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Original Research

Dicliptera Chinensis polysaccharides target TGF-β/Smad pathway and inhibit stellate cells activation in rats with dimethylnitrosamine-induced hepatic fibrosis

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Abstract: This study aims to study impact of *Dicliptera chinensis* polysaccharide (DCP) on hepatic fibrosis (HF) and activation of hepatic stellate cells (HSCs). Liver fibrosis model was induced by intraperitoneal injection of dimethyl nitrosamines (DMN) in rat. Rats in treatment group were administrated with different concentrations of DCP (0, 100, 300 mg/kg) by intraperitoneal injection. Hematoxylin and eosin (H&E) and Masson's trichrome staining were used to assess histo-pathological change. α-SMA, TGF-β1 and pSmad 2/3 were assayed by immuno-histochemistry. HSC-T6 cells were stimulated by recombined rat TGF-β1 (1 ng/mL) to simulate an activating model *in vitro* and then interfered with DCP (concentration of 0, 25, 50, 100, 200, 400 μg/ml). MTT assay was used to determine cell proliferation and western blotting was used to detect α-SMA and pSmad 2/3 expression. Results demonstrated that DCP alleviated DMN-induced liver fibrosis in rat and significantly down-regulated TGF-β1 expression, pSmad2/3 and α-SMA in liver tissue in a dose-dependent way. DCP inhibited proliferation and activation of TGF-β1-stimulated HSC-T6 *in vitro* and significantly down-regulated α-SMA and pSmad2/3 expression. In conclusion, this study revealed that DCP attenuates progression of liver fibrosis through suppressing TGF-β/Smad pathway. DCP is a potential botanical polysaccharide to management liver fibrosis.

Key words: *Dicliptera chinensis* polysaccharide, TGF-β1, HSCs, α-SMA, pSmad 2/3.

Introduction

Increasing evidence shows that hepatic fibrosis ascribes to the accumulation of excessive extra-cellular matrix proteins including collagen that occurs in most types of chronic liver injury. Advanced liver fibrosis results in cirrhosis, portal hypertension, and liver failure and often requires liver transplantation. Hepatic fibrosis is a wound healing response in which damaged regions are encapsulated by the excessive accumulation of extra-cellular matrix (ECM) and activated hepatic stellate cells (HSCs) that are experiencing myofibroblast transition identified by α-SMA expression (1,2). The present study investigated whether HSCs play a role liver in fibrosis. Therefore, Inhibition of the activation and function of HSCs has become the most important treatment strategy for hepatic fibrosis (3).

In healthy liver, HSCs are usually quiescent cells, but in response to chronic liver damage they undergo an activation process in which they become highly proliferative and synthesize a fibrotic matrix rich in type I collagen (4). Many studies have shown that TGF-β1 mediator is considered to be the most important in the process of liver fibrosis including HSCs activation and ECM remodeling (5,6). It has been widely accepted that the TGF-β 1 downstream mediators, such as Smad 2 and Smad 3, could mediate the fibrosis (7). It appears that collagen expression by TGF-beta is mediated by the phosphorylation of Smad 2 and Smad 3, and subsequent nuclear translocation of the Smad complex (8). Therefore, several studies have focused on the inhibition of TGF-beta activation and intervention of the TGF-β/Smad signaling pathways to treat liver fibrosis.

Zhuang medicine *Dicliptera chinensis* (L.) Ness annual or perennial herbs. *Dicliptera chinensis* contain a variety of active ingredients through teaching and research hospital of natural medicinal chemistry. Pharmacological study found that *Dicliptera chinensis* polysaccharide (DCP) on DMN induced liver injury model has anti-fibrosis effect (9). However, there is little known about anti-fibrosis mechanism of DCP. This study was designed to investigate the anti-fibrosis activity of DCP in rats induced by DMN and in HSCs activation.

Materials and Methods

Dicliptera chinensis polysaccharide

The HPLC-Purified nature product of DCP (90%) was obtained from Guilin Medical University (Guangxi, China) and was used for in vivo treatment as described below. Meanwhile, the some kind of HPLC-purified DCP was used for in vitro studies.

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Animal model of DMN-induced hepatic fibrosis and DCP treatment

Male wista rats (6-8 weeks of age, 170-230 g) were obtained from the Guilin Medical Laboratory Animal Center, fed with a standard laboratory diet and tap water in a temperature-and humidity-controlled animal house under 12 hours light-dark cycles. Sixty-four rats were divided randomly into four groups (n=16 for each group) including: 1) normal control, 2) model group, and 3) two DCP treatment groups at doses of 100 mg/kg, and 300 mg/kg, respectively. In addition, one group of normal 16 rats was treated with distilled water. Except the normal control groups, all animals were treated with intra-peritoneal injection of 1.6 ml/kg of DMN (diluted in 0.5% distilled water) thrice per week for 4 weeks to induce hepatic fibrosis. For those received the DCP treatment, animals were given with two different doses of DCP (100 mg/kg and 300 mg/kg) suspended in distilled water by intra-peritoneal injection daily for the 4 week-period, while rats from model group were received with equivalent volumes distilled water. Normal control group were also treated the same volumes of distilled water equivalent to the model group. At the end of the fourth week, all of rats were sacrificed under anesthesia with 3% sodium pentobarbital. Blood samples and liver specimens were obtained for analyses of liver functions, protein expression of fibrotic indexes by Western blot, histology, and immuno-histochemistry. All experimental procedures were approved by the Animal Experimental Committee at the Guilin Medical College affiliate Hospital.

Liver Function Test

Aspartate transaminase (AST) and serum alanine transaminase (ALT) activities, markers for hepatotoxicity, were detected with an automatic detection kit (NanJing JianCheng Bioengineering Institute).

Histopathology and immunohistochemistry

Changes in liver morphology were examined in methyl Carnoy's fixed, paraffin-embedded tissue sections (3 micrometer) stained with hematoxylin and eosin (HE) and Masson's trichrome staining. The histopathological scores of fibrosis were evaluated following the published criteria (10): ① normal liver; ② an increase in collagen matrix accumulation without the formation of septa (small stellate expansions of the portal fields); ③ formation of incomplete septa from the portal tract to the central vein (septa that do not interconnect with each other); ④ complete but thin septa interconnecting with each other to divide the patenchyma into separate fragments; ⑤ same as grade 3, except for the presence of thick septa (complete cirrhosis).

The TGF-β1, α-SMA and pSmad 2/3 antibodies for immuno-histochemistry were purchased from (Santa Curz, CA, USA). Slides of the tissue micro-array underwent heat incubated with the primary antibody for 1 h at room temperature. Secondary antibody incubation and DAB coloring (Aldrich-Sigma, CA, USA) were conducted following the manufacturer's instruction (11). Expression of TGF-β1, α-SMA and pSmad2/3 in the liver cross-sections was determined using the quantitative Image Analysis System (HPIAS-1000). Briefly, 8 fields (×40) were randomly selected from each section and positive signals within the section were highlighted, measured, and expressed as percent positive area of the entire liver tissues examined.

Cell culture

The HSC cells line was purchased from Zhong Shang Medical University. HSCs were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, America) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.01% penicillin and streptomycin at 37°C in a humidified incubator with 5% CO_2.

We first determined the safe dosages of DCP for the study, and HSCs were resuspended into single-cell suspensions and cultured at a density of 5×10^4 cells/mL in 100 μL DMEM containing 0.2% FBS in 96-well microplates. The cells were incubated overnight to adherent monolayer of cells. DCP as control in various dosages (25, 50, 100, 200, 400 mg/mL) was added to the culture for 48 h. A dosage-dependent cytotoxicity of DCP was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay kit (Amresco, America) following the manufacturer's instructions.

To determine the optimal dose of TGF-beta 1, HSCs were treated with TGF-beat (NOVOPROTEIN, Shanghai) at dosages of 1, and 5 ng/mL for various time of 0, 12, 24, 48h. The proliferation of HSCs was determined by MTT.

To investigate the inhibitory effect and mechanism of DCP on TGF- beta 1-mediated fibrosis, HSCs were pre-treated with DCP at dosages of 0, 25, 50, 100, 200, 400 μg/ml for 48h, followed by addition of an optimal dose of TGF-beta 1 (1 ng/ml).

Western blot analysis

The total protein from cultured HSCs was extracted with lysis buffer (Bioss, Beijing). The proteins were separated by a 12% SDS-PAGE gel and then transferred onto polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat milk in TBST for 1 h. After incubation with 10% nonfat milk, the membranes were probed with the rabbit anti pSmad 2/3 (Santa Cruz, Shanghai, China) and rat anti-α-SMA (BOSTER, Wuhan, China) and β-actin (ZsBio, Beijing, China) antibody (1:1500) overnight at 4°C. After washing for 5×3 min with TBST buffer, membranes were incubated with horseradish peroxidase-conjugated goat-anti-rabbit or goat-anti-rat secondaty antibodies (ZsBio) for 1 h at room temperature and detection was carried out by enhanced chemiluminescence detection system (MultiScience Biotech, Shanghai, China). Intensity of the bands was quantified by densitometry. The relative expression was measured according to the reference blots ofβ-actin (11).

Statistical Analyses

Data are expressed as mean ± SD. Statistical analyses were performed by independent t test. All statistical analyses were performed using SPSS 17.0 statistical softwate. $P<0.05$ were considered to be statistically significant difference.

Results

Effects of DCP on DMN-induced hepatic functional and histological damage in rats

Administration of DMN for 4 weeks caused liver injury as demonstrated by the development of severe liver damage with thick fibrotic septa and pseudolobular formation (Fig. 1A and B). Serologically, levels of ALT and AST were also highly significantly elevated in disease control group when compared to normal control group (Fig. 1 C). In contrast, treatment with DCP resulted in attenuation of both histological and functional injury in a dosage-dependent manner, being significant at doses of 300 mg/kg (Fig, 1 A , B and C). Normal rats treated with DCP (300 mg/kg) exhibited normal histololgical and serological changes similar to the normal control group (data not shown).

Effects of DCP on the expression and distribution of α-SMA, TGF-β1 and pSmad 2/3 in DMN-induced liver fibrosis tissue

Immuno-histochemical analysis was used to detect the expression and distribution of α-SMA, TGF-β1 and pSmad 2/3 in liver tissue. There were few α-SMA-positive regions in the normal control group (Fig. 2A). In contrast, the expression of α-SMA was significantly increased in the DMN-induced disease control group (only treated with DMN, and without CDP treatment), and α-SMA-positive regions can be seen around the periportal fibrotic band areas, central vein and fibrous septa (Fig. 2A), whereas they were sharply down-regulated in the DCP-treated group (Fig. 2B). The expression of TGF-β1 and pSmad 2/3 was consistent with α-SMA (Fig. 2C and D). However, the expression of the total Smad2 and Smad3 were not changed in DCP-treated group and the DMN group (Supplementary Fig. 1).

Effect of DCP on proliferation of TGF-β1-induced activation of HSCs

Normal HSCs treated with DCP (0, 25, 50, 100, 200, 400 mg/ml) exhibited OD value similar to the normal control group (Fig. 3 A). As we all known, TGF-β1 has been considered as a key mediator in the pathogenesis of liver fibrosis (6), We firstly determined an optimal dose of TGF-β1 in fibrosis response on HSCs. MTT analyses detected that 1 ng/ml and 5 ng/ml of TGF-β1 could induce HSCs fibrosis at 48h. Finally, we decided to choose an optimal dose of TGF-β1 at 1 ng/ml with the peaked time for MTT at 48h (Fig. 3 B). Therefore, safe dose of DCP (0, 25, 50, 100, 200, 400 mg/ml) were used for studying the inhibitory effect of DCP on TGF-β1 (1 ng/ml)-induced HSCs activation. MTT demonstrated that DCP inhibited the proliferation of TGF-β1-induced HSCs (Fig. 3 C).

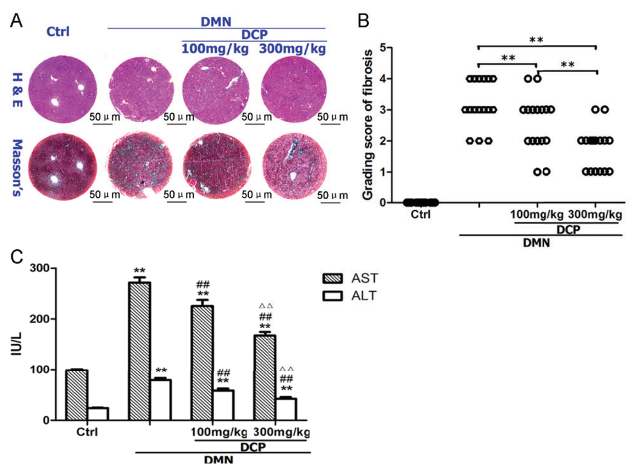

Figure 1. DCP treatment attenuates DMN-induced liver damage and functional impairment in a dosage-dependent manner in rats. A. H&E staining and Masson`s trichrome staining, Magnification: ×40. B. Fibrosis score, **P<0.01. C. Liver function tests. Data represent as mean ± SD for group 16 animals. *P<0.05, **P<0.01 compared to normal control; #P<0.05, ##P<0.01, compared to DMN-treated group.

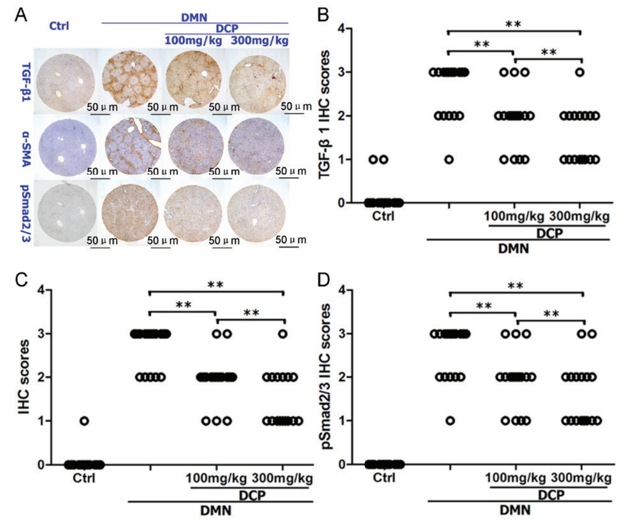

Figure 2. Immuno-histochemistry showed that DCP treatment down-regulated TGF-β1/pSmad 2/3 signaling and αSMA in DMN-induced liver fibrosis in rats. A. Immuno-histochemistry assay for TGF-β1, αSMA and pSmad 2/3. The α-SMA-positive regions can be seen around the periportal fibrotic band areas, central vein and fibrous septa. B. Statistical analysis forα-SMA expression. C. Statistical analysis for TGF-β1 expression. D. Statistical analysis for pSmad 2/3 expression. *P<0.05, **P<0.01 compared to normal control; #P<0.05, ##P<0.01 compared to DMN-treated group. Magnification: ×40.

Effect of DCP on expression of α-SMA and pSmad 2/3 in TGF-β1-induced activation of HSCs

To further investigate the molecular mechanism involved in anti-fibrosis effects of DCP of α-SMA and pSmad 2/3 in TGF-β1-induced the activation of HSCs by western blot, respectively. The protein expression of α-SMA and pSmad 2/3 in TGF-β1-induced activated HSCs were significantly increased in the TGF-β1-induced model control group compared with the normal control group (Fig. 4). However, DCP apparently decreased the expression of α-SMA and pSmad 2/3 compared with the TGF-β1-induced model control group. However, the western blot assays showed that the expression of total Smad2 and Smad 3 were not changed in TGF-β1-treated and DCP-treated group and the DMN group (Supplementary Fig. 2). For the αSMA expression, there were no significant at 12 hours (data not shown). However, the levels of αSMA expression at 24 hours were similar to 48 hours (data not shown). Therefore, DCP could significantly reduce the expression of α-SMA which may be associated with decreased TGF-β1-induced the activation of HSCs.

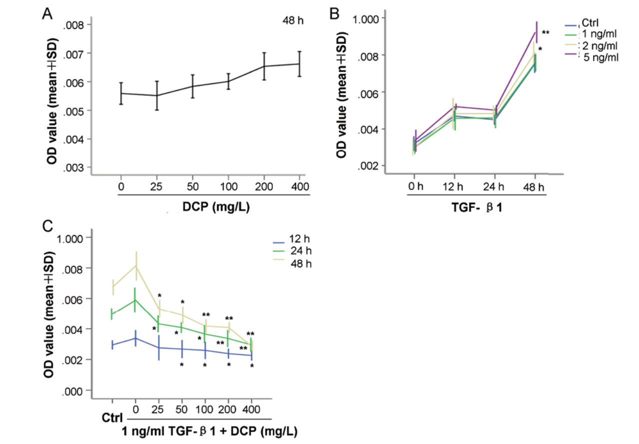

Figure 3. DCP inhibits TGF-β1-induced HSC-T6 cells in a dosage-dependent manner. A. DCP showed faint pro-proliferation function on HSC-T6 cells by MTT assays at 48 hours. B. TGF-β1 showed weak inhibition on cell proliferation in HSC-T6 cells by MTT assays. Data represent as mean ± 1 SD for 3 independent experiments. *P<0.05, **P<0.01 compared to Ctrl group. C. While DCP showed mild inhibitory effect on TGF-β1-stimulated HSC-T6 cells. Data represent as mean ± 1 SD for 3 independent experiments. *P<0.05, **P<0.01 compared to only TGF-β1-treated (0 mg/L) group.

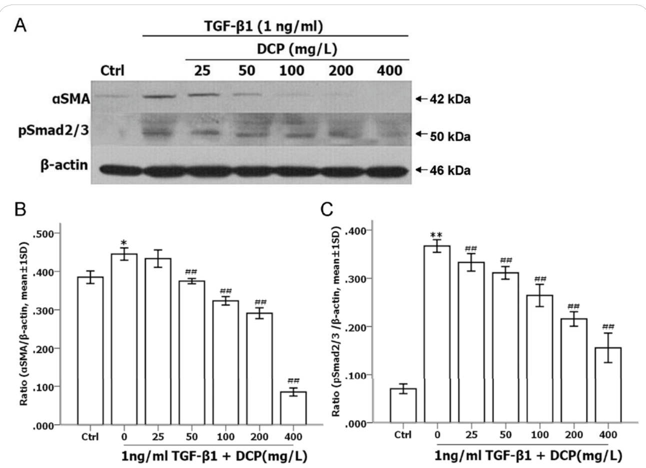

Figure 4. DCP inhibits TGF-β1-induced α-SMA and pSmad 2/3 expression in HSC-T6 cells. A. Western blot analysis for α-SMA and pSmad 2/3 expression. B & C. Column bar for relative expression of α-SMA and pSmad 2/3 respectively. Data represent as mean ± 1 SD for 3 independent experiments. *P<0.05, **P<0.01 compared to normal control; #P<0.05, ##P<0.01 compared to TGF-β1-induced cells.

Discussion

Hepatic fibrosis describes the presence of excess deposition of ECM, laid down as part of the tissue wound-healing response of chronic liver injury. The causes of hepatic injury include toxins, alcohol and disorders of the immune system (12). Although it is now well accepted that TGF-β/Smad signaling pathway plays key roles in the progress of hepatic fibrosis including HSC activation (13,14). Activated HSCs could produce many cytokines and ECM remodeling in hepatic, thus further boosting inflammatory process and activating HSCs (15). Many evidences demonstrated that reducing the release of cytokines and inflammatory cells could prevent and reverse the liver fibrosis (16,17). Recently, some studies show that DCP have anti-fibrosis (9). However, there is little known about anti-fibrosis mechanism.

In this study, we found that DCP may be a novel therapeutic agent for hepatic fibrosis. Our data showed that the serum levels of AST and ALT in rats were significantly reduced by DCP compared to the disease control group. Histological examination also demonstrated that there were more collagen fibers in DMN-treated rats compared with normal control group. In contrast, DCP treatment remarkably reduced the deposition of collagen fibers compared with the disease control group. Immuno-histochemical analysis showed that the expression of TGF-β1, α-SMA, pSmad2/3 decreased significantly by DCP treatment.

MTT data showed that DCP had inhibited the proliferation of the HSC in dose dependent way. But DCP has not effect on non-activated HSC. As we all known that α-SMA was a specific marker of HSCs activation. Western blot data indicated that considerable protein expression ofα-SMA and pSmad 2/3 was observed in the disease control group compared with normal control group, while it significantly reduced by DCP suggesting that anti-fibrosis effects of DCP were through down-regulating the TGF-β/Smad 2/3 signaling pathway.

In summary, the results showed that DCP significantly inhibited DMN-induced activation of HSCs and liver fibrosis and largely improved liver functional injury in a dosage-dependent manner in rats. The anti-fibrosis of DCP maybe associated with its inhibitory effects on HSCs activation by down-regulating the TGF-β/Smad 2/3 signaling pathway.

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