

Original Article

S-allyl-cysteine attenuates carbon tetrachloride-induced liver fibrosis in rats by targeting STAT3/SMAD3 pathway

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Abstract: S-allyl-cysteine (SAC) is one of the major compounds in aged garlic extract, and has been proved to be an endogenous donor of hydrogen sulfide (H₂S), which plays emerging roles in the gastrointestinal tract and liver. In this study, Sprague-Dawley rats were intraperitoneally injected with a mixture of carbon tetrachloride (CCl₄, 1 mL/kg body weight) and olive oil (1:1 v/v) every other day for 8 weeks to induce liver fibrosis. Treatment of SAC (50 mg/kg/day) could attenuate CCl₄-induced liver fibrosis, with improved semi-quantitative scores of fibrosis severity based on the staining of H&E, Oil Red O, and Sirius Red. SAC attenuated CCl₄-induced transaminase elevation in the plasma of the rats. In the liver, SAC could reduce the mRNA expression of inflammatory and fibrogenic cytokines, including interleukin 6, interferon γ , tumor necrosis factor α , and transforming growth factor β (TGF β), as well as induce the mRNA expression of antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase. The mRNA expression of biomarkers of liver fibrosis, including α -smooth muscle actin, fibronectin and collagen I, were also decreased after SAC treatment. In addition, SAC reduced the phosphorylation of SMAD3 and signal transducers and activators of transcription 3, and further inhibited their binding ability to transcription promoters. Taken together, SAC attenuated CCl₄-induced liver fibrosis in rats with anti-oxidant, anti-inflammatory and anti-fibrotic effects, and targeted STAT3/SMAD3 pathway to inhibit gene transcription.

Keywords: Liver fibrosis, S-allyl-cysteine, oxidation, inflammation, TGF β , SMAD3

Introduction

Liver fibrosis and its end stage, cirrhosis, represent the final common pathways of virtually all chronic liver diseases, in which cirrhosis affected about 2.8 million people in 2015 [1]. Liver fibrosis, characterized by the excess production and deposition of extracellular matrix (ECM) components, is the result of wound-healing responses stimulated by various liver injury, including alcohol abuse, viral infection, and cholestasis. In response to liver injury, quiescent hepatic stellate cells (HSC) are activated and undergo morphological transition to myofibroblast-like cells that express α -smooth muscle actin (α SMA) and profibrogenic genes. HSC activation, the most important event in liver fibrosis, is mediated by many inflammatory and fibrogenic cytokines, or reactive oxygen species (ROS) released from the damaged hepatocytes

and Kupffer cells [2-4]. Therefore, all these cytokines/enzymes involved in the complicated cross-talking of various cell types are targets for pharmacological interventions for the treatment of hepatic fibrosis [5].

Among these cytokines, transforming growth factor β (TGF β) is the most well-clarified signal molecule in pathogenesis of liver fibrosis [6, 7]. High concentration of TGF β was reported in serum and liver biopsy samples of patients with hepatic fibrosis [8]. TGF β binds to TGF β receptor on the HSC, and acts through phosphorylation of intracellular SMAD proteins, which can transduce extracellular signals from TGF β to the nucleus where they activate downstream gene transcription [9, 10]. SMAD proteins are divided into three functional classes: receptor-regulated (SMAD 1, 2, 3, 5, and 8/9), common mediator (SMAD4), and inhibitor (SMAD 6 and

7) [11]. Signal transducers and activators of transcription 3 (STAT3) belongs to another family of transcription factors, which can be activated by cytokine receptors, particularly interferon and interleukin receptors [12]. STAT3 could enhance hepatic fibrosis through the upregulation of TGF β expression [13].

Hydrogen sulfide (H₂S) is the third gasotransmitter in biology and medicine after nitrogen oxide and carbon monoxide, and displays antioxidant, anti-inflammatory and cytoprotective activities [14-16]. H₂S is endogenously produced in mammalian tissues by cystathionine γ -lyase (CSE) and cystathionine β -synthase (CBS), but CSE accounts for more than 96% of H₂S production in livers [17]. Hydrogen sulfide attenuated carbon tetrachloride (CCl₄)-induced hepatotoxicity, liver cirrhosis and portal hypertension in rats [18]. *In vitro*, exogenous H₂S inhibited proliferation and induced cell cycle arrest and apoptosis in activated HSC [19, 20]. S-allyl-cysteine (SAC) is one of the major compounds in aged garlic extract, which is derived from the degradation of S-alk(en)yl-cysteine sulfoxide, has been proved to be an endogenous H₂S donor [21]. In this study, we aimed to test the protective effects of SAC in a rat model of CCl₄-induced liver fibrosis, and try to explore its molecular mechanisms.

Materials and methods

Reagents

CCl₄ and SAC were purchased from Sigma-Aldrich Co. (St Louis, MO).

Animals

Male Sprague-Dawley rats (200-250 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Animals were housed in a temperature-controlled room (22°C) with 12-hour-light/12-hour-dark cycling, and had free access to food and water. All the animals received humane care. All experimental protocols complied with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH) of the United States and were approved by the Institutional Animal Care and Use Committee of Guangxi Traditional Chinese Medical University.

Model and treatment

After one week acclimation, thirty rats were randomly divided into three groups (N=10). Group 1 was the naïve control in which rats were intraperitoneally injected with the vehicle olive oil. Group 2 was the model group in which rats were intraperitoneally injected with CCl₄. Group 3 was the treatment group in which rats were intraperitoneally injected with both CCl₄ and SAC (50 mg/kg/day). Rats in group 2 and 3 were injected with a mixture of CCl₄ (1 mL/kg body weight) and olive oil (1:1 v/v) every other day for 8 weeks to induce liver fibrosis [22].

Sample collection

After 8 weeks of treatment, all rats were euthanized by CO₂ inhalation. Blood was obtained by cardiac puncture and collected into EDTA-containing tubes, then centrifuged to get plasma for biochemical analysis. Livers were removed and cut into 2 parts, one of the parts was fixed in 4% formalin in room temperature for histopathological evaluation, and the other part was stored in -80°C for other studies.

Histopathologic evaluation

For hematoxylin and eosin (H&E) and Sirius Red collagen staining, formalin-fixed liver was embedded into paraffin and cut into 5 μ m sections. For Oil Red O staining, liver tissue was dehydrated before embedded into OCT compound (Sakura, Tokyo, Japan), and cut into 10 μ m frozen sections. Commercially available kits (Beyotime, Shanghai, China) were used to stain sections. An expert pathologist evaluated the slides in a blinded fashion with Olympus microscope (Tokyo, Japan) in 100 \times (for Oil Red O) or 200 \times (for H&E and Sirius Red) augmentation, and assigned scores for fibrosis severity [23]: 0, no fibrosis and normal liver architecture; 1, fibrosis localized only in portal zone and had the inclination of extension into lobule; 2, fibrosis extended into the lobule of its 2/3 and had the same changes as 1; 3, fibrosis extended into lobule to the periphery of its central vein; 4, fibrosis extended into the whole lobule, pseudolobule formation and had the same changes as 3.

Biochemical analysis

Plasmatic levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and

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alkaline phosphatase (ALP) were determined by using Hitachi 7180 autoanalyzer (Hitachi, Tokyo, Japan) with Cobas kits (Roche Diagnostics, Indianapolis, IN).

Real-time polymerase chain reaction (RT-PCR)

Trizol reagent (Takara, Dalian, China) was used for isolating total RNA from liver tissue. 50-100 mg of tissue was directly lysed by mixing with 1 mL of Trizol reagent and homogenized using a homogenizer. Then 0.2 mL of chloroform was added to the homogenized sample, and incubated for 20 minutes at room temperature. Subsequently, RNA was precipitated by mixing with isopropyl alcohol. Total RNA yield was quantified by microplate reader (Molecular Devices, Sunnyvale, CA) measured at 260 nm. Then mRNA was isolated from total RNA by using Oligo (dT), and reverse transcribed into first-strand complement DNA (cDNA) and amplified using a PrimeScript 1st strand cDNA synthesis kit (Takara). Reaction system included 2 μ L of cDNA, 12.5 μ L of 2 \times SYBR Green 1 Master Mix (Takara), and 1 μ L of each primer. The PCR condition was as follows: pre-incubation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 5 seconds, and annealing/extension at 60°C for 30 seconds using iQ5 RT-PCR detection system (Bio-Rad, Hercules, CA). The primers were listed as below [22, 24-27]:

SOD1: Forward 5'-GCAGGACCTCATTTAATCC-TCACCT-3', Reverse 5'-AGGTCTCCAACATGCCT-CTCTTC-3'; *CAT*: Forward 5'-GGAGGCAGAAAC-TTCCCATT-3', Reverse 5'-GGCCAAACCTTGG-TCAGATC-3'; *GPx*: Forward 5'-CAGTTCGGACA-TCAGGAGAAT-3', Reverse 5'-AGAGCGGGTGAG-CCTTCT-3'; *IL6*: Forward 5'-GGAGTTCGGTTT-CTACCTGG-3', Reverse 5'-GCCGAGTAGACCTC-ATAGTG-3'; *IFN γ* : Forward 5'-TCAAGTGGCATA-GATGTGGAAGAA-3', Reverse 5'-TGGCTCTGCAG-GATTTTCATG-3'; *TNF α* : Forward 5'-CCACGTCG-TAGCAAACCACCAAG-3', Reverse 5'-CAGGTAC-ATGGGCTCATACC-3'; α SMA: Forward 5'-CCGA-CCGAATGCAGAAGGA-3', Reverse 5'-ACAGAGT-ATTTGCGCTCCGGA-3'; *TGF β* : Forward 5'-TGC-TCGCTTTGTACAACAGCAC-3', Reverse 5'-CGTT-CCCGAATGTCTGACGTAT-3'; *Fibronectin*: Forward 5'-TGTCACCCACCACCTTGA-3', Reverse 5'-CTGATTGTTCTTCACTGCGA-3'; *Collagen I*: Forward 5'-CCTCAAGGGCTCCAACGAG-3', Reverse 5'-TCAATCACTGTCTTGCCCCA-3'; *MMP2*:

Forward 5'-ACAGGTGTGCCAAGGTGGAA-3', Reverse 5'-CGCCGGAACCATCACTAGGCG-3'; *TIMP1*: Forward 5'-CGAGACCACCTTATACCAGC-GTTA-3', Reverse 5'-CGCCATTTCCGTTTCTTAG-GCG-3'.

Western blot

Liver tissue was rinsed in ice-cold PBS, and lysed in lysis buffer (Beyotime). Equal amounts of protein were separated by 10% SDS-PAGE, transferred to PVDF membrane (Millipore, Billerica, MA), and blocked with 5% nonfat dry milk in TBS-Tween 20 for 1 hour at room temperature. The membrane was incubated with primary antibody overnight. Antibodies to p-STAT3, STAT3 and GAPDH were purchased from Cell Signaling Technology (Danvers, MA), and antibodies to SMAD3, p-SMAD3 and CSE were purchased from Abcam (Cambridge, MA). After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 hour. Following several washes, the blots were developed by Immobilon solution (Millipore).

Chromatin immunoprecipitation (ChIP)

ChIP was performed using STAT3 antibody (Cell Signaling Technology), SMAD3 antibody (Abcam) or control rabbit IgG using Agarose ChIP Kit (Pierce, Rockford, IL). Briefly, liver tissue was homogenized and sonicated to shear the chromatin. The immunoprecipitated chromatin fragments were eluted, treated with proteinase K. The eluted DNA fragments were quantified by RT-PCR using primers specific to the *JUNB* promoter as follows [28]:

-321 bp~-44 bp: Forward 5'-CCAGTGGACTC-CAGGGAAATC-3', Reverse 5'-GCGCTAGTCAGC-CACGGAAG-3'; -2858 bp~-2718 bp: Forward 5'-CTGAATTACTGTGGCCTCCT-3', Reverse 5'-GCTAATAACTGCAGCTGACATC-3'.

Measurement of H₂S level

To measure H₂S concentration, 50 μ L of plasma and 450 μ L of distilled water were added to a microtube containing 250 μ L of zinc acetate (1% w/v). Subsequently, 133 μ L of N,N-dimethyl-p-phenylenediamine sulphate (NNDPD, 20 μ M) in 7.2 M HCl was added, then followed by 133 μ L of FeCl₃ (30 μ M) in 1.2 M HCl. 250 μ L

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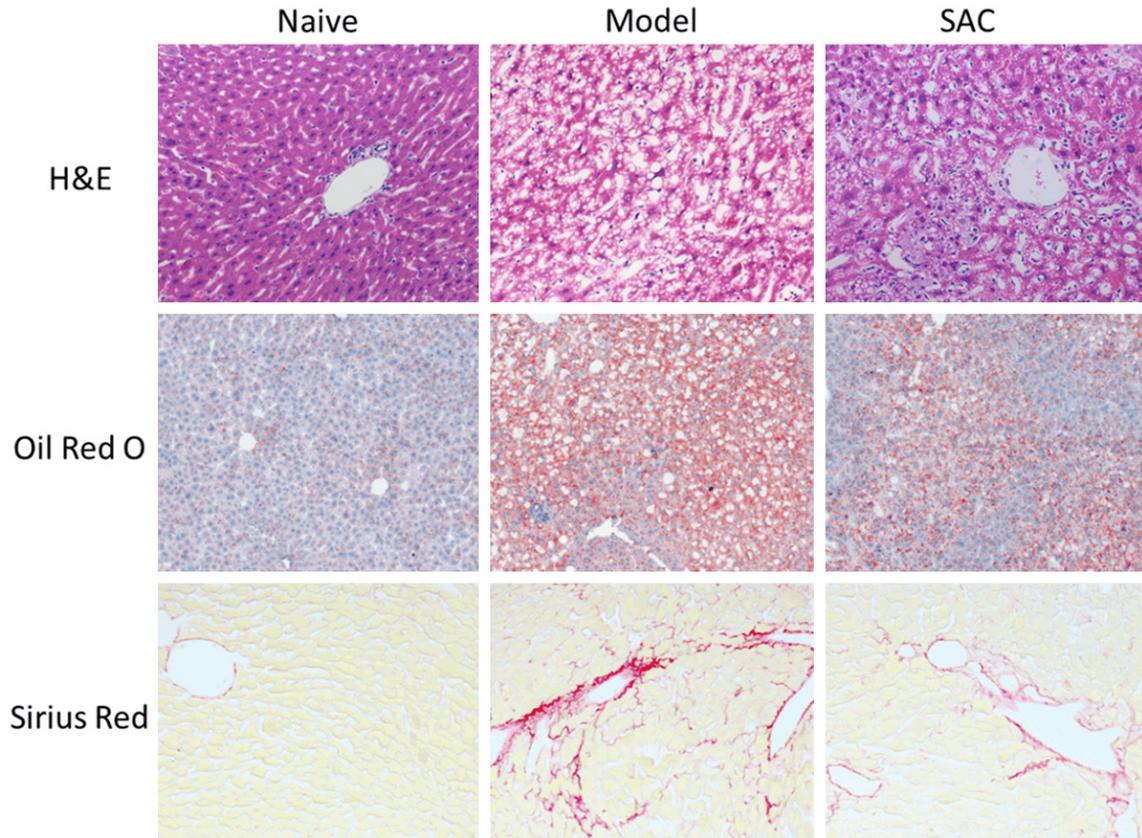


Figure 1. SAC attenuated CCl_4 -induced liver fibrosis in rats. The rats were intraperitoneally injected with a mixture of CCl_4 (1 mL/kg body weight) and olive oil (1:1 v/v) every other day for 8 weeks to induce liver fibrosis. SAC (50 mg/kg/day) was intraperitoneally injected into rats for 8 weeks. At the termination, liver was removed, fixed, and cut into sections for histopathological evaluation. 100 \times augmentation for Oil Red O staining, and 200 \times augmentation for H&E and Sirius Red staining.

Table 1. Semi-quantitative scoring system for fibrosis severity

	Animal Number						Fibrosis Score
	Total	Fibrosis Grade					
		0	1	2	3	4	
Naïve	10	8	2	0	0	0	0.20 \pm 0.42
Model	10	0	0	2	5	3	3.10 \pm 0.74 ^{###}
SAC	10	0	4	6	0	0	1.60 \pm 0.52 ^{***}

Data of fibrosis score were presented as Mean \pm SD. N=10. ^{###} $P < 0.001$ vs. naïve group. ^{***} $P < 0.001$ vs. model group.

of trichloroacetic acid (TCA, 10% w/v) was then used to precipitate any protein. This final solution was centrifuged at 24,000 g for 5 min at 4°C. The optical absorbance was measured at 670 nm using a 96-well microplate reader (Molecular Devices).

Statistical analysis

Data were presented as Mean \pm standard deviation (SD). Significance of difference between groups was analyzed by performing one-way ANOVA with Dunnett's multiple comparison test or unpaired Student's t test. P value less than 0.05 was considered statistically significant. Data were analyzed and graphed by Prism 6.0 (GraphPad Software, La Jolla, CA).

Results

SAC attenuated CCl_4 -induced liver fibrosis in rats

In model group, H&E staining showed moderate inflammatory infiltration and ballooning degeneration of hepatocytes; Oil Red O staining showed significant fat deposition; Sirius Red

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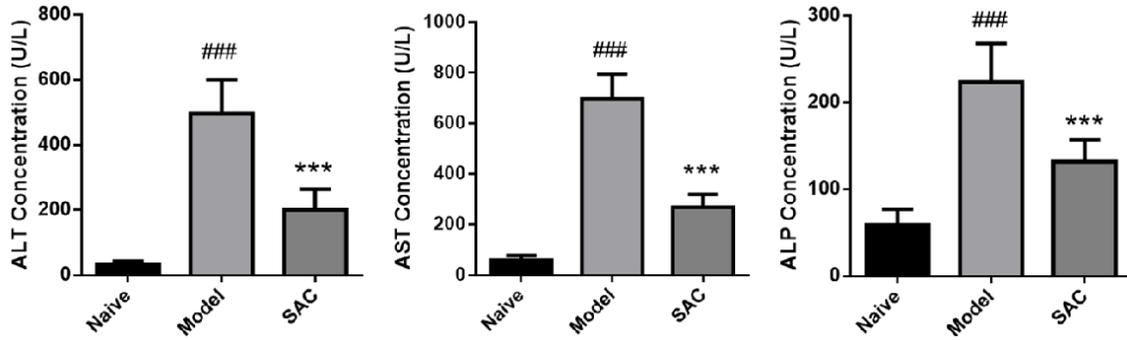


Figure 2. SAC attenuated CCl₄-induced transaminase elevation. Plasmatic levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were determined by autoanalyzer. Data were presented as Mean \pm SD. N=10. ###*P* < 0.001 vs. naïve group. ****P* < 0.001 vs. model group.

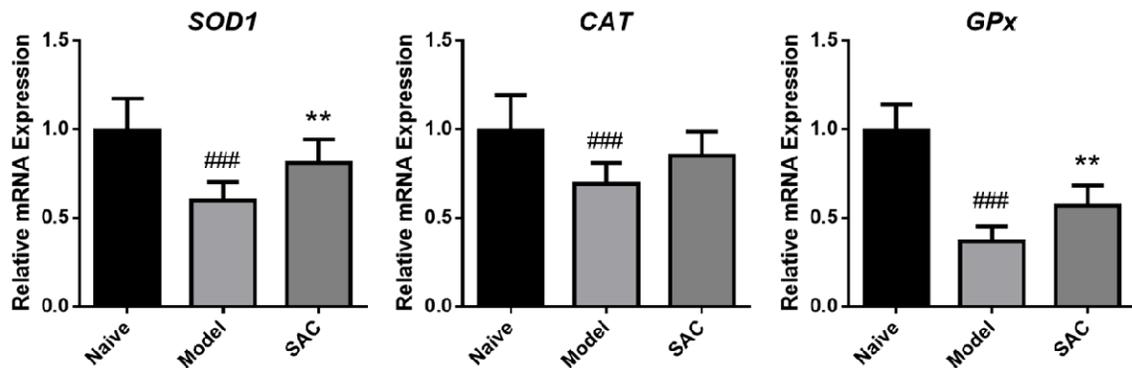


Figure 3. SAC modulated the expression of genes related to oxidation. Livers were homogenated for RNA extraction and real-time PCR analysis of mRNA expression. SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase. Data were presented as Mean \pm SD. N=10. ###*P* < 0.001 vs. naïve group. ***P* < 0.01 vs. model group.

staining revealed marked pericellular fibrosis around central veins (**Figure 1**). These histopathological data demonstrated that 8-week-administration of CCl₄ successfully induced liver fibrosis. Using a semi-quantitative scoring system for fibrosis severity, we observed that the score of model group was much higher than that of naïve group (3.10 ± 0.74 vs. 0.20 ± 0.42 , *P* < 0.001), while SAC could significantly attenuate fibrosis severity (1.60 ± 0.52 vs. 3.10 ± 0.74 , *P* < 0.001; **Table 1**).

SAC attenuated CCl₄-induced transaminase elevation

The plasmatic transaminase levels of rats in model group were elevated by CCl₄ administration compared with those in naïve group (496.3 ± 104.7 U/L vs. 32.10 ± 10.01 U/L, *P* < 0.001 for ALT; 697.7 ± 98.09 U/L vs. 58.90 ± 18.02 U/L, *P* < 0.001 for AST; 223.7 ± 44.06 U/L vs. 58.90 ± 18.02 U/L, *P* < 0.001 for ALP; **Figure**

2). SAC could preserve the transaminases to lower levels (200.7 ± 63.59 U/L, *P* < 0.001 vs. model group for ALT; 268.8 ± 50.75 U/L, *P* < 0.001 vs. model group for AST; 131.8 ± 25.17 U/L, *P* < 0.001 vs. model group for ALP; **Figure 2**).

Anti-oxidant, anti-inflammatory and anti-fibrotic effects of SAC

The mRNA expression of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), in the liver was reduced by CCl₄ administration, while SAC could preserve the expression to a higher level (**Figure 3**). The mRNA expression of pro-inflammatory mediators, including interleukin 6 (IL6), interferon γ (IFN γ), and tumor necrosis factor α (TNF α), in the liver was induced by CCl₄ administration, while SAC could lower the expression (**Figure 4**).

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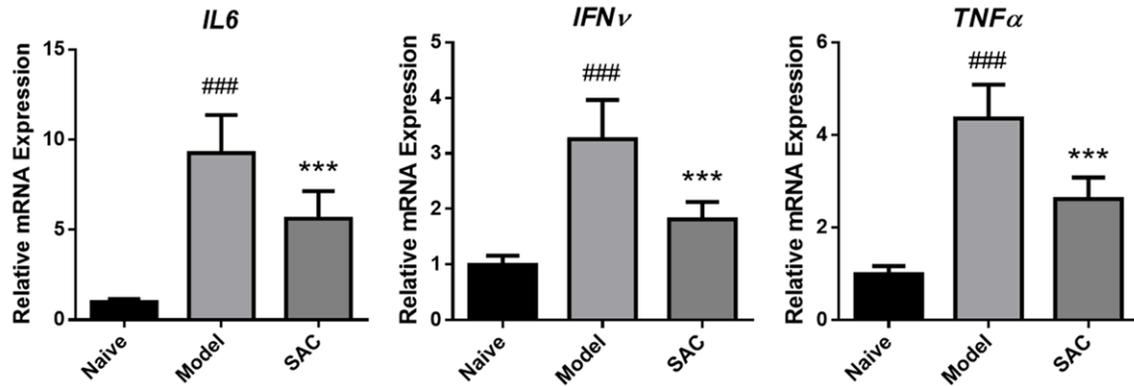


Figure 4. SAC modulated the expression of genes related to inflammation. Livers were homogenated for RNA extraction and real-time PCR analysis of mRNA expression. IL6, interleukin 6; IFN γ , interferon γ ; TNF α , tumor necrosis factor α . Data were presented as Mean \pm SD. N=10. ### P < 0.001 vs. naive group. *** P < 0.001 vs. model group.

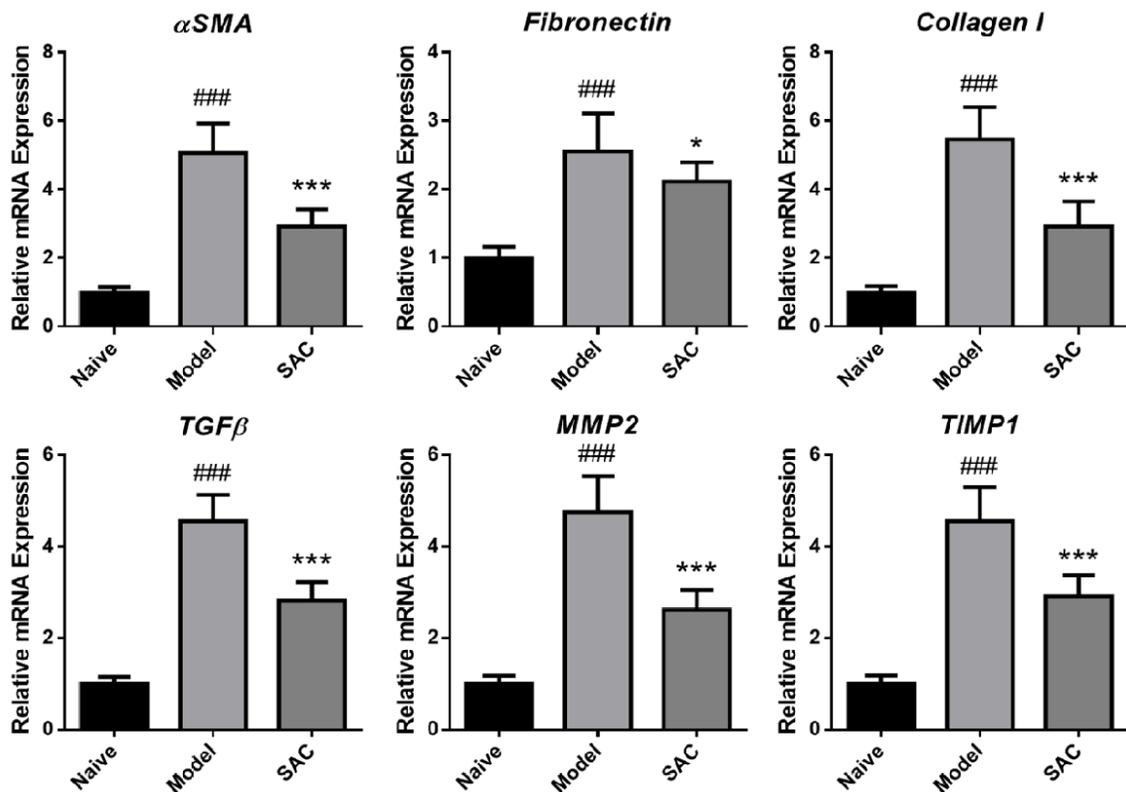


Figure 5. SAC modulated the expression of genes related to fibrosis. Livers were homogenated for RNA extraction and real-time PCR analysis of mRNA expression. α SMA, α -smooth muscle actin; TGF β , transforming growth factor β ; MMP2, matrix metalloproteinase 2; TIMP1, tissue inhibitor of metalloproteinases 1. Data were presented as Mean \pm SD. N=10. ### P < 0.001 vs. naive group. *** P < 0.001 vs. model group. * P < 0.05 vs. model group.

The mRNA expression of biomarkers of liver fibrosis, including α SMA, fibronectin and collagen I, and pro-fibrotic mediators, including TGF β , matrix metalloproteinase 2 (MMP2), and

tissue inhibitor of metalloproteinases 1 (TIMP1), in the liver was also induced by CCl $_4$ administration, which could be reduced by SAC treatment (Figure 5).

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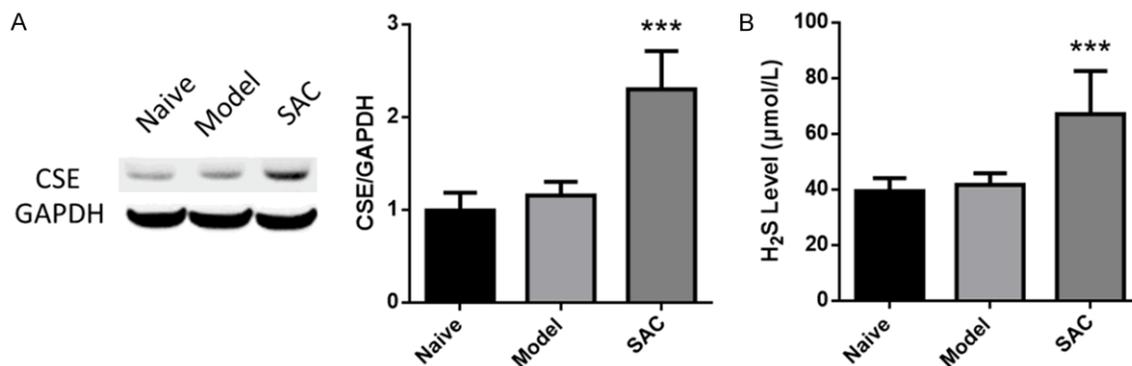


Figure 6. SAC acted through elevating H₂S level. A. Livers were homogenated for Western blot. B. Plasmatic level of H₂S was determined by colorimetric method. Data were presented as Mean ± SD. N=10. ****P* < 0.001 vs. model group.

SAC acted through elevating H₂S level

In order to make sure that the protective effect of SAC was mediated by elevating H₂S level, we determined the protein expression of CSE, producing enzyme of endogenous H₂S, in the liver and H₂S level in the plasma. The model did not change the protein expression of CSE and plasmatic level of H₂S, while both of them were significantly induced after SAC treatment (**Figure 6**).

SAC targeted STAT3/SMAD3 pathway

SAC did not change the protein expression of STAT3 and SMAD3, but significantly reduced the phosphorylated protein (**Figure 7A**). To investigate possible regulatory changes of STAT3 and SMAD3 in transcription, we used ChIP to examine their ability to bind *JUNB* promoter, which contained both well-characterized proximal STAT3 binding site and distal SMAD3 binding site [28]. Our results showed that specific binding of STAT3 or SMAD3 to their respective sites was reduced after SAC treatment (**Figure 7B**). All data suggested inhibitory effect of SAC on STAT3/SMAD3 pathway.

Discussion

Emerging roles of H₂S in the gastrointestinal tract and liver has been clarified in the past two decades [16]. SAC is a natural product in aged garlic extract with both long-term stability in storage and high bioavailability in subjects [29, 30], and could produce endogenous H₂S in freshly collected human blood to mediate vasodilatation *ex vivo* [31]. It was reported that SAC

could increase CSE activity in myocardial tissue and plasma H₂S concentration [21]. Our study observed induced CSE activity in liver tissue and elevated plasma H₂S concentration after SAC treatment, which was consistent with the previous study.

The pathogenesis of liver fibrosis is complicated, in which the activation of HSC via transformation to myofibroblast-like cells that express α SMA is a key issue [4]. It was reported that in the normal rat liver, α SMA was observed only in vascular smooth muscle cells. With the progression of fibrosis induced by CCl₄ injection, α SMA-positive cells appeared in the perisinusoidal space and the fibrous septa, and ultimately surrounded regenerative nodules [32]. Therefore, α SMA is a good biomarker of liver fibrosis. In our study, we observed the induced mRNA expression of α SMA in model group, which could be reduced after SAC treatment.

The activation of HSC is mediated by various cytokines and ROS released from the damaged hepatocytes and activated Kupffer cells [4]. TGF β is the most potent profibrogenic factor that activates HSC [6]. High levels of TGF β were widely reported in many CCl₄-induced rat model [33, 34]. TGF β transgenic mouse is also an inducible animal model to study liver fibrosis [35]. In contrast, blockade of TGF β signaling prevented liver fibrosis and dysfunction in the rat [36]. Besides, many inflammatory cytokines, such as IL6, IFN γ , and TNF α , as well as ROS, were also secreted by the damaged hepatocytes and activated Kupffer cells to activate HSC [6, 7]. SAC treatment could reduce the mRNA expression of these fibrogenic and

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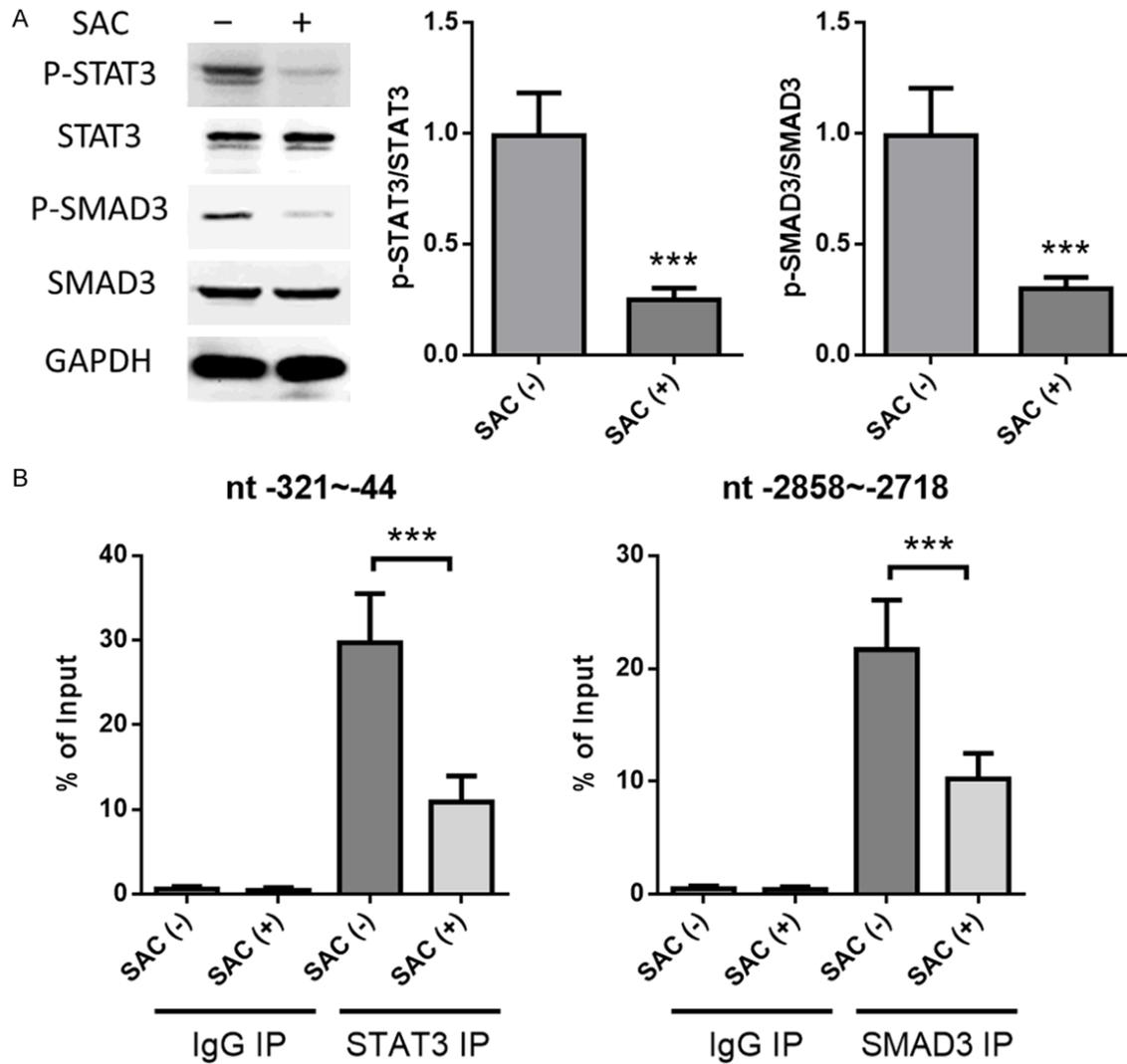


Figure 7. SAC targeted STAT3/SMAD3 pathway. A. Livers were homogenated for Western blot. B. Livers were homogenated for chromatin immunoprecipitation and analyzed by real-time PCR. Data were presented as Mean \pm SD. N=10. *** $P < 0.001$.

inflammatory cytokines in the liver, while induce that of antioxidant enzymes, such as SOD, CAT, and GPx, to resist the oxidative stress.

The events subsequent to HSC activation, including the augmented production and disposition of ECM, are crucial for the hepatic fibrogenesis cascade [6]. In advanced stages, the liver contains approximately 6 times more ECM than normal, including collagens (I, III, and IV), fibronectin, undulin, elastin, laminin, hyaluronan, and proteoglycans [7]. Accumulation of ECM results from both increased synthesis and decreased degradation, including the induced activity of TIMPs and reduced activity of MMPs

[6, 7]. In our study, we observed accumulated collagen I and fibronectin in the liver, which could be attenuated by SAC treatment. These data were also consistent with that of Sirius Red staining of liver tissue.

TGF β /SMAD pathway is the most extensively investigated molecular mechanism in hepatic fibrogenesis [37]. Synergistic cooperation between SMAD3 and other transcription factors can mediate the stimulatory effect of TGF β on transcription of collagen in activated HSC [37]. Cell type-specific intervention of TGF β /SMAD signaling suppresses collagen gene expression and hepatic fibrosis in mice [38]. In

addition, SMAD has some cross-talk with STAT proteins [9, 37]. Activation of STAT3 can activate the canonical TGF β signaling cascade via SMAD3 activation that leads to collagen expression in an autocrine loop [39]. On the other hand, STAT1 and STAT3 can induce the expression of inhibitory SMAD7, leading to inhibition of TGF β signaling [9, 37, 40]. Here in this study, we observed an inhibitory effect of SAC on the binding ability of STAT3 and SMAD3 to *JUNB* promoter. However, whether there was interaction between STAT3 and SMAD3 and how SAC effected on these two transcription factors were still unknown, and needed to investigate in the following studies.

In conclusion, SAC could attenuate CCl₄-induced liver fibrosis in rats with anti-oxidant, anti-inflammatory and anti-fibrotic effects, and target STAT3/SMAD3 pathway to inhibit gene transcription. These findings provided new insight into the translational research of liver fibrosis.

Acknowledgements

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Disclosure of conflict of interest

None.

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