α-lipoic acid protects against carbon tetrachloride-induced liver cirrhosis through the suppression of the TGF-β/Smad3 pathway and autophagy

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Abstract. α-lipoic acid (ALA) is a naturally occurring anti-oxidant with protective effects against various hepatic injuries. The aim of the present study was to investigate the mechanisms by which ALA protects the liver from carbon tetrachloride (CCl_4)-induced liver cirrhosis. The widely used liver cirrhosis rat model was established via an intraperitoneal injection of 2 mg/kg 50% CCl_4, three times/week for 8 weeks. Simultaneously, 50 or 100 mg/kg ALA was orally administrated to the rats every day for 8 weeks. The activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was detected in the serum. The pathological liver injuries were analyzed using hematoxylin and eosin and Masson's trichrome staining. The principal factors involved in the transforming growth factor-β (TGF-β)/mothers against decapentaplegic homolog 9 (Smad3) and protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathways and in autophagy were examined using reverse transcription-quantitative polymerase chain reaction or western blot analysis. The results demonstrated that the administration of ALA alleviated CCl_4-induced liver injury, as demonstrated by decreased ALT and AST activity, improved pathological injuries and reduced collagen deposition. The CCl_4-induced increase in TGF-β and phosphorylated-Smad3 expression levels was additionally inhibited by treatment with ALA. Furthermore, the administration of ALA reversed the CCl_4-induced upregulation of light chain 3II and Beclin-1, and downregulation of p62. The CCl_4-induced suppression of the AKT/mTOR pathway was additionally restored following treatment with ALA. In combination, the results of the present study demonstrated that ALA was able to protect CCl_4-induced liver cirrhosis, an effect that may be associated with inactivation of the TGF-β/Smad3 pathway and suppression of autophagy.

Introduction

Liver cirrhosis, caused by progressive hepatocyte injury-induced conversion of normal liver architecture into abnormal nodules, is a common chronic liver disease characterized by hepatic fibrosis. There are a number of factors that may lead to cirrhosis, including alcohol abuse, viral infection, administration of drugs or chemicals (1). In total, one million people succumb to liver cirrhosis every year, accounting for 2% of the total global mortality in 2010 (2,3). Therefore, liver cirrhosis is a significant global health burden. Although the survival of patients with liver cirrhosis has been improved with the development of medicine and surgical procedures (including liver transplantation), developing effective therapeutic agents to prevent the progression of early-stage cirrhosis remains crucial.

Autophagy is a metabolic process occurring in all cell types. Under conditions of cellular stress, defective organelles and excessive components are eliminated to maintain cell survival and cellular activities through autophagy (4,5). Previous studies demonstrated that autophagy may drive the activation of hepatic stellate cells (HSCs) (6,7), a critical event in liver fibrogenesis (8). Furthermore, inhibition of autophagy may reduce the expression of fibrosis-associated genes, including COL1, α-SMA, β-platelet derived growth factor receptor and matrix metalloproteinase-2 (7). Therefore, selective suppression of autophagic activity may be a promising therapeutic strategy for the prevention of early fibrosis in liver cirrhosis.

α-lipoic acid (ALA) is a naturally occurring thiol anti-oxidant that protects against acetaminophen-induced liver damage (9), high-fat diet induced-fatty liver (10), concanavalin A-induced hepatitis (11), lipopolysaccharide induced-acute liver injury (12) and liver cirrhosis (13). ALA was able to regulate autophagic activity in certain cases (14,15). However, whether ALA may protect the liver through the regulation of autophagy remains unclear. In the present study, a carbon tetrachloride (CCl_4)-induced liver cirrhosis rat model was

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established and the mechanism of the hepatoprotective effect of ALA was investigated.

Materials and methods

Animals and experimental design. A total of 30 Sprague-Dawley rats (male; 6 week old; weight, 180-200 g) obtained from the Liaoning Chang Sheng Biotechnology Co., Ltd. (Benxi, China) were used in the experiment. After adapting for 1 week under standard animal laboratory conditions ($25\pm2°C$; air humidity $50\pm10\%$; 12-h light/dark cycle) with free access to food and water, the rats were randomly divided into five groups (6/group): Control group, 100 mg/kg ALA group, CCl_4-treated group, CCl_4+50 mg/kg ALA group and CCl_4+100 mg/kg ALA group. The animal raising and handling procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (16) and approved by the Animal Experimental Ethics Committee of the Henan University of Chinese Medicine (Zhengzhou, China).

CCl_4-induced liver cirrhosis model and treatments. A CCl_4-induced liver cirrhosis model was established as previously described (17,18). Rats were intraperitoneally injected with 50% CCl_4 (purchased from Shanghai Aladdin Bio-Chem Technology Co. Ltd., Shanghai, China; 1:1 diluted with olive oil) three times a week for 8 consecutive weeks. In addition to treatment with CCl_4, rats in the CCl_4+50 mg/kg ALA or CCl_4+100 mg/kg ALA groups received 50 or 100 mg/kg ALA (Sangon Biotech Co. Ltd., Shanghai, China) orally every day for 8 weeks. Rats in the control group were intraperitoneally injected with an equal volume of olive oil instead of CCl_4 3 times a week and received 0.5% sodium carboxymethylcellulose (the solvent of ALA) 0.5 ml orally every day. Rats in the 100 mg/kg ALA group were intraperitoneally injected with an equal volume of olive oil instead of CCl_4 three times a week and received 100 mg/kg ALA orally every day for 8 weeks. At the end of treatment, rats were anesthetized with isoflurane (2.5-3%), subsequently, blood was collected from the orbital sinus as Parasuraman *et al* (19) described previously. The rats were sacrificed with 200 mg/kg pentobarbital sodium by intraperitoneal injection and the liver tissues were harvested.

Histopathological and immunohistochemical analysis. Liver tissues were fixed in 4% paraformaldehyde for 48 h at 4°C, and were subsequently embedded in paraffin and cut into 5-μm thick sections. Following washing with xylene and hydrating in graded ethanol, the sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome, according to standard procedures (20). For immunohistochemical analysis, the deparaffinized liver sections were incubated with rabbit anti-α-smooth muscle actin (α-SMA) antibody or rabbit anti-transforming growth factor (TGF)-β antibody (both 1:50; α-SMA, cat. no. 55135-1-AP; TGF-β, cat. no. 21898-1-AP; Wuhan Sanying Biotechnology, Wuhan, China) at 4°C overnight. Subsequently, the specific proteins were detected with biotinylated goat anti-rabbit immunoglobulin G antibody (1:200; cat. no. A0208; Beyotime Institute of Biotechnology, Haimen, China) at 37°C for 30 min. The reactions were finally analyzed with horseradish peroxidase-conjugated streptavidin (Beyotime Institute of Biotechnology). Following visualization using a diaminobenzidine substrate kit (Beijing

Table I. Primer sequences used in the present study.

Gene	Sequences (5'-3')
COL1A1	Forward: AGAGGCATAAAGGGTCATCGTG
	Reverse: CAGGAGAACCAGCAGAGCCA
TGF-β	Forward: CAACAATTCCTGGCGTTACCT
	Reverse: AGCCCTGTATTCCGTCTCCTT
Beclin-1	Forward: CAGCAGTTCAAAGAAGAGGTG
	Reverse: GAGGACACCCAAGCAAGAC
p62	Forward: AGCGGGTACTGATCCCTGTC
	Reverse: TCTTCCTCCTTGGCTTTGTC
β-actin	Forward: GGAGATTACTGCCCTGGCTCCTAGC
	Reverse: GGCCGGACTCATCGTACTCCTGCTT

TGF-β, transforming growth factor-β; COL1A1, Collagen α-1(I) chain.

Solarbio Science & Technology Co., Ltd., Beijing, China), the sections were imaged under a light microscope (BX53; Olympus Corporation, Tokyo, Japan; magnification, x100-200).

Biochemical measurement. Serum was isolated from blood samples by centrifugation at 1,100 x g for 10 min at 4°C. The levels of alanine transaminase (ALT) and aspartate transaminase (AST) in the serum were detected using commercial kits (ALT, cat. no. C009-2; AST, cat. no. C010-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's protocol. The hydroxyproline level in liver tissues was measured using a hydroxyproline assay kit (cat. no. A030-2; Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from liver tissues was extracted using the China TRIpure Total RNA Extraction kit (BioTeke Corporation, Beijing, China). cDNA was synthesized using Super M-MLV Reverse Transcriptase (BioTeke Corporation) according to the manufacturer's protocol and the temperature protocol was: 25°C for 10 min, 42°C for 50 min and 80°C for 10 min. The primer sequences used are presented in Table I. RT-qPCR reactions were performed on an Exicycler™ 96 (Bioneer Corporation, Daejeon, Korea) with the 2X Power Taq PCR Master Mix (BioTeke Corporation) and SYBR Green (Beijing Solarbio Science & Technology Co., Ltd.), according to the manufacturer's protocol. The thermocycling conditions were 94°C for 5 min, 94°C for 10 sec, 60°C for 20 sec, 72°C for 30 sec, 40 cycles; 72°C for 2.5 min; 40°C for 1.5 min; melting at 60-94°C, every 1°C per second; incubation at 25°C for 2 min. The relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Western blot analysis. Total protein was isolated from liver tissues using radioimmunoprecipitation assay solution (Beyotime Institute of Biotechnology). Protein quantitation was conducted using the enhanced Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Subsequently, 40 μg protein was separated by SDS-PAGE on 10-15% gels and electrotransferred onto polyvinylidene difluoride
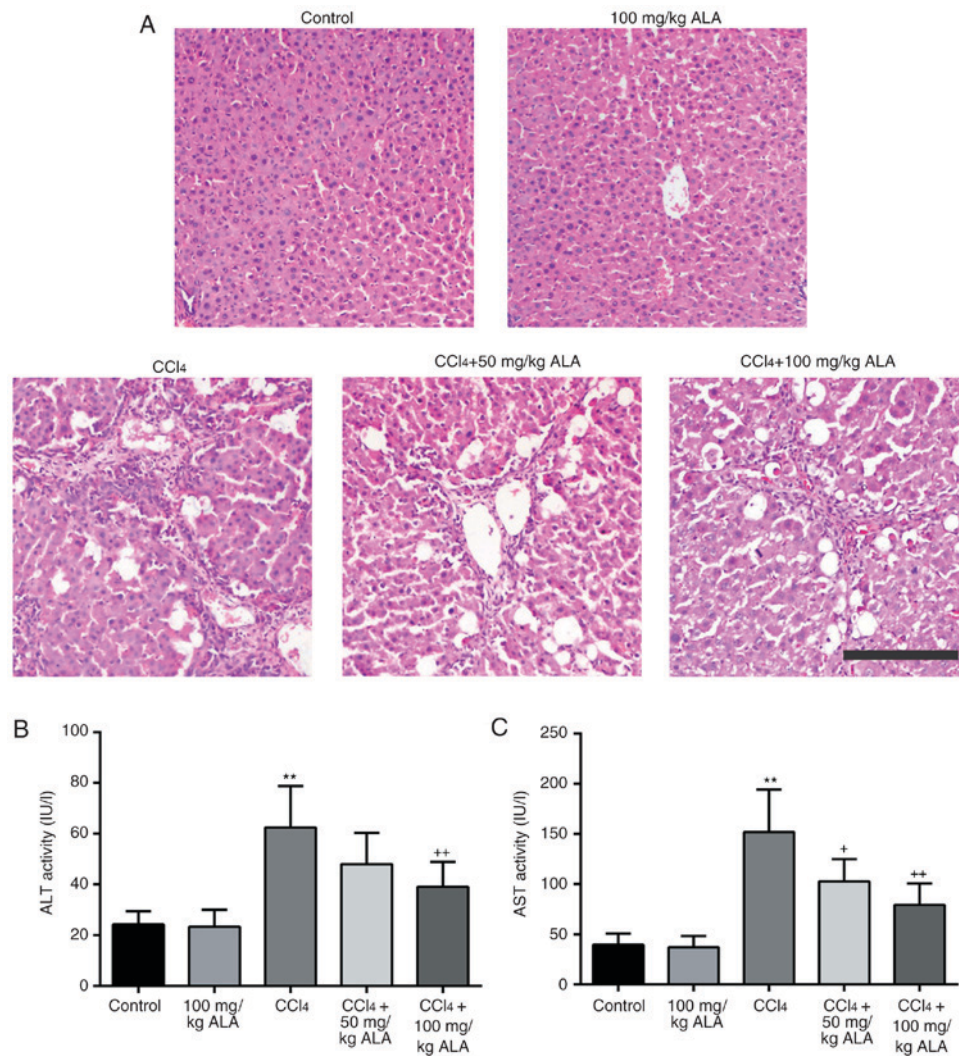
Figure 1. Protective effects of ALA on CCl₄-induced liver cirrhosis. (A) Pathological alterations were detected by hematoxylin and eosin staining. Representative images from six independent experiments are presented. Serum (B) ALT and (C) AST activities were determined using commercial kits. Data are expressed as the mean ± standard deviation of six independent experiments. Scale bar, 200 μm. **P<0.01 vs. the control group; $^+$P<0.05, $^{++}$P<0.01 vs. the CCl₄-treated group. ALA, α-lipoic acid; CCl₄, carbon tetrachloride; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

membranes (EMD Millipore, Bedford, MA, USA). Subsequent to blocking with 5% nonfat milk at room temperature for 1 h, the membranes were incubated with specific primary antibodies against α-SMA (1:1,000; cat. no. 55135-1-AP; Wuhan Sanying Biotechnology), TGF-β (1:500; cat. no. 21898-1-AP; Wuhan Sanying Biotechnology), phosphorylated (p)-Smad3 (1:1,000; cat. no. 9520; Cell Signaling Technology, Inc., Danvers, MA, USA), Smad3 (1:1,000; cat. no. 9523; Cell Signaling Technology, Inc.,), Beclin-1 (1:1,500; cat. no. 11306-1-AP; Wuhan Sanying Biotechnology), light chain (LC)3II/LC3I (1:1,000; cat. no. 2775; Cell Signaling Technology, Inc.), p62 (1:1,000; cat. no. 39749; Cell Signaling Technology, Inc.), protein kinase B (AKT; 1:2,000; cat. no. 9272; Cell Signaling Technology, Inc.), p-AKT (1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.), mammalian target of rapamycin (mTOR; 1:1,000; cat. no. 2972; Cell Signaling Technology, Inc.), p-mTOR (1:1,000; cat. no. 2971; Cell Signaling Technology, Inc.) or β-actin (1:500; cat. no. bsm-33139M; BIOSS, Beijing, China) at 4℃ overnight. The membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:5,000; HRP-conjugated goat anti-rabbit

antibody, cat. no. A0208; HRP-conjugated goat anti-mouse antibody, cat. no. A0216; Beyotime Institute of Biotechnology) at 37℃ for 45 min. Finally, the specific proteins were detected using the Beyo Enhanced Chemiluminescent kit (Beyotime Institute of Biotechnology). The relative protein expression level was detected by densitometry using Gel-Pro Analyzer Version 3.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are presented as the mean ± standard deviation of six independent experiments. Differences between groups were analyzed using one-way analysis of variance, followed by the Bonferroni post hoc test using Image-pro plus 6.0 software (Media Cybernetics, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

ALA alleviates CCl₄-induced liver injury. Histopathological alterations in the liver following treatment with CCl₄ and different doses of ALA were examined by H&E staining (Fig. 1). The livers of healthy and 100 mg/kg ALA-treated
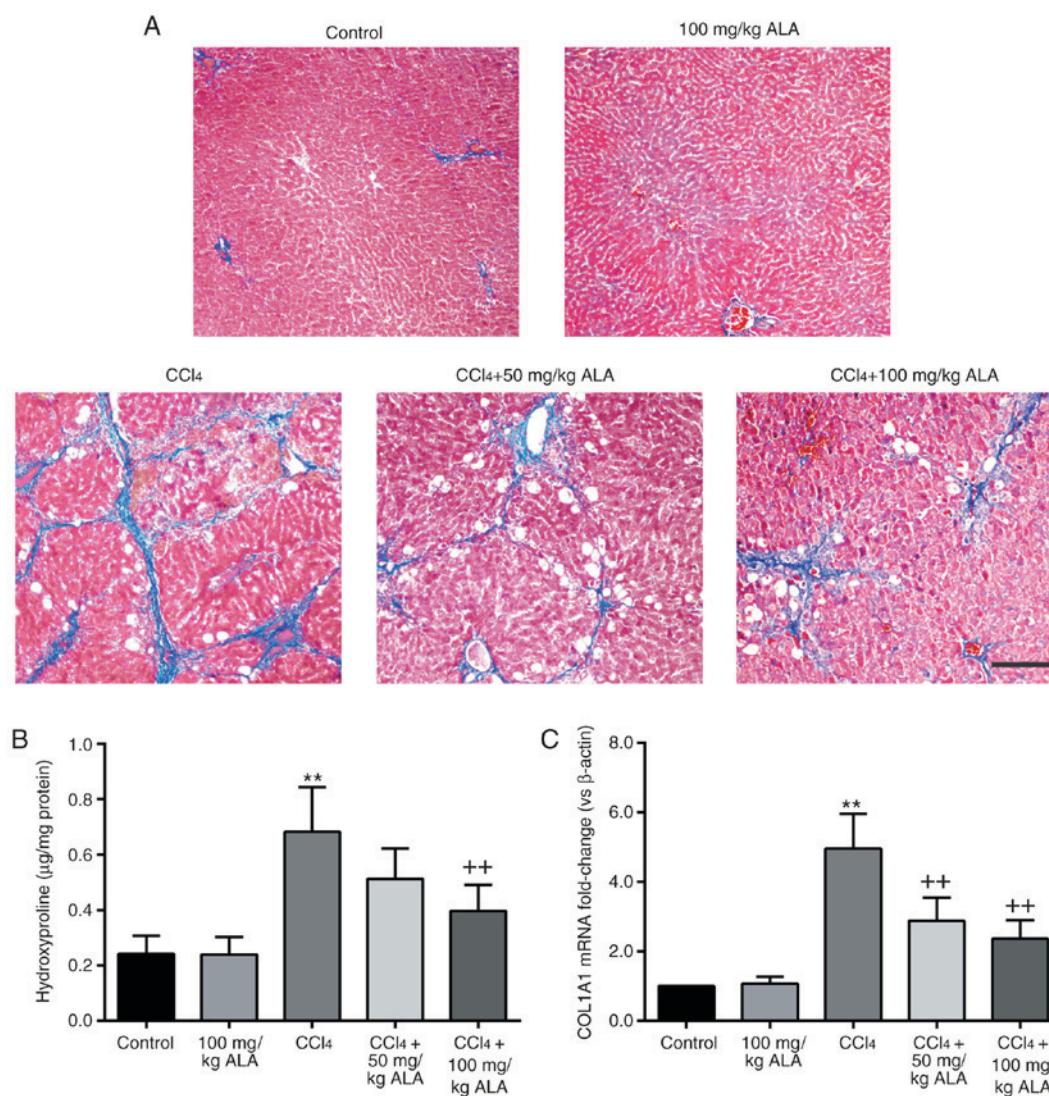
Figure 2. Treatment with ALA attenuates CCl$_4$-induced collagen deposition in the liver. (A) Collagen was detected by Masson's trichrome staining. Representative images from six independent experiments are presented. (B) Liver hydroxyproline level was detected using a commercial kit. (C) Expression of COL1A1 was examined by reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean ± standard deviation of six independent experiments. Scale bar, 200 μm. **P<0.01 vs. the control group; ++P<0.01 vs. the CCl$_4$-treated group. ALA, α-lipoic acid; CCl$_4$, carbon tetrachloride.

rats exhibited normal liver architecture. Treatment with CCl$_4$ resulted in visible lesions with the formation of fibrotic septa, the congestion of cytoplasmic vacuolation and hepatocyte necrosis. However, these histopathological alterations induced by CCl$_4$ were alleviated in the liver of 50- or 100 mg/ml-ALA treated rats. To further examine liver function, the activity of two key enzymes, ALT and AST, in the serum were determined. As presented in Fig. 1B and C, the activity of ALT and AST in CCl$_4$-treated rats were significantly increased 2.58- and 3.83-fold, respectively, compared with the control rats (P<0.01). By contrast, the activity of ALT and AST was reduced in a dose-dependent manner following treatment with ALA. These results suggested that ALA attenuated CCl$_4$-induced liver injury.

ALA attenuates CCl$_4$-induced hepatic fibrosis. The collagen deposition of livers was determined using Masson's trichrome staining (Fig. 2A). Liver tissues from normal rats and 100 mg/kg ALA-treated rats demonstrated little collagen

deposition, whereas those from CCl$_4$-treated rats demonstrated dense bundles of collagen fibers around lobules with disordered liver structure. The liver of CCl$_4$-exposed rats treated with 50 or 100 mg/ml demonstrated decreased collagen deposition. Furthermore, the marker of collagen deposition, hydroxyproline, was additionally significantly increased in the liver of CCl$_4$-treated rats and decreased in ALA-treated liver-cirrhosis-rats (P<0.01; Fig. 2B). Consistent with these alterations, the mRNA expression level of collagen α-1(I) chain (COL1A1) was significantly upregulated in the liver of rats exposed to CCl$_4$ and significantly reduced following treatment with 50 or 100 mg/ml ALA compared with the CCl$_4$ group (P<0.01; Fig. 2C). These data indicated that CCl$_4$-induced severe fibrosis was improved by treatment with ALA.

ALA suppresses CCl$_4$-induced activation of HSCs. The marker of activated HSCs, α-SMA, was detected for the purpose of evaluating the effect of ALA on HSCs. Immunohistochemical
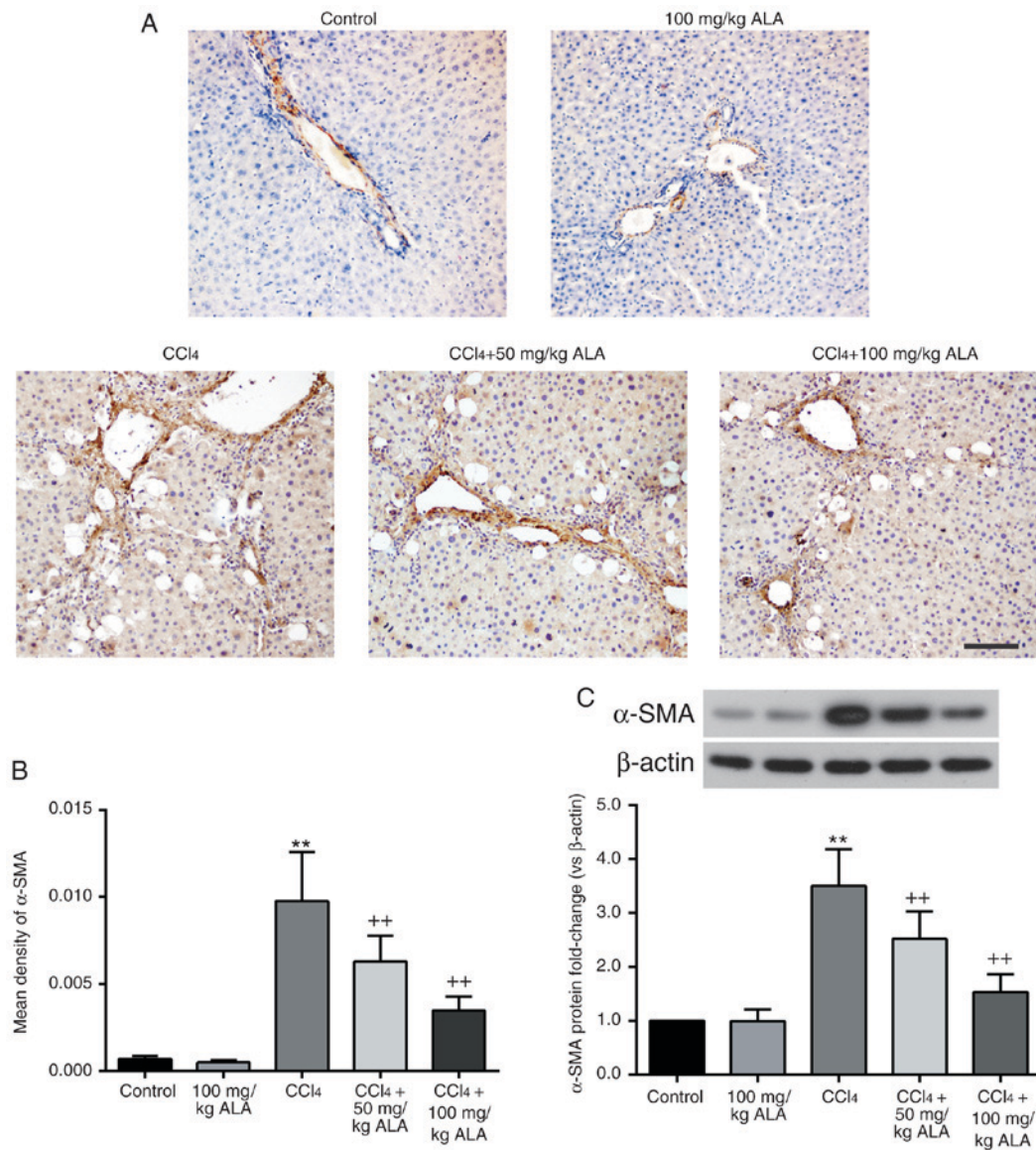
Figure 3. Treatment with ALA suppresses the CCl$_4$-induced activation of hepatic stellate cells. Expression of α-SMA was detected by (A) immunohisto-chemical staining (B) that was quantitatively analyzed and (C) western blotting. Representative images from six independent experiments are presented. Scale bar, 100 μm. Data are expressed as the mean ± standard deviation of six independent experiments. **P<0.01 vs. the control group; ++P<0.01 vs. the CCl$_4$-treated group. ALA, α-lipoic acid; CCl$_4$, carbon tetrachloride; α-SMA, α-smooth muscle actin.

staining results demonstrated that the expression level of α-SMA was significantly increased in the liver of CCl$_4$-treated rats and was significantly reduced following treatment with ALA (P<0.01; Fig. 3A and B). These alterations in the α-SMA level were additionally confirmed by western blot analysis (Fig. 3C). Therefore, the administration of ALA was demonstrated to result in a decrease in the degree of HSC activation in the liver of CCl$_4$-treated rats.

ALA inhibits CCl$_4$-induced activation of the TGF-β/Smad3 pathway. To investigate the mechanism behind the hepato-protective effect of ALA, the activation of the TGF-β/Smad3 pathway, an important mediator of liver fibrosis, was detected (22). As presented in Fig. 4A, immunohisto-chemical analysis demonstrated an increased TGF-β level in the liver tissue of CCl$_4$-treated rats, when compared with that in the liver tissue of the control rats, while the elevation

of TGF-β was decreased by ALA. These alterations in the TGF-β levels were additionally confirmed by RT-qPCR and western blot analysis (Fig. 4B and C). Consistent with the results of TGF-β, the protein level of p-Smad3 in the liver was significantly elevated by CCl$_4$ (P<0.01) and decreased by ALA. However, the level of Smad3 remained unaltered (Fig. 4D). These results demonstrated that ALA inhibited the activation of the TGF-β/Smad3 pathway in the liver of CCl$_4$-treated rats.

ALA suppresses CCl$_4$-induced autophagy in the liver. Autophagy has been implicated in CCl$_4$-induced liver cirrhosis (18) and thus, the expression of autophagy-associated factors was examined in the present study. RT-qPCR and western blot analysis demonstrated that the mammalian autophagy protein, Beclin-1, was significantly increased in the liver of CCl$_4$-treated rats (P<0.01), and was decreased
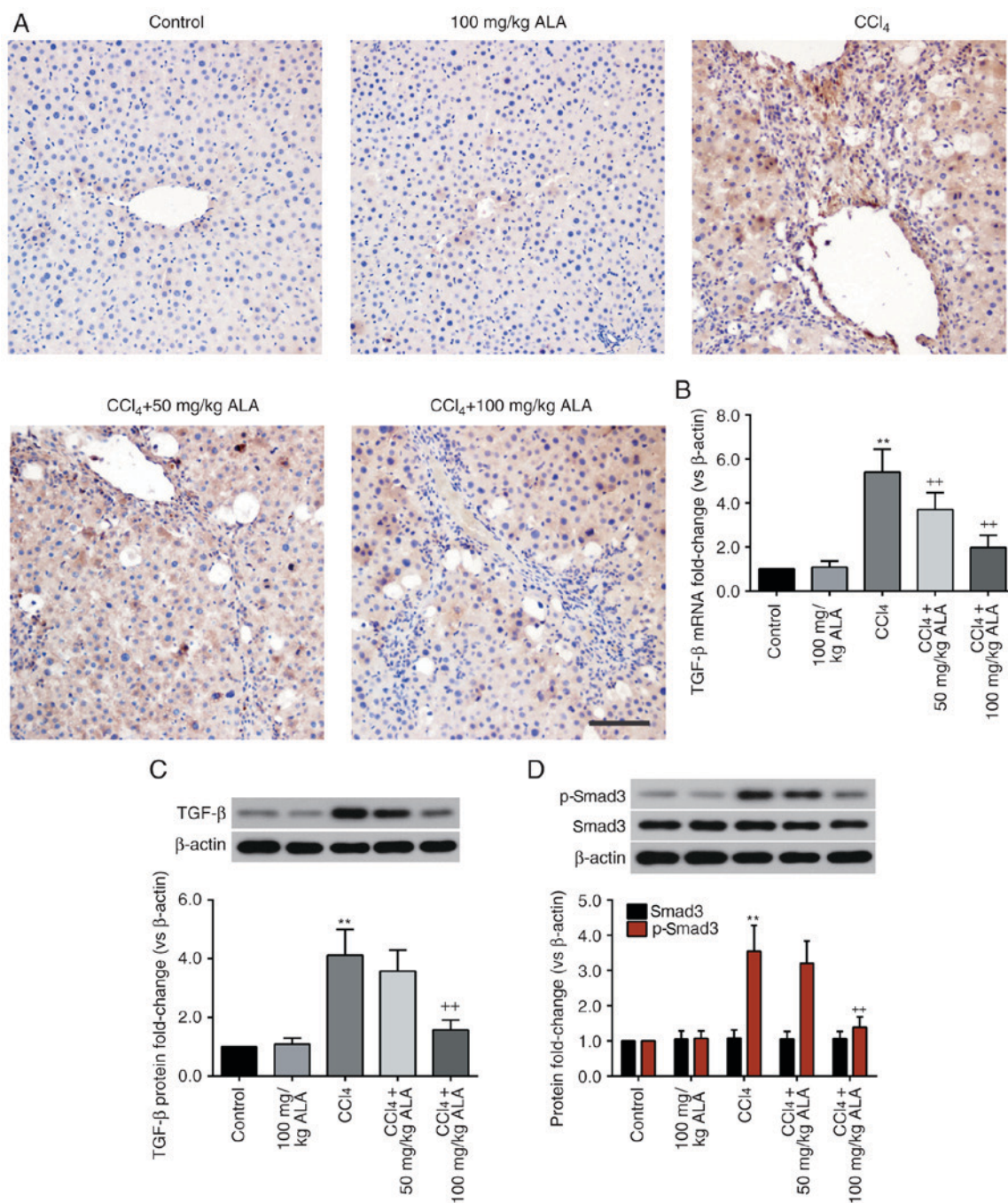
Figure 4. Treatment with ALA inhibits CCl$_4$-induced activation of TGF-β/Smad3 pathway in the liver. The expression of TGF-β was detected by (A) immunohistochemical staining, (B) reverse transcription quantitative polymerase chain reaction and (C) western blot analysis. (D) Expression of p-Smad3 and Smad3 were determined by western blotting. Representative images from six independent experiments are presented. Scale bar, 100 µm. Data are expressed as the mean ± standard deviation of six independent experiments. **P<0.01 vs. the control group; $^{++}$P<0.01 vs. the CCl$_4$-treated group. ALA, α-lipoic acid; CCl$_4$, carbon tetrachloride; p-Smad, phosphorylated mothers against decapentaplegic homolog 9; TGF-β, transforming growth factor-β.

by ALA in a dose-dependent manner (Fig. 5A). In addition, the marker of autophagosome formation, LC3II, which was significantly upregulated following treatment with CCl$_4$, was additionally significantly reduced following treatment with ALA (P<0.01; Fig. 5B). Furthermore, the expression of the autophagy substrate p62 was significantly decreased following CCl$_4$ treatment (P<0.01) and increased following the administration of ALA, in a dose-dependent manner (Fig. 5C). In combination, these results suggested that treatment with ALA reversed CCl$_4$-induced hepatocyte autophagy.

ALA activates the AKT/mTOR pathway in the liver of CCl$_4$-treated rats. The upstream signaling pathway of autophagy, the AKT/mTOR pathway, was further examined (23). As expected, the p-AKT and p-mTOR expression levels were significantly reduced in the liver of CCl$_4$-treated rats (P<0.01), and alterations were prominently restored by the administration of ALA (Fig. 6). These results indicated that ALA was able to activate the AKT/mTOR pathway in the liver, which had previously been repressed by treatment with CCl$_4$.
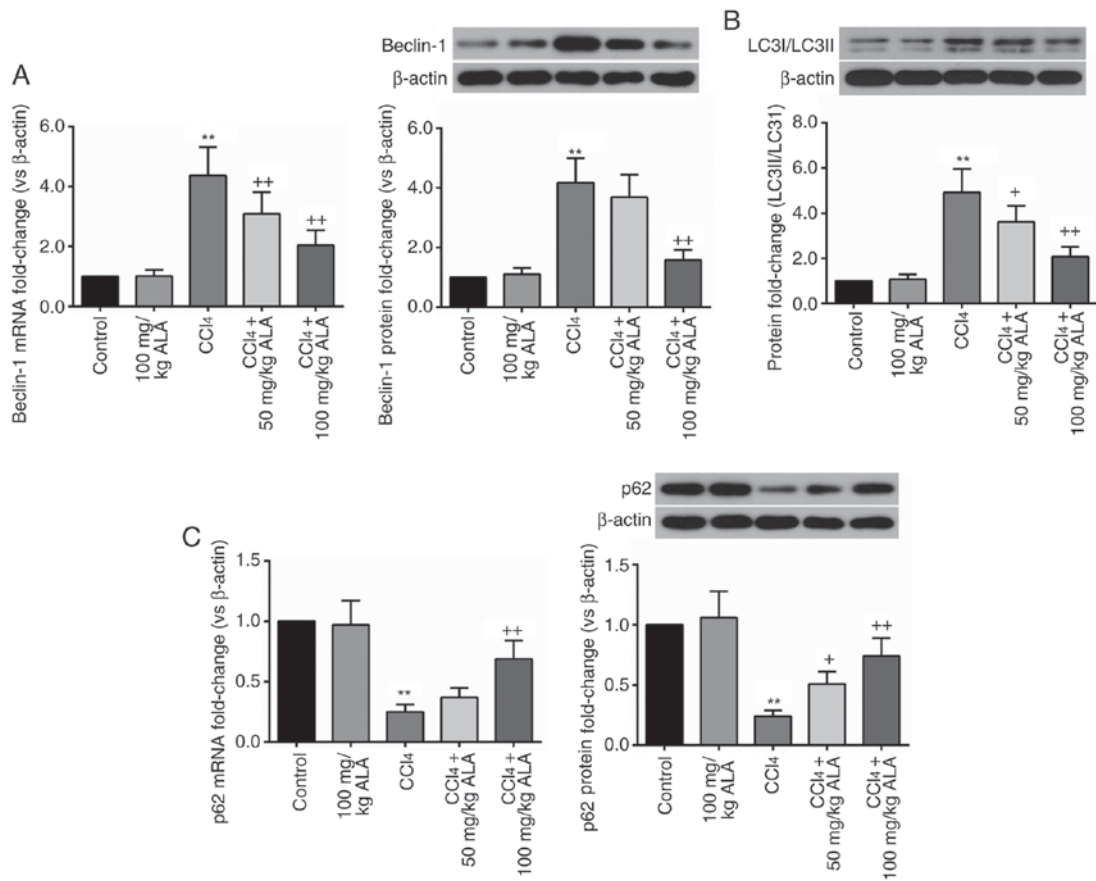
Figure 5. Treatment with ALA inhibits CCl$_4$-induced autophagy in the liver. Expression of (A) Beclin-1, (B) LC3II and (C) p62 were examined by reverse transcription-quantitative polymerase chain reaction or western blot analysis. Representative protein bands from six individual experiments are presented. Data are expressed as the mean ± standard deviation of six independent experiments. **P<0.01 vs. the control group; +P<0.05 and ++P<0.01 vs. the CCl$_4$-treated group. ALA, α-lipoic acid; CCl$_4$, carbon tetrachloride; LC3, light chain 3.
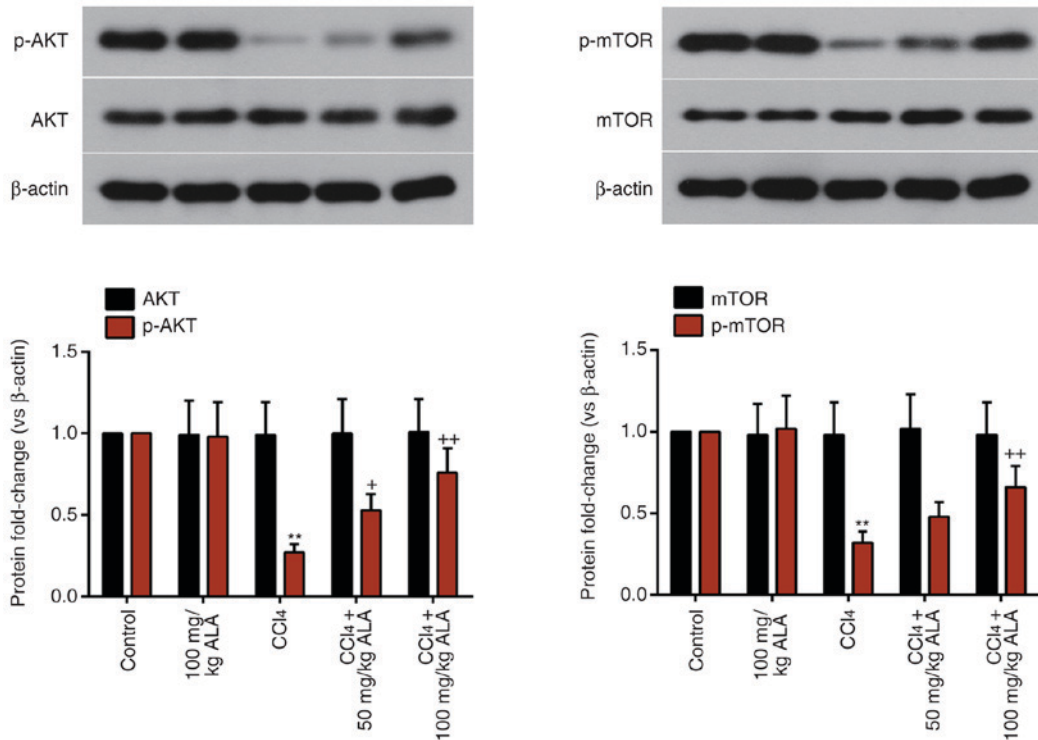

Figure 6. ALA suppresses the AKT/mTOR pathway in the liver of CCl$_4$-treated rats. Expression of p-AKT, AKT, p-mTOR and mTOR were detected by western blot analysis. Data are expressed as the mean ± standard deviation of six independent experiments. **P<0.01 vs. the control group; +P<0.05 and ++P<0.01 vs. the CCl$_4$-treated group. p, phosphorylated; mTOR, mammalian target of rapamycin; ALA, α-lipoic acid; CCl$_4$, carbon tetrachloride; AKT, protein kinase B.

Discussion

The results of the present study demonstrated that treatment with ALA markedly alleviated CCl_4-induced liver cirrhosis, which became evident through positive histopathological alterations, reduced liver fibrosis and decreased ALT, AST and HSCs activity. Furthermore, it was demonstrated that ALA inhibited the activation of the TGF-β/Smad3 pathway in the liver of CCl_4-treated rats. CCl_4-induced hepatocellular autophagy was suppressed by ALA. The administration of ALA additionally promoted the activation of the AKT/mTOR pathway in the liver of CCl_4-treated rats. These results suggested that the inhibition of autophagy through the regulation of the AKT/mTOR pathway and the repression of the TGF-β/Smad3 pathway may be a mechanism potentially responsible for the protective effect of ALA against CCl_4-induced liver cirrhosis.

Fibrosis is the final stage of chronic liver injury, characterized by excessive extracellular matrix (ECM) deposition, particularly that of the fibrous protein collagen I. The excessive ECM may destroy normal liver architecture and cause hepatic dysfunction (24). TGF-β is the most powerful cytokine triggering fibrosis-associated signaling in the liver. TGF-β initiates intracellular signaling through the phosphorylation of Smad2/3, which serve as transcription factors and mediate the transcription of COL1A1 (25). Previous studies identified abnormally elevated TGF-β and p-Smad3 expression levels in chronic fibrotic liver disease (26-28). Accordingly, interfering with the TGF-β/Smad3 pathway results in reduced collagen I and ECM production in livers that have been exposed to CCl_4 (27,29,30). Min *et al* (31) identified that ALA disrupted the TGF-β/Smad3 pathway in the bile duct ligation-induced hepatic fibrosis mouse model. ALA additionally decreased the TGF-β expression level in the liver that had been exposed to thioacetamide (32). Similar results were obtained in the present study; the administration of ALA suppressed the TGF-β/Smad3 pathway in the liver of CCl_4-treated rats. In addition, CCl_4-induced collagen deposition and pathological liver injuries were improved by ALA. These results indicated that ALA was able to protect the liver against cirrhosis through the regulation of the TGF-β/Smad3 pathway.

Insulin plus ALA reduced the mRNA expression level of TGF-β in peripheral blood mononuclear cells of patients with type 1 diabetes, compared with the patients in the insulin treatment group (33). Administration of ALA attenuates glomerular injury in diabetes mellitus by the reduction of nephrogenous TGF-β (34,35). Although the inhibitory effects of ALA on TGF-β expression have been widely reported, the precise mechanisms by which ALA regulate the TGF-β signaling are still not fully understood. Min *et al* (31) demonstrated that ALA was able to suppress the activity of Smad3 and reduce the binding activity of AP-1 and Sp1, which are downstream mediators of TGF-β. Therefore, it was hypothesized that ALA may decrease the TGF-β expression level through negative feedback regulation. Considering that ALA additionally reduced the mRNA expression level of TGF-β (32), the modulation appears to occur on the transcriptional level.

Under normal circumstances, there is a basal level of autophagy in approximately all cell types; however, when cells were threatened by cellar stress (including cytotoxins and hypoxia), autophagy was rapidly enhanced to provide intracellular nutrients and energy to maintain cell survival (36,37). In the liver, intensive autophagy may drive the activation of HSCs (6,7), which serve a critical role in liver fibrosis. Blocking autophagy was able to inhibit HSC activation and fibrogenesis in cultured cells. Furthermore, the knockout of the HSC-specific Atg7 attenuated CCl_4/thioacetamide-induced liver fibrosis in mice (6). When autophagy is activated, LC3 transforms from soluble LC3 I to lipid-bound LC3 II, indicating the formation of autophagosomes (38). Beclin-1 is another autophagy marker involved in the formation and maturation of autophagosomes. p62 is a scaffolding protein that may be degraded by autophagy. In the present study, treatment with ALA suppressed CCl_4-induced autophagy flow, as demonstrated by the decreased Beclin-1 and LC3II/LC3I and increased p62 expression. Therefore, the hepatoprotective effect of ALA appears to be associated with the inhibition of autophagy. The autophagy-inhibitory effect of ALA was additionally reported in 3T3-L1 pre-adipocytes (14), Parkinson's cellular models (39) and hypoxia/reoxygenation-treated H9c2 cells (15).

mTOR, which consists of the two complexes mTORC1 and mTORC2, is known to be a principal negative regulator of autophagy (40). mTORC1 is sensitive to rapamycin and serves as a pivotal checkpoint in modulating the balance between cell growth and autophagy. The PI3K/AKT pathway, which is involved in cell survival, is an upstream modulator of mTORC1 (41). Morales-Ruiz *et al* (42) suggested that the activation of the PI3K/AKT pathway was disturbed in the livers that had been exposed to CCl_4. In accordance with previous results, it was demonstrated in the present study that the p-AKT expression level in the liver of CCl_4-treated rats was reduced compared with the control rats. It was previously demonstrated that ALA was able to protect nerve cells from H_2O_2-induced cell death through the activation of the PI3K/AKT/mTOR pathway (43). Pre- or post-treatment with ALA was demonstrated to upregulate p-AKT and p-mTOR in ischemia/reperfusion-injured cerebral endothelial cells (44). Herein, treatment with ALA reversed the CCl_4-induced inhibition of the AKT/mTOR pathway in the liver, suggesting that ALA may suppress autophagy through the regulation of the AKT/mTOR pathway.

Liver cirrhosis is frequently accompanied by enhanced oxidative stress that is demonstrated by the increase of lipid peroxidation marker, malondialdehyde (MDA) and the decrease of the most studied antioxidant, glutathione (GSH) (45,46). Previously, accumulating evidence suggested that reactive oxygen species (ROS) are the primary intracellular signal transducer in inducing and sustaining autophagy (47-49). Furthermore, ROS was able to inhibit the activation of the AKT/mTOR pathway in vascular smooth muscle cells (50) and human hepatoma cells (51). ALA was originally used as an antioxidant, treatment with ALA significantly restored GSH and antioxidant capacity levels in liver fibrosis rat models (52). Supplementation with ALA additionally reduced MDA level, total oxidant status and lipid peroxides concentration in liver homogenates from high-fat diet rats (53). Therefore, as Rudich *et al* (54) suggested, ALA may activate the AKT/mTOR pathway and inhibit autophagy by the suppression of oxidative stress.

In conclusion, the present study demonstrated that ALA effectively attenuates liver cirrhosis in CCl$_4$-poisoned rats. Additionally, ALA administration suppressed CCl$_4$-induced autophagy in the liver. CCl$_4$-induced dysregulation of the TGF-β/Smad3 and AKT/mTOR pathways was restored by ALA. These data suggested that ALA may alleviate liver cirrhosis through the inhibition of the TGF-β/Smad3 pathway and autophagy.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GL designed the present study, performed the animal experiments, analyzed the data and wrote the manuscript. JL and LP performed the animal experiments, the physiological test and the pathological experiments. SG analyzed the data, organized the images and improved the language. BL performed the molecular and protein detection. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal raising and handling procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Experimental Ethics Committee of the Henan University of Chinese Medicine (Zhengzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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