Oroxylin A inhibits ethanol-induced hepatocyte senescence via YAP pathway

Huanhuan Jin¹ | Naqi Lian¹ | Mianli Bian¹ | Chenxi Zhang¹ | Xingran Chen¹ | Jiangjuan Shao^{2,3} | Li Wu¹ | Anping Chen⁴ | Qinglong Guo⁵ | Feng Zhang^{1,2,3} | Shizhong Zheng^{1,2,3}

¹Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, Nanjing University of Chinese Medicine, Nanjing, China

²Jiangsu Key Laboratory of Therapeutic Material of Chinese Medicine, Nanjing University of Chinese Medicine, Nanjing, China

³State Key Laboratory Cultivation Base for TCM Quality and Efficacy, Nanjing University of Chinese Medicine, Nanjing, China

⁴Department of Pathology, School of Medicine, Saint Louis University, St Louis, MO, USA

⁵Jiangsu Key Laboratory of Carcinogenesis and Intervention, China Pharmaceutical University, Nanjing, China

Correspondence

Shizhong Zheng, Department of Pharmacology, School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China.
Email: nytws@163.com

Funding information

the National Natural Science Foundation of China, Grant/Award Number: 31571455, 31401210, 31600653, 81600483 and 81270514; the Natural Science Foundation of Jiangsu Province, Grant/Award Number: BK20140955; the Open Project Program of Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, Grant/Award Number: JKLPS 201502; the Project of the Priority Academic Program Development of Jiangsu Higher Education Institutions, Grant/Award Number: PAPD

Abstract

Objectives: Oroxylin A, a natural flavonoid isolated from *Scutellaria baicalensis*, has been reported to have anti-hepatic injury effects. However, the effects of oroxylin A on alcoholic liver disease (ALD) remains unclear. The aim of this study was to elucidate the effects of oroxylin A on ALD and the potential mechanisms.

Materials and methods: Male ICR mice and human hepatocyte cell line LO₂ were used. Yes-associated protein (YAP) overexpression and knockdown were achieved using plasmid and siRNA technique. Cellular senescence was assessed by analyses of the senescence-associated β -galactosidase (SA- β -gal), senescence marker p16, p21, Hmga1, cell cycle and telomerase activity.

Results: Oroxylin A alleviated ethanol-induced hepatocyte damage by suppressing activities of supernatant marker enzymes. We found that oroxylin A inhibited ethanol-induced hepatocyte senescence by decreasing the number of SA- β -gal-positive LO₂ cells and reducing the expression of senescence markers p16, p21 and Hmga1 in vitro. Moreover, oroxylin A affected the cell cycle and telomerase activity. Of importance, we revealed that YAP pharmacological inhibitor verteporfin or YAP siRNA eliminated the effect of oroxylin A on ethanol-induced hepatocyte senescence in vitro, and this was further supported by the evidence in vivo experiments.

Conclusion: Therefore, these aggregated data suggested that oroxylin A relieved alcoholic liver injury possibly by inhibiting the senescence of hepatocyte, which was dependent on its activation of YAP in hepatocytes.

Abbreviations: ALD, alcoholic liver disease; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FBS, foetal bovine serum; GAPDH, glyceraldehyde phosphate dehydrogenase; γ -H2AX, phospho-histone H2AX, gamma-H2AX; H&E, haematoxylin and eosin; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; SA- β -gal, senescence-associated β -galactosidase; TERT, telomerase reverse transcriptase; YAP, Yes-associated protein.

1 | INTRODUCTION

Alcoholic liver disease (ALD) is a major health problem in both developed and developing countries. Considerable evidence suggests that ALD is associated with liver injury such as fibrosis, cirrhosis and

hepatocellular cancer in humans. It is demonstrated that cellular senescence happened in diverse liver disorders and in a range of cell types, including hepatocytes,¹⁻³ hepatic stellate cells^{4,5} and cholangiocytes.⁶ Recent studies proved that senescence of hepatocytes is linked to fibrosis stage and disease progression.^{7,8} Elimination of senescent hepatocyte may be a novel therapeutic strategy to reduce steatosis in non-alcoholic fatty liver disease.⁹ However, the effects of cellular senescence on ALD progression and potential mechanisms remain unclear.

Cellular senescence is defined as an anti-proliferative programme that leads to the stable form of cell cycle arrest in the cell.¹⁰ Senescent cells is typically characterized by telomere shortening or telomerase system dysfunction,^{11,12} activation of senescence-associated β -galactosidase (SA- β -gal) and accumulation of DNA damage¹³ that distinguishes them from most quiescent cells. Besides, the senescence of cell mediated by interplay between multiple pathways, most notably, the p16/pRb and p53/p21 signal pathways.^{14,15} The Hippo/YAP signalling pathway has been well established as a growth-suppressive pathway. Hippo pathway kinases inhibited the downstream effector Yes-associated protein (YAP) by promoting its cytoplasmic localization in a S127 phosphorylation-dependent manner. The activation of YAP stimulates genes expression of the cell fate acts mainly through TEAD family transcription factors.^{16,17} Previous studies have discovered that YAP plays an important role in the regulation of cellular senescence, except in the regulation of cell proliferation and apoptosis.^{18,19}

Oroxylin A (5, 7-dihydroxy-6-methoxyflavone, $C_{16}H_{12}O_5$), the active component of *Scutellaria baicalensis*, is one of the most important medicinal herbs in traditional Chinese medicine. Oroxylin A has been reported to have antioxidant,²⁰ anti-inflammation,²¹ anti-cancer,²² antiviral and anti-bacterial infections.²³ Although oroxylin A has been reported to have anti-hepatic injury effects, the hepatoprotective role of oroxylin A in alcoholic liver injury remains unclear. The purpose of this study was to investigate the effects of oroxylin A on alcoholic liver injury and the senescence of hepatocyte.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

The following compounds were used in this study: oroxylin A was generously presented by Qinglong Guo (China Pharmaceutical University, Nanjing, China), and verteporfin was obtained from Cayman Chemical (Ann Arbor, MI, USA). They were dissolved in dimethylsulfoxide (DMSO; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for the vitro experiments. Treatment with DMSO alone was used as vehicle control in vitro experiments throughout the current study. The primary antibodies were used in this study: p16, γ -H2AX, YAP and β -actin (Cell Signaling Technology, Danvers, MA, USA); p21, TERT, TRF1, TRF2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Hmga1 (Abcam Technology, Abcam, Cambridge, UK).

2.2 | Experimental animal procedures

All experimental procedures were approved by the institutional and local committee on the care and use of animals of Nanjing University of Chinese Medicine (Nanjing, China), and all animals received humane care according to the National Institutes of Health (USA) guidelines. Male ICR mice (20-25 g body weight) were obtained from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China). Animals were housed in animal facilities in standardized conditions ($20 \pm 2^\circ\text{C}$ room temperature, $40 \pm 5\%$ relative humidity and a 12 hours light/dark cycle with dawn/dusk effect). Water and standard pathogen-free chow diet were provided ad libitum. After adaptive feed for a week, they were randomly divided into 4 groups ($n = 10$) and given administration of vehicle, ethanol (i.g., 56% v/v, 5 mL/kg, once per 2 days), ethanol+oroxylin A (i.p., 30 mg/kg, once per 2 days), ethanol+oroxylin A+verteporfin (i.v., 50 mg/kg, once a week) respectively. Mice in groups 2-4 were administrated with ethanol for 8 weeks. During the last 4 weeks, mice in groups 3 and 4 were administrated with corresponding dose of oroxylin A and verteporfin. At the end of the experiment, all mice were intraperitoneally injected with pentobarbital (50 mg/kg) for anaesthesia at the end of the experiment. Next, mice's blood was collected from eye socket vein clump for biochemical analyses. Liver were fixed in 4% buffered paraformaldehyde for histological analysis and immunostaining analysis.

2.3 | Isolation and culture of hepatocytes

Human hepatocyte cell line LO₂ (Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium with 10% foetal bovine serum, 1% antibiotics and grown in a 5% CO₂ atmosphere at 37°C.

2.4 | Cell transfection with YAP plasmid or YAP siRNA

YAP CRISPR Activation Plasmid (h) (sc-400040-ACT; Santa Cruz Biotechnology) of 4 μg was added to 200 μL medium without serum and antibiotic and incubated at room temperature for 5 minutes. Lipofectamine[®] 2000 reagent (Life Technologies, Grand Island, NY, USA) of 8 μL was added to 200 μL medium without serum and antibiotics and incubated at room temperature for 5 minutes. The above 2 solutions were mixed well at room temperature for 20 minutes and about 400 μL transfection complex was obtained. Cells were incubated with the transfection complex solution at 37°C for 6 hours, and then were re-incubated in complete medium at 37°C for an additional 18 hours. Control CRISPR Activation Plasmid (Santa Cruz Biotechnology; sc-437275) is provided as a negative control for CRISPR Activation Plasmid.

YAP siRNA (Santa Cruz Biotechnology; sc-38637) of 100 pmol was added to 200 μL medium without serum and antibiotic and incubated at room temperature for 5 minutes. Lipofectamine[®] 2000 reagent (Life Technologies) of 8 μL was added to 200 μL medium without serum and antibiotics and incubated at room temperature for 5 minutes. The

above 2 solutions were mixed well at room temperature for 20 minutes and about 400 μ L transfection complex was obtained. Cells were incubated with the transfection complex solution at 37°C for 6 hours, and then were re-incubated in complete medium at 37°C for an additional 18 hours. Control siRNA (Santa Cruz Biotechnology; sc-37007) is a non-targeting 20-25 nt siRNA designed as a negative control.

2.5 | Immunofluorescence staining

Immunofluorescence staining with liver tissues or treated cells were performed as we previously reported.²⁴ 4',6-Diamidino-2-phenylindole was used to stain the nucleus of cells in liver tissues and LO₂ in vitro.

2.6 | Analysis of LO₂ cell senescence

SA- β -gal staining kit was used to determine the senescence of LO₂ cell by the detection of SA- β -gal activity. Briefly, adherent cells were fixed with 0.5% glutaraldehyde in PBS for 15 minutes, washed with PBS containing 1 mmol/L MgCl₂, and stained overnight in PBS containing 1 mmol/L MgCl₂, 1 mg/mL X-Gal and 5 mmol/L potassium ferricyanide. Photographs were captured with a light microscope and representative images were shown.

2.7 | Real-time polymerase chain reaction

Trizol reagent (Sigma, Saint Louis, MO, USA) was used for total RNA extraction from treated LO₂ cells. After isolation, 1 μ g of total RNA was transcribed into cDNA by using the superscript reverse transcriptase kit (Vazyme Biotech Co., Ltd, Nanjing, China). The detection of cDNA expression for the specific genes was performed using quantitative polymerase chain reaction (PCR) (Vazyme Biotech Co., Ltd). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the invariant control. The following primers of genes (GenScript, Nanjing, China) are available upon request:

p16: (forward) 5'-GGAGTTAATAGCACCTCTCC-3', (reverse) 5'-TTCAATCGGGGATGTCTGAGG-3';

p21: (forward) 5'-GTCAGTTCCTTGTTGGAGCCG-3', (reverse) 5'-GAAGGTAGAGCTTGGGCAGG-3';

Hmga1: (forward) 5'-AGGAGCAGTGACCCATGCGT-3', (reverse) 5'-TGATGGTGGGCTGGGGAAG-3';

GAPDH: (forward) 5'-CTTCTTTTGCCTCGCCAGCCGA-3', (reverse) 5'-ACCAGGCGCCCAATACGACCAA-3'.

2.8 | Western blot analysis

The whole cell protein extracts were prepared from treated LO₂ cells. Cells were lysed in ice-cold RIPA lysis buffer and subjected to centrifugation (10 000 g) at 4°C for 15 minutes. Then, the supernatants were used for quantification of the total protein concentration by using a BCA Protein Assay Kit (Beyotime Biotechnology, Haimen, China) according to the manufacturer's protocol. Western blot analysis was performed as we described previously.²⁴ Briefly, hepatocyte lysate (50 μ g/well) was separated by SDS-PAGE and the proteins

were transferred onto the nitrocellulose membrane. β -Actin was used as an invariant control for the target proteins. Representative blots were shown.

2.9 | Biochemical analyses

Mice serum and cell supernatant were collected for the detection of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) or lactate dehydrogenase (LDH) by using commercial assay kits according to the protocols from the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.10 | Histological analyses and immunohistochemistry

Liver tissues fixed in 4% buffered paraformaldehyde were embedded in paraffin and stained with haematoxylin and eosin (H&E). Immunohistochemistry of liver section was performed by using antibody against γ -H2AX and Ki67 according to the previous study.²⁵

2.11 | Statistical analysis

Data were presented as mean $\pm$ SD, and results were analysed using SPSS 16.0 software. Histograms were created using GraphPad Prism 5 software (San Diego, CA, USA). The significance of difference was determined by 1-way ANOVA with the post hoc Dunnett's test. Values of $P < .05$ were considered to be statistically significant.

3 | RESULTS

3.1 | Ethanol induced cellular senescence in human hepatocyte LO₂ cells

To confirm the role of YAP in protecting hepatocyte against ethanol damage, immortalized human hepatocyte LO₂ cells were incubated with 100 mmol/L ethanol for 24 hours to establish an in vitro model of ALD (Figure 1A).²⁶⁻²⁹ LO₂ cells were derived from primary normal human hepatocytes and maintained the biological features and ultrastructures of normal adult hepatocytes. After stable transfection with human telomerase reverse transcriptase (TERT) gene, LO₂ cells were immortalized and widely used as an in vitro model of liver tissues for studying the pathophysiology of hepatocytes.³⁰ Compared with the control group, the activities of ALT, AST and LDH were elevated when LO₂ cells were treated with ethanol (Figure 1B). Previous studies showed that ethanol induced hepatocyte apoptosis,^{31,32} but we also observed the senescence of hepatocyte in ethanol-stimulated LO₂ cells. SA- β -gal staining was used to detect the cellular senescence in ethanol-treated hepatocyte. As shown in Figure 1C, ethanol increased the number of SA- β -gal-positive LO₂ cells. Then, senescence marker p16, p21 and Hmga1 were used to determine the effect of ethanol on cellular senescence by real-time PCR analyses. The results showed that the mRNA levels of senescence marker p16, p21 and Hmga1 were elevated in ethanol-stimulated hepatocytes (Figure 1D). The

FIGURE 1 Ethanol induced cellular senescence in human hepatocyte LO₂ cells. A, Cell count kit-8 analyses of cell viability in LO₂ cells treated with ethanol for the indicated concentration. Data are represented as mean $\pm$ SD. Significance: * P < .05 vs ddH₂O, ** P < .01 vs ddH₂O, *** P < .001 vs ddH₂O. B, Determination of supernatant ALT, AST and LDH levels in LO₂ cells treated with ethanol for 24 h. Data are represented as mean $\pm$ SD. Significance: * P < .05 vs ddH₂O, ** P < .01 vs ddH₂O, *** P < .001 vs ddH₂O. C, Representative images of the β -galactosidase staining of cells under the indicated conditions. Percentages of cells positive for SA- β -gal were scored; data are represented as mean $\pm$ SD. Significance: *** P < .001 vs ddH₂O. D, Real-time PCR analyses of genes relevant to senescence in control or ethanol-treated LO₂ cells, including p16, p21, Hmga1. Data are represented as mean $\pm$ SD. Significance: * P < .05 vs ddH₂O, ** P < .01 vs ddH₂O, *** P < .001 vs ddH₂O

above results showed that ethanol damages hepatocyte and makes them senesce.

3.2 | YAP expression levels correlated with cellular senescence in ethanol-treated hepatocytes

It has been reported that YAP binds to the transcription factor TEAD directly to inhibit cellular senescence.¹⁹ So we investigated the effect of YAP on ethanol-induced cellular senescence in LO₂ cells. Interestingly, the protein level of YAP was significantly down-regulated in ethanol-treated LO₂ cells (Figure 2A). As reported, YAP plays a critical role in cellular senescence. To further investigate the potential role of YAP in hepatocyte senescence, we further overexpression of YAP using the standard protocol for YAP CRISPR Activation Plasmid transfection of hepatocytes, in which western blot analyses indicated that overexpression of YAP was able to inhibit the protein levels of senescence marker p16, p21, Hmga1 and DNA damage marker γ -H2AX (Figure 2B). Furthermore, immunofluorescence staining showed that YAP plasmid abrogated the effects of ethanol on p21 and γ -H2AX (Figure 2C).

To determine how the telomerase activity was affected by YAP plasmid in ethanol-treated hepatocyte, real-time PCR and western blot experiments were performed to exam the expression of TERT. The results revealed the mRNA and protein levels of TERT were elevated by YAP plasmid in ethanol-treated hepatocytes (Figure 2D-E). We also analysed telomere-associated proteins TRF1 and TRF2, which might affect telomerase activity. Previous reports indicated that TRF1

negatively control the maintenance of telomerase-associated telomere length.³³ On the other hand, although TRF2 is associated with stabilizing the telomere structure,^{34,35} TRF2 is also related to telomere shortening.³⁶ Western blot experiment indicated that the expression of TRF1 was elevated by ethanol treatment, but overexpression of YAP could reserve this effect. In addition, TRF2 expression did not change significantly by YAP plasmid in ethanol-treated hepatocyte (Figure 2E). Together, these data displayed that YAP overexpression inhibited the senescence phenotype in ethanol-damaged hepatocyte.

3.3 | Oroxylin A inhibited cellular senescence in ethanol-treated hepatocytes

Oroxylin A, a traditional medicinal herb, has been studied in liver disease, especially in the field related to liver cancer.^{37,38} Cell Counting Kit-8 assay showed that oroxylin A (0-60 μ mol/L) treatment have no effect on cell viability of hepatocyte (Figure 3A). On the other hand, ethanol at 100 mmol/L concentration inhibited hepatocyte viability obviously, and oroxylin A restored cell viability in a dose-dependent manner when the dose was between 20 and 40 μ mol/L (Figure 3B). Additionally, oroxylin A could dose-dependently reduce the activities of supernatant AST, ALT and LDH, which were elevated by ethanol treatment. (Figure 3C). Next, the effect of oroxylin A on cellular senescence in ethanol-treated LO₂ cells were investigated. The results indicated oroxylin A dose-dependently decreased the number of SA- β -gal-positive LO₂ cells (Figure 4A). In addition, although ethanol elevated the protein levels

FIGURE 2 YAP expression levels correlated with cellular senescence in ethanol-treated hepatocytes. A, Western blot analyses of protein expression of YAP in control or ethanol-treated LO₂ cells. All the in vitro experiments were repeated at least 3 times independently. Data are expressed as mean $\pm$ SD. Significance: *** P < .001 vs ddH₂O. B, Western blot analyses of YAP, senescence molecules p16, p21, Hmga1 and DNA damaged molecule γ -H2AX in cells transfected with control plasmid or YAP plasmid. Representative blots were from 3 independent experiments. Data are expressed as mean $\pm$ SD. Significance: *** P < .001 vs control plasmid, ## P < .01 vs control plasmid + ethanol, ### P < .001 vs control plasmid + ethanol. C, Representative images of γ -H2AX and p21 staining by immunofluorescence in plasmid-transfected cells. 4',6-Diamidino-2-phenylindole reagent was used to stain the nucleus. Scale bar, 50 μ m. D, Relative telomerase (TERT) mRNA levels in cells transfected with the indicated plasmids analysed by real-time PCR in control or ethanol-treated cells. Data are represented as mean $\pm$ SD. Significance: *** P < .001 vs control plasmid, # P < .05 vs control plasmid + ethanol. E, Protein levels of telomere-associated protein TERT, TRF1 and TRF2 in cells transfected with the indicated plasmids analysed by Western blot in control or ethanol-treated cells. Representative blots were from 3 independent experiments. Data are expressed as mean $\pm$ SD. Significance: *** P < .001 vs control plasmid, # P < .05 vs control plasmid + ethanol, ### P < .001 vs control plasmid + ethanol

of p16, p21 and γ -H2AX, oroxylin A suppressed the expression of senescence marker in ethanol-stimulated hepatocyte (Figure 4B). Furthermore, flow cytometric analysis showed G0/G1 cell cycle

arrest by ethanol was eliminated by oroxylin A in a dose-dependent manner (Figure 4C). Altogether, oroxylin A could suppress ethanol-induced cellular senescence in vitro.

FIGURE 3 Oroxylin A suppressed ethanol-induced cell damage in LO_2 cells. A,B, Cell count kit-8 analyses of cell viability in LO_2 cells treated with ethanol (100 mmol/L) and/or oroxylin A for the indicated concentration. Data are represented as mean $\pm$ SD. Significance: * $P < .05$ vs DMSO, *** $P < .001$ vs DMSO, # $P < .05$ vs ethanol, ## $P < .01$ vs ethanol, ### $P < .001$ vs ethanol. C, Determination of supernatant ALT, AST, LDH levels in LO_2 cells treated with ethanol and/or oroxylin A. Significance: * $P < .05$ vs DMSO, ** $P < .01$ vs DMSO, # $P < .05$ vs ethanol, ## $P < .01$ vs ethanol

FIGURE 4 Oroxylin A inhibited cellular senescence in ethanol-treated hepatocytes. A, Representative images of the β -galactosidase staining of cells under the indicated conditions. Percentages of cells positive for SA- β -gal were scored; data are represented as mean $\pm$ SD. Significance: *** $P < .001$ vs DMSO, ## $P < .01$ vs ethanol, ### $P < .001$ vs ethanol. B, Western blot analyses of protein expression of p16, p21 and γ -H2AX in LO_2 cells treated with ethanol (100 mmol/L) and/or oroxylin A (10, 20, 40 $\mu\text{mol/L}$). Representative blots were from 3 independent experiments. Data are expressed as mean $\pm$ SD. Significance: ** $P < .01$ vs DMSO, *** $P < .001$ vs DMSO, # $P < .05$ vs ethanol, ## $P < .01$ vs ethanol, ### $P < .001$ vs ethanol. C, Cell cycle analysis by flow cytometry. Percentages of cell cycle distributions were determined. Data are represented as mean $\pm$ SD. Significance: ** $P < .01$ vs DMSO, # $P < .05$ vs ethanol, ## $P < .01$ vs ethanol

FIGURE 5 Activation of YAP is required for oroxylin A to prevent cellular senescence in ethanol-treated hepatocyte. A, Western blot analyses of protein expression of YAP in LO₂ cells treated with ethanol (100 mmol/L) and/or oroxylin A (10, 20, 40 μ mol/L). Representative blots were from 3 independent experiments. Data are expressed as mean $\pm$ SD. Significance: * P < .05 vs DMSO, ## P < .01 vs ethanol. B, Representative images of the β -galactosidase staining of cells under the indicated conditions. Percentages of cells positive for SA- β -gal were scored; data are represented as mean $\pm$ SD. Significance: *** P < .001 vs DMSO, ## P < .01 vs ethanol, \$ P < .05 vs ethanol + oroxylin A. C, Real-time PCR analysis of the mRNA levels of telomerase (TERT) in LO₂ cells treated with ethanol (100 mmol/L), oroxylin A (20 μ mol/L) and/or verteporfin (1 μ mol/L). Data are represented as mean $\pm$ SD. Significance: *** P < .001 vs DMSO, ## P < .01 vs ethanol, \$ P < .05 vs ethanol + oroxylin A. D, Real-time PCR analyses of genes relevant to senescence in LO₂ cells treated with ethanol (100 mmol/L), oroxylin A (20 μ mol/L) and/or verteporfin (1 μ mol/L), including p16, p21, Hmga1. Data are represented as mean $\pm$ SD. Significance: *** P < .001 vs DMSO, ### P < .001 vs ethanol, \$ P < .05 vs ethanol + oroxylin A, \$\$\$ P < .01 vs ethanol + oroxylin A. E, Protein expression of YAP, p16 and p21 in cells transfected with the indicated siRNAs analysed by Western blot in ethanol and/or oroxylin A-treated cells. Representative blots were from 3 independent experiments. Data are expressed as mean $\pm$ SD. Significance: *** P < .001 vs control siRNA, ### P < .001 vs control siRNA + ethanol, \$ P < .05 vs control siRNA + ethanol + oroxylin A, \$\$\$ P < .001 vs control siRNA + ethanol + oroxylin A.

3.4 | Activation of YAP is required for oroxylin A to prevent cellular senescence in ethanol-treated hepatocyte

Furthermore, the inhibition of hepatocyte senescence by oroxylin A via YAP activation was investigated in depth. The data showed that YAP expressions were down-regulated in ethanol-treated LO₂ cells. However, oroxylin A could reserve the protein level of YAP (Figure 5A). To further evaluate the role of YAP in cellular senescence, LO₂ cells were co-treated with or without YAP inhibitor verteporfin in the presence or absence of oroxylin A (20 µmol/L) for 24 hours. Verteporfin can increase the number of SA-β-gal-positive LO₂ cells when compared to the oroxylin A treatment (Figure 5B). Meanwhile, we found that oroxylin A activation of telomerase was suppressed by verteporfin in ethanol-treated hepatocyte (Figure 5C). Besides, verteporfin partially reversed the levels of p16, p21 and Hmga1, which inhibited by oroxylin A in ethanol-treated hepatocyte (Figure 5D). We further knock down YAP using the standard protocol for siRNA transfection of hepatocytes, in which hepatocytes are transfected after 6 hours of in vitro culturing. Consistently, down-regulation of YAP significantly disturbed oroxylin A inhibition of cellular senescence in ethanol-treated hepatocyte (Figure 5E). In sum, these results showed that YAP played a critical role in oroxylin A regulation of cellular senescence in ethanol-treated hepatocyte.

3.5 | YAP inhibition abrogated the protective effect of oroxylin A on alcohol-induced liver injury

Next, we investigated the role of YAP disruption in the effect of oroxylin A on alcohol-induced liver injury in vivo. Our data demonstrated that oroxylin A reduced serum levels of ALT, AST and alkaline phosphatase (ALP) in alcohol-induced liver injury. However, suppression of YAP weakened the effects of oroxylin A on the levels of ALT, AST and ALP (Figure 6A). Disordered hepatic lobule structure and lipid vacuolation were discovered in ethanol-treated liver by H&E staining. However, administration of oroxylin A significantly suppressed these histological changes. Verteporfin-mediated YAP inhibition impaired hepatoprotective effects of oroxylin A (Figure 6B).

3.6 | YAP suppression abolished the repression effect of oroxylin A on alcohol-induced hepatocyte senescence

Additionally, compared with the ethanol treatment group, oroxylin A decreased the number of foci containing γ-H2AX⁺ hepatocytes. However, this influence could be reserved by verteporfin (Figure 6C). At the same time, the expression of proliferation marker Ki67 is opposite to the level of γ-H2AX (Figure 6D). Furthermore, immunofluorescence analysis was conducted. Strong fluorescence of p16 and p21 was observed in nuclei of ethanol-treated cells, and oroxylin A treatment attenuated the fluorescence intensity in the nuclei. But verteporfin could reserve the effects of oroxylin A on p16 and p21 (Figure 6E).

4 | DISCUSSION

In the present study combining cell culture experiments and animal models, we identified a protective mechanism of oroxylin A against ALD that may rely on the anti-senescent impact of YAP in ethanol exposed hepatocyte. YAP, a major target of the Hippo pathway, played an important role in liver regeneration,³⁹ liver fibrosis^{18,40} and liver cancer.⁴¹ However, the role of YAP in ALD is not known. Human hepatocyte LO₂ cells were incubated with 100 mmol/L ethanol for 24 hours to establish an in vitro model of ALD as described in quite a few researches.^{26,29,42} In this study, we observed that ethanol directly damaged hepatocyte by the examination of intracellular enzymes, including ALT, AST and LDH. In addition, we found a novel phenomenon that ethanol induced the senescence of hepatocyte via detecting the accumulation of SA-β-gal⁺ cells and the expression of p16, p21 and Hmga1. The accumulation of SA-β-gal⁺ cells is the hallmark of cellular senescence. We also used p16, p21 (cell cycle regulators) and Hmga1 as the markers of cellular senescence.^{4,43,44} γ-H2AX is a special marker for DNA damage; increasing evidence has demonstrated that the expression of γ-H2AX is up-regulated in senescent cells.⁴⁵

We then need to examine the level of YAP in ethanol-damaged hepatocyte and test the effect of YAP on hepatocyte senescence. The results manifested that YAP overexpression could reserve the pro-senescent response of ethanol in LO₂ cells, as evaluated by SA-β-gal staining and p16, p21, Hmga1, γ-H2AX expression. Western blot analyses and immunofluorescence staining showed that YAP overexpression inhibited the expression of senescence markers induced by ethanol treatment. It has been well-known that cellular senescence is accompanied by the reduction in telomerase activity and telomere length. Real-time PCR and Western blot analyses indicated that telomerase activity was decreased by ethanol treatment, whereas elevated by YAP plasmid. These discoveries supported the possibility that suppression of ethanol-induced hepatocyte senescence by YAP may improve alcohol-induced hepatocellular injury.

Previous studies declared that oroxylin A could improve LPS and/or D-galactosamine-induced acute liver injury.⁴⁶ In additions, oroxylin A could suppress the growth of hepatoma carcinoma cells⁴⁷ and accelerates liver regeneration in CCl₄-induced acute liver injury mice.⁴⁸ In this study, we investigated the protective effects of oroxylin A on alcoholic liver injury. First, we used the vitro experiment to study the role of oroxylin A in alcohol-induced hepatocellular injury and cellular senescence. Our results showed that oroxylin A decreased the levels of ALT, AST and LDH in ethanol-treated hepatocyte. Senescence marker analyses demonstrated that oroxylin A had a protective effect on ethanol-induced hepatocyte senescence. On the one hand, SA-β-gal staining provided the evidence that ethanol induction of hepatocyte senescence could be inhibited by oroxylin A treatment in vitro. On the other hand, oroxylin A suppressed the protein expression of senescence markers p16, p21 and γ-H2AX in ethanol-stimulated hepatocyte. Furthermore, we found that ethanol blocked cell cycle at the G0/G1 checkpoint. But oroxylin A could attenuate the effect of ethanol on cell cycle arrest. Herein, our results revealed that oroxylin A could

FIGURE 6 YAP inhibition abrogates the effect of oroxylin A on alcohol-induced liver injury and hepatocyte senescence. Mice were grouped: group 1, vehicle control (no ethanol, no treatment); group 2, model (with ethanol, no treatment) group; group 3, ethanol + oroxylin A (30 mg/kg)-treated group; group 4, ethanol + oroxylin A (30 mg/kg) + verteporfin (50 mg/kg)-treated group. A, Determination of serum ALT, AST, ALP levels. Data are represented as mean $\pm$ SD. Significance: ** $P < .01$ vs control group, *** $P < .001$ vs control group, # $P < .05$ vs ethanol group, ## $P < .01$ vs ethanol group, ### $P < .001$ vs ethanol group, \$ $P < .05$ vs ethanol + oroxylin A group. B, Liver sections were stained with H&E. Representative photographs are shown. Scale bar, 100 μ m. C,D, Protein expression of γ -H2AX and Ki67 in vivo was investigated by immunohistochemistry on paraffin sections of mouse livers. Scale bar, 100 μ m. E, Liver sections were stained with immunofluorescence by using antibodies against p16 and p21. 4',6-Diamidino-2-phenylindole was used to stain the nucleus. Scale bar, 50 μ m

ameliorate alcohol-induced hepatocellular injury through inhibiting the senescence of hepatocyte.

The above results have showed the critical role of YAP in regulating ethanol-induced senescence in LO₂ cells; further studies need to investigate whether oroxylin A regulated cellular senescence was dependent on YAP. In the present study, we examined the effect of oroxylin A on the protein levels of YAP. Western blot analyses exhibited that oroxylin A dose-dependently promoted the expression of YAP. SA- β -gal staining revealed that a middle concentration of oroxylin A

(20 μ mol/L) decreased the number of SA- β -gal⁺ LO₂ cells when compared to the ethanol-treated group, but YAP inhibitor verteporfin eliminated this effect of oroxylin A. Telomerase system dysfunction is the critical feature of cellular senescence, real-time PCR analyses indicated verteporfin could weaken the effect of oroxylin A on telomerase activity in ethanol-treated hepatocyte. Besides, examination of senescence marker p16, p21 and Hmga1 showed that anti-senescence effect of oroxylin A was abated by verteporfin or YAP siRNA in ethanol-treated hepatocyte.

To prove the functional role of YAP in inhibition of hepatocyte senescence in vivo, mice were orally administrated with ethanol (56%, v/v, 5 mL/kg) for 8 weeks to establish an ALD model. Subsequently, mice were subjected to administration of oroxylin A or verteporfin for the last 4 weeks. The examinations of ALT, AST, ALP and H&E staining showed that oroxylin A's suppression of ethanol-stimulated hepatocyte damage could be reversed by verteporfin. Immunohistochemistry analyses demonstrated that verteporfin could reserve the effects of oroxylin A, which increased the number of proliferated hepatocyte and decreased the number of DNA damaged hepatocyte. Furthermore, immunofluorescence staining showed that verteporfin weakened the oroxylin A inhibition of expression of senescence marker p16 and p21 in vivo. These results were consistent with other prior observations.

In summary, the aggregate data in this study demonstrated that oroxylin A may ameliorate alcoholic liver injury via the inhibition of ethanol-induced hepatocyte senescence. Moreover, the molecular mechanism underlying oroxylin A inhibition of ethanol-induced hepatocyte senescence possibly by regulating the expression of YAP. However, our results do not rule out the possible involvement of any other signalling mechanisms in the oroxylin A inhibition of hepatocyte senescence. Our observations indicated that inhibition of ethanol-induced hepatocyte senescence could be a new strategy for the improvement of alcoholic hepatocellular injury by oroxylin A.

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (31571455, 31401210, 31600653, 81600483 and 81270514), the Natural Science Foundation of Jiangsu Province (BK20140955), the Open Project Program of Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica (JKLPSE 201502) and the Project of the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ORCID

Shizhong Zheng <http://orcid.org/0000-0002-7092-7843>

REFERENCES

- Richardson MM, Jonsson JR, Powell EE, et al. Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction. *Gastroenterology*. 2007;133:80-90.
- Nakajima T, Moriguchi M, Katagishi T, et al. Premature telomere shortening and impaired regenerative response in hepatocytes of individuals with NAFLD. *Liver Int*. 2006;26:23-31.
- Aravinthan A, Mells G, Allison M, et al. Gene polymorphisms of cellular senescence marker p21 and disease progression in non-alcohol related fatty liver disease. *Cell Cycle*. 2014;13:1489-1494.
- Krizhanovsky V, Yon M, Dickins RA, et al. Senescence of activated stellate cells limits liver fibrosis. *Cell*. 2008;134:657-667.
- Schrader J, Fallowfield J, Iredale JP. Senescence of activated stellate cells: not just early retirement. *Hepatology*. 2009;49:1045-1047.
- Sasaki M, Ikeda H, Yamaguchi J, Nakada S, Nakanuma Y. Telomere shortening in the damaged small bile ducts in primary biliary cirrhosis reflects ongoing cellular senescence. *Hepatology*. 2008;48:186-195.
- Marshall A, Rushbrook S, Davies SE, et al. Relation between hepatocyte G1 arrest, impaired hepatic regeneration, and fibrosis in chronic hepatitis C virus infection. *Gastroenterology*. 2005;128:33-42.
- Kim MY. The progression of liver fibrosis in non-alcoholic fatty liver disease. *Korean J Gastroenterol*. 2017;69:341-347.
- Ogrodnik M, Miwa S, Tchkonja T, et al. Cellular senescence drives age-dependent hepatic steatosis. *Nat Commun*. 2017;8:15691.
- Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol*. 2007;8:729-740.
- von Figura G, Hartmann D, Song Z, Rudolph KL. Role of telomere dysfunction in aging and its detection by biomarkers. *J Mol Med (Berl)*. 2009;87:1165-1171.
- Karlseder J, Smogorzewska A, de Lange T. Senescence induced by altered telomere state, not telomere loss. *Science*. 2002;295:2446-2449.
- Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. *Genes Dev*. 2010;24:2463-2479.
- Beausejour CM, Krtolica A, Galimi F, et al. Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J*. 2003;22:4212-4222.
- Brookes S, Rowe J, Gutierrez Del Arroyo A, Bond J, Peters G. Contribution of p16(INK4a) to replicative senescence of human fibroblasts. *Exp Cell Res*. 2004;298:549-559.
- Aragona M, Panciera T, Manfrin A, et al. A mechanical checkpoint controls multicellular growth through YAP/TAZ regulation by actin-processing factors. *Cell*. 2013;154:1047-1059.
- Zhao B, Ye X, Yu J, et al. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev*. 2008;22:1962-1971.
- Mannaerts I, Leite SB, Verhulst S, et al. The Hippo pathway effector YAP controls mouse hepatic stellate cell activation. *J Hepatol*. 2015;63:679-688.
- Xie Q, Chen J, Feng H, et al. YAP/TEAD-mediated transcription controls cellular senescence. *Cancer Res*. 2013;73:3615-3624.
- Han Q, Wang H, Xiao C, Fu BD, Du CT. Oroxylin A inhibits H2O2-induced oxidative stress in PC12 cells. *Nat Prod Res*. 2017;31:1339-1342.
- Li J, Tong D, Liu J, Chen F, Shen Y. Oroxylin A attenuates cigarette smoke-induced lung inflammation by activating Nrf2. *Int Immunopharmacol*. 2016;40:524-529.
- Shen L, Zhang LL, Li H, et al. Oroxylin A inhibits the generation of Tregs in non-small cell lung cancer. *Oncotarget*. 2017;8:49395-49408.
- Kwon BE, Song JH, Song HH, et al. Antiviral activity of Oroxylin A against Coxsackievirus B3 alleviates virus-induced acute pancreatic damage in mice. *PLoS ONE*. 2016;11:e0155784.
- Zhang F, Zhang Z, Chen L, et al. Curcumin attenuates angiogenesis in liver fibrosis and inhibits angiogenic properties of hepatic stellate cells. *J Cell Mol Med*. 2014;18:1392-1406.
- Antoniades CG, Quaglia A, Taams LS, et al. Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans. *Hepatology*. 2012;56:735-746.
- Wang H, Zhang Y, Bai R, Wang M, Du S. Baicalin attenuates alcoholic liver injury through modulation of hepatic oxidative stress, inflammation and sonic hedgehog pathway in rats. *Cell Physiol Biochem*. 2016;39:1129-1140.
- Xu W, Lu C, Yao L, Zhang F, Shao J, Zheng S. Dihydroartemisinin protects against alcoholic liver injury through alleviating hepatocyte steatosis in a farnesoid X receptor-dependent manner. *Toxicol Appl Pharmacol*. 2017;315:23-34.

28. Lu C, Xu W, Shao J, Zhang F, Chen A, Zheng S. Nrf2 activation is required for ligustrazine to inhibit hepatic steatosis in alcohol-preferring mice and hepatocytes. *Toxicol Sci*. 2017;155:432-443.
29. Lu C, Xu W, Zhang F, Shao J, Zheng S. Nrf2 knockdown disrupts the protective effect of curcumin on alcohol-induced hepatocyte necrosis. *Mol Pharm*. 2016;13:4043-4053.
30. Cheng B, Zheng Y, Guo X, Wang Y, Liu C. Hepatitis B viral X protein alters the biological features and expressions of DNA repair enzymes in LO2 cells. *Liver Int*. 2010;30:319-326.
31. Deaciuc IV, Fortunato F, D'Souza NB, Hill DB, McClain CJ. Chronic alcohol exposure of rats exacerbates apoptosis in hepatocytes and sinusoidal endothelial cells. *Hepatol Res*. 2001;19:306-324.
32. Cardin R, D'Errico A, Fiorentino M, Cecchetto A, Naccarato R, Farinati F. Hepatocyte proliferation and apoptosis in relation to oxidative damage in alcohol-related liver disease. *Alcohol Alcohol*. 2002;37:43-48.
33. van Steensel B, de Lange T. Control of telomere length by the human telomeric protein TRF1. *Nature*. 1997;385:740-743.
34. van Steensel B, Smogorzewska A, de Lange T. TRF2 protects human telomeres from end-to-end fusions. *Cell*. 1998;92:401-413.
35. Zhang P, Dilley C, Mattson MP. DNA damage responses in neural cells: focus on the telomere. *Neuroscience*. 2007;145:1439-1448.
36. Smogorzewska A, van Steensel B, Bianchi A, et al. Control of human telomere length by TRF1 and TRF2. *Mol Cell Biol*. 2000;20:1659-1668.
37. Dai Q, Yin Y, Liu W, et al. Two p53-related metabolic regulators, TIGAR and SCO2, contribute to oroxylin A-mediated glucose metabolism in human hepatoma HepG2 cells. *Int J Biochem Cell Biol*. 2013;45:1468-1478.
38. Zou M, Lu N, Hu C, et al. Beclin 1-mediated autophagy in hepatocellular carcinoma cells: implication in anticancer efficiency of oroxylin A via inhibition of mTOR signaling. *Cell Signal*. 2012;24:1722-1732.
39. Grijalva JL, Huizenga M, Mueller K, et al. Dynamic alterations in Hippo signaling pathway and YAP activation during liver regeneration. *Am J Physiol Gastrointest Liver Physiol*. 2014;307:G196-G204.
40. Machado MV, Michelotti GA, Pereira TA, et al. Accumulation of duct cells with activated YAP parallels fibrosis progression in non-alcoholic fatty liver disease. *J Hepatol*. 2015;63:962-970.
41. Yimlamai D, Fowl BH, Camargo FD. Emerging evidence on the role of the Hippo/YAP pathway in liver physiology and cancer. *J Hepatol*. 2015;63:1491-1501.
42. Lu C, Jiang Y, Zhang F, et al. Tetramethylpyrazine prevents ethanol-induced hepatocyte injury via activation of nuclear factor erythroid 2-related factor 2. *Life Sci*. 2015;141:119-127.
43. Burd CE, Sorrentino JA, Clark KS, et al. Monitoring tumorigenesis and senescence in vivo with a p16(INK4a)-luciferase model. *Cell*. 2013;152:340-351.
44. Munoz-Espin D, Canamero M, Maraver A, et al. Programmed cell senescence during mammalian embryonic development. *Cell*. 2013;155:1104-1118.
45. Serrano M. Cancer: a lower bar for senescence. *Nature*. 2010;464:363-364.
46. Huang H, Zhang X, Li J. Protective effect of oroxylin A against lipopolysaccharide and/or D-galactosamine-induced acute liver injury in mice. *J Surg Res*. 2015;195:522-528.
47. Hu Y, Yang Y, You QD, et al. Oroxylin A induced apoptosis of human hepatocellular carcinoma cell line HepG2 was involved in its antitumor activity. *Biochem Biophys Res Commun*. 2006;351:521-527.
48. Zhu R, Zeng G, Chen Y, et al. Oroxylin A accelerates liver regeneration in CCl(4)-induced acute liver injury mice. *PLoS ONE*. 2013;8:e71612.

How to cite this article: Jin H, Lian N, Bian M, et al.

Oroxylin A inhibits ethanol-induced hepatocyte senescence via YAP pathway. *Cell Prolif*. 2018;51:e12431.

<https://doi.org/10.1111/cpr.12431>