



Original Article

Protective effect of *Schisandra chinensis* total lignans on acute alcoholic-induced liver injury related to inhibiting CYP2E1 activation and activating the Nrf2/ARE signaling pathway

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ABSTRACT

Schisandra chinensis (Turcz.) Baill., Schisandraceae, 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 *S. chinensis* total lignans on the liver injury induced by alcoholic. *S. chinensis* total lignans were extracted with ethanol extraction. The liver index, alanine aminotransferase and aspartate aminotransferase levels in serum of the rat culture supernatant were examined. The malondialdehyde level and superoxide dismutase activities in serum and liver tissue, and triacylglyceride content in liver tissue were tested. Western blot was conducted to determine cytochrome P450 2E1 expression in liver tissue of rats. The results showed that *S. chinensis* total lignans administration significantly inhibited alcohol-induced liver injury. In exploring the underlying mechanisms of *S. chinensis* total lignans action, we found that it significantly decreased Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AKP), Glutamyl transpeptidase (γ -GT), reactive oxygen species (ROS) and malondialdehyde (MDA) level in livers/serum and inhibited the gene expression level of CYP2E1 in rat livers. The Nuclear factor erythroid-2 related factor 2 (Nrf2) gene expression and Nuclear factor erythroid-2 related factor 2 (Nrf2) protein nuclear transfer increased significantly, and significantly increased the expression of downstream target gene and protein heme oxygenase-1 (HO-1), Glutamate-cysteine ligase regulatory subunit (GCLM), NAD(P)H:quinone oxidoreductase 1 (NQO1). Moreover, *S. chinensis* total lignans decreased production of pro-inflammatory markers including Tumor Necrosis Factor- α (TNF- α), Interleukin-1beta (IL-1 β) and Interleukin-6 (IL-6). In conclusion, these results suggested that the inhibition of alcohol-induced liver injury by *S. chinensis* total lignans is associated with its ability to inhibiting CYP2E1 activation and activating the Nrf2/Antioxidant response element (ARE) signaling pathway.

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Introduction

The dry ripe fruits of *Schisandra chinensis* (Turcz.) Baill., Schisandraceae (Wuweizi in China), officially listed as a sedative and tonic in the China Pharmacopoeia, has been used as an important component in various prescriptions in Traditional Chinese medicine (TCM). *S. chinensis* has been used as a hepatoprotective herb in clinical for a long time. The results of many studies showed that the chemical constituents of *S. chinensis* were mainly lignans, polysac-

charides, volatile oils, three terpenoids, sesquiterpene and organic acids (Shi et al., 2011; Hu et al., 2013; Zhu et al., 2015). The content of Schisandrae lignans were the highest, and they are the main active components of anti-liver injury (Pan et al., 2012; Park et al., 2014; Mocan et al., 2016). Studies have showed that a variety of ingredients in *S. chinensis* can enhance liver detoxification function, with specific hepatoprotective function (Su et al., 2013a; Xie et al., 2014; Jiang et al., 2015). It is well accepted that the efficacy of TCM is largely based on the synergistic effects of multi-components on multi-targets.

Liver is an important barrier of the organism, and its function of phagocytosis and detoxification is of great significance to the protection of the body. At present, alcoholic liver injury is one of

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the difficult problems in the world, which is seriously harmful to human health. Furthermore, harmful alcohol intake is ranked as one of the top five risk factors for death and disability worldwide with 2.5 million deaths and 69.4 million annual disability adjusted life years (Pan et al., 2012). In recent years, many studies have shown that Nrf2/ARE signaling pathway has an important protective effect in avoiding alcohol induced oxidative stress liver injury (Gong and Cederbaum, 2006; Yao et al., 2007; Wu et al., 2012). When an organism or cell attacked by a large number of ROS and other oxidative stress stimulation, Keap1 undergone conformational changes, thus Nrf2 ubiquitination and proteasomal degradation was inhibited, and Keap1 showed strong expression of uncoupling, transferring into the nucleus in translation and protein substitution on Bach1 combined with ARE gene, expression of ARE related regulation of downstream resistance starting oxidase gene and phase II detoxification enzyme gene, or enhance cell antioxidant ability to activate the Nrf2 signaling pathway (Huang et al., 2002; McMahon et al., 2003; Furukawa and Xiong, 2005). So, we focus on the Nrf2/ARE signal pathway to carry out this study.

The mechanism of the ethanol extract of the *S. chinensis* (SCTL) to acute alcohol-induced liver damage has not been study deeply. In this study, the main idea is liver specific role by studying the Nrf2/ARE signaling pathway and metabolism of CYP2E1 enzyme analysis of biological information to evaluate the potential mechanism of SCTL on ALI.

Materials and methods

Chemicals, reagents and instruments

Schisandra chinensis (Turcz.) Baill., Schisandraceae, pieces were purchased from Anhui Fengyuan Tongling Herbal Pieces Co. Ltd., which grows in Changbai Mountain, Jilin Province. And the origin was identified by Professor Liu Shengjin of College of Pharmacy of Nanjing University of Traditional Chinese Medicine. Red Star Erguotou (56°, one kind of Chinese spirits) purchased from Beijing Red Star Limited by Share Ltd. Sodium carboxymethyl cellulose (CMC-Na) purchased from Chinese Medicine Group Chemical Reagent Co., Ltd.

Bicinchoninic acid (BCA) protein, alanineaminotransferase (ALT), γ -glutamyltransferase (γ -GT or GGT) and malondialdehyde (MDA) assay kits were purchased from the Jiancheng Bioengineering Institute of Nanjing (Nanjing, China). Alkaline phosphatase (AKP), reactive oxygen species (ROS), tumor necrosis factor α (TNF- α), interleukin 1 β (IL-1 β) and interleukin6 (IL-6) ELISA assay kits were provided by Nanjing Austrian Green Biotechnology Co., Ltd. (Nanjing, China).

FA1104 type Electronic analytical balance and 722 type UV spectrophotometer (Shanghai Precision Scientific Instrument Co., Ltd., China), Milli-Q Gradient A10 Ultra-pure water meter (Merck Millipore, USA), ALLEGRA X-22 type High speed refrigerated centrifuge (Beckman Coulter, Germany), Synergy HT type Microplate Reader (BioTek, USA), DHG-9140A type Electric heating constant temperature drying box (Shanghai Jing Hong experimental equipment company, China).

Extraction of SCTL

The *S. chinensis* was prepared according to the methods in the Chinese Pharmacopoeia. The *S. chinensis* had any impurities removed, and was washed before use. Then, the *S. chinensis* was extracted twice by refluxing with boiling water for 2 h with eight times amount of 85% ethanol, respectively. After merging the filtrate and condensing to certain concentration under reduced pressure, the extractum was then dissolved into 0.5% sodium

carboxymethyl cellulose (CMC-Na) to obtain the target concentration 1.5 g/ml (the amount of crude herb or pieces), the solution of intragastric administration (SIA), the prepared drugs were stored at 4 °C until administration for rats.

Determination of the main chemical constituents in SCTL

In addition, the eight lignans (the main chemical constituents of SCTL), schizandrin, gomisin J, schisandrin B, gomisin G, schisantherin A, deoxyschisandrin, schisandrin B and schisandrin C, in the extract (SCTL) were determined by HPLC/UV detector in different batches (Supplementary data).

High Performance Liquid Chromatography (HPLC) method was performed to identify the main chemical constituents of SCTL. The reference standards of schizandrin, gomisin J, schisandrin B, gomisin G, schisantherin A, deoxyschisandrin, schisandrin B and schisandrin C (purity >98%) were purchased from National Institutes for Food and Drug Control (Beijing, China). HPLC was performed on Waters 2695-2998 (Waters, USA) equipped with a Kromasil C₁₈ (4.6 mm \times 250 mm, 5 μ m). The mobile phase included deionized water (A) and acetonitrile (B) at a flow speed of 1 ml/min. The detection wavelength was set at 217 nm and the sample injection volume was 10 μ l. In addition, the temperature of the column was set at 30 °C. The gradient elution was as follows: 0–30 min, 51% B, 30–60 min, 51%–80% B, 60–70 min, 80%–51% B.

Animals, treatment and biological samples collection

A total of forty male Sprague-Dawley rats (200 \pm 20 g, aged approximately 8 weeks) were purchased from Shanghai Sipper-BK Lab Animal Co. Ltd. The rat were maintained under constant conditions (24 \pm 1 °C and 60% humidity) with free access to rodent food and tap water under 12 h light/dark schedule (lights on from 8:30 a.m. to 8:30 p.m.).

The rats could acclimate for a period of one week before randomly divided into five groups of eight rats each. The method of administration of intragastric (i.g.) was used in this experiment. Group I: the rat served as control (treated with CMC-Na solution for two weeks); Group II: the rat served as model received alcohol (10 ml/kg, once daily, at 9 a.m.); Groups III: the rat served as positive received silymarin (200 mg/kg, once daily, at 3 p.m.); Groups IV and V: the rat received L-SCTL and H-SCTL (400 and 800 mg/kg, once daily, at 3 p.m.). The Groups III to Groups V were given the alcohol according to the method of model group at the same time. The approximately same volume of vehicle as the SCTL groups was administered orally to the control and model groups. L-SCTL means low dose of SCTL, H-SCTL means high dose of SCTL.

The experiment lasted for two weeks. At the end of treatment, rats were sacrificed, and blood samples were drawn by cardiac puncture with heparinized tubes. Liver tissues were quickly collected, placed in ice-cold 0.9% NaCl solution, perfused with the physiological saline solution to remove blood cells, blotted on filter paper, and stored at –80 °C for later use.

Histological evaluations

The histological changes of liver were evaluated as described in reference report (Leung and Nieto, 2013). A portion of liver tissue of the median and left lateral liver lobes was fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into sections, and stained with hematoxylin and eosin (H&E). The histological changes were scored according to the following criteria: 0, absent; 1, mild; 2, moderate; 3, severe; and 4, irreversible.

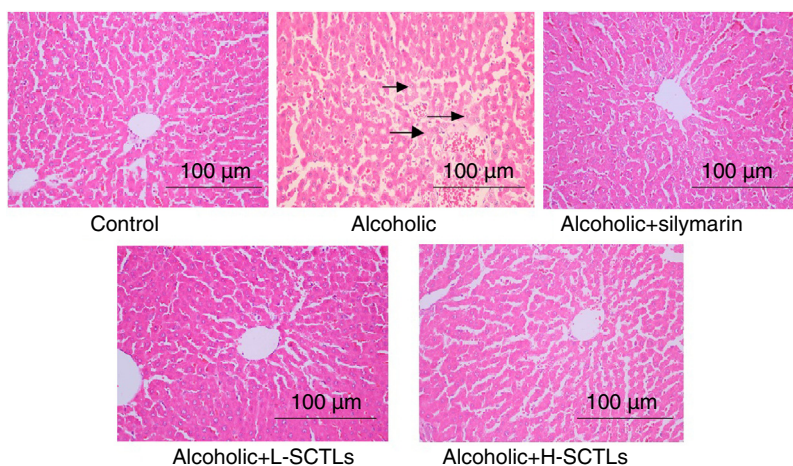


Fig. 1. Morphological and histological evaluation of liver in rats. Original magnification, 100 μm . ** $p < 0.01$, compared with the control group; ### $p < 0.01$, compared with the alcohol-treated group.

Measurement of serum and liver biochemical assays

The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AKP), γ -glutamyltransferase (γ -GT or GGT), reactive oxygen species (ROS) and malondialdehyde (MDA) in serum and liver were estimated spectrophotometrically using commercial diagnostic kits.

Western blot analyses

Liver tissues (80 mg each) were homogenized in ice-cold lysis buffer (RIPA, 1 mM PMSF, PMSF/RIPA = 1/100). Homogenates were centrifuged at $12,000 \times g$ for 15 min at 4°C . The supernatants were collected and centrifuged again, and the final supernatants were collected. Nuclear and cytoplasmic extracts for western blotting were obtained by using a nuclear/cytoplasmic isolation kit (Solarbio® Biotechnology, Beijing, China). Protein levels were determined using the BCA assay kit (Yi Fei Xue Biotechnology, Nanjing, China). Samples (50 μg each) were separated by denaturing SDS-PAGE and collected on a PVDF membrane (0.45 μm , Merck Millipore, USA) by electrophoretic transfer (Mini-Protean® 3 Cell, Bio-Rad, USA). The membrane was pre-blocked with 5% BSA and 0.1% Tween-20 in Tris-buffered saline (TBST) and incubated overnight with the primary antibody (in TBST with 5% BSA). Each membrane was washed three times for 30 min and incubated with the secondary horseradish peroxidase-linked antibodies (Aff-biotech, USA). Quantitation of detected bands was performed with the ImageQuant™ TL analysis software (General Electric, USA). To correlate protein loading, the blots were analyzed for β -actin expression using an anti- β -actin antibody (Affbiotech, USA). Each density was normalized using each corresponding β -actin density as an internal control and averaged from three samples. The density of the control was set at 1.0 to compare other groups.

Real-time PCR analysis

Total RNA was isolated from liver tissues treated with alcohol, RSC and VPC for 8 weeks. Quantitative real-time PCR assays for liver tissues CYP2E1 mRNA levels were performed using a Real-time PCR Master Mix (SYBR® Green, KeyGen) according to the manufacturer's instructions. PCR was performed in a volume of reaction mixture (20 μl) for each sample containing SDW (8 μl), Power SYBR® Green Master Mix (10 μl), Forward Primer (0.5 μl), Reverse Primer (0.5 μl) and the cDNA (1 μl). After incubation at 55°C for 2 min and 95°C for 10 min, the PCR reaction was

performed for 40 cycles: denaturation at 95°C for 30 s, anneal at 63°C for 30 s and extension at 72°C for 30 s. Primers for CYP2E1 were: sense primer, GACTTTGGCCGACCTGTCTTT, and anti-sense primer, CATGAGGATC-AGGAGCCCATATCT. Primers for the housekeeping β -actin gene were: sense primer, GAATTC-CCAGTAAGTGCCGGTCATA, and anti-sense primer, CGAGGG-CCTCACTAAACCATC. β -Actin was used to normalize gene expression in all the samples. All samples were run in triplicate simultaneously with negative controls. Melting curve analyses were performed in each real-time RT-PCR experiment.

Statistical analysis

All statistical analyses were performed using the SPSS software (version 16.0, SPSS Inc., Chicago, USA). A one-way analysis of variance (ANOVA; $p < 0.05$) was used to determine significant differences between groups and the individual comparisons were obtained by Turkey's HSD *post hoc* test. Statistical significance was set at $p \leq 0.05$.

Results

SCTL alleviated alcohol-induced histology changes in the liver

As shown in Fig. 1, alcohol treatment caused several visible histology liver changes, including fatty degeneration, necrosis of focal hepatocytes and infiltration of inflammatory cells. Moreover, the score for liver injury was markedly higher in the alcohol-treated rat as compared with controls ($p < 0.01$). However, SCTL treatment alleviated the alcohol-induced damage in rat livers. And the H-SCTL treatment significantly alleviated the alcohol-induced damage in rat livers than L-SCTL.

SCTL protects against alcohol-induced hepatic dysfunction

In order to determine whether SCTL can attenuate the liver damage in the alcohol-treated rats, we measured the activities of ALT, AST, AKP, GGT, as well as the levels of ROS and MDA in serum and liver (Fig. 2). In alcohol-treated rat (Model group), the activities of ALT, AST, AKP, GGT, as well as the levels of ROS and MDA in serum/liver markedly increased, as compared with these of the controls ($p < 0.01$). Interestingly, treatment with low and high dose of SCTL in alcohol-treated rat significantly decreased the activities of ALT and AST in serum ($p < 0.01$).

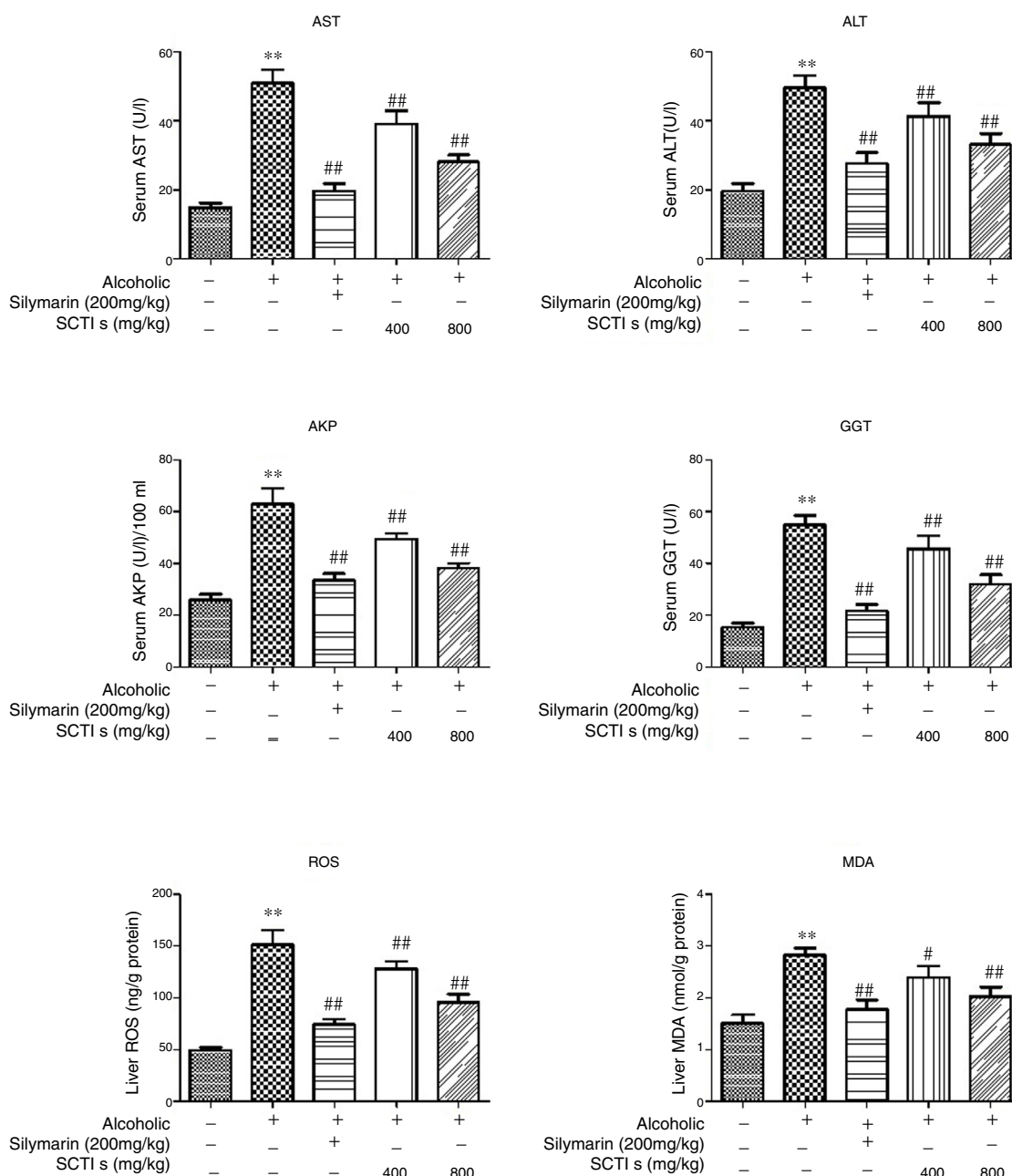


Fig. 2. Effect of the ethanol extract of the *Schisandra chinensis* on alcohol-induced changes in hepatic functional markers. (A) AST, ALT, AKP and GGT in serum; (B) ROS and MDA in liver. All values are expressed as mean \pm SD ($n=8$). ** $p < 0.01$, compared with the control group; ## $p < 0.01$, # $p < 0.05$, vs. the alcohol-treated group.

SCTL reduce the expression of inflammatory factors in the serum and livers of rats

We further examined the expression of inflammatory factors, including TNF- α , IL-1 β and IL-6, in the serum/liver of each group by western blot analysis. As shown in Fig. 3, the expressions of TNF- α , IL-1 β and IL-6 were markedly decreased in the serum/livers of alcoholic-induced liver injury rat, as compared with the controls ($p < 0.01$). However, all treatment groups could inhibit the inflammatory response in the serum/liver of rat. And, the H-VPSC administration markedly inhibited the inflammatory response in the serum/liver of rat ($p < 0.01$).

The hepatoprotective effect of SCTL by inhibiting the expression of CYP2E1

The expression level of CYP2E1 mRNA in liver tissue of the rat was shown in Fig. 4. After alcohol oral administration in 2 weeks, the liver CYP2E1 mRNA expression level of model group was significantly higher than the normal control group ($p < 0.01$). Instead, each treatment group significantly inhibited the expression of CYP2E1 mRNA ($p < 0.05$, $p < 0.01$), and CYP2E1 mRNA expression level in H-SCTL decreased significantly better than L-SCTL group. The results showed that the relative expression level of CYP2E1 mRNA in rats of liver injury was inhibited, and the relative expression level was measured.

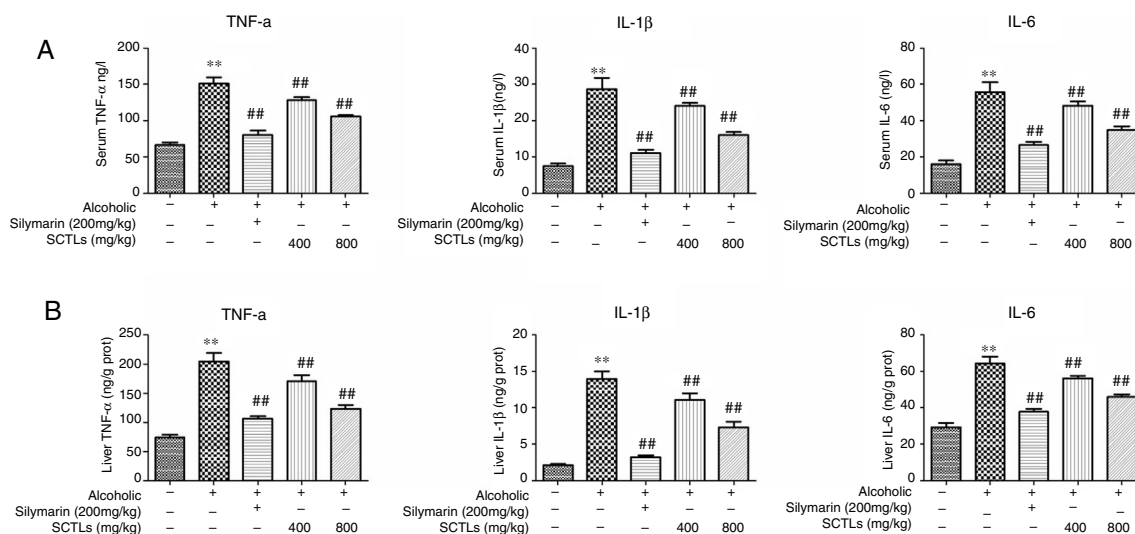


Fig. 3. Ethanol extract of the *Schisandra chinensis* reduced the levels of inflammatory cytokines in the serum and livers of rat induced by alcohol. (A) Serum; (B) liver; each value is expressed as mean \pm SD ($n=8$). ** $p < 0.01$, compared with the control group; ## $p < 0.01$, * $p < 0.05$, vs. the alcohol-treated group.

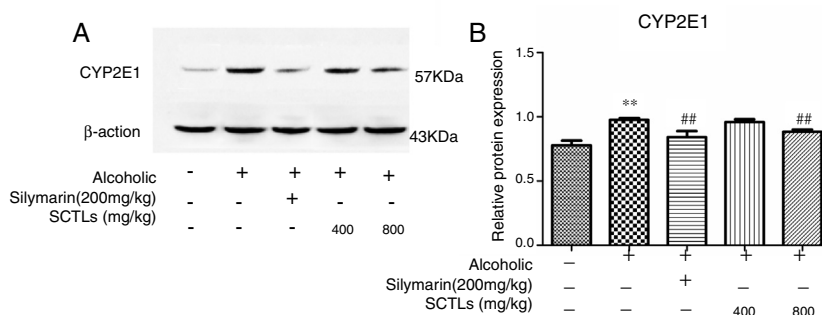


Fig. 4. Ethanol extract of the *Schisandra chinensis* inhibited the expression level of CYP2E1 protein and mRNA in alcohol-induced liver. A. Western blot analysis of the CYP2E1; B. The expression level of CYP2E1 mRNA. ** $p < 0.01$, compared with the control group; ## $p < 0.01$, * $p < 0.05$, vs. the alcohol-treated group.

The hepatoprotective effect of SCTL involves Nrf2, HO-1, GCLM and NQO1 activation

To further investigate the molecular mechanism of inflammation in rat livers, we measured the expression levels of Nuclear factor E2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), glutamate-cysteine ligase modifier subunit (GCLM) and NAD (P) H quinone oxidoreductase 1 (NQO1). As shown in Figs. 5 and 6, the translocation of Nrf2 from the cytosol to the nuclear fraction was significantly decreased in alcohol-induced rats ($p < 0.01$). The HO-1, GCLM and NQO1 expression level was decreased in rats treated with alcohol alone as compared with controls ($p < 0.01$). However, administration with H-SCTL markedly increased the HO-1, GCLM and NQO1 expression level and Nrf2 activation in the livers of alcohol-induced rats ($p < 0.01$).

Discussion

Schisandra chinensis has been reported to have many benefits and medicinal properties, such as anti-oxidative, immunomodulatory, anti-hepatic injury. This study showed that SCTL could protect acute alcoholic liver injury in rats by activating the Nrf2/ARE signaling pathway and inhibiting CYP2E1 activation. Previous studies showed that alcohol-induced could cause liver injury and dysfunction, such as mild alcoholic liver disease, alcoholic hepatitis, alcoholic fatty liver, alcoholic liver fibrosis, alcoholic cirrhosis and liver cancer. The present study also revealed that the

activities of AST, ALT, AKP, GGT, ROS and MDA in the serum/liver of alcohol-treated rat were markedly increased (Fig. 2). Moreover, the histological changes of liver, such as structure damage, fatty degeneration, hydropic degeneration, hepatocellular necrosis and leukocyte infiltration had been observed in alcohol-treated rat. The score for liver injury was also higher in the treatment rat as compared with controls (Fig. 1). However, liver injury rats treated with SCTL effectively protected organism against alcohol-induced liver damage by reducing increased serum aminotransferase activities and alleviating hepatic histological changes.

Many studies indicated that alcohol could induce inflammatory responses by activating the innate immune system (Pan et al., 2012; Park et al., 2014). TNF- α , IL-1 β and IL-6 are the important members of a class of signaling molecules known as pro-inflammatory cytokines, which play an important role in initiating the body's inflammatory response to infections, injuries, or stress (Liu et al., 2011; Gonzalez et al., 2008; Siednienko et al., 2011). The present study showed that alcohol-induced significantly up-regulated the TNF- α , IL-1 β and IL-6 levels in the rat livers. However, SCTL markedly attenuated this regulation (Fig. 3). These results suggested that SCTL could alleviate liver injury caused by alcohol through suppressing inflammatory response.

Among the cytochrome P450 family, CYP2E1 has been identified as the most relevant for alcoholic liver disease (ALD) as it is highly inducible, and it has high catalytic activity for alcohol (Lieber, 1997). CYP2E1 is mainly expressed in the liver, with hepatocytes showing the highest expression (Lu, 2008; Lu et al., 2008). During the catalytic cycle of CYP2E1, significant amounts of ROS

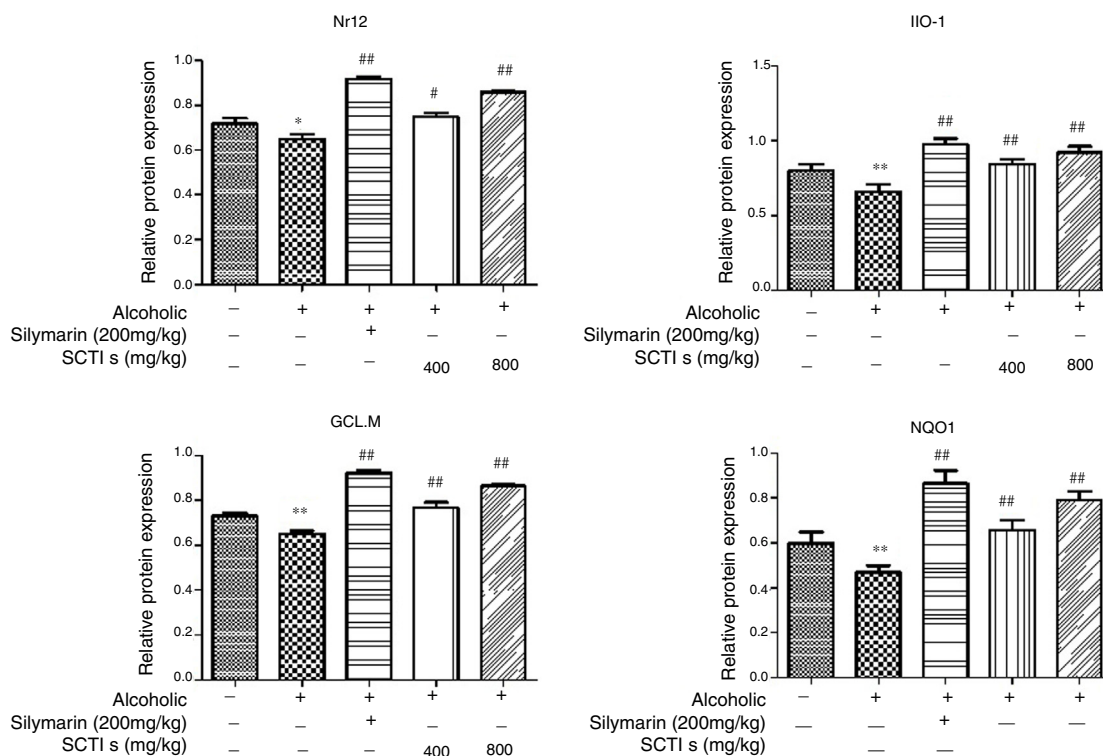


Fig. 5. The western blot analysis of the Nrf2, HO-1, GCLM and NQO1 in alcohol-induced liver tissues. ** $p < 0.01$, compared with the control group; ## $p < 0.01$, # $p < 0.05$, vs. the alcohol-treated group.

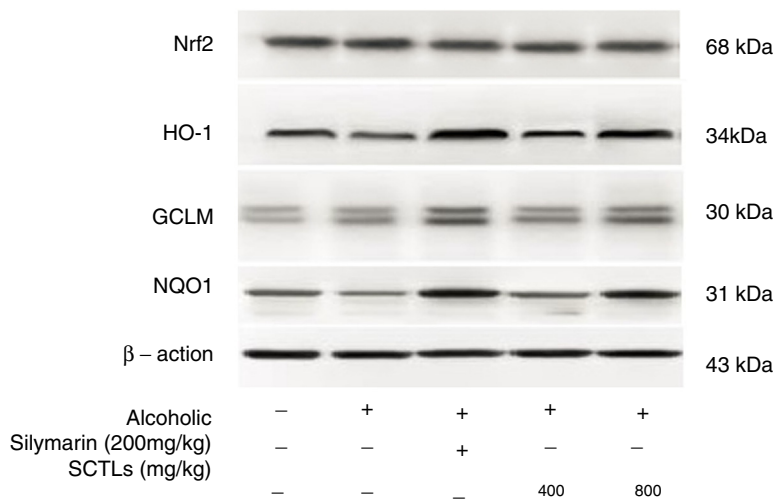


Fig. 6. The relative expression level of Nrf2, HO-1, GCLM and NQO1 protein. ** $p < 0.01$, compared with the control group; ## $p < 0.01$, # $p < 0.05$, vs. the alcohol-treated group.

are generated, which subsequently cause liver-cellular damage. Previous studies showed that increased cellular injury, lipid peroxidation, oxidant and nitrosative stress, and mitochondrial damage occurred in livers from acute alcohol-fed rat compared with pair-fed rat. One of the reasons for the increase in CYP2E1 protein during chronic ethanol intake is decreased proteasomal degradation, which increases CYP2E1 protein stability (Roberts et al., 1995; Leung and Nieto, 2013). Our results indicated that low and high dose of SCTL could inhibit the expression and activity of CYP2E1, and H-SCTL was the most effective one.

The organism produces a large number of hydroxyethyl radical, H_2O_2 , O_2^- , HO^- and other products of ROS oxidative stress under internal environment of alcohol metabolism produce excessive acetaldehyde poison and the expression of CYP2E1 increased

in liver tissue (Leung and Nieto, 2013). And these free radicals can inhibit the ubiquitination mediated by kelch-like ECH-associated protein 1 (Keap1), result in the phenomenon of Nrf2 and Keap1 uncoupling been activated, and then the nuclear translocation of Nrf2 were increased, enter nucleus, finally combined with the downstream antioxidant response element (ARE). Nrf2 is referred as the master regulator of the antioxidant response, which modulates a series of phase II detoxification enzymes and antioxidant genes, as well as anti-inflammatory, energy metabolism, cell survival and other groups of genes that contain a cis-acting element in their promoter region recognized as ARE (Kobayashi et al., 2004; Marhenke et al., 2008; Lewis et al., 2010; Copple, 2012). Under physiological condition, Nrf2 is sequestered in the cytoplasm by binding to the cytoskeleton-binding Keap1 protein. However, stim-

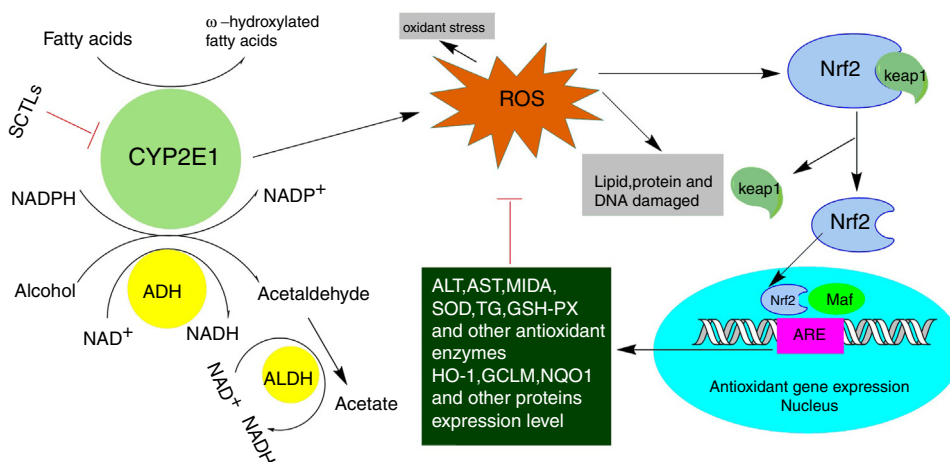


Fig. 7. The schematic diagram showing protective signaling of ethanol extract of the *Schisandra chinensis* in alcohol induced liver inflammation. The → indicates activation or induction, and indicates inhibition or blockade.

ulated by inducers, Nrf2 dissociates from Keap1 and translocated into the nucleus where it dimerizes with some cofactors and binds to ARE (Taguchi et al., 2011; Magesh et al., 2012). The anti-oxidative protein HO-1, which contains ARE in its promoter, is regulated at the transcriptional level through antioxidants interacting directly or indirectly to the cis-acting ARE (Giudice et al., 2010; Su et al., 2013b). Nrf2 can inhibit the expression of pro-inflammatory mediators, including cytokines, chemokines, adhesion molecules, matrix metalloproteinases (MMP-9), COX-2 and iNOS by regulation of anti-inflammatory enzymes, such as HO-1 (Kim et al., 2010). The present study showed that alcohol increased the expression levels of TNF- α , IL-1 β , IL-6 and decreased the activation of HO-1 and Nrf2 in the livers of rat. However, we found that SCTL markedly inhibited these events in alcohol-induced liver injury rat. Thus, our findings indicate that SCTL might enhance protecting alcoholic liver injury in rats by activating the Nrf2/ARE signaling pathway and inhibiting CYP2E1 activation.

Conclusions

In summary, we propose *S. chinensis* can protect acute alcoholic liver injury (Fig. 7). This study demonstrates for the first time that SCTL has potent significant protective effects against alcohol-induced oxidative stress and inflammatory response associated with inhibiting CYP2E1 activity and modulating the Nrf2/ARE pathway in the liver of rat. Here we demonstrated that H-SCTL administration attenuated alcohol-induced hepatic dysfunction than L-SCTL. SCTL treatment could effectively inhibit alcohol-induced inflammation in livers associated with decreasing expression levels of TNF- α , IL-1 β , IL-6 and inhibiting CYP2E1 activity and increasing activation of Nrf2, HO-1, GCLM and NQO1. SCTL certainly enhance protecting acute alcoholic liver injury in rats, therefore, the clinical treatment of liver injury with the common use of SCTL has the scientific principle.

Ethical statement

All applicable international, national, and institutional guidelines for the care and use of animals were followed. All of the protocols on living animals used in this paper were come from the Experimental animal center of Nanjing University of Chinese Medicine (license no. SYXK (Su) 2014-0001). Moreover, all efforts were made to minimize suffering of the rats.

Author contributions

Conceptualization, LS and TL; investigation, PL, MH; data curation, DJ, CM; writing-original draft preparation, LS and ZH; writing-review and editing, TL; supervision, CM; project administration, TL.

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Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2019.01.008.

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