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The protective role of protocatechuic acid against chemically induced liver fibrosis *in vitro* and *in vivo*

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Liver fibrosis is the result of long-term liver injury and has a high incidence worldwide. Protocatechuic acid (PCA) is ubiquitous in vegetables, nuts, brown rice and herbal medicines, which is reported to possess anti-asthmatic, anti-cancer, and anti-oxidation properties. Our research aimed to investigate the effect of PCA on liver fibrosis. *In vitro*, TNF-α-induced hepatic stellate cell (HSC) model was used to assess the anti-fibrosis effects of PCA. *In vivo*, mice were treated with thioacetamide (TAA) to develop liver fibrosis. Body weight, organ index, histological changes, and proteins alteration of factors associated with TGF-β signaling pathway were used to assess the anti-fibrosis effects of PCA. Our results showed that PCA not only inhibited cell viability in TNF-α activated HSC-T6 cells *in vitro*, but also efficiently mitigated TAA-induced liver damage and fibrosis *in vivo*. Further experiments indicated that PCA played a protective role in liver fibrosis through regulation of the TGF-β signaling pathway downregulating the protein expression of p-Smad2, p-ERK, c-Jun. In summary, our findings provide a pharmacological justification for the clinical application of PCA in preventing or treating liver fibrosis.

1. Introduction

Liver fibrosis caused by chronic liver injury represents a significant health problem estimated to affect over 100 million people worldwide (Li et al. 2011a). With the development of liver fibrosis, the risk of acute chronic liver failure and hepatocellular carcinoma is increased, which leads to large morbidity and mortality in patients all over the world (Zoubek et al. 2017). Infection, drug toxicity, alcoholic diseases, cholestasis and metabolic disorders are common causes of liver fibrosis (Cohen-Naftaly and Friedman 2011). Although liver fibrosis and advanced fibrosis can be reversed, cirrhosis, which is the end-stage event of fibrosis, is generally irreversible (Friedman 2000). In addition, effective therapy for liver fibrosis is not yet available. Therefore, there is an urgent need to develop effective strategies for preventing and treating liver fibrosis.

The liver fibrosis process is related to the hepatic stellate cells, parenchymal and non-parenchymal cells. Hepatic stellate cells (HSCs), the main storage sites for retinoids in healthy liver, account for 15% of the number of resident hepatocytes. After liver injury with any cause, biliary cells, hepatocytes, Kupffer cells (which could produce TNF-α) and T-cells produce an inflammatory environment that stimulates the conversion of hepatic stellate cells (HSCs) from quiescent cells to activated myofibroblasts (a-SMA-expressing) (Canbay et al. 2003; Deng et al. 2013; Tomita et al. 2006). When liver injury occurs, the TGF-β signaling pathway in stellate cells is enhanced. Increasing evidence suggests that the role of inhibiting HSC proliferation during the reversal of liver fibrosis is fatal. Hence, in addition to inhibiting HSC activation, reduction of HSC cell proliferation could be a potential treatment for liver fibrosis (Li et al. 2011b; Orr et al. 2004).

Protocatechuic acid (PCA, 3,4-dihydroxybenzoic acid) is a major metabolite of anthocyanins (Vitaglione et al. 2007), and its daily intake is much higher than that of other polyphenols. It is ubiquitous in nuts, vegetables, and cereals. PCA possesses numerous pharmacologic properties, containing anti-asthmatic, anti-cancer, antioxidant, anti-inflammatory and anti-hyperglycemia activities (Liu et al. 2019; Tseng et al. 1998; Wei et al. 2012). In addition, there is no scientific information about the influence of PCA on liver fibrosis. In this research, we investigated the effects of PCA on liver fibrosis. Our results demonstrated that PCA inhibited the TGF-β signaling pathway which plays a vital role in improving liver fibrosis and indicated that PCA is likely to be used to treat liver fibrosis in the future.

2. Investigations and results

2.1. PCA regulates cell viability in TNF-α-induced HSC-T6 cells via regulation on TGF-β signaling pathway

Activated hepatic stellate cells (HSCs) play a critical role in liver fibrosis. To activate HSC-T6 cells, they were incubated with 10 ng/ml TNF-α. MTT assay and morphological observation results showed that PCA at 3 mM exerted a significant proliferation inhibition effect on TNF-α-induced HSC-T6 cells (Figs. 1A, B). In the control group, α-SMA was expressed more than that in the mock group indicating that HSC-T6 cells were activated (Trasino et al. 2016) (Fig. 1C, D). Because the TGF-β signaling pathway is related to cell survival, we then examined the expression of TGF-β-related proteins. Interestingly, results revealed that PCA attenuated the increased expression of TGF-β, p-Smad2, p-ERK, and c-Jun in TNF-α-stimulated HSC-T6 cells (Fig. 1C, E). All those results indicate that PCA reduces the expression of TGF-β-related proteins, thereby regulating cell viability in HSC-T6 cells to prevent liver fibrosis.

2.2. PCA attenuates the alteration of phenotype associated with TAA-induced liver damage and fibrosis in mice

In order to explore the therapeutic effect of PCA on liver damage and fibrosis, we used TAA to induce liver fibrosis in mice.

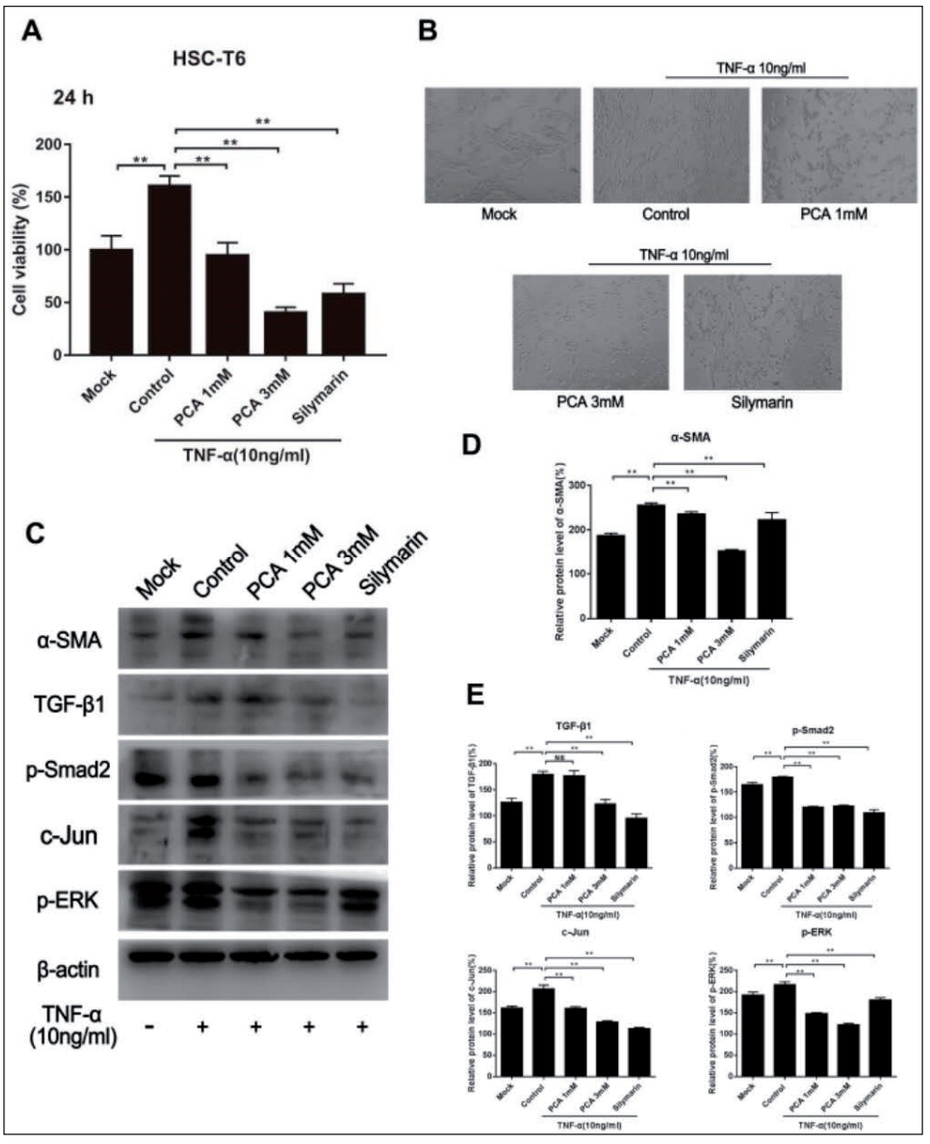

Fig. 1: PCA inhibits HSC-T6 cell viability through TGF-β signaling pathway. (A) Effect of PCA on cell viability of activated HSC-T6 cells. HSC-T6 celss were incubated with PCA for 24 h. was measured with MTT assay. (B) Morphological changes of HSC-T6 cells treating with PCA, magnification: ×200. (C-E) The expression of TGF-β-related proteins in activated HSC-T6 cells. Data are expressed as the mean±S.D. $*p < 0.05$, $**p < 0.01$, not significant (NS).

The mice injected with TAA developed marked liver damage. Compared with the mock group, all the treatment groups showed an observably decrease in body weight, at the end of treatment, PCA treatment mice had higher body weights than TAA treatment mice (Fig. 2A). However, PCA treatment decreased spleen and kidney index scores (Figs. 2B-D). This showed that PCA improves the enlargement of liver and restores liver tissue structure (Zhang et al. 2014). Further, the histopathological analysis showed that the TAA treatment resulted in severe hepatic necrosis, ballooning degeneration of hepatocytes, inflammatory cell infiltration. And PCA 150 mg/kg treatment alleviated liver fibrosis, with a significant decrease of collage (Fig. 2E).

2.3. PCA attenuates TAA-induced liver damage and fibrosis in mice

To evaluate the synthetic function of the liver in TAA-induced mice, the liver and serum levels of GOT and GPT were analyzed. Compared to the mock group, the levels of GOT and GPT in the liver and serum were increased in the control group (Fig. 3A-D). However, by comparing with TAA-induced mice, we found that administration of PCA partially recovered the liver synthetic function (Fig. 3A-D). Moreover, in the PCA (75 mg/kg) group and the model group, masson-staining showed that collagen deposition was quite severe, indicating that liver fibrosis was serious. However, compared with the model group, PCA administration (150 mg/kg) and silymarin administration greatly reduced collagen deposition (Fig. 3E). In summary, all the results illustrate that PCA can reduce TAA-induced liver injury and fibrosis *in vivo*.

2.4. PCA inhibits the protein level of factors associated with TGF-β signaling pathway

TGF-β signaling plays a crucial role in liver fibrosis. TNF-α-induced liver fibrosis is related to the activation of Smad and non-Smad (JNK, ERK, PI3K/Akt and RhoA) (Nakamura et al. 2004). Hence, we investigated whether the TGF-β signaling pathway was associated with PCA-mediated anti-liver fibrosis. The results showed that compared with the normal group, the levels of TGF-β, p-Smad2, p-ERK and c-Jun were increased in the control group (Fig. 4A, B). However, PCA inhibits TAA-induced activation of the TGF-β signaling and decreases high expression levels of p-Smad2 and p-ERK (Fig. 4A, B).

2.5. PCA inhibits the mRNA level of IL-6 and TNF-α in TAA-induced mice

Further we evaluated inflammatory cytokines in the liver and higher mRNA levels in IL-6 and TNF-α were observed in liver

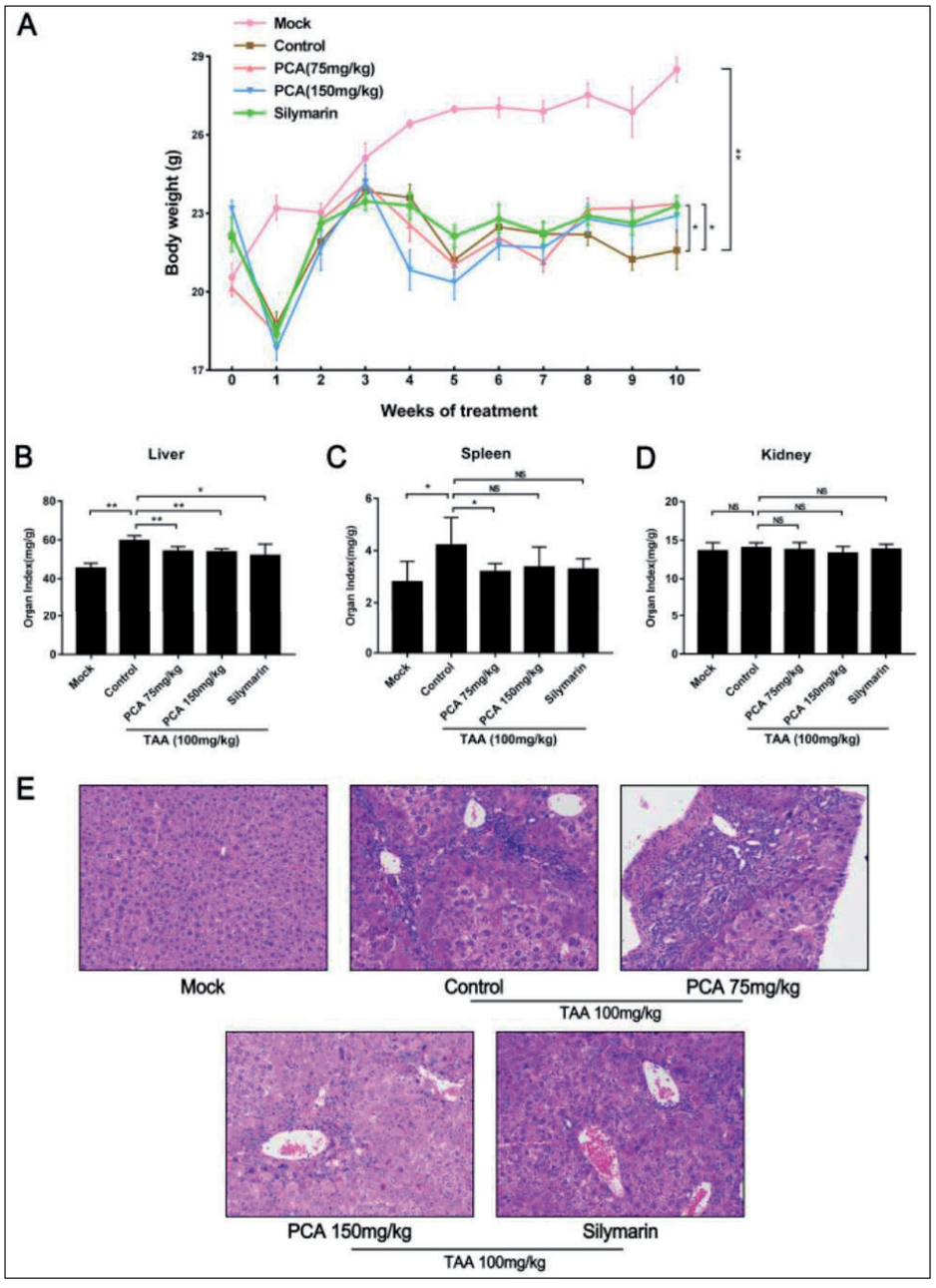

Fig. 2: Effects of PCA on TAA-induced mice. (A) Body weight at the end of the experiment. (B-D) Mice organ index at the end of the experiment. (E) H&E-staining of liver sections, magnification: ×200. H&E stain: hematoxylin and eosin stain. Body weight are expressed as the mean±S.E., and other data are expressed as the mean±S.D. $*p < 0.05$, $**p < 0.01$, not significant (NS).

fibrosis mice. Then we found that treatment with PCA partially relieved liver inflammation (Fig. 5A, B). These results suggest that PCA mitigates inflammation and prevents liver fibrosis by suppressing abnormal activation of the TGF-β signaling pathway.

3. Discussion

Chronic liver injury leads to liver fibrosis with increased activation of hepatic stellate cells. Advanced fibrosis may still be reversed. The main mechanisms of hepatic fibrosis reversal include the deactivation of HSCs, the switch of inflammatory environment and the degradation of extracellular matrix (Zoubek et al. 2017). Protocatechuic acid is widely found in many plants as active phytoconstituent imparting various pharmacological potentials (Herrmann 1989). PCA has special nutritional value. Studies have shown that after 12 weeks of supplementation with PCA in the diet, the level of PCA was increased in blood and organs, such as the brain, heart, liver and kidneys (Lin et al. 2011). In addition, as metabolite of anthocyanins, PCA has been identified in rat plasma and tissues, and in human blood, following administration of anthocyanins (Vitaglione et al. 2007). Therefore, we tried to evaluate the protective effects of PCA on liver fibrosis.

As the key role of causing liver fibrosis, activated HSCs have been regarded as a target for anti-fibrotic treatment. High expression of α-SMA is the characteristic marker of HSC activation (Benyon and Arthur 2001; Friedman 2008; Seki and Brenner 2015). In the study of Jiang et al. (2017), the inhibition of C3G and its metabolite procatechin acid (PCA) on the activation of hepatic stellate cells (HSCs) was found. The inhibition of hepatic stellate cell (HSC) proliferation has been considered as an effective therapeutic target for the treatment of liver fibrosis (Jeong et al. 2015). In the present research, we examined whether PCA can inhibit the cell viability of activated HSCs. It was also found that PCA significantly decreased the cell viability of HSCs and downregulated the expression of TGF-β-related proteins *in vitro*. The results suggested that PCA significantly decreased activation of HSCs. In addition, PCA reduces cell viability in activated HSCs.

TGF-β signaling in most cases is over-activated in the process of liver fibrosis (Meindl-Beinker and Dooley 2008), which can activate Smad-dependent pathways and MAPK-dependent pathways

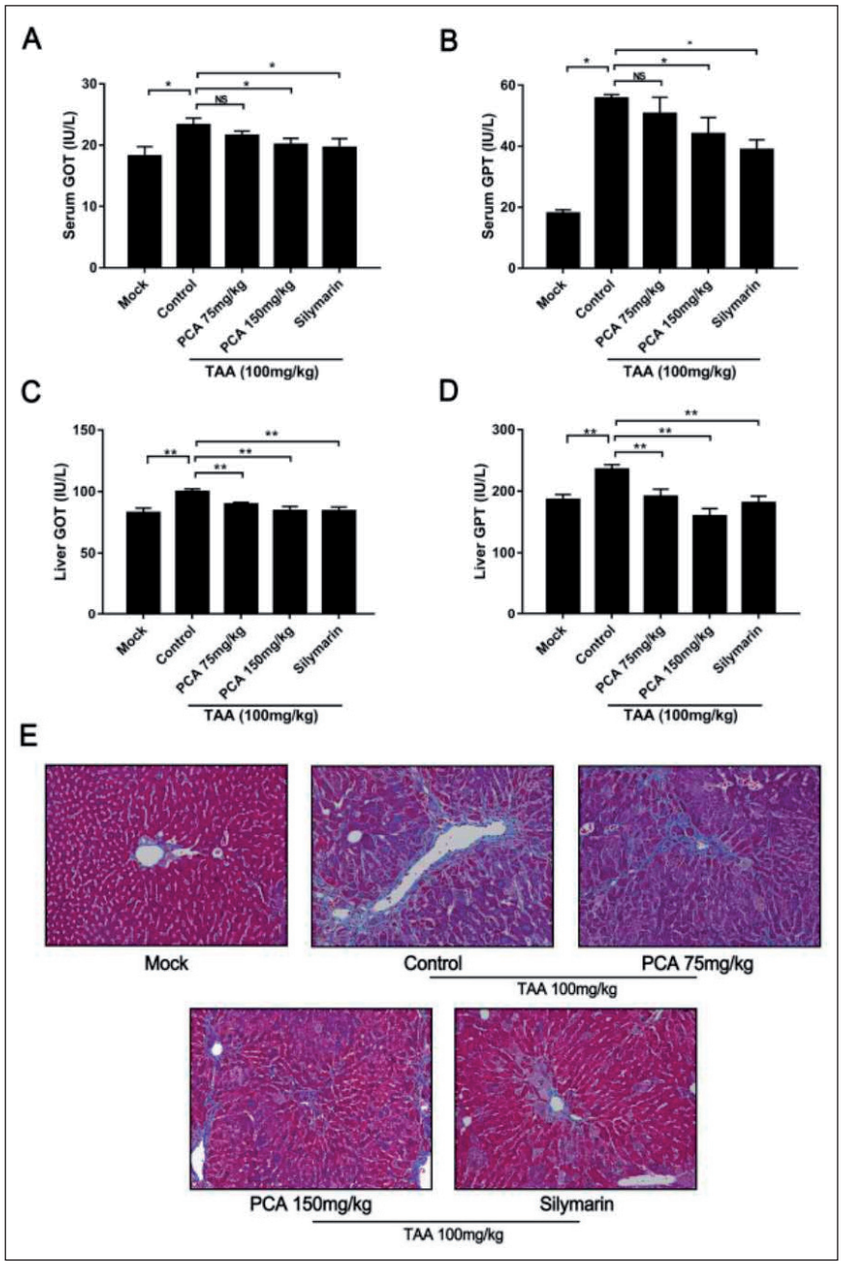

Fig. 3: PCA mitigates liver fibrosis and restores the synthetic function in TAA-induced liver. (A-B) the levels of GOT and GPT in serum. (C-D) The levels of GOT and GPT in liver. (E) Masson-staining of liver sections, magnification: ×200. Data are expressed as the mean±S.D. $*p < 0.05$, $**p < 0.01$, not significant (NS).

(Nakamura et al. 2004). As a kind of MAPKs, ERK activation is required for fibrosis (Yoshikawa et al. 2010). ERK can regulate cell adherents junctions and migration (Davies et al. 2005). What's more, ERK can also phosphorylate Smads (Matsuura et al. 2005). Therefore, inhibiting Smads and ERK activation is necessary for suppression and reversal of liver fibrosis. Our research showed that PCA inhibits HSCs activation and proliferation through downregulation on ERK and Smad2 signaling pathway. To explore the mechanism of PCA in liver fibrosis, we investigated whether the TGF-β pathway was inhibited when mice were treated with PCA. Western blot showed that the phosphorylation levels of Smad2, ERK and TGF-β were obviously decreased in mice treated with PCA, indicating that the therapeutic effect for fibrosis of PCA is dependent on the inhibition of the TGF-β signaling pathway.

In summary, our research provides original evidence about the protective role of PCA against liver fibrosis *in vivo* and *in vitro via* downregulation of TGF-β signaling pathway, which has potential implications for the development of an anti-fibrotic therapy.

4. Experimental

4.1. Chemicals and antibodies

Thioacetamide (TAA) (purity > 99%, modeling level), protocatechuic acid (PCA) (purity > 98%), and silymarin (purity≥80 purity) were purchased from Dalian Meilun Biology (Dalian, China). TGF-β recombinant protein was from Thermo Fischer Scientific (Fremont, CA, USA). Cloud-Clone Corp (Katy, TX, USA) provided TNF-α. TGF-β, HRP-conjugated goat anti-rabbit or mouse IgG was got from Cell Signaling Technology, Inc. (Danvers, MA, USA). β-actin, c-Jun were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). α-SMA, Snail was obtained from Abcam Inc. (MA, USA). Santa Cruz Biotechnology provided p-Smad2, p-ERK. PCA was firstly prepared with sterile water into mother liquor with a concentration of 100 mM, and TNF-α was prepared with 1×PBS into stock liquor with a concentration of 10 μg/mL. Finally, the stock liquor was diluted with medium into working fluid.

4.2. Cell culture and treatment

Culturing HSC-T6 in DMEM (Hyclone, Thermo Fisher, Waltham, MA), which contained 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were cultured in an atmosphere of 37 °C with 5% CO_2. 5000 HSC-T6 cells were plated in 96-well plates and treated with FBS-free DMEM medium for 12 h, then the experiment group was treated with PCA (1 mM and 3 mM)

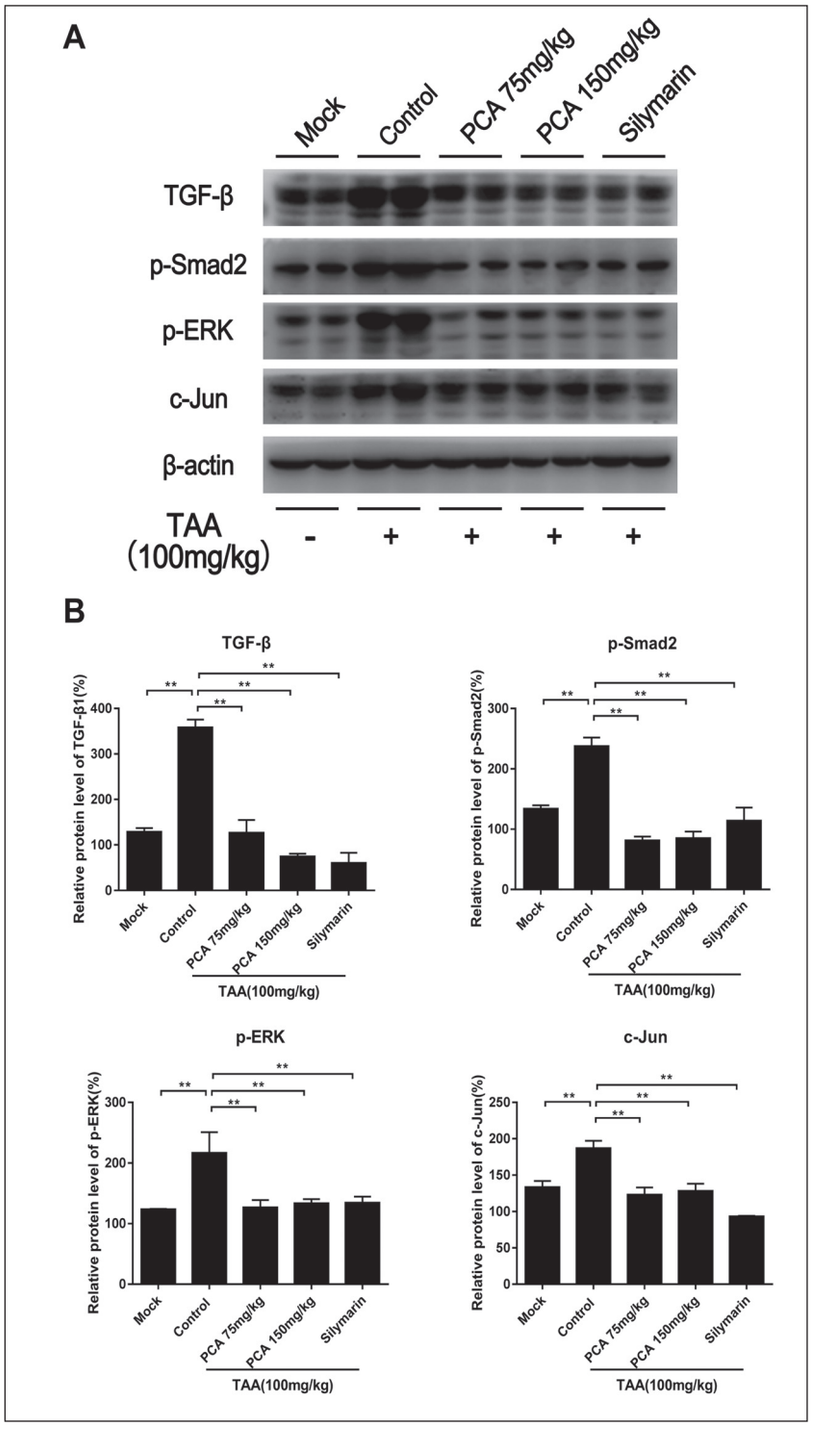

Fig. 4: PCA depresses the TGF-β signaling pathway in TAA-induced liver fibrosis. (A-B) Effects of PCA on the expression of TGF-β-related protein in the liver tissues were measured by western blot analysis. Data are expressed as the mean ± S.D. $*p < 0.05$, $**p < 0.01$, not significant (NS).

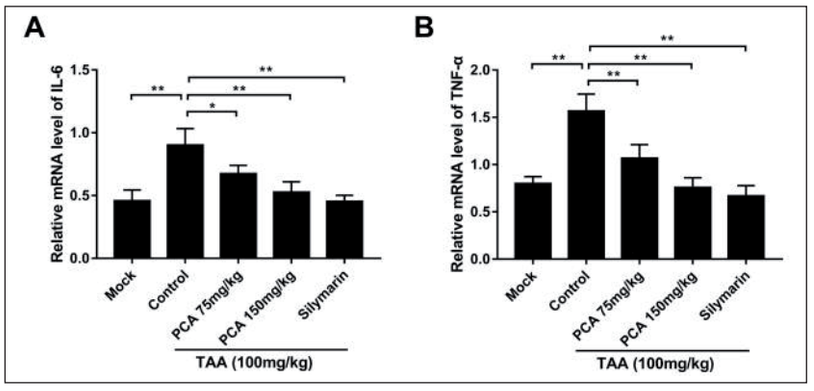

Fig. 5: PCA mitigates liver inflammation in TAA-induced liver fibrosis. (A-B) The mRNA expression of TNF-α and IL-6 were assessed in different groups. Data are expressed as the mean ± S.D. $*p < 0.05$, $**p < 0.01$, not significant (NS).

containing 10 ng/ml TNF-α for 24 h (Paik et al. 2006), the model group was treated with 10 ng/ml TNF-α only to establish a fibrotic model, and the positive control group was treated with 40 μM silymarin (Ezhilarasan et al. 2017; Shin et al. 2018).

4.3. Cell proliferation assays

Cell viability was measured by MTT assay. For MTT in HSC-T6, cells were transplanted into 96-well plates at the appropriate seeding density of 5000 cells/well, after treatment with TNF-α and PCA for 24 h, the HSC-T6 cells were treated with 0.5 mg/ml MTT for 4 h, then 100 μl dimethylsulfoxide (DMSO) were added to incubate for 20 min. The absorbance of each well in plate was measured at 490 nm (Hu et al. 2015; Xu et al. 2013; Yao et al. 2014).

4.4. Animals and experimental design

Male C57BL/6 mice which were five weeks old (18-20 g) were purchased from Benxi Changsheng Laboratory Animal Technology Co., Ltd. The mice were maintained in controlled environment at 21±2.0 ℃ and had free access to water and food. All animal experiment protocols were approved by the Ethics Committee of Liaoning University of Traditional Chinese Medicine (Shenyang, China).

After one week of adaptation, the mice were separated into five groups: one normal group (mock group), one model control group (control group), two PCA-treated groups (PCA group) and one positive control group (Silymarin group), every group contained eight mice. Except those in the mock group, all mice were intraperitoneally injected with 10 mg/ml TAA (dissolved in 0.9% saline) to induce liver injury (100 mg/kg every 3 days for 10 weeks) (Chang et al. 2014). The mock group received saline instead of TAA. The PCA group and the silymarin group received TAA plus PCA (75 mg/kg, 150 mg/kg) and TAA plus silymarin (100 mg/kg, suspended in 0.5% CMC mixture), respectively, by gastric gavage after three weeks treating with TAA (El-Sisi et al. 2017). At the end of the tenth week, mice were sacrificed. The blood samples were stored at 4 °C to analyze GOT and GPT. The liver was weighed and divided into two parts, one part was fixed in 10% paraformaldehyde and the other part was immediately frozen in liquid nitrogen then stored at -80 °C.

4.5. Serum and liver glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) analysis

Mice blood was taken by removing the eyeball and kept at 4 °C for 30 min. The serum was separated by centrifugation at 2500 r/min for 10 min. The level of liver and serum GPT was measured by Reit's method (C009-2, Njjcbio, China), and GOP was detected using colorimetry method (C010-1, Njjcbio, China).

4.6. Quantitative RT-PCR

The total RNA was extracted from liver tissues with TRIzol Reagent (ComWin Biotech Co., Ltd., Beijing, China). The concentration and the quantity and quality of RNA were determined by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA). Total RNA (1000 ng) was reverse-transcribed to complementary DNA (cDNA) using cDNA synthesis kit (Takara, Dalian, China). Quantification of mRNA expression was performed by quantitative real-time PCR (qRT-PCR) using SYBR Green Supermix kit (Takara) and Applied Biosystems 7500 Real-Time PCR system. The measurement was conducted at least three times. The sequences of the primers were:

IL-6: forward 5'-TAGTCCTTCCTACCCCAATTTCC-3',
reverse 5'-TTGGTCCTTAGCCACTCCTTC-3';
TNF-α:forward 5'-CCCT -CACACTCAGATCATCTTCT-3',
reverse 5'-GCTACGACGTGGGCTA -CAG-3'.

4.7. Histological analysis

Liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin then made into 4 μm sections. The haematoxylin/eosin (H&E) and Masson's trichrome were used to stain sections, and then examined under light microscopy by an experienced pathologist.

4.8. Western blot analysis

Total proteins were quantified by a BCA protein assay kit (Thermo Fisher, Waltham, MA). The BCA assay was used to determine the protein concentration. SDS-PAGE (10%) was used to separate protein and the wetting transfer system was used to transfer protein onto NC membranes. The membrane was blocked with 5% BSA in TBS-Tween 20 (0.1% TBST) at room temperature for 1 h. After a brief rinse, the membrane was incubated overnight at 4 °C in TBST with corresponding primary antibodies including mAb of TGF-β (1:1000), α-SMA (1:1000), p-Smad2 (1:1000), p-ERK (1:1000), c-Jun (1:1000) and β-actin (1:1000). After that, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:5000) for 2 h at room temperature. Then, the membrane was washed in TBST and protein was detected by enhanced chemical luminescence (ECL). Each sample was analyzed in triplicate.

4.9. Statistical method

Each cell experiment was repeated at least three times. Student's *t* test and one-way ANOVA was used to compare differences between groups. (mean±SD). Statistical significance was assumed for $*P < 0.05$, $**P < 0.01$, not significant (NS).

Authors contributions: Bo Cui: Data development, Methodology, Writing-original draft. Zhe Yang: Data development, Software, Validation. Shuning Wang: Data development, Analysis. Mengnan Guo: Software, Methodology, Resource. Qianqian Li: Data development, Software. Qiuhua Zhang: Data development, Investigation. Xiuli Bi: Project administration, Supervision, Conceptualization, Methodology, Writing-original draft.

Conflict of interest: The authors declare that there is no conflict of interest.

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