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Protective effect of acidic polysaccharide from *Schisandra chinensis* on acute ethanol-induced liver injury through reducing CYP2E1-dependent oxidative stress

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ABSTRACT

Aim: *Schisandra chinensis* is a well-known traditional Chinese medicine used mainly as a recipe for hepatoprotection. Modern researches have revealed that the hepatoprotection is related to its lignans and crude polysaccharide. In this study, we examined the effect and mechanism of *Schisandra chinensis* acidic polysaccharide (SCAP) on the liver injury induced by ethanol.

Main methods: SCAP was extracted with water extraction and ethanol precipitation. Liver injury models of both mice and HepG2 cells were produced by ethanol. The liver index, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in serum of the mice and cell culture supernatant were examined; HE staining was performed for observing pathological changes of liver. The malondialdehyde (MDA) level and superoxide dismutase (SOD) activities in serum, liver tissue and HepG2 cells, and triglyceride (TG) content in liver tissue were tested. Western blot was conducted to determine cytochrome P450 2E1 (CYP2E1) expression in liver tissue of mice and HepG2 cells.

Key findings: SCAP significantly reduced serial AST and ALT levels in the injuried liver and HepG2 cells induced by ethanol and also decreased TG level in the liver tissue. SCAP also obviously improved the hepatopathological changes and decreased MDA level as well as increased SOD activities in the serum, liver tissue and HepG2 cells induced by ethanol. Furthermore, Western blot analysis indicated that SCAP significantly inhibited the upregulation of CYP2E1 protein.

Significance: SCAP has a protective effect on ethanol-induced liver injury in mice and cells, and the mechanism underlying may be via inhibiting the expression of CYP2E1 protein and then alleviating oxidative stress injury induced by ethanol.

1. Introduction

Alcoholic liver disease (ALD) is a disorder caused by a long-term heavy alcohol drinking, and can progress to alcoholic hepatitis and alcoholic liver fibrosis, finally even alcoholic liver cirrhosis and liver cancer [1,2]. In recent years, with the improvement of living standards, the incidence of ALD is rising year by year and ALD has become the second major cause of liver injury following viral hepatitis [3]. The pathogenesis of ALD is not completely clear, and it is considered now that its pathogenesis is mainly associated with oxidative stress and lipid peroxidation, cytokines and mitochondrial dysfunction, of which oxidative stress plays a key role in the occurrence and development of acute alcoholic liver injury [4,5]. Currently, the treatment of alcoholic liver injury is unsatisfied and there is no effective drug for its treatment in clinic yet [6].

Schisandra chinensis (turcz.) baill (*Schisandra chinensis*, *Schisandra*) is a traditional Chinese medicine and has been used in clinic for thousands of years, and usually used for the treatment of viral and chemical liver

Abbreviations: ALD, alcoholic liver disease; SCAP, *Schisandra chinensis* acidic polysaccharide; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglyceride; MDA, malondialdehyde; SOD, superoxide dismutase; HE, hematoxylin and eosin; ADH, alcohol dehydrogenase; CYP2E1, cytochrome P450 2E1; MEOS, microsomal ethanol oxidizing system; ROS, reactive oxygen species; SD, standard deviation; ANOVA, analysis of variance

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injuries, with an obvious therapeutic effect [7]. The main active constituents of *Schisandra* include lignans and polysaccharides, and most of the current studies focus on ethanol extracts and lignans of *Schisandra* [8,9]. Plant polysaccharides have attracted more and more attention due to their antioxidant, hypoglycemic, immunoregulation functions [10]. The content of polysaccharide accounts for about 10% of *Schisandra*, the results of our previous studies showed that *Schisandra* polysaccharide had a significant protective effect on the liver injury induced by both CCL_4 and high-fat diet, and a significant antioxidant effect, suggesting that *Schisandra* polysaccharide might be one of components that could play a protective effect of liver injury [11,12]. However, whether *Schisandra* polysaccharide has a protective effect on the liver injury induced by alcohol has not been reported yet. In this study, ethanol-induced mouse liver injury and liver cell injury models were established, then the preventive and therapeutic effect of *Schisandra chinensis* acidic polysaccharide (SCAP) on the alcoholic liver injury was investigated, and the underlying mechanism was further explored, to provide a theoretical basis for the further development of *Schisandra* new drugs used for the prevention and treatment of ALD and health foods with the auxiliary function.

2. Materials and methods

2.1. Reagents

Schisandra chinensis was purchased from Jian *Schisandra* Seedlings Base of Jilin Province and identified by Professor Lihua Zhang at the College of Pharmacy, Beihua University, according to the identification standard of the Pharmacopoeia of the People's Republic of China (2015 Edition). Kits for measuring serum and tissue levels of ALT, AST, TG, SOD and MDA were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). BCA kit was purchased from Beyotime Institute of Biotechnology (Jiangshu, China). Human hepatic cancer cell lines HepG2 cells were obtained from American Type Culture Collection (ATCC, USA). DMEM medium and other culture reagents were obtained from Hyclone (Logan, Utah, USA). Polyclonal antibody against CYP2E1 was purchased from Abcam (Cambridge, MA, USA). Reagents for Western Blot were purchased from Bio-Rad (California Hercules, USA).

2.2. Extraction and characterization of SCAP

2.2.1. Extraction of SCAP

SCAP was prepared in The Center of Life Science, Northeast Normal University. Briefly, *Schisandra* was steeped in 10 times volume of distilled water overnight and extracted twice by boiling it in 100 °C hot water. The concentrated extract was added with 95% ethanol to be adjusted to one containing of 75% ethanol, precipitated overnight, and then centrifuged (4500 rpm, 15 min). The precipitation was collected and washed with 95% ethanol and absolute ethyl alcohol in turn, and then dried routinely to obtain a powder-like *Schisandra* polysaccharide, with a yield of 8.55% polysaccharide. A 0.5% *Schisandra* polysaccharide solution was prepared and loaded on a balanced DEAE-cellulose ion-exchange chromatographic column (20mL, Cl-type), eluted with distilled water to remove the neutral polysaccharide. Then 0–1.0 M NaCl solution was used for the linear gradient elution at a flow rate of 1 mL/min to obtain the eluent, which was concentrated and dried routinely to obtain the *Schisandra chinensis* acidic polysaccharide, with a yield about 2.8%.

2.2.2. Characterization of SCAP

Using D-glucose as the reference substance, total sugar content was determined with phenol-sulfuric acid method [13]. Using galacturonic acid as the reference, uronic acid content was measured with meta-hydroxydiphenyl method [14]. Each monosaccharide composition of SCAP was analyzed by PMP derivatization and high performance liquid chromatography (HPLC).

2.3. Animal experimentation

Male ICR mice, weighing 19–21 g, were provided by Changchun Yisi Laboratory Animal Technology Co. Ltd. (Changchun, China) [license number: SCXK (Ji) 2015-0001, SPF]. The mice were raised in separate cages at 18–22 °C (relative humidity 40-60%), under normal lighting conditions, and with ad libitum access to water. The normal diet for the experimental mice was purchased from Changchun Yisi Laboratory Animal Technology Co. Ltd (Changchun, China). All procedures were approved by the Ethics Committee for Use of Experimental Animals at Beihua University (Jilin, China).

Fifty male mice were randomly divided into normal control group (CON), model group (MOD), low-dose SCAP group (SCAP-L, 5 mg/kg), middle-dose SCAP group (SCAP-M,10 mg/kg) and high-dose SCAP group (SCAP-H, 20mg/kg), 10 in each group. Mice in the CON and MOD groups were administered equal volumes of water and those in SCAP-treated groups were intragastrically given 5, 10, 20 mg/kg of SCAP once daily successively for 15 days.

One hour after the last administration, mice in the MOD group and SCAP-treated groups were intragastrically given 50% ethanol solution (12 mL/kg), and those in the CON group were intragastrically given the same volume of solvent. All the mice were fasted, but with free access to water for 16 h, and anesthetized with ether, then 0.8–1.0 mL of blood was collected from each mouse by removing eyeballs. The serum was separated by centrifugation at 4000 rpm, 10 min and stored at −80 °C. The peritoneal cavity was opened along the abdominal middle line, and the liver carefully isolated and removed to calculate liver index (wet liver weight/body weight × 100%). The hepatic tissue was washed with cold saline and divided into three parts: the first part was fixed with 10% neutral formaldehyde for histopathological examination, the second part was prepared into homogenates for the detection of relative index, and the third part was preserved at −80 °C for analyzing the mechanism of drug action.

2.4. Cell culture and administration

HepG2 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were incubated in humidified atmosphere of 5% CO_2 at 37 °C and passaged according to the recommended procedures of ATCC, and used for experiments from the logarithmic phase of growth, seeded into 96-well plates (1×10^4 cells per well, 100 μL). Cells were exposed to different concentrations of ethanol (2%, 3%, 4%, 5%, 6%, v/v) or SCAP (3.12, 6.25, 12.5, 25, 50, 100 μg/mL) for 24 h for determining the concentration of ethanol and SCAP. The final concentration of ethanol was 3% for inducing liver cell injury model, and cells in SCAP-treated groups were treated with 3.12, 6.25 and 12.5 μg/mL of SCAP, respectively.

2.5. Determination of cell viability

Cell viability was determined by MTT assay method [15]. Briefly, MTT (5 mg/mL, 20 μL/per well) were added into the cell-seeded 96-well plates and incubated at 37 °C for 4 h. Then the solutions were removed and DMSO (100 μL/well) was added into the wells. The absorbance was measure at 490 nm using a 96-well plate reader.

2.6. Detection of ALT and AST

Cell culture supernatants were collected from different groups after the treatment for 24 h. Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the mice's serum and cell culture supernatant were determined using the commercial kits.

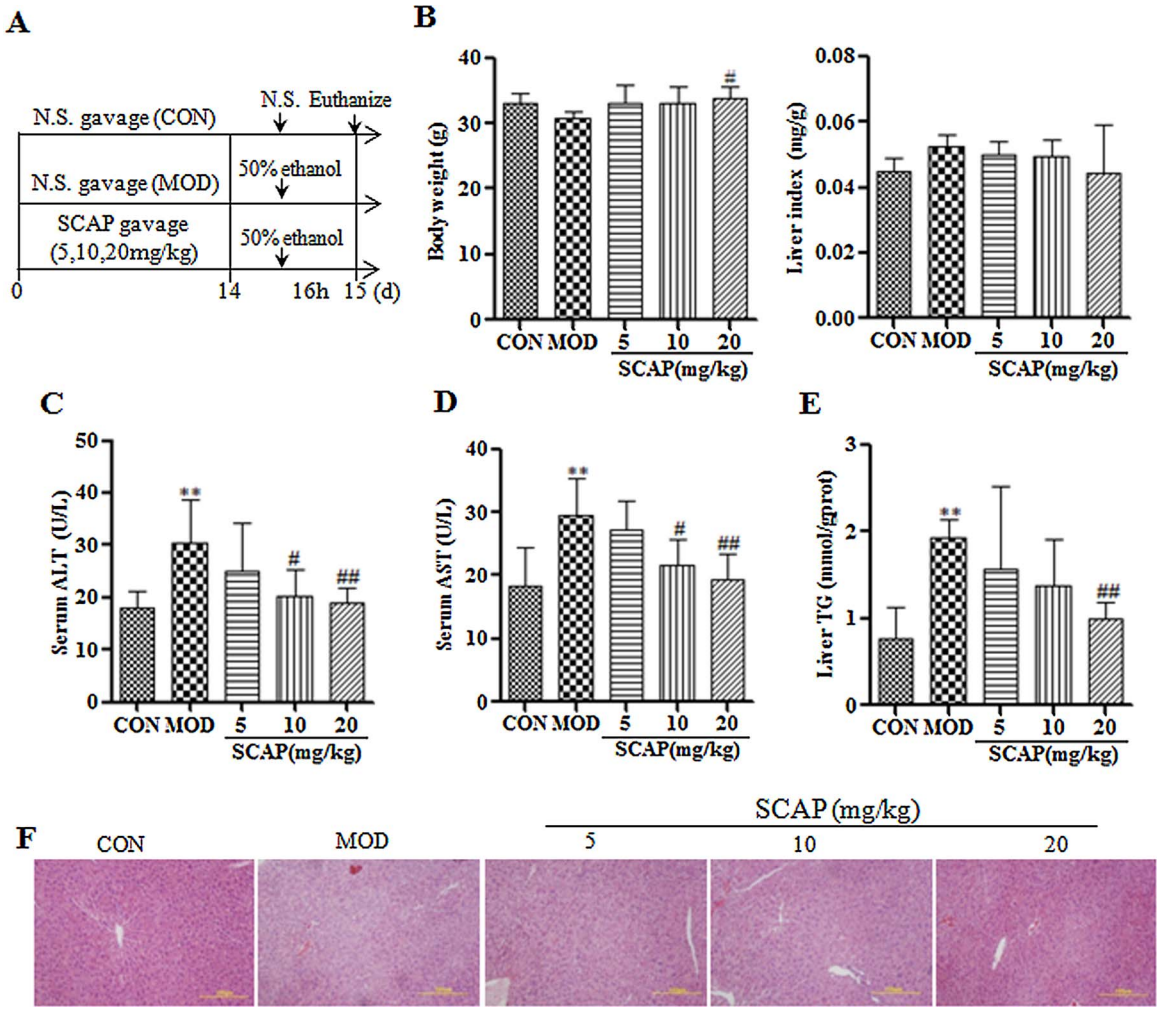

Fig. 1. Effects of SCAP on liver index, serum aminotransferases levels, hepetic TG content and the pathomorphology of liver in mice.(A) Animal group and administration; (B) Body weight and liver index; (C–E) Quantitative analysis of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and triglyceride (TG) content; (F) H&E stained sections of liver tissue (×100),$^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* the CON group; $^{\#}P < 0.05$,$^{\#\#}P < 0.01$ *vs* the MOD group.

2.7. Detection of MDA, SOD and TG

HepG2 cells in the logarithmic growth phase were seeded into 6-well plate at 1×10^5 cells/well, incubated with the corresponding agents for 24 h. The cells were digested with trypsin and centrifuged (1000 rpm, 5 min). Then, 0.5 mL of PBS was added into the precipitated cells and the cells were cracked by ultrasonic method in the ice water bath (300 W power, 3–5 s each time, 4 intervals, 30 s each interval). The liver tissue of mice was added with nine times amount of saline to prepare 10% tissue homogenateinon in the ice water bath. MDA levels and SOD activities in the liver tissue, HepG2 cells and serum, and TG levels in the liver tissue of mice, were measured by enzymic method according to the kit instructions.

2.8. Histopathological analysis of liver

The liver tissue samples were fixed in 10% neutral formaldehyde, washed in PBS and eluted with alcohol gradient elution. After paraffin-embedded and cut into slices. The slices were dewaxed with xylene, eluted with alcohol gradient elution, stained with hematoxylin-eosin, eluted with alcohol gradient elution again, clarified with xylene and mounted by adding neutral balata. Pathological changes in the morphology of cells were observed under alight microscope.

2.9. Western blotting analysis

Cells in each group were collected, added with 100 μL of cell lysis buffer, cracked on the ice for 30 min, and centrifuged (12,000 rpm, 15 min, 4 °C) to obtain the supernatants. Protein samples from liver tissues were prepared by homogenization with ice-cold lysate buffer for 1 h, and tissue lysate samples were centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatants were collected and the protein concentrations were measured using Bradford assay (Bio-rad protein assay kit).

Protein samples (30 μg) were denatured by boiling for 5 min, separated by 10% SDS-polyacrylamide gel, and then transferred onto polyvinylirdenediflouride (PVDF) membranes at 4 °C(Millipore, USA). The membranes were blocked in 5% non-fat milk for 2 h at room temperature and then incubated with rabbit polyclonal antibodies against CYP2E1 (diluted 1:2,000) with gentle agitation overnight at 4 °C. The membranes were washed three times for 10 min each with 15 ml of TBST and then incubated with the second antibody (1:2000, goat Anti-rabbit IgG Horseradish Peroxidase Conjugate, Santacruz Biotechnology, CA, USA) at room temperature for 1 h. The protein was visualized with enhanced chemiluminescene solution and taken image using chemiluminescent imaging system (Protein simple, FluorChem, CA, USA). The relative expression of proteins was expressed by the target protein band/GAPDH band, a loading control.

2.10. Statistical analysis

Data are presented as mean ± *S.D*. derived from at least three separate experiments. A comparison of the results was performed with one-way ANOVA using GraphPad Prism program (GraphPad Software, Inc., San Diego, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of SCAP

SCAP was a milk white powder and its yield was 2.8% (the percentage of SCAP to the dry weight of raw medicinal material). The total sugar content and uronic acid content of SCAP were 61.4% and 52.2%, respectively. The monosaccharide composition analysis showed that SCAP was mainly composed of GalA (53.60%) and Glc (30.19%), and a small amount of Gal (7.25%), Ara (4.30%), Rha (3.68%) and Man (1.36%).

3.2. Effects of SCAP on acute alcoholic liver injury in mice

Compared with those in CON group, ALT and AST levels in the serum and TG levels in the liver tissue of mice were increased significantly in MOD group ($P < 0.01$) (Fig. 1C–E). The pathological results showed that hepatic cords in hepatic tissues of mice in MOD group were irregular, and the cytoplasm of liver cells presented a ballooning degeneration fatty change, suggesting that the ethanol-induced acute liver injury model was successfully established in mice (Fig. 1F). Compared with those in MOD group, the ALT and AST levels in the serum and TG levels in the liver tissue of mice were decreased significantly in SCAP-treated groups ($P < 0.05$ or $P < 0.01$) (Fig. 1C–E).Moreover, the of mice liver cells arranged regularly, the ballooning degeneration of liver cell cytoplasm was significantly alleviated in SCAP-treated groups(Fig. 1F). These results suggest that SCAP has a certain protective effect on the alcoholic liver injury in mice.

3.3. Effects of SCAP on the injury of HepG2 cells induced by ethanol

With the increase of ethanol concentration, the viability of HepG2 cells was decreased gradually. When the ethanol concentration in the culture medium was greater than 3%, the cells viability was less than 70%, showing a significant difference compared with CON group ($P < 0.01$). In this study, 3% of ethanol was selected as the concentration to induce the injury of liver cells (Fig. 2A). Due to different effects produced by different concentrations of SCAP on cell viability, 3.12, 6.25 and 12.5 μg/mL of SCAP were chosen as the concentrations of SCAP treatment (Fig. 2B). Compared with that in CON group, the viability of HepG2 cells was decreased significantly, and the levels of ALT and AST in the culture supernatant was increased significantly in MOD group ($P < 0.05$ or $P < 0.01$). However, SCAP treatment increased cell viability, decreased significantly ALT and AST levels in the supernatant ($P < 0.05$ or $P < 0.01$), suggesting that SCAP has a protective effect on ethanol-induced HepG2 cells injury (Fig. 2C–E).

3.4. Effects of SCAP on MDA levels and SOD activities

As shown in Fig. 3, compared with those in CON group, MDA levels in the serum, liver tissue of mice and HepG2 cells were significantly increased, while SOD activities obviously were decreased in MOD group ($P < 0.05$ or $P < 0.01$). Compared with those in MOD group, MDA levels in the serum, liver tissue of mice and HepG2 cells were decreased significantly, while SOD activities were increased significantly in SCAP-treated groups ($P < 0.05$ or $P < 0.01$).

3.5. Effects of SCAP on the expression of CYP2E1 protein

To further analyze the mechanism of SCAP on inhibiting oxidative stress, CYP2E1 was analyzed in mice and cells model induced by ethanol. Compared with that in CON group, the expression of CYP2E1 in the liver tissue of mice and HepG2 cells was significantly increased ($P < 0.01$). While SCAP treatment decreased the expression of CYP2E1 ($P < 0.01$). These results indicated that SCAP inhibited oxidative stress through decreasing CYP2E1 expression (Fig. 4).

4. Discussion

Schisandra is the dried ripe fruit of a *Schisandraceae* plant *Schisandra chinensis*(Turcz.) Baill, originally was recorded and listed as a top-grade herbal medicine in Shen Nong's Herbal Classic (Chinese Herbals Editor Board, 1999). As a traditional Chinese medicine, *Schisandra* is known as "the king of protecting the liver and reducing the enzymes", and often used in the prescription of traditional Chinese medicine for the treatment of liver diseases [16,17]. Polysaccharide is one of the main active constituents of *Schisandra chinensis*, which accounts for about 10% of *Schisandra chinensis* with a high development value due to its low toxicity and high curative effect [18,19]. The results of this study showed that SCAP could significantly reduce the levels of AST and ALT, the content of TG, and ameliorate the injured liver in pathology, showing a good protective effect on the acute ethanol-induced liver injury, which was consistent with our previous studies.

Oxidative stress plays an important role in alcoholic liver injury [20]. Ninety to ninety five percentage of the alcohol absorbed into the blood is oxidized by the microsomal ethanol oxidizing system (MEOS), which mainly contains alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1) in the cytosol of liver cells, to promote the generation of a large amount of ROS [21]. The excessive ROS can activate the lipid peroxidation of liver cells to cause the increase in the generation of malondialdehyde (MDA) and other lipid peroxides, which can damage the structure of cell membrane, leading to the swelling and necrosis of cells [22,23]. So the content of MDA can reflect the degree of cell injury induced by peroxidation. Antioxidant enzymes, especially superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), can reduce the level of free radicals and the formation of active oxygen in cells, and play an important role in the improvement of lipid peroxidation and the maintenance of cell membrane structure and function [24,25]. Due to these reasons, we observed the protective effect of SCAP on ethanol liver injury by detecting MDA level and SOD activity. The results showed that SCAP could significantly reduce the level of MDA and increase the activity of SOD in the serum, liver tissue of mice and HepG2 cells, suggesting that the protective effect of SCAP on ethanol liver injury may be associated with its antioxidant activity.

CYP2E1 is an important member of cytochrome P450 superfamily, involved in the metabolism of many poisons and carcinogens [26,27].CYP2E1 can be induced by ethanol and some drugs, which may increase the capacity of its metabolism, to promote the generation of a large number of reactive oxygen species (ROS), triggering the lipid peroxidation to damage the structure and function of various organelles and enzymes, and finally leading to the injury of liver cells [28–30]. Studies have shown that both expression level and gene polymorphism of CYP2E1 can affect the ethanol-induced toxicities on the liver, and CYP2E1 inhibitors, such as disulfiram, have a protective effect on the ethanol-induced liver injury [31,32]. In order to further investigate the mechanism of SCAP against the liver injury, the expression of CYP2E1 in the liver tissue and HepG2 cells was detected. The results showed that the upregulation of CYP2E1 induced by ethanol was significantly inhibited in the mice and cells treated with SCAP, suggesting that SCAP may protect against the ethanol liver injury by inhibiting the expression of CYP2E1 protein to decrease ROS generation and increase anti-oxidative activity.

In this study, we for the first time found that the protective effect

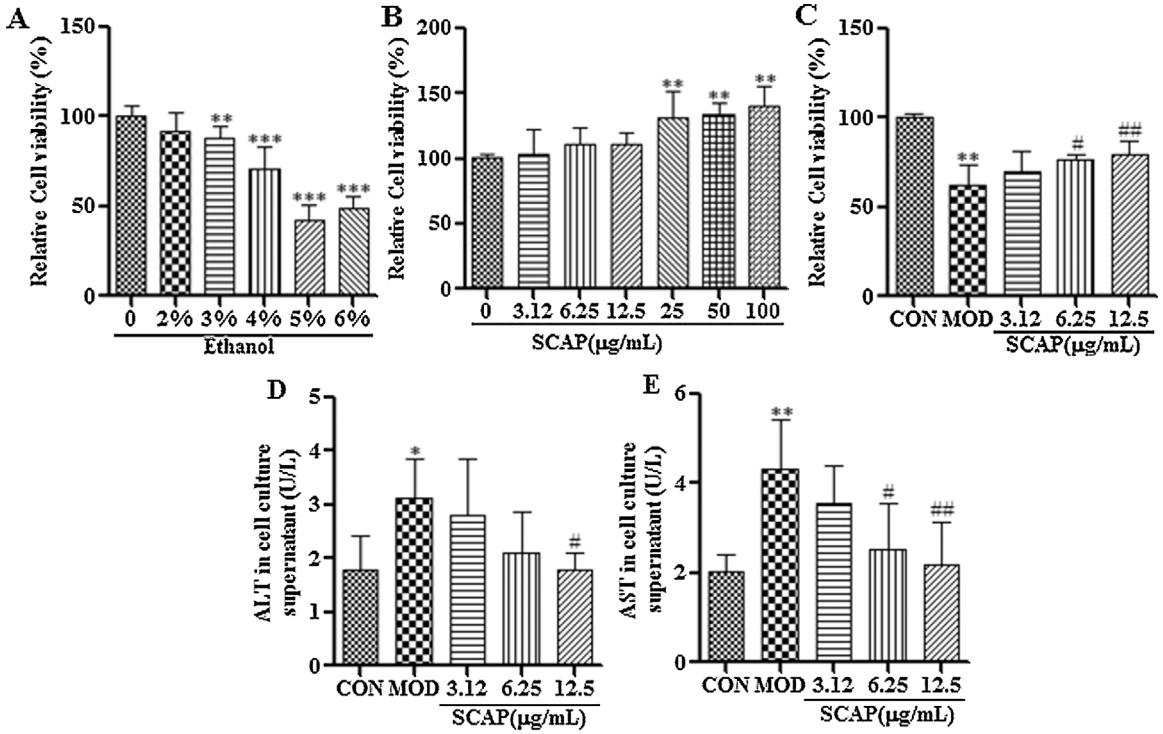

Fig. 2. Effects of SCAP on cell viability and aminotransferases levels in culture supernatant in HepG2 cells. (A) Effects of different concentration of ethanol on cell viability; (B) Effects of different concentration of SCAP on cell viability; (C) Cell viability in different groups; (D–E) Quantitative analysis of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), $^{*}P < 0.05$,$^{**}P < 0.01$ *vs* the CON group; $^{\#}P < 0.05$,$^{\#\#}P < 0.01$ *vs* the MOD group.

and mechanism of SCAP against acute ethanol-induced liver injury. In fact, long-term ethanol liver injury model is more consistent with the pathological process of alcoholic liver injury, which could be further research direction. Additionally, how SCAP, a polysaccharide macromolecule could be absorbed and circulated into blood to play a protective function on liver injury after oral administration could be investigated in future.

5. Conclusions

In summary, this study demonstrated that SCAP as one of the main active constituents of *Schisandra chinensis* has a significant protective effect on the liver injury induced by ethanol, and its mechanisms may be related to its reducing the oxidative stress and regulating the expression of CYP2E1. These results may provide a theoretical basis for

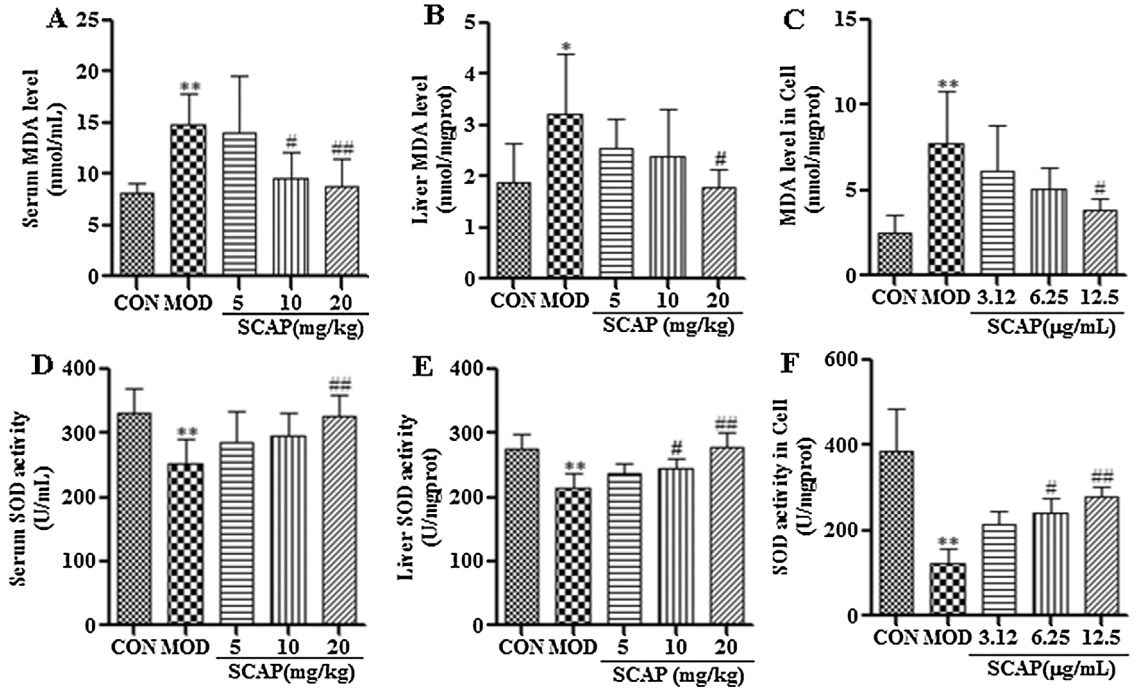

Fig. 3. Effects of SCAP on MDA levels and SOD activity. (A)Serum MDA level; (B) Liver MDA level ; (C) MDA level in HepG2 cells; (D) SOD activity in serum; (E) SOD activity in liver; (F) SOD activity in HepG2 cells, $^{*}P < 0.05$,$^{**}P < 0.01$ *vs* theCON group;$^{\#}P < 0.05$,$^{\#\#}P < 0.01$ *vs* the MOD group.

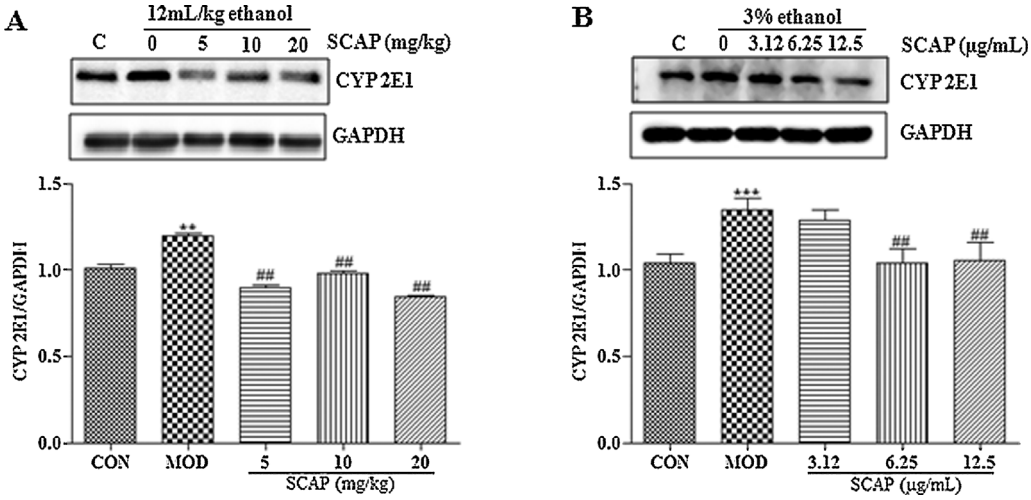

Fig. 4. Effects of SCAP on protein expression of CYP2E1.(A) Protein expression of CYP2E1 in the liver of mice; (B) The protein expression of CYP2E1 in HepG2 cells, $^{*}P < 0.05$,$^{**}P < 0.01$ *vs* the CON group;$^{\#\#}P < 0.01$ *vs* the MOD group.

the application of *Schisandra* in the prevention and control of ALD.

Authors contribution

Rongshuang Yuan – Conducts of the study.
Xue Tao – Animal experiment.
Shuang Liang – HepG2 cell experiment.
Yan Pan – Animal experiment.
He Li – Data collection.
Jinghui Sun – Data collection.
Wenbo Ju – Animal experiment, Data analysis, manuscript writing.
Xiangyan Li – Manuscript writing and English polishing.
Jianguang Chen – Design of the study.
Chunmei Wang – Design of the study and manuscript writing.

Conflict of interest

All authors declare that they have no conflict of interest.

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