

# Sinomenine Attenuates Acetaminophen-Induced Acute Liver Injury by Decreasing Oxidative Stress and Inflammatory Response via Regulating TGF- $\beta$ /Smad Pathway in vitro and in vivo

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**Introduction:** Liver disease is common and often life-threatening. Sinomenine (SIN) is an active ingredient extracted from *Sinomenium acutum*. This study investigated the protective effect and mechanism of sinomenine (SIN) on acetaminophen (APAP)-induced liver injury from in vitro and in vivo.

**Methods:** In vivo experiments, mice were randomly divided into six groups (n=10): control group, model group, SIN (25 mg/kg) group, SIN (50 mg/kg) group, SIN (100 mg/kg) group and SIN (100 mg/kg) + SRI-011381 group. Alanine transaminases (ALT), aspartate transaminases (AST) and alkaline phosphatase (ALP) were detected. The pathological lesion was measured by HE staining. Apoptosis was measured by TUNEL staining. In vitro experiments, BRL-3A cells were treated with APAP (7.5 mM) and then subjected to various doses of SIN (10, 50 and 100  $\mu$ g/mL) at 37°C for 24 h. Inflammatory factors and oxidative stress index were measured by ELISA. The expression of proteins was detected by Western blot.

**Results:** The results showed that compared with the control group, the levels of ALT, AST and ALP in the serum of APAP-induced mice were significantly increased, followed by liver histological damage and hepatocyte apoptosis. Besides, APAP reduced the activity of SOD and GSH-Px, while increasing the content of MDA and LDH. Notably, APAP also promoted the expression of NLRP3, ASC, caspase-1 and IL-1 $\beta$ . Interestingly, SIN treatment dose-dependently reduced APAP-induced liver injury and oxidative stress, inhibited the activation of NLRP3 inflammasomes, and reduced the levels of inflammatory cytokines. In vitro studies have shown that SIN treatment significantly reduced the viability of BRL-3A cells and oxidative stress and inflammation. In addition, the Western blotting analysis showed that SIN inhibited the activation of TGF- $\beta$ /Smad pathway in a dose-dependent manner in vitro and in vivo. These effects were significantly reversed by TGF- $\beta$ /Smad activator SRI-011381 or TGF- $\beta$  overexpression.

**Discussion:** The study indicates that SIN attenuates APAP-induced acute liver injury by decreasing oxidative stress and inflammatory response via TGF- $\beta$ /Smad pathway in vitro and in vivo.

**Keywords:** Sinomenine, acetaminophen, inflammatory response, oxidative stress, TGF- $\beta$ /Smad pathway, acute liver injury

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## Introduction

Liver disease is common and often life-threatening.<sup>1</sup> Carbon tetrachloride (CCl<sub>4</sub>),<sup>2</sup> paracetamol<sup>3</sup> and lipopolysaccharide<sup>4</sup> may cause acute liver injury. The occurrence of liver injury may be related to viral infection, alcohol and drugs.<sup>5</sup> Drug-induced liver

injury is a direct or indirect side effect of long-term high-dose administration.<sup>6</sup> Acetaminophen (APAP), also known as paracetamol, is a common over-the-counter drug.<sup>7</sup> APAP is considered to be a safe and effective antipyretic analgesic. However, prolonged or excessive use of APAP may lead to liver damage.<sup>8</sup> N-acetyl-p-benzoquinone imine (NAPQI) produced by excessive APAP may deplete 85-glutathione (GSH) cells in the liver, resulting in oxidative stress-induced liver damage.<sup>9–11</sup> Besides, excessive APAP can also cause cellular inflammation.<sup>12,13</sup> Therefore, it is of great significance for the clinical application of APAP to investigate the potential molecular mechanism of liver injury caused by APAP.

Sinomenine (SIN) is the main active ingredient in the rhizome of *Sinomenine sinensis*.<sup>14</sup> In China, SIN is used to treat rheumatoid arthritis.<sup>15</sup> Numerous studies have shown that SIN has anti-inflammatory, antioxidant, immunosuppressive, and analgesic effects.<sup>16,17</sup> In addition, SIN can reduce the fulminant hepatitis caused by endotoxin and has a protective effect on the liver.<sup>18</sup> However, the effect of SIN on liver injury caused by APAP has not been reported. In this study, we examined the effects of SIN on acute liver injury induced by APAP in mice and its underlying molecular mechanisms.

TGF- $\beta$  signaling pathway plays an important role in regulating stem cell activity and organ formation. Smad protein is the downstream transmembrane receptor of TGF- $\beta$  and is an important regulatory molecule of TGF- $\beta$  superfamily signaling. Studies have shown that TGF- $\beta$ /Smad signaling pathway plays an important role in liver fibrosis<sup>19</sup> and acute liver injury.<sup>20</sup> However, whether the TGF- $\beta$ /Smad pathway is involved in the regulation of SIN in APAP-induced acute liver injury remains unknown.

This study investigated the effects of different doses of SIN on APAP-induced acute liver injury and its potential molecular mechanisms in vivo and in vitro. The results showed that SIN alleviated APAP-induced acute liver injury by inhibiting the TGF- $\beta$ /Smad signaling pathway and then reducing the oxidative stress and inflammatory responses induced by APAP.

## Materials and Methods

### Cell Culture and Treatment

The rat hepatocyte cell line BRL-3A was obtained from West China Hospital, Sichuan University, and maintained in DMEM supplemented with 1% penicillin–streptomycin solution and 10% FBS at 37°C under a humidified

atmosphere of 5% CO<sub>2</sub>. Cells were grown to 90% confluence then passaged and subcultured using 0.25% trypsin every 1–3 days. Cells were treated with APAP (7.5 mM) and then subjected to various doses of SIN (10, 50 and 100  $\mu$ g/mL) at 37°C for 24 h.

### Animals and Groups

All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by Hubei Center for Disease Control and Prevention (SYXK (鄂) 2015—0027). Male C57BL/6 mice (6–8 weeks, 20 g  $\pm$  2) were obtained from the animal center of Hubei Center for Disease Control and Prevention. All mice were raised under standard animal-holding conditions (12 h light/dark cycle, relative humidity 60  $\pm$  5%, and 25  $\pm$  2°C) for 1 wk. Afterwards, the mice were randomly divided into six groups (n=10): control group, model group, SIN (25 mg/kg) group, SIN (50 mg/kg) group, SIN (100 mg/kg) group and SIN (100 mg/kg) + SRI-011381 group. Mice in the administration group were continuously administered SIN (sigma-aldrich) for 7 days. Meanwhile, mice in the control group and model group were treated with 0.9% saline. After final administration, acute liver injury was induced by intraperitoneal injection of APAP (250 mg/kg) in the APAP group and the APAP + SIN group. Meanwhile, the control group was given 0.9% saline. SIN + SRI-011381 group: Mice treated with APAP were administered SIN (100mg/kg) and SRI-011381 hydrochloride (30mg/kg). The serum was then collected. The mice were sacrificed by cervical dislocation, and the left liver lobe of the mice was removed for subsequent study.

### Serum Biochemical Indicators

The serum alanine transaminases (ALT), aspartate transaminases (AST) and alkaline phosphatase (ALP) activities in each group were measured using the Catalyst DxTM biochemical analyzer.

### ELISA

The levels of TNF- $\alpha$  (PT512, Beyotime, Shanghai, China), IL-1 $\beta$  (PI301, Beyotime, Shanghai, China) and IL-6 (PI326, Beyotime, Shanghai, China) in mice and cells were determined by ELISA kits according to the manufacturer's. The optical density (OD) value was measured by a Microplate Reader at the corresponding wavelength. Draw the standard curve of OD value vs concentration, and calculate the sample concentration according to the standard curve.

## Indicators of Oxidative Stress

Liver tissues of each group were accurately weighed and saline (1:19) was added to prepare liver homogenate (5%, weight ratio). SOD (S0109, Beyotime, Shanghai, China), MDA (S0131, Beyotime, Shanghai, China), GSH-Px (S0052, Beyotime, Shanghai, China) and LDH (C0016, Beyotime, Shanghai, China) were detected according to the instructions provided by the manufacturer. For SOD, the samples were incubated at 37°C for 30 min with NBT/enzyme working solution and reaction start-up working solution. Absorbance was determined at 560 nm. Total SOD activity was calculated according to the instructions of the kit. For MDA, MDA detection solution was added to the sample, and the mixture was heated in a boiling water bath for 15 min. After cooling to room temperature in a water bath, the mixture was centrifuged at 1000 g for 10 min. The supernatant was collected and the absorbance at 532 nm was determined. Calculate the MDA content according to the instructions of the kit. For GSH-Px, add the detection buffer, sample and GPx detection working solution to the 96-well plate in sequence and mix well. Subsequently, 4  $\mu$ L of 15 mM peroxide reagent solution was added and the reaction was carried out at 25°C for 20 min. The absorbance at a wavelength of 340 nm was measured with a microplate reader. The obtained data calculates the activity of GSH-Px according to the following formula: [GSH-Px activity in the sample] = [GSH-Px in the detection system] X [Dilution factor]/[Protein concentration in the sample]. For LDH, add 60 $\mu$ L LDH detection working solution to the sample. Mix well and incubate at 25°C in the dark for 30 min. The absorbance was then measured at 490 nm. Draw the absorbance vs concentration curve. From this, the LDH activity can be calculated according to the following formula: (absorbance of the treated sample-absorbance of the sample control)/(absorbance of the maximum enzyme activity of the cell-absorbance of the sample control)  $\times$  100.

## HE Staining

The left liver lobe of the mice was fixed with 4% paraformaldehyde and embedded in paraffin. After dewaxing with xylene for 10 min, the paraffin sections (3–4  $\mu$ m) were hydrated with ethanol at different concentrations (100%, 95%, 90%, 85%) for 1 min. The sections were then stained with hematoxylin for 4 min and desensitized in a hydrochloric acid ethanol mixture for 3–5 min. The sections were then stained with eosin for 2 min and sealed

with neutral rubber. The morphological changes of the left liver lobe were observed under the microscope.

## TUNEL Staining

Apoptosis was measured using TUNEL staining with in situ cell death detection kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions. First, paraffin-embedded sections were dewaxed and then rehydrated. Thereafter, the sections were permeabilized with proteinase K at 37°C for 15 min and then treated with TdT. Finally, apoptotic cells were observed and photographed using a common optical microscope.

## Western Blot

The expression of NLRP3, ASC, caspase-1, IL-1 $\beta$ , Smad2, Smad3, p-Smad2, p-Smad3 and TGF- $\beta$  in tissues and cells was detected by Western blotting. Total protein was extracted from BRL-3A cells and the left liver lobe tissues and quantified by the BCA protein analysis kit. The proteins were separated by SDS-PAGE and transferred to the PVDF membrane. The GAPDH gene acts as a reference gene. Subsequently, the membrane was sealed with 5% skim milk powder and incubated overnight with primary antibodies NLRP3 (ab214185, 1: 500, Abcam, UK), ASC (ab168811, 1: 2000, Abcam, UK), caspase-1 (ab62698, 1: 500, Abcam, UK), IL-1 $\beta$  (ab2105, 1: 1000, Abcam, UK), Smad2 (ab40855, 1: 2000, Abcam, UK), Smad3 (ab40854, 1: 1000, Abcam, UK), p-Smad2 (ab53100, 1: 500, Abcam, UK), p-Smad3 (ab63403, 1: 500, Abcam, UK) and TGF- $\beta$  (ab92486, 0.5–4  $\mu$ g/mL, Abcam, UK) at 4°C. The next day, after washing with TBST for 3 times, the membrane was incubated with horseradish peroxidase-labeled secondary antibody at room temperature for 1 h. Proteins were visualized using enhanced chemiluminescence kits and gel imaging systems. The results are analyzed by Image Tools.

## TGF- $\beta$ Overexpression

TGF- $\beta$  lentivirus particles were obtained from GenePharma. By subcloning TGF- $\beta$  cDNA to the pSLIK lentivirus expression system, the lentivirus expressing TGF- $\beta$  was generated. For lentiviral packaging, HEK293T cells were co-transfected with lentiviral particles. For transduction, the cells were incubated with virus-containing supernatant in the presence of 5  $\mu$ g/mL polypropylene. After 48 h, the infected cells were selected with puromycin (2 g/mL).

## Statistical Analysis

Data are expressed as mean  $\pm$  standard deviation. SPSS 22.0 (SPSS Inc., Chicago, Illinois, USA) was used to analyze the differences among the groups by one-way ANOVA following Student-Newman-Keul's test.  $P < 0.05$  was considered significant.

## Results

### SIN Reduces APAP-Induced Liver Dysfunction in Mice with Acute Liver Injury

HE staining showed normal liver structure in the control group without hepatocyte edema, degeneration or necrosis. In the model group, there was obvious hepatocyte edema, necrosis and liver structure destruction, accompanied by a large number of inflammatory cell infiltration. Compared with the model group, SIN treatment reduced APAP-induced cell edema and necrosis and reduced inflammatory cell infiltration in a dose-dependent manner. In particular, the SIN (100 mg/kg) treatment reduced liver tissue damage in mice, with an orderly arrangement of liver cells, and reduced the infiltration of inflammatory cells (Figure 1A). These results suggested that SIN significantly improved the hepatic lesions induced by APAP in a dose-dependent manner. In addition, APAP treatment significantly increased ALT, AST and ALP levels in serum. However, serum ALT, AST and ALP levels were decreased in a dose-dependent manner after SIN treatment. These results suggest that SIN reduced APAP-induced liver injury (Figure 1B).

### SIN Alleviates APAP-Induced Oxidative Stress in Mice with Acute Liver Injury

Besides, compared with the control group, the activity of SOD and GSH-Px in the model group was significantly reduced, while the activity of MDA and LDH was significantly increased. After SIN treatment, the activity of MDA and LDH were decreased in a dose-dependent manner, while the activity of SOD and GSH-Px were increased (Figure 2). These results showed that SIN alleviated APAP-induced oxidative stress in mice with acute liver injury.

### SIN Inhibited APAP-Induced Secretion of Pro-Inflammatory Factors and Activation of NLRP3 Inflammasome in Mice with Acute Liver Injury

The levels of pro-inflammatory factors (TNF- $\alpha$ , IL-1 $\beta$ , IL-6) were significantly higher in the model group than in the

control group. Compared with the model group, the levels of pro-inflammatory factors were decreased in a dose-dependent manner after treatment with SIN (Figure 3A), suggesting that SIN inhibited APAP-induced secretion of pro-inflammatory factors. Besides, the model group had higher levels of NLRP3, ASC, caspase-1 and IL-1 $\beta$  than the control group. Compared with the model group, SIN dose-dependently inhibited the expression of NLRP3, ASC, caspase-1 and IL-1 $\beta$  (Figure 3B), suggesting that SIN inhibited APAP-induced activation of NLRP3 inflammasome. In summary, these results showed that SIN inhibited APAP-induced pro-inflammatory factors secretion and NLRP3 inflammasome activation in mice with acute liver injury.

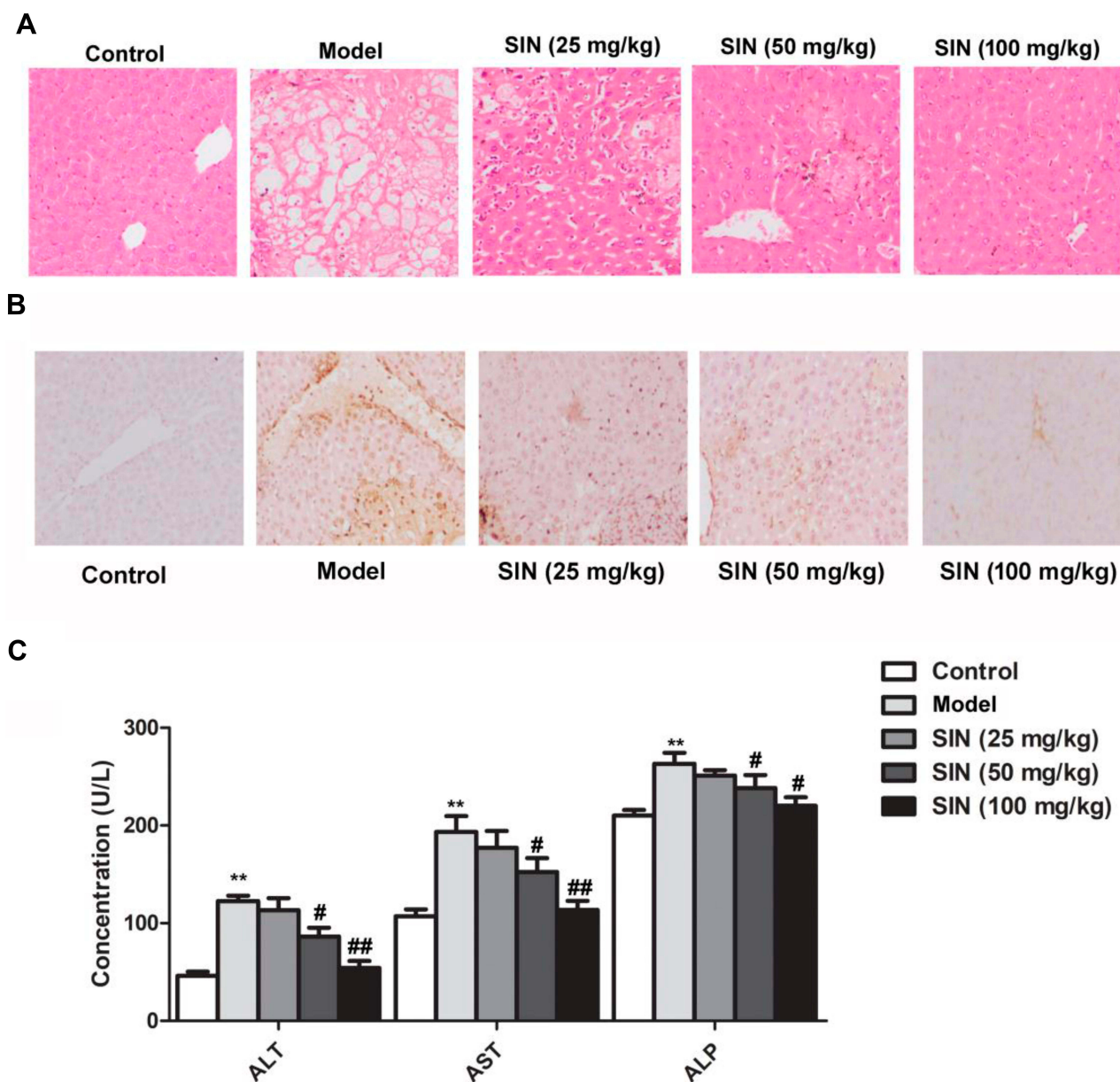
### SIN Inhibited the Activation of the TGF- $\beta$ /Smad Signaling Pathway

This study investigated the effect of SIN on the TGF- $\beta$ /Smad pathway. As shown in Figure 4, APAP treatment significantly promoted the phosphorylation of Smad2 and Smad3 and the expression of TGF- $\beta$ . It is worth noting that the SIN treatment inhibited the phosphorylation of Smad2 and Smad3 and the expression of TGF- $\beta$  in a dose-dependent manner. These results suggested that SIN inhibited the activation of TGF- $\beta$ /Smad signaling pathway.

### SIN Attenuated APAP-Induced Acute Liver Injury by Decreasing Oxidative Stress and Inflammatory Response via Regulating TGF- $\beta$ /Smad Signaling Pathway

In addition, the levels of p-smad2, p-smad3 and TGF- $\beta$  were significantly increased after treatment with TGF- $\beta$ /Smad activator SRI-011381, suggesting that SRI-011381 significantly reversed the inhibitory effect of SIN on the TGF- $\beta$ /Smad pathway (Figure 5A). Further functional analysis showed that SRI-011381 treatment similarly restored the levels of ALT, AST and ALP in serum, suggesting that SRI-011381 reversed SIN-induced improvements in liver function (Figure 5B). Notably, the activity of SOD and GSH were significantly decreased after SRI-011381 treatment, while the activity of MDA and LDH were significantly increased, indicating that SRI-011381 reversed the inhibitory effect of SIN on APAP-induced oxidative stress (Figure 5C–F). Similarly, the expression of NLRP3, ASC, caspase 1 and IL-1 $\beta$  was significantly inhibited after SRI-011381 treatment, suggesting that SRI-011381 reversed the inhibitory effect of SIN on the activation of NLRP3 inflammasome and the expression of IL-1 $\beta$  (Figure 5G). In summary, these results showed that SIN





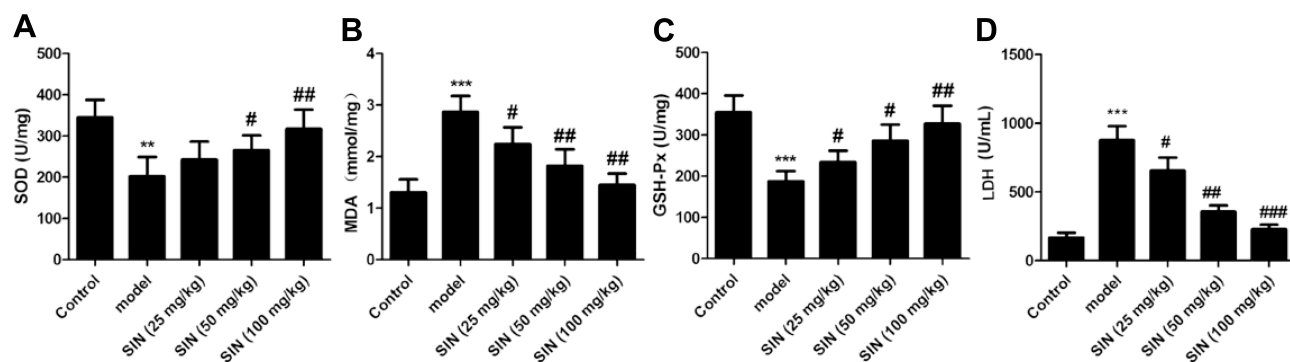
**Figure 1** SIN reduced APAP-induced liver dysfunction in mice with acute liver injury. The mice were randomly divided into five groups (n=10): control group, model group, SIN (25 mg/kg) group, SIN (50 mg/kg) group and SIN (100 mg/kg) group. (A) The liver injury was detected by HE staining. (B) Apoptosis was measured by TUNEL. (C) The contents of ALT, AST and ALP in serum were measured by Catalyst DxTM automatic biochemical analyzer; \*\*P < 0.01 vs control group; #P < 0.05, ##P < 0.01 vs model group.

attenuated APAP-induced acute liver injury by decreasing oxidative stress and inflammatory response via regulating TGF- $\beta$ /Smad signaling pathway.

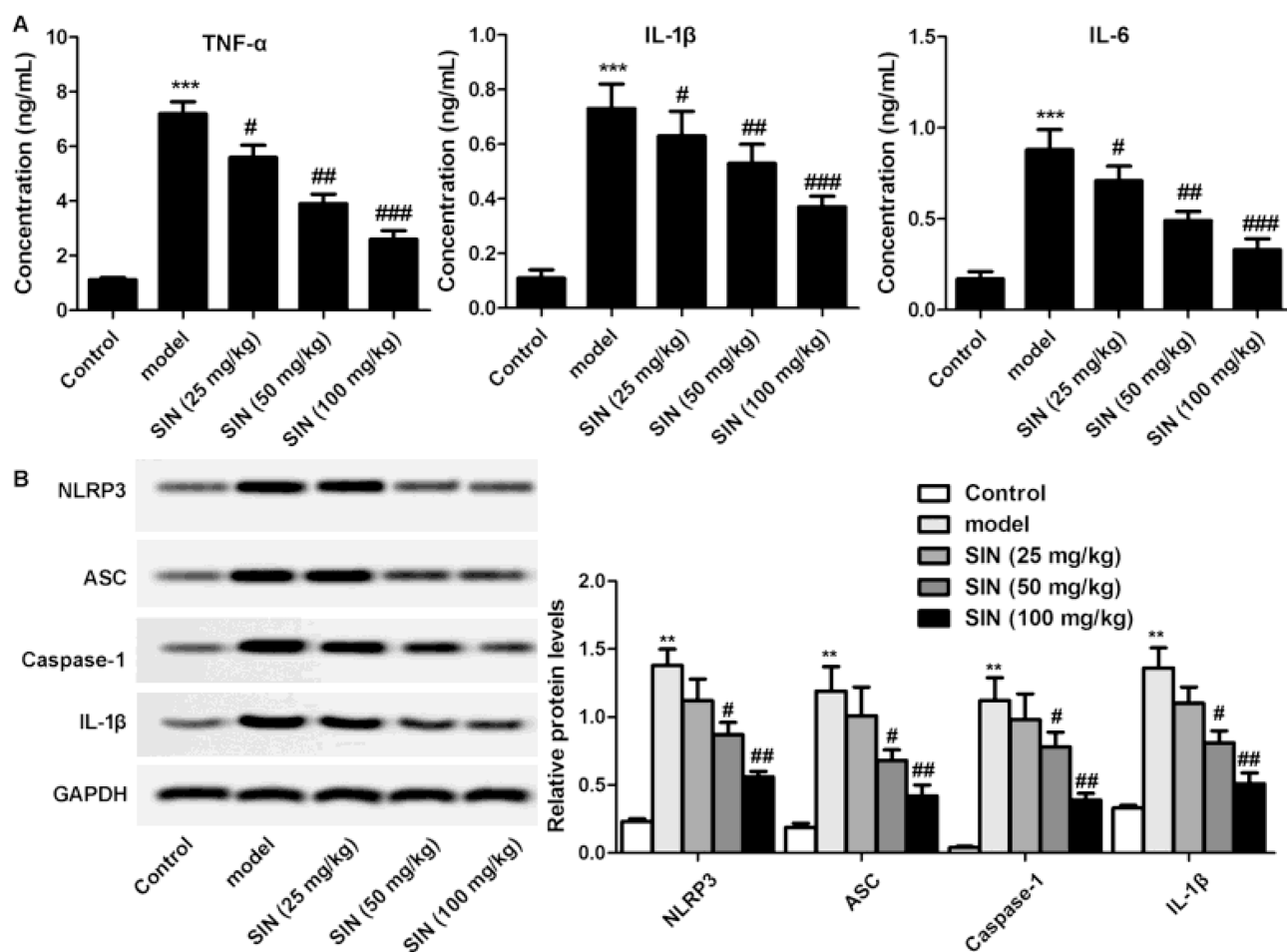
### SIN Alleviated APAP-Induced Oxidative Stress and Inflammatory Response via Regulating TGF- $\beta$ /Smad Signaling Pathway in vitro

The effect of SIN on the liver injury was also investigated in vitro. As shown in Figure 6A, compared with the

control group, SIN treatment alone had no obvious toxic and side effects on BRL-3A cells. However, cell viability was significantly reduced after APAP treatment, while SIN treatment reversed the APAP-induced decrease in cell viability. Besides, this study explored the effects of SIN on cellular oxidative stress and inflammation. As shown in Figure 6B and C, compared with the control group, APAP significantly inhibited the activity of SOD and increased the content of MDA. As expected, SIN treatment significantly increased the activity of SOD and decreased the content of MDA in a dose-dependent manner. Western



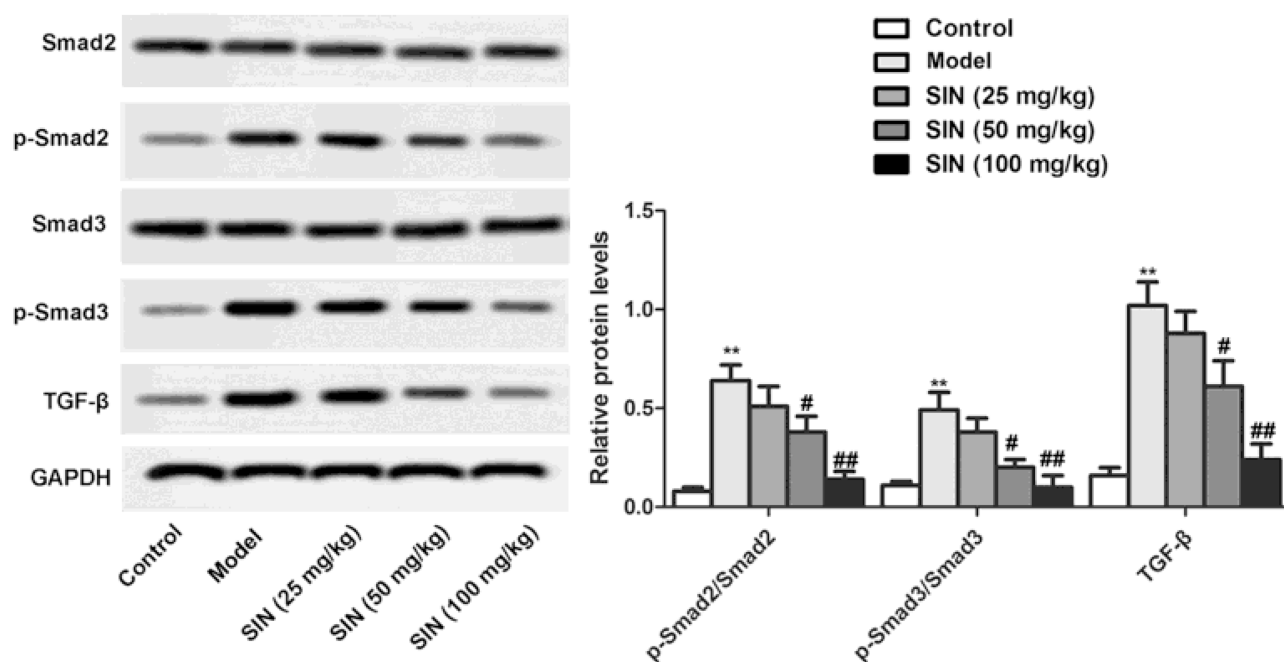
**Figure 2** SIN alleviated APAP-induced oxidative stress in mice with acute liver injury. The mice were randomly divided into five groups (n=10): control group, model group, SIN (25 mg/kg) group, SIN (50 mg/kg) group and SIN (100 mg/kg) group. (A) SOD; (B) MDA; (C) GSH-Px; (D) LDH. \*\*P < 0.01, \*\*\*P < 0.001 vs control group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs model group.



**Figure 3** SIN inhibited APAP-induced secretion of pro-inflammatory factors and activation of NLRP3 inflammasome in mice with acute liver injury. The mice were randomly divided into five groups (n=10): control group, model group, SIN (25 mg/kg) group, SIN (50 mg/kg) group and SIN (100 mg/kg) group. (A) The levels of pro-inflammatory factors (TNF- $\alpha$ , IL-1 $\beta$  and IL-6) in serum were detected by ELISA. (B) The expression of NLRP3 inflammasome (NLRP3, ASC and Caspase 1) and their downstream inflammatory factor IL-1 $\beta$  were measured by Western blot in liver tissues. \*\*P < 0.01 vs control group; #P < 0.05, ##P < 0.01 vs model group; \*\*\*P < 0.001 vs control group; ###P < 0.001 vs model group.

blotting showed that SIN similarly reversed the activation of NLRP3 inflammasomes in BRL-3A cells and reduced levels of inflammatory cytokines (TNF- $\alpha$  and IL-1 $\beta$ ) (Figure 6D-F). Furthermore, SIN also reversed the

activation of APAP to the TGF- $\beta$ /Smad pathway in BRL-3A cells (Figure 6G). Notably, when TGF- $\beta$  was over-expressed by lentivirus, the levels of p-smad2, p-smad3, and TGF- $\beta$  in BRL-3A cells significantly increased, along



**Figure 4** SIN inhibited the activation of the TGF- $\beta$ /Smad signaling pathway. The mice were randomly divided into five groups (n=10): control group, model group, SIN (25 mg/kg) group, SIN (50 mg/kg) group and SIN (100 mg/kg) group. The expression of TGF- $\beta$ /Smad signaling pathway-related proteins (Smad2, p-Smad2, Smad3, p-Smad3 and TGF- $\beta$ ) was measured by Western blotting. \*\*P < 0.01 vs control group; #P < 0.05, ###P < 0.01 vs model group.

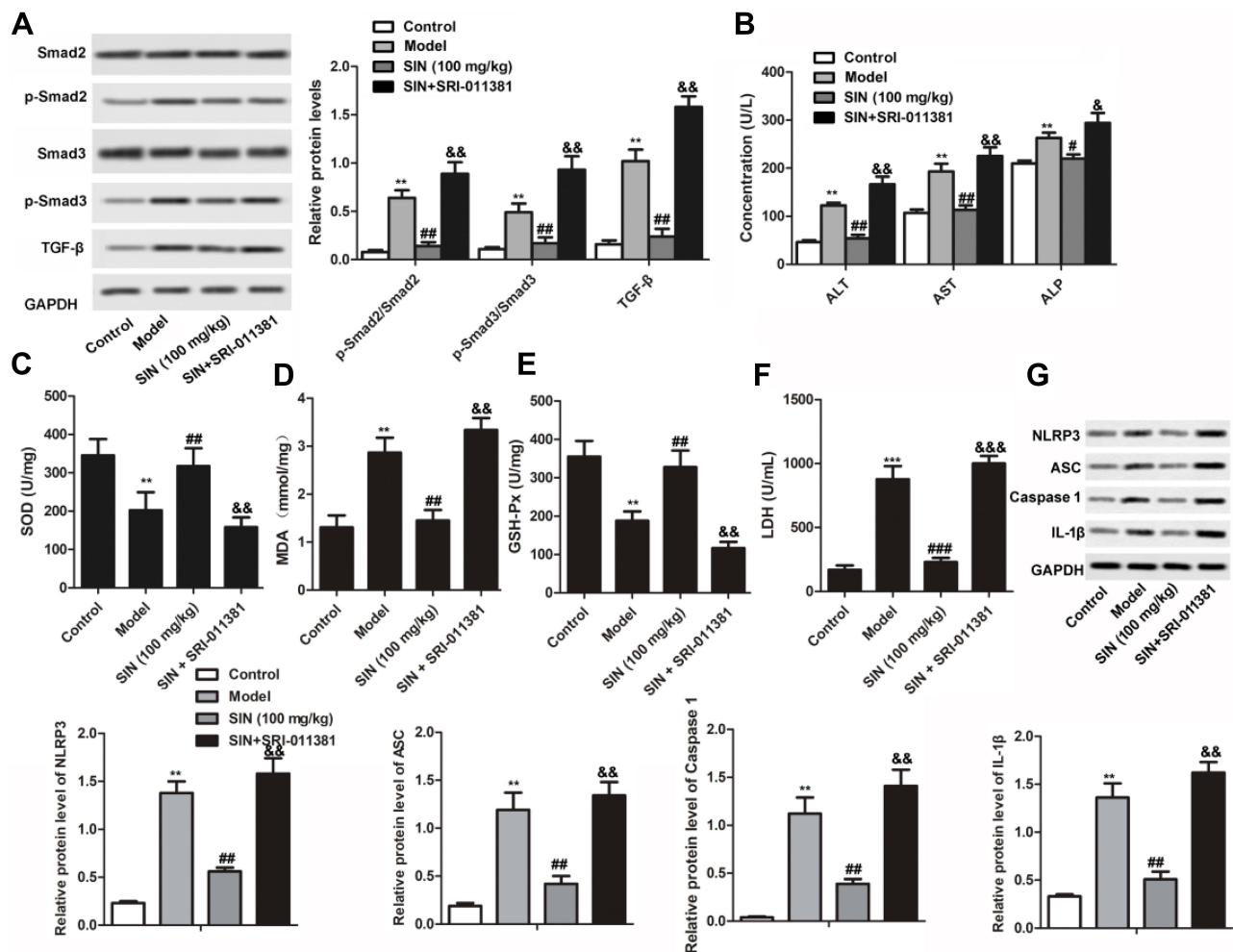
with the levels of MDA and the expression of inflammasomes (NLRP3, ASC, and caspase-1). However, after TGF- $\beta$  overexpression, SOD activity was significantly reduced, indicating that TGF- $\beta$  overexpression reversed the improvement effect of SIN on liver injury (Figure 6H–N). Taken together, these results suggest that SIN alleviated APAP-induced oxidative stress and inflammatory response via regulating TGF- $\beta$ /Smad signaling pathway in vitro.

## Discussion

APAP-induced acute liver failure is not only a long-standing clinical problem but also a typical drug-induced liver injury model.<sup>21,22</sup> APAP poisoning is associated with intense liver necrotizing inflammation. The key feature of APAP-induced ALI is lobule hepatocellular necrosis driven by NAPQI, an APAP metabolite produced by liver Cyp2e1 and Cyp1a2. Under the influence of NAPQI liver cells, oxidative stress, abnormal mitochondrial respiratory function, ATP decline and cell necrosis were observed.<sup>22</sup> In this study, APAP (250 mg/kg) was injected into mice to establish APAP-induced acute liver injury model. The results showed that the model mice showed significant liver injury and hepatocellular necrosis. In addition, serum levels of ALT, AST and ALP were significantly increased, and oxidative stress and inflammation were

significantly increased. NLRP3 inflammasome is significantly activated in liver tissue. These results indicate that the establishment of the acute liver injury model in this study is successful. Surprisingly, this study found that the natural bioactive substance SIN has an obvious repair effect on APAP-induced acute liver injury.

Previous studies have shown that ALT is mainly distributed in the liver, and AST is mainly distributed in the myocardium, while ALP is mainly distributed in the liver and bone.<sup>23,24</sup> When liver cells are damaged, ALT, AST and ALP are released into the blood and increased the levels of ALT, AST and ALP in serum.<sup>25</sup> Coincidentally, our study found that SIN significantly reduced serum ALT, AST, and ALP levels in mice with acute liver injury. Previous studies have shown that GSH-Px was an antidote, and SOD can effectively remove oxygen free radicals.<sup>26,27</sup> MDA is the final product of lipid peroxides, which may damage the structure of cell membranes, leading to cell swelling and necrosis.<sup>28,29</sup> In addition, an increase in LDH can proportionately damage hepatocytes.<sup>30</sup> Our study found that SIN increased the levels of GSH-Px and SOD and decreased the levels of MDA and LDH in mice with liver injury. The results showed that SIN could significantly improve the liver detoxification ability of mice, enhance the resistance to free radical damage, inhibit lipid peroxidation and significantly reduce liver cell



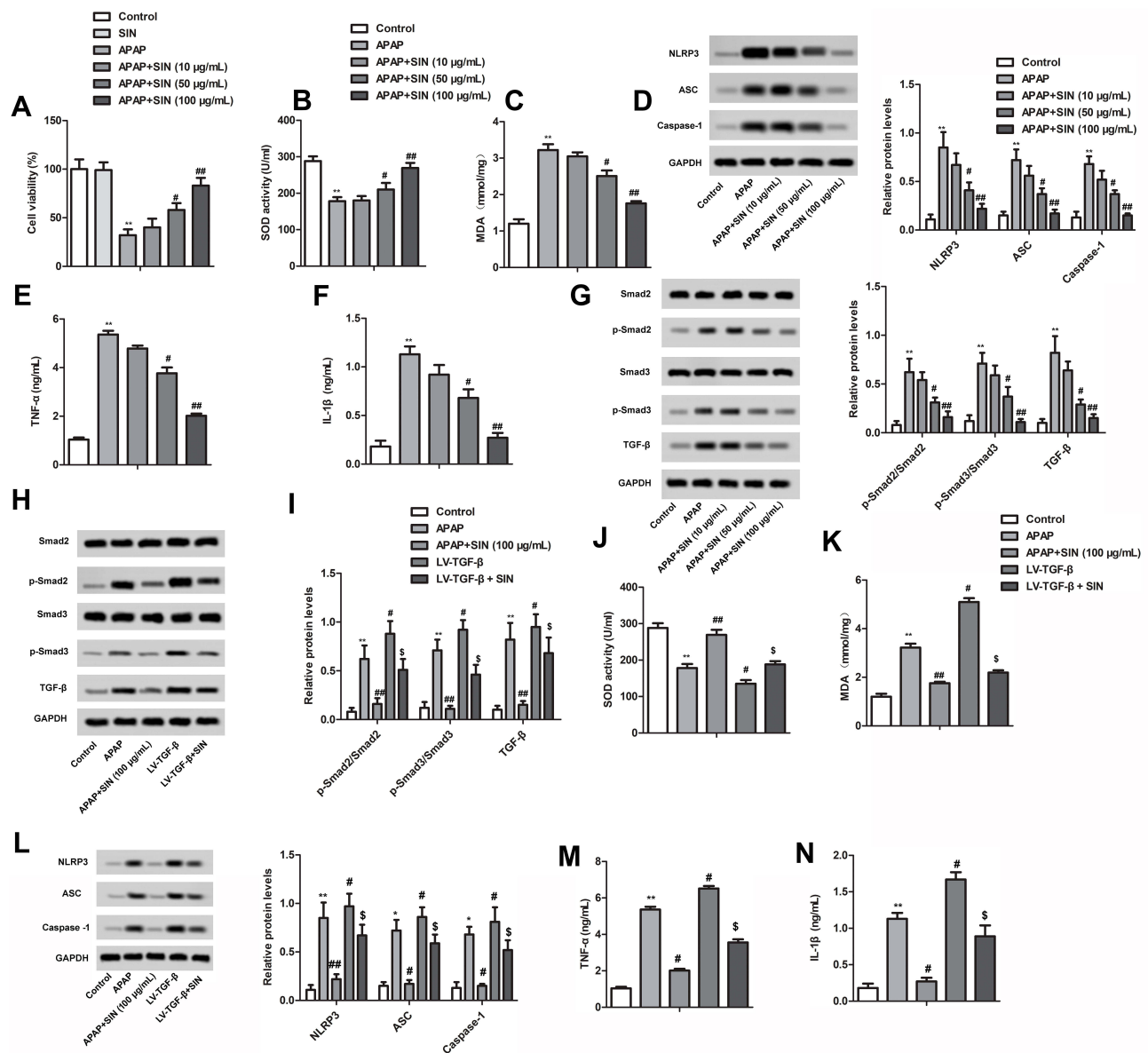
**Figure 5** SIN attenuated APAP-induced acute liver injury by decreasing oxidative stress and inflammatory response via regulating TGF- $\beta$ /Smad signaling pathway. The mice were randomly divided into four groups (n=10): control group, model group, SIN (100 mg/kg) group and SIN+SRI-011381 group. **(A)** The expression of TGF- $\beta$ /Smad signaling pathway-related proteins (Smad2, p-Smad2, Smad3, p-Smad3 and TGF- $\beta$ ) was measured by Western blotting. **(B)** The contents of ALT, AST and ALP in serum were measured by Catalyst DxTM automatic biochemical analyzer. **(C)** SOD. **(D)** MDA. **(E)** GSH-Px. **(F)** LDH. **(G)** The expression of NLRP3 inflammasome (NLRP3, ASC and Caspase 1) and their downstream inflammatory factor IL-1 $\beta$  were measured by Western blot in liver tissues. \*\*P < 0.01, \*\*\*P < 0.001 vs control group; #P < 0.05, ##P < 0.01 vs model group; &P < 0.01 vs SIN (100mg/kg) group; &&P < 0.001 vs control group; &&&P < 0.001 vs model group.

damage in mice, suggesting that SIN might prevent liver cell damage by enhancing the defense function of the cell membrane. In addition, inflammatory factors such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 also play a role in acute liver injury caused by APAP. Zhu et al found that the upregulation of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in serum promoted the production of NO, histamine and leukotriene, leading to hepatocyte necrosis.<sup>31</sup> Similarly, our study found that SIN significantly promoted the secretion and expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in serum and cells. Notably, SIN significantly reversed these effects, suggesting that SIN prevented APAP-induced liver damage from worsening by inhibiting the inflammatory response.

NLRP3 inflammasomes include NLRP3, ASC and pro-caspase-1.<sup>32</sup> When the danger signal is present, NLRP3

inflammasome aggregated and cleaved pro-caspase-1.<sup>33</sup> Subsequently, caspase-1 cleaves the inflammatory mediators pro-IL-1 $\beta$  and pro-IL-18 into the mature state by proteolysis, and then secretes them to extracellular. Studies by Martinon and Tschopp have shown that caspase-1 initiated or executed cellular processes that mediated inflammation or cell death.<sup>34</sup> In addition, caspase-1 and IL-1 $\beta$  are typical pro-inflammatory cytokines,<sup>35</sup> which promote the release of inflammatory cytokines (IL-3, IL-5, IL-6, IL-13) by activating the NF- $\kappa$ B signaling pathway, resulting in inflammation and targeted cell damage.<sup>36</sup> In this study, in vivo and in vitro experiments showed that SIN significantly reduced the inflammatory response by inhibiting the activation of NLRP3 inflammasomes and the production of inflammatory factors, thereby inhibiting liver injury.





**Figure 6** SIN alleviated APAP-induced oxidative stress and inflammatory response via regulating TGF- $\beta$ /Smad signaling pathway in vitro. A-G. Cells were treated with APAP (7.5 mM) and then subjected to various doses of SIN (10, 50 and 100  $\mu$ g/mL) at 37°C for 24 h. (A) Viability of BRL-3A cells was measured by CCK8 assay. (B) SOD in BRL-3A cells. (C) MDA in BRL-3A cells. (D) The expression of NLRP3 inflammasome (NLRP3, ASC and Caspase1) in BRL-3A cells was measured by Western blotting. (E) TNF- $\alpha$  in BRL-3A cells. (F) IL-1 $\beta$  in BRL-3A cells. (G) The expression of TGF- $\beta$ /Smad signaling pathway-related proteins (Smad2, p-Smad2, Smad3, p-Smad3 and TGF- $\beta$ ) in BRL-3A cells was measured by Western blotting. (H-N) Cells treated with APAP (7.5 mM) were subjected to SIN (100  $\mu$ g/mL) or infected with LV-TGF- $\beta$ . (H) The expression of TGF- $\beta$ /Smad signaling pathway-related proteins (Smad2, p-Smad2, Smad3, p-Smad3 and TGF- $\beta$ ) in BRL-3A cells was measured by Western blotting. (I) SOD. (J) MDA. (L) The expression of NLRP3 inflammasome (NLRP3, ASC and Caspase1) in BRL-3A cells was measured by Western blotting. (M) TNF- $\alpha$ . (N) IL-1 $\beta$ . \*\*P < 0.01 vs control group; #P < 0.05, ##P < 0.01 vs model group; \$P < 0.05 vs LV-TGF- $\beta$  group.

TGF- $\beta$  is a group of multifunctional protein peptides that have a wide range of effects on cell growth, cell differentiation, and immune response. TGF- $\beta$ /Smad signaling pathway plays an important role in the progression of liver injury. Park et al found that Arazyme protected damaged liver cells by inhibiting TGF- $\beta$ /Smad pathway and increasing antioxidant protein expression.<sup>37</sup> Similarly, in this study, in vivo and in vitro experiments showed that SIN significantly decreased

the expression of p-Smad2, p-Smad3, and TGF- $\beta$ , suggesting that SIN inhibited TGF- $\beta$ /Smad signaling pathway, thereby inhibiting APAP-induced liver injury. Notably, TGF- $\beta$ /Smad pathway activator SRI-011381 reversed the inhibitory effect of SIN on the TGF- $\beta$ /Smad signaling pathway. But not only that, TGF- $\beta$  overexpression significantly reversed the inhibitory effect of SIN on the TGF- $\beta$ /Smad signaling pathway in vitro. More importantly, SRI-011381 reversed the

protective effect of SIN against APAP-induced acute liver injury. However, this study only explored the role of SIN on several proteins in the TGF pathway at the protein level. The underlying molecular mechanism of the effect of SIN on APAP-induced acute liver injury still needs further investigation.

## Conclusion

In short, in vivo and in vitro studies have shown that SIN significantly improved APAP-induced liver dysfunction, oxidative stress, and inhibit the activation of NLRP3 inflammasomes and the expression of inflammatory factors. The mechanism is associated with the activation of the TGF- $\beta$ /Smad signaling pathway, which may be valuable for the development of novel diagnostic markers and targeted therapies.

## Disclosure

The authors report no conflicts of interest in this work.

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