti-p-Smad2/3 (1:1000) or Smad7 (1:200) antibodies. Goat anti-mouse antibody (1:1000) or goat anti-rabbit antibody (1:1000) was used as the secondary antibodies. The membranes were developed with the Millipore’s enhanced ECL kit and quantified using gel imaging system (Sage Creation Technology Company, Beijing, China).

Establishment of Chronic Pancreatitis (CP) Rat Models and Treatment

Thirty SD rats weighing 200 g were randomly allocated into five groups: control group, CP group, Salvianolic acid B group (SA-B), low dose- (L-Sc) and high dose of scoparone (H-Sc) groups. A rat model of chronic pancreatitis was established by caudal vein injection of dibutyltin dichloride (DBTC). Dibutyltin dichloride (Sigma-Aldrich, Chemie GmbH, Steinheim) was dissolved in 100% ethanol (Changshu Yangyuan Chemical Co. China, Changshu, China) and mixed with glycerol (Amresco, Ohio), with the ethanol: glycerol volume ratio of 2:3 and the final DBTC concentration of 8 mg/mL. The CP group and each experimental group was injected with 200 μl DBTC solutions at a dose of 8 mg/kg [14]. The control group was injected with 200μl 100% ethanol and glycerol mixed solvent. One day after DBTC infusion, the rats randomly received 1.5ml saline containing high or low dose scoparone (60 mg/kg, 30 mg/kg, respectively) or salvianolic acid B (10 mg/kg) by oral administration. The control and CP groups were given the same volume of saline. After 4 weeks, pancreatic tissue and serum were obtained for experiments.

Weigh the body weight of rats and pancreas and histological examinations

Animals were sacrificed at eight weeks. Serum was stored at -20°C until the assays were carried out. Pancreases were quickly removed, isolated from fat and lymph nodes and weighed. Portions were immediately snap-frozen and stored at -80°C. Two pathologists scored on six indicators of inflammation, edema, abnormal architecture, glandular atrophy, fibrosis, and pseudotubular complexes.

Detect biochemical parameters of serum

We assayed the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) according to the manufacturers’ specifications (Jiancheng Bioengineering Institute, Nanjing, China).

Detection the levels of Intrapancreatic MDA and SOD

To identify the oxidative stress, the levels of MDA and SOD were quantified by commercially available detection kits (Jiancheng Bioengineering Institute, Nanjing, China), according to Jiang et al. [2] and the manufacturer’s instructions. The content of MDA in the pancreas was calculated as nanomoles per mg of protein, and the content of SOD was expressed as U of SOD per ml of pancreatic tissue.

Detection of α-SMA, collagen I, and TGF-β/Smad activation in pancreas

As detailed previously, the expression of α-SMA, collagen I, and TGF-β/Smad signaling pathway protein in pancreas were determined by Western blotting.
Statistical analysis

All results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student's t-test for comparison of two groups, and one-way ANOVA for multiple comparisons. In both cases, differences of \( P < 0.05 \) were considered statistically significant.

Results

Isolation, Culture and identification of PSCs

Small tissue blocks were cut and seeded in cell culture bottles. Primary PSCs grew out from the tissue blocks 1-3 days later (Fig. 1A, B). After passaging, the viability of PSCs was identified by immunofluorescence. Almost all activated PSCs were found to express the myofibroblastic markers, α-SMA, desmin, and GFAP, after passage (Fig. 1C-E).

Scoparone inhibits proliferation of PSCs, decreases MDA, and increases SOD

To examine the effect of scoparone on PSC proliferation, different concentrations of scoparone and salvianolic acid B were added after 24 h and 48 h. PSCs were treated with various concentrations of scoparone for 24 h and 48 h. The results showed that high doses of scoparone (0.4 mmol/L) produced the most inhibition on PSC proliferation compared with
other groups at 24 and 48 h after treatment ($P < 0.01$) (Fig. 2A). However, compared with the treatment with low and medium concentrations of scoparone (L-Sc and M-Sc, respectively) was not significantly different from salvianolic acid B treatment (Fig. 2A).

Compared with control group, the levels of intracellular MDA in the different doses of scoparone and salvianolic acid B group decreased significantly at 48 h after treatment (Fig. 2B), while the levels of SOD increased ($P < 0.01$) (Fig. 2C). Moreover, H-Sc treatment resulted in the lowest levels of MDA and highest levels of SOD compared to the other treatment groups (SA-B, M-Sc, and L-Sc group) ($P < 0.05$).

**Scoparone represses epithelial-mesenchymal transition components in PSCs.**

The expression levels of α-SMA, Collagen I, Vimentin and E-cadherin were identified by immunofluorescence. Compared with the control group, the levels of α-SMA, Collagen I, and Vimentin decreased significantly in salvianolic acid B and with different doses of scoparone while E-cadherin levels increased. Immunofluorescence assay results showed that high doses of scoparone had a more obvious effect than other experimental groups (Fig. 3A). Furthermore, western blot analysis showed that salvianolic acid B and different concentrations of scoparone could significantly reduce the expression of α-SMA, Collagen I, and vimentin, while concomitantly increasing the expression of E-cadherin (Fig. 3B).

When compared to the control group, treatment with salvianolic acid B or different concentrations of scoparone resulted in decreased expression of TGF-β and p-Smad2/3,
while Smad7 levels increased. H-Sc resulted in higher levels of TGF-β compared to SA-B; however, p-Smad2/3 and Smad7 levels were similar between the two groups (Fig. 3C).

Scoparone protects against pancreatic damage.

Total pancreatic weight is a simple but reliable method for estimating the degree of pancreatic regeneration after injury [15]. Scoparone administration greatly ameliorated the pancreatic weight loss induced by DBTC in pancreatitis rats. The pancreatic wet weight of CP rats was far below the weight observed in control or a high dose of scoparone treated rats at week 4 ($P < 0.05$). Compared with the CP group, the ratio of pancreas wet weight/body weight in each experimental group and the control group significantly increased ($P < 0.05$). However, no significant difference was detected between the experimental groups ($P > 0.05$) (Fig. 4A). The differences in the levels of serum AST, ALT, BUN, and Cr in each group was insignificant ($P > 0.05$) (Fig. 4B).

Compared with the control rats, DBTC can induce significant histological changes in inflammation, edema, abnormal architecture, glandular atrophy, fibrosis, and pseudotubular complexes at week 4. Scoparone can significantly protect against the pancreatic damage. Compared with the CP rats, all markers of pancreatic damage were significantly attenuated in rats treated with high doses of scoparone or salvianolic acid B ($P < 0.05$). However, in
agreement with the weight changes, there is no significant difference between each experimental group ($P > 0.05$) (Fig. 4C).

**Scoparone reduces the level of oxidative stress markers of pancreatic tissues in rats**

DBTC induction resulted in a significant increase in the concentration of MDA and a significant decrease in SOD in pancreatic tissues compared with the controls (Fig. 5A, B) ($P < 0.05$). Moreover, treatment with SA-B or Sc significantly reduced the levels of MDA and increased the levels of SOD compared with rats with DBTC induction (Fig. 5A, B) ($P < 0.05$). High concentration of Sc caused significant decreases in MDA and increases in SOD compared to SA-B or L-Sc ($P < 0.05$). However, MDA and SOD concentrations between low dose scoparone or salvianolic acid B treatment were unaffected (Fig. 5).

**Scoparone inhibits the activation of PSCs and the TGF-β/Smad signaling activation in pancreas**

Chronic pancreatitis resulted in significant increases in the expressions of α-SMA and collagen I in the pancreas. However, high concentration of scoparone or salvianolic acid B...
treatment significantly lowered the expression levels. The effects of scoparone are dose-dependent. Low doses of scoparone (L-Sc) are significantly different from high doses of scoparone (H-Sc); however, H-Sc and SA-B are similar (Fig. 6A).

Scoparone or salvianolic acid B treatment resulted in significant down regulation of TGF-β and p-Smad2/3 levels. Furthermore, Smad7 expression increased significantly in all the experimental groups. There was no significant difference in expression of TGF-β/Smad between the salvianolic acid B group and the high dose scoparone group (Fig. 6B).

Discussion

The present study suggests that scoparone can attenuate the level of oxidative stress, repress pancreatic stellate cells activation, and alleviate pancreatic fibrosis by decreasing the activity of TGF-β/Smad pathway. The effect of the high dose scoparone group was significantly superior to salvianolic acid B group in inhibiting the level of oxidation, but there is no significant difference in alleviating fibrosis in vivo between the two groups.

The pathogenesis of chronic pancreatitis is not completely understood. There is growing recognition that persistent oxidative stress may play a role in the development and maintenance of the disease. Several lines of evidence arising from clinical settings and experimental animal models reinforce this argument. Antioxidants, such as α-tocopherol [2] and epigallocatechin gallate [3], can effectively inhibit pancreatic fibrosis. In recent years, increasing numbers of researchers have begun to study the effects of traditional Chinese herbal medicines and their main components on the function and molecular mechanisms of chronic diseases around the world. The traditional Chinese herb, Artemisia capillaris, has beneficial effect on the gall bladder, protects liver function, in addition to its anti-inflammatory and lipid-lowering roles. Lee et al. [16] found that the herbal medicine, Yin-Chen-Hao-Tang (the soap with Artemisia capillaris), ameliorates hepatic fibrosis in bile duct ligation rats. They have failed to report on the anti-oxidative or anti-fibrotic effects of it in PSCs or an in vivo model of CP. This study focused on scoparone, which is the main component of Artemisia capillaris, to have a better understanding of its role and the related molecular mechanisms on CP. Salvianolic acid B has been reported to be effective in ameliorating oxidative damage [17], suppressing the EMT in activated hepatic stellate cells (HSCs) and ameliorating liver fibrosis [18]. Additionally, salvianolic acid B can inhibit the activation of PSCs and pancreatic fibrosis [8]. Therefore, salvianolic acid B was chosen as a positive control in this study.

Oxidative stress is intimately linked to the development of inflammation and fibrosis in CP, which regulates critical pathophysiological aspects in the whole process. We observed that scoparone treatment dilutes the level of oxidative stress, depresses pancreatic stellate cells activation, and alleviates fibrosis. The anti-oxidative or anti-fibrotic effects of high concentration of scoparone group were better than other treatment groups in PSCs. Scoparone treatment in the animal model of chronic pancreatitis reproduced the anti-oxidative results; however, there was no significant difference in the anti-fibrotic effects of scoparone and salvianolic acid B.

The epithelial-mesenchymal transition (EMT) is a process reflected by higher levels of N-cadherin, fibronectin, and vimentin, which are expressed in mesenchymal cells, and a lower level of E-cadherin is observed, which is expressed in epithelial cells. EMT is associated with fibrosis and is frequently observed in chronic diseases of the lung, liver, kidney, and heart [12, 19]. The expression of EMT markers is often seen before histological signs and is correlated with the risk of progression to chronic fibrosis, suggesting that EMT may be used to predict progression towards interstitial fibrosis [20]. The results of immunofluorescence or western blotting in this study indicate that scoparone may reverse the process of EMT.

TGF-β is a prominent regulator of EMT during developmental morphogenesis and migration of cells [21]. TGF-β is deemed to be the critical regulator of fibrosis in many organs, like renal, cardiac, lung [22-27]. TGF-β intracellular signaling is mediated and modulated primarily by Smads. Upon TGF-β binding to its receptor, Smad2 and Smad3 are phosphorylated.
by the receptor. High concentrations of scoparone inhibited the phosphorylation of Smad2/3. On the basis of the above-mentioned evidence, we postulate that scoparone exerts its effects on PSCs and the pancreas by regulating the TGF-β/Smad pathway.

Our study on body weight and pancreatic weight in a rat model of chronic pancreatitis suggests that the high dose of scoparone has a high degree of pancreatic regeneration after injury. Furthermore, serum levels of several key markers of liver and kidney function show no significant differences between rats treated with none, low, and high doses of scoparone, suggesting that scoparone has no negative impact on liver and kidney function. Moreover, scoparone improved inflammation and fibrosis in CP rats induced by DBTC.

In conclusion, we have found that both scoparone and salvianolic acid B inhibited the proliferative, profibrotic phenotype of PSCs by down-regulating the expression of α-SMA and collagen I; diminishing the level of oxidative stress; inhibiting components of EMT; and alleviating pancreatic fibrosis via the TGF-β/Smad pathway. The anti-oxidative effects of scoparone are superior to salvianolic acid B, while no anti-fibrotic differences were observed. Our study provides evidence for the potential clinical use of scoparone to reduce the progression of pancreatic oxidative stress and fibrosis.

On the basis of the above-mentioned evidence, we hypothesize that scoparone prevents or mitigates pancreatic oxidation, fibrosis, and epithelial-to-mesenchymal (EMT) by regulating TGF-β/Smad pathway.

Acknowledgments

This study was supported by grants from the Natural Science Foundation of Jiangsu Province (BK20131247) and the Research Project of "169 project" in Zhenjiang City.

Disclosure Stement

The authors declare no conflict of interest.

References


