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Wogonin attenuates liver fibrosis *via* regulating hepatic stellate cell activation and apoptosis

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ABSTRACT

Liver fibrosis is the representative features of liver chronic inflammation and the characteristic of early cirrhosis. To date, effective therapy for liver fibrosis is lacking. Recently, Traditional Chinese Medicine (TCM) has attracted increasing attention due to its wide pharmacological effects and more uses in clinical. Wogonin, as one major active constituent of Scutellaria radix, has been reported it plays an important role in anti-inflammatory, anti-cancer, anti-viral, anti-angiogenesis, anti-oxidant and neuro-protective effects. However, the anti-fibrotic effect of wogonin is never covered in liver. In this study, we evaluated the protect effect of wogonin in liver fibrosis. Wogonin significantly attenuated liver fibrosis both in CCl_4-induced mice and TGF-β_1 activated HSCs. Meanwhile, wogonin can enhances apoptosis of TGF-β_1 activated HSC-T6 cell from rat and LX-2 cell from human detected by flow cytometry. Additionally, wogonin can largely enhances cle-caspase3, cle-caspase9 expression and the ratio of Bax/Bcl-2 in T6 cells. Pro-apoptosis effect of wogonin *in vivo* was further verified *in situ*. In conclusion, wogonin can attenuate liver fibrosis *via* regulating the activation and apoptosis of hepatic stellate cells, and may be an effective drug to treat and prevent liver fibrosis.

1. Introduction

Liver fibrosis is an adaptive response to liver injuries to maintaining liver integrity [1]. Clinically, the progression of liver fibrosis including cirrhosis and liver cancer has become a serious social problem [2]. Many causes including viral infection, non-alcoholic fatty liver disease, alcoholic liver disease, drug toxicity, metabolic disorders, autoimmune disease can result in liver fibrosis [3,4]. In the recent years, liver fibrosis attracted increasing attention because it is a major cause of liver cirrhosis, with organ failure and high mortality [5,6]. It is characterized with activation of hepatic stellate cells (HSCs) and excessive deposition of extracellular matrix (ECM) [7,8]. Quiescent HSCs in the perisinusoidal space of Disse filled with thin permeable connective tissue are activated and differentiated into myofibroblasts characterized by up-regulation of α-SMA and Coll-α_1 during liver fibrosis [9,10]. Therefore, the activation and proliferation of HSCs play crucial roles in the development of liver fibrosis [11]. Evidences reveal that inhibiting proliferation, activation of HSCs and promoting HSCs apoptosis may become a key therapy strategy for liver fibrosis [12,13].

Apoptosis, a unique style of cell death, regulate programmed cell death process [14], accompanied by DNA division, cell atrophy, and other phenotypes [15]. There are many enzymatic bases of apoptosis, among which caspase family [16–18] and Bcl-2 [16,19] family are closely related to apoptosis.

With the increasing emphasis, many TCMs have been reported to promote cell apoptosis, among of them wogonin has also been reported to promote apoptosis in liver cancer cells [20]. Wogonin (5,7-dihydroxy-8-methoxyflavone), one of the main active ingredients of Scutellaria radix, is a kind of flavonoid compound [21,22]. It has been involved in the occurrence and treatment of many diseases and has wide ranges of pharmacological activities including anti-inflammation [23–25], anti-angiogenesis [26], anti-virus [21,27], anti-fibrosis [28,29], anti-cancer [30] and leukemia [31,32]. Studies have showed that wogonin plays a role in promoting apoptosis of liver cancer cells through Bax/Bcl-2 [20]. In addition, wogonin can inhibit the fibrosis of renal tubular epithelial cells [28], but wogonin has never been studied in liver fibrosis. Thus, we studied the protective role of wogonin in liver fibrosis by regulating the apoptosis of hepatic stellate cells.

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Table 1
Primers sequences used for real-time q-PCR.

Gene	Forward	Reverse
Mouse α-SMA	5′-CGGGAGAAAATGACCCAGATT-3′	5′-AGGGACAGCACAGCCTGAATAG-3′
Human α-SMA	5′-CAGGAAGGACCTGTATGCCA-3′	5′-TGATCTTCATGGTGCTGGGT-3′
Rat α-SMA	5′-CAATGGCTCCGGGCTCTGTA-3′	5′-CTCTTGCTCTGCGCTTCGTC-3′
Mouse Coll-α_1	5′-GGAGAGTACTGGATCGACCCTAAC-3′	5′-ACACAGGTCTGACCTGTCTCCAT-3′
Human Coll-α_1	5′-CACCAATCACCTGCGTACAG-3′	5′-GCAGTTCTTGGTCTCGTCAC-3′
Rat Coll-α_1	5′-ACCTCAGGGTATTGCTGGAC-3′	5′-GACCAGGGAAGCCTCTTTCT-3′
Mouse β-actin	5′-CATTGCTGACAGGATGCAGAA-3′	5′-ATGGTGCTAGGAGCCAGAGC-3′
Human β-actin	5′-GGGAAATCGTGCGTGACATTAAGG-3′	5-CAGGAAGGAAGGCTGGAAGAGTG-3′
Rat β-actin	5′-CCCATCTATGAGGGTTACGC-3′	5′-TTTAATGTCACGCACGATTTC-3′

2. Materials and methods

2.1. Reagents and antibodies

2.1.1. Reagents

Reagents	Supplier	Cat. no.
Wogonin (purity > 98%)	Meilune Biology Technology (DaLian, China)	MB6663, CAS 632-85-9
Colchicine (purity ≧ 98%)	Target Molecule (Boston, USA)	T0320, CAS 64-86-8
TGF-β_1	Pepro Tech Inc. (Rocky Hill, USA)	500-M66
Dimethyl sulfoxide	Sigma Chemical (Sigma-Aldrich, USA)	67-68-5
Methyl thiazolyl tetrazolium	Sigma Chemical (Sigma-Aldrich, USA)	57,360–69-7
ALT assay kit	Jiancheng Biology Institution PeproTech (Nanjing, Jiangsu, China)	C009-2
AST assay kit	Jiancheng Biology Institution PeproTech (Nanjing, Jiangsu, China)	C010-2
Annexin V-FITC Cell apoptosis kit	BestBio (Shanghai, China)	BB-4101-3
Cell cycle and apoptosis analysis kit	Beyotime (China)	C1052
In Situ Cell Death Detection Kit	Roche (Switzerland)	11684817910
Pronase E	Sigma Chemical (Sigma-Aldrich, USA)	P6911
Collagenase IV	Sigma Chemical (Sigma-Aldrich, USA)	C5138
Hepes	Caisson Labs (USA)	H006-100GM
Nycodenz	Axis-shield (Norway)	AS1002424

2.1.2. Antibodies

Antibody	Supplier	Cat. no.
α-SMA	CST (USA)	19245S
Coll-α_1	Bioss (China)	bs-7158R
Caspase3	CST (USA)	9662S
Caspase9	CST (USA)	9504S
Bax	Abcam (USA)	ab32503
Bcl-2	Abcam (USA)	ab59348
β-Actin	ZSGB-BIO (Beijing, China)	TA-09

2.2. Animal treatment

Six-week-old male C57BL/6 mice (n = 72) weighting 18–22 g, were provided from the Experimental Animal Center of Anhui Medical University. The animal experimental protocol was reviewed and approved by the University Animal Care and Use Committee. And all mice were housed in comfortable environment and were acclimatized for 3 days before the experiment. Total mice were randomly divided into 6 groups (n = 12 per group) including control group, CCl_4 group, CCl_4 group treated with wogonin (10 mg/kg, 20 mg/kg, 40 mg/kg), CCl_4 group treated with colchicine (0.32 mg/kg). CCl_4 group and treatment group were subjected intraperitoneal injections of olive oil with 20% CCl_4 (2.5 ml/kg weight) for 2 times per week up to 4 weeks to induce liver fibrosis model, and before intraperitoneal injection of CCl_4, mice in treatment group were injected intraperitoneally different concentrations of wogonin and colchicine for 5 days, control group were injected intraperitoneally the same olive oil.

2.3. ALT/AST activity assay

The levels of ALT and AST in serum from each group mice were assayed by using the corresponding activity assay kits according to the manufacturer's instruction. The absorbance was detected with a Multiskan MK3 (Biotek, USA) at 510 nm.

2.4. Histopathology and immunohistochemistry staining

The suitable size of each group was soaked in 4% polyformaldehyde for 24 h, then were paraffin embedding. Permanent tissue was cut (5 μm thick) to stain with hematoxylin and eosin (H&E), masson, and immunohistochemical (IHC) staining of α-SMA. Uniform distribution of hepatocytes, size of intercellular spaces and infiltration of inflammatory cells in portal area were evaluated by HE staining. The formation of fibrosis connective tissue and destruction of hepatic lobule structure were observed by Masson staining. Fibrosis indicator α-SMA was detected by IHC. Finally, the dyeing section were observed and photographed by light microscopy.

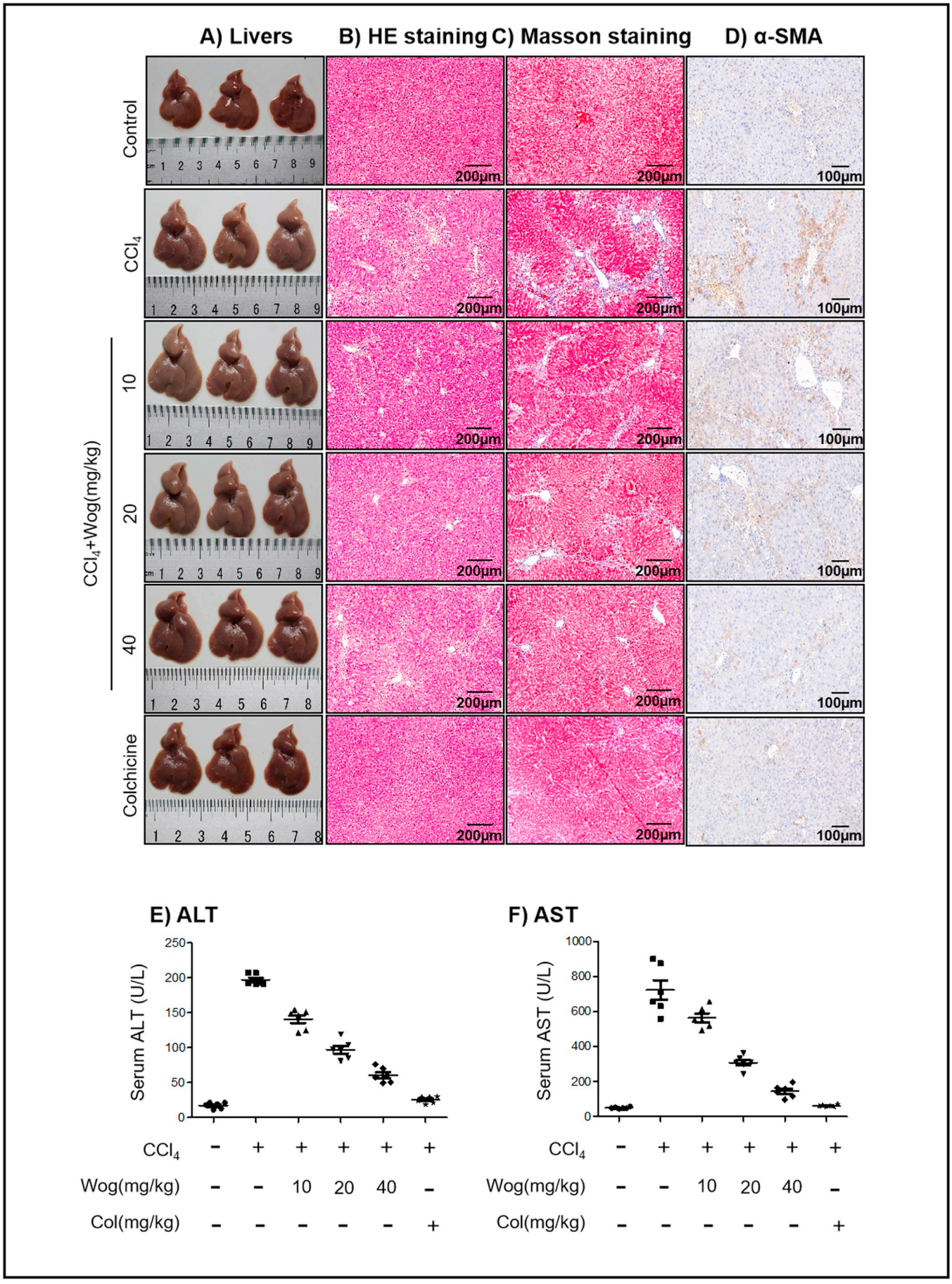

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Fig. 1. Wogonin attenuates liver injury induced by CCl_4 in mice. A: Liver appearance of mice in each group, including control group, CCl_4-treated mice, 10 mg/kg, 20 mg/kg, 40 mg/kg wogonin-treated mice and positive-treated mice (colchicine, 0.32 mg/kg). B and C: Hematoxylin and eosin (H&E) staining and Masson staining of liver tissues in different groups. D: Immunohistochemistry of α-SMA in different groups of liver tissues. E and F: Serum ALT and AST levels of mice in each group. The values were represented by means ± SD at least 6 separate experimental. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control mice. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 compared to CCl_4-treated mice. Wog: wogonin, Col: Colchicine.

2.5. TUNEL staining

The paraffin-embedded liver tissue of each group were dewaxed and washed slices before repaired with protease K at 37 °C for 25 min. Then them were covered with the membrane breaking solution and incubated at room temperature for 20 min, and washed 3 times with PBS. Each time for 5 min. Slices were incubated with mixture of TdT and dUTP mixed 1:9 at 37 °C for 2 h in a humid environment. Then washed 3 times with PBS and each time for 5 min. Slices were incubated with DAPI dye at room temperature for 10 min. Finally, the dyeing section were observed and photographed by light microscopy.

2.6. Hepatic stellate cells were perfusion in situ

Hepatic stellate cells were isolated from liver of mice as previously described. The technology mainly adopted perfusion *in situ* after opening the abdominal cavity by using collagenase IV and Pronase E, then the hepatic stellate cells were isolated by gradient-density centrifugation. Firstly, anesthetic immobilizes the mice, and open abdomen to fully expose the liver. Secondly, insert a 20-G catheter through the portal vein of mice, and cut the inferior vena cava as the liver bulges. Thirdly, the liver was perfused with PB, a kind of buffer solution containing NaCl, KCl, Hepes and NaOH. Fourthly, perfusion of the liver with digestion buffer [1 × PBC, Pronase E (Sigma Chemical (Sigma-Aldrich, USA)) and collagenase IV (Sigma Chemical (Sigma-Aldrich, USA)) and 4.76 mM $CaCl_2$]. After digestion, the liver was dissociated and broken in 1% BSA solution. The single cells were filtered by 200-meshsieve cell strainer, and the cell suspension was layered by Nycodenz (Axis-shield, Norway) density gradient centrifugation according to manufacture protocol. The supernatant after separated contained the freshly isolated HSCs. While HSCs stick to the bottle for 1 h, unattached cells and impurities were washed off by PBS. Protein cracking liquid and Trizol were respectively added to each group cells, total protein and total RNA were extracted for subsequent experiments.

2.7. Cell culture and cell treatment

T6-hepatic stellate cells of rats, LX2-liver stellate cells of humans were obtained from the Chinese Academy of Science (Shanghai, China). T6 cells cultured in DMEM (HyClone, USA) supplemented with 5% FBS (Every Green, China) and LX-2 cells cultured in DMEM supplemented with 10% FBS (Bovine, China), cells were incubated at 37 °C, 5% CO_2. When cells grow steadily, the cells of the treatment groups were replaced with culture media containing respective concentrations of wogonin, while the other groups were still replaced with normal culture media for 8 h, then the treatment groups and the model group were given TGF-β_1 (10 ng/ml) to stimulate for 48 h.

2.8. Total RNA extraction and interference analysis (real-time PCR analysis)

Total RNA was extracted from T6 cells, LX-2 cells, liver tissues and HSCs isolated from mice liver by using Trizol according to the instruction. After quantified by Nanodrop 2000 (Thermo Scientific, USA), cDNA was produced by reverse transcription using TAKARA kit (QIAGEN, Japan). Then, the mRNA expression of α-SMA, Coll-α_1, Bax/Bcl-2, and β-actin was detected by Piko-real 96 system (Thermo Scientific, USA) and SYBR-Green (Takara, Japan) real-time quantitative PCR analyses in real-time Thermal Cycler. β-Actin was considered an internal reference gene. cDNA was amplified by the program (95 °C for 10 min, 40 cycles at 95 °C for 15 s and at 60 °C for 1 min) of Piko-real 96 real-time PCR system (Thermo Scientific, USA). The expression level of target gene relative to β-actin was evaluated according to the formula: $2^{-\Delta\Delta Ct}$. The primers sequences were listed in Table 1.

2.9. Protein extraction and western blot analysis

T6 cells, LX-2 cells, HSCs from perfusion *in situ* and liver tissues from mice were lysed with RIPA (Beyotime, Jiangsu, China) and PMSF (Beyotime, Jiangsu, China) lysate buffer. The protein concentration was

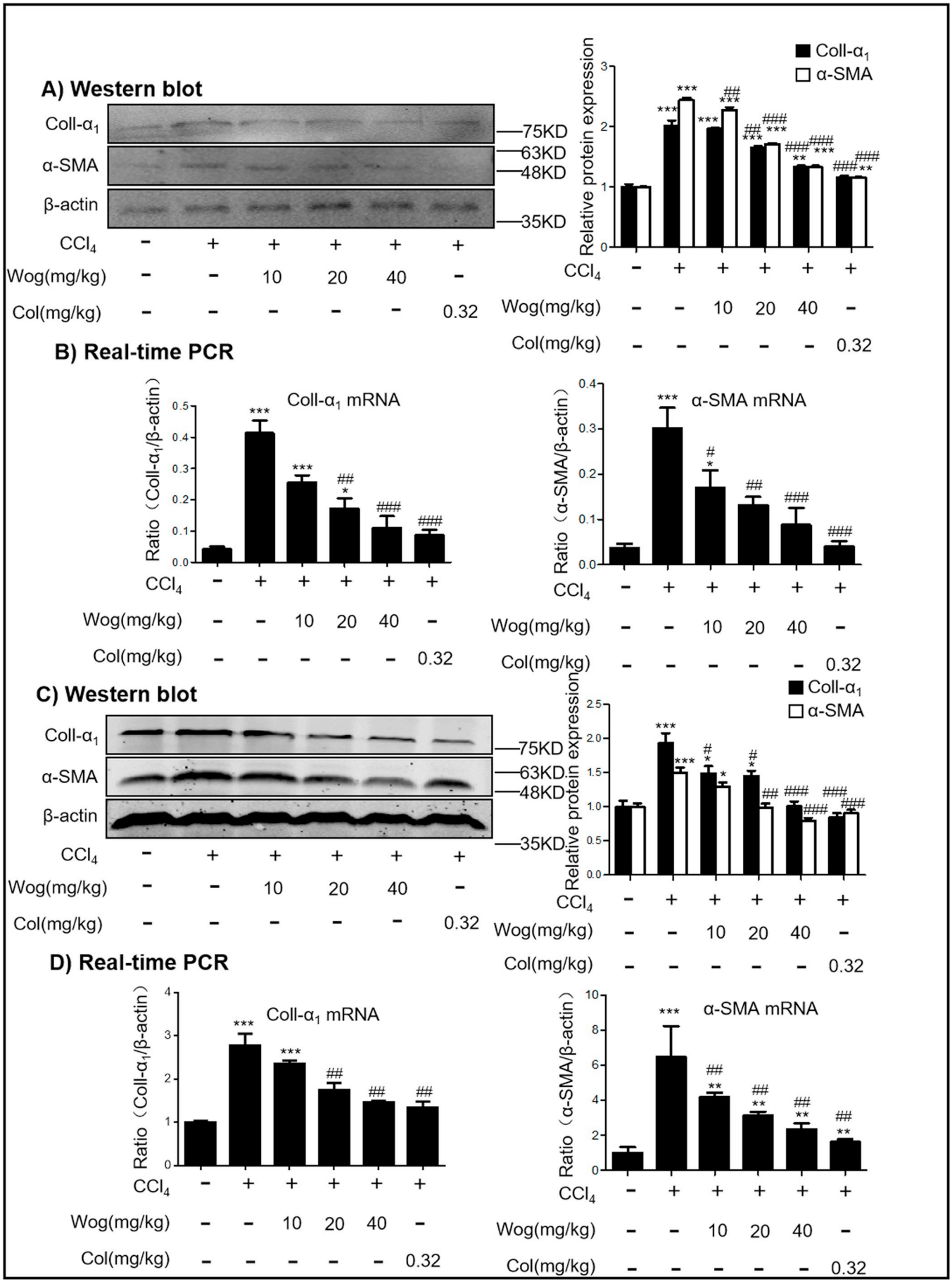

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Fig. 2. Wogonin alleviates CCl_4-induced liver fibrosis *in vivo*. A and B: the expression of fibrosis indicators Coll-α_1 and α-SMA from mice primary HSCs was detected by western blot and real-time PCR. C and D: the expression of fibrosis indicators Coll-α_1 and α-SMA from liver tissue was detected by western blot and real-time PCR. The values were represented by means ± SD at least 6 separate experimental. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control mice. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 compared to CCl_4-treated mice. Wog: wogonin, Col: Colchicine.

measured with the BCA assay kit (Beyotime, Jiangsu, China) according to the manufacturer's instruction. Then, proteins were separated by using sodium dodecyl sulfate-polyacrylamide gel and transferred to NC membranes. Next, nonspecific proteins were binding sealed with TBS/Tween 20 (0.075%) solution containing 5% skim milk for 3 h at room temperature. The NC membranes were washed 3 times with TBS/Tween 20 and each time for 15 min, then incubated with the special primary antibody against α-SMA, Coll-α_1, Bax/Bcl-2, Caspase3, Caspase9, β-actin at 4 °C. After 24 h, NC membranes were washed 3 times with TBS/Tween 20 and each time for 15 min, then avoided light incubated with IRDye 800-conjugated secondary antibody (Rockland immunochemical, Gilbertsville, PA) for 1.5 h at room temperature. Protein signatures were detected by LiCor/Odyssey infrared image system (LI-COR Bioscience, Lincoln, NE) and protein was quantitatively analyzed by using the Image Lab software (Bio-Rad, USA).

2.10. MTT assays

Cell activity was determined by standard 3-(4,5-dimethylthiazol-2-yl)-2, 4-diphenyl-tetrazolium bromide (MTT) assay. When cells grew steadily, 200ul cell suspension with 5×10^4 cells/ml was transferred to 96-well plate and incubated 8 h at 37 °C and 5% CO_2 atmosphere. Cells were treated with different concentrations of wogonin (0, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160 μg/ml) for 48 h. Then 20 μl PBS containing with 5 mg/ml MTT was added to each well and incubated for 4 h. The medium containing MTT was replaced with 150ul DMSO and dissolve the purple formazan crystals on a shaking table for 15 min. The optical density (OD) was measured with Thermomax microplate reader (BioTek, Winooski, VT, USA).

2.11. Cell apoptosis analysis

Cell apoptosis was detected by Annexin-V-FITC Apoptosis Detection Kit (Bestbio, China) and cell apoptosis analysis according to the manufacturer's instruction. Results showed that cells were divided into normal cells, dead cells, early apoptosis cells and late apoptosis cells (apoptosis cells were tagged by Annexin-V). Apoptosis detection was performed on BD FACS Verse (BD Biosciences, USA) and cells apoptosis was analyzed with FlowJo data analysis software package (TreeStar, USA).

2.12. Cell cycle analysis

Cell cycle was detected by cell cycle and apoptosis analysis kit (Beyotime, China). HSC-T6 cells (LX-2 cells) were collected and washed with pre-cooled PBS twice before fixed in 70% ethanol at 4 °C overnight. Then, cells were washed twice with cold PBS, 0.5 ml propidium iodide (PI) staining buffer was added and incubated at 37 °C for 30 min in the dark. Cell cycle detection was performed on a BDLSR (BD Biosciences, USA) and cells rested on the G1, S, G2/M phase were analyzed by the ModFit analysis software (Verity Software House, USA).

2.13. Statistical analysis

All experiments were performed at least 3 times and statistical analysis was performed by using GraphPad Prism 5. All data was reported as mean ± SD and statistical significance was determined by one-way ANOVA with the post-hoc test. All cases significance levels were set at 0.05.

3. Results

3.1. Wogonin attenuates liver injury in CCl_4-induced mice

To investigate the liver protective effect of wogonin in liver fibrosis,

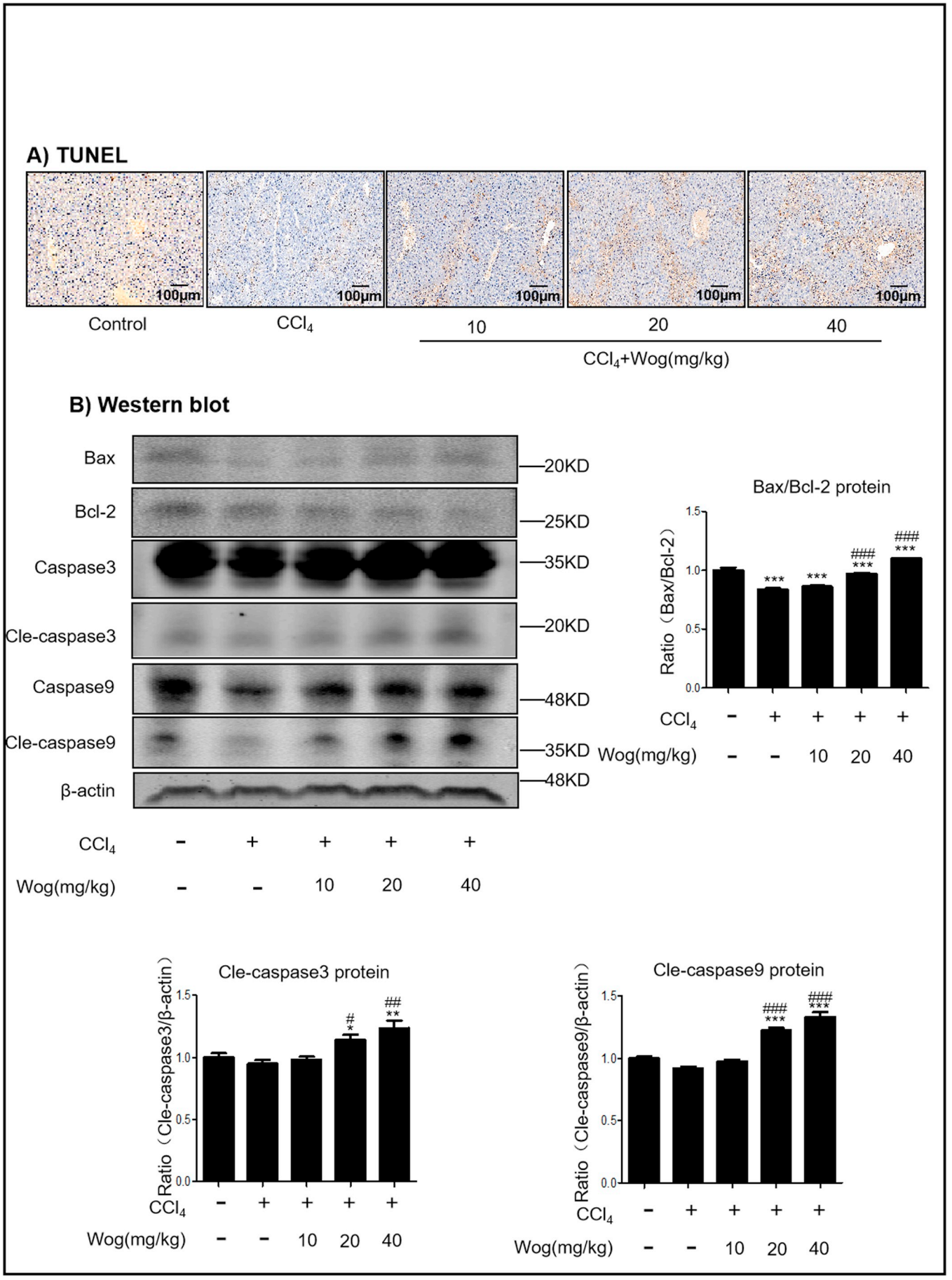

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Fig. 3. Wogonin activates apoptosis index *in vivo*. A: the expression of apoptosis indicators Cle-caspase3, Cle-caspase9 and the ratio of Bax/Bcl-2 from liver tissue was detected by western blot. B: TUNEL staining of liver tissues in control, CCl_4-treated mice, 10 mg/kg, 20 mg/kg, 40 mg/kg wogonin-treated mice groups. The values were represented by means ± SD at least 6 separate experimental. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control mice. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 compared to CCl_4-treated mice. Wog: wogonin.

the essential histopathology was performed *in vivo*. Liver images of each group were showed in Fig. 1A. H&E staining results showed that CCl_4-induced mice were shown with increased cell space, destruction of hepatic lobule structure and abundant inflammatory cells infiltrated in the portal area compared to normal mice (Fig. 1B). Masson staining showed that there were more fibroblasts in the model group with abundant collagen deposition and collagen fibrillation leading to damage of the lobule structure compared to the normal group (Fig. 1C). Aboved liver injuries were alleviated while treated with wogonin by dose-dependent manner, and the therapeutic effect at 40 mg/kg was close to the positive control group. The therapeutic effect of wogonin was further detected with ALT and AST assay, wogonin significantly decreased the levels of ALT and AST in serum induced by CCl_4 in a dose-dependent manner (Fig. 1E and F). Preliminary results confirmed that wogonin can attenuates liver injury in CCl_4-induced mice.

3.2. Wogonin reduces CCl_4-induced liver fibrosis in vivo

The result of α-SMA IHC staining showed wogonin can significantly reduce CCl_4-induced α-SMA expression and attenuate liver fibrosis in dose-dependent manner (Fig. 1D). The therapeutic effect of 40 mg/kg was almost consistent with the positive control group. Primary HSCs isolated from mice and the liver tissues of mice were used to detect the expression of fibrosis indicators α-SMA and Coll-α_1 in each group (Fig. 2A, B, C and D). Western blot and real-time PCR results suggested that wogonin can effectively inhibit CCl_4-induced α-SMA and Coll-α_1 expression in a dose-dependent manner, and the therapeutic effect of 40 mg/kg almost reach to the normal group. Above results preliminary proved that wogonin can effectively attenuate liver fibrosis *in vivo*.

3.3. Effect of wogonin on caspase3/9-mediated apoptosis signaling pathway in CCl_4-induced liver fibrosis

TUNEL results showed that wogonin promoted apoptosis *in situ* in a dose-dependent manner (Fig. 3A). Apoptosis level in CCl_4-induced group was lower than that in the normal group while significantly elevated in wogonin treated group. In addition, caspase3/9 axis is a frequently reported apoptotic pathway [33,34]. Inhibition of HSCs activation and promotion of HSCs apoptosis can be explored as a research direction for the treatment of liver fibrosis. Inhibition of hepatic fibrosis progression by wogonin might be related to caspase3/9 signaling pathway. We verified caspase3/9 expression by western blot (Fig. 3B). Compared with the normal group, the expression of cle-caspase9 and cle-caspase3 in CCl_4-induced group is slightly decreased, whereas wogonin can significantly enhance the expression of cle-caspase3/9 in dose-dependent manner, thereby promoting the apoptosis of HSCs. Meanwhile, the expression ratio of Bax/Bcl-2 support that wogonin promotes apoptosis of HSC *in vivo* (Fig. 3B).

3.4. Wogonin attenuates liver fibrosis by promoting apoptosis of HSC-T6 cells and LX-2 cells induced by TGF-β_1 in vitro

The maximum safe dose of wogonin was measured by MTT assay in HSC-T6 cells and LX-2 cells, and results showed that the highest concentration of non-toxic side effects on HSC-T6 cells and LX-2 cells respectively were 1.25 μg/ml and 20 μg/ml (Fig. 4A and B). The concentrations of wogonin we used in the subsequent experiment were 1.25 μg/ml on HSC-T6 cells and 20 μg/ml on LX-2 cells. The concentration of TGF-β_1 (10 ng/ml) was confirmed in previous literature [18]. Compared with the normal group, the expression of α-SMA and

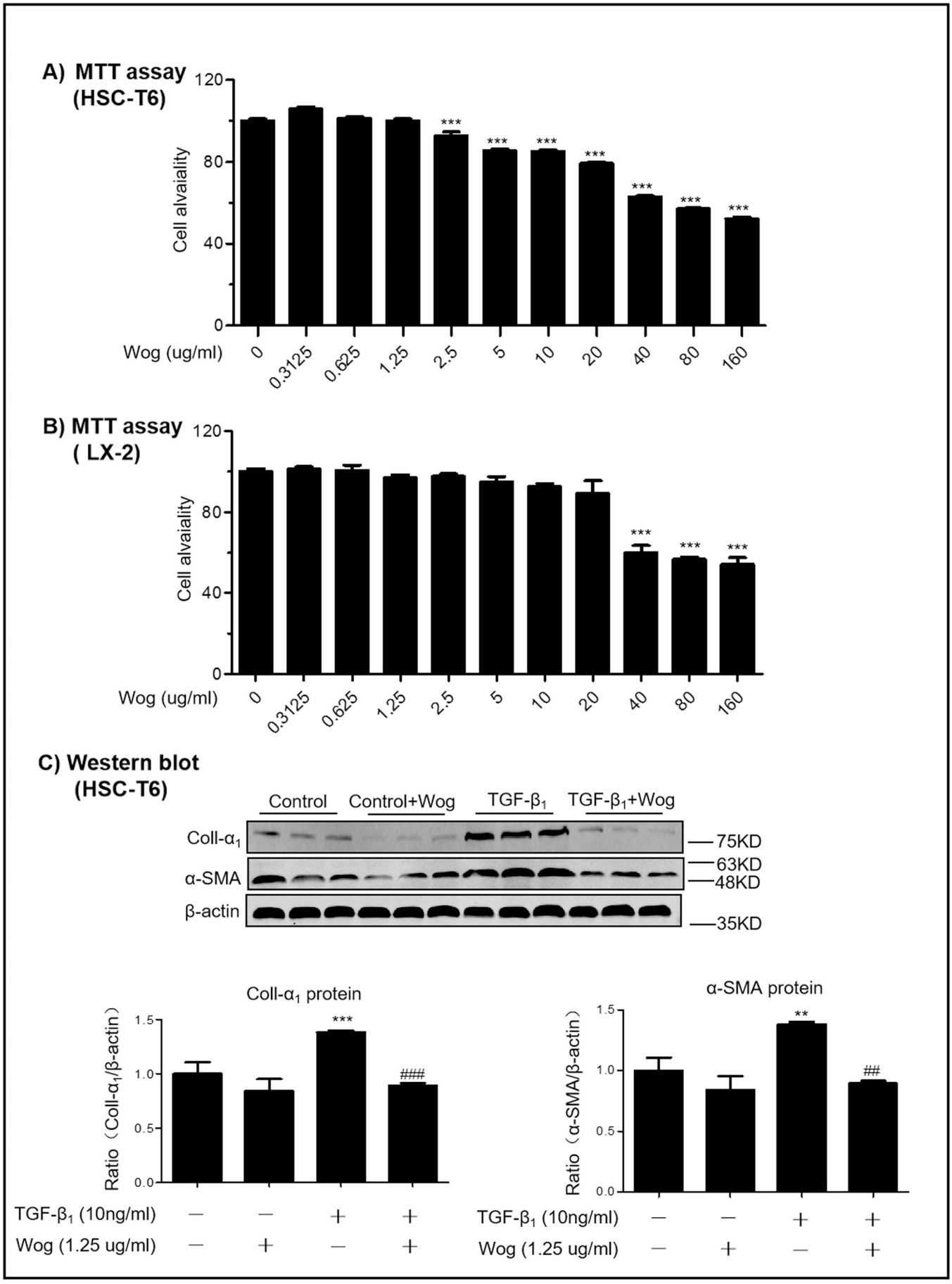

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Fig. 4. Wogonin inhibits the activation of TGF-β_1 induced HSCs *in vitro*. A and B: the safe dose of wogonin in HSC-T6 and LX-2 cells was detected by MTT assay. C and D: T6 cells were cultured with 1.25 μg/ml while stimulated by TGF-β_1 for 36 h. The expression of fibrosis indicators Coll-α_1 and α-SMA from these T6 cells was detected by western blot and real-time PCR. E and F: LX-2 cells were cultured with 20 μg/ml while stimulated by TGF-β_1 for 36 h. The expression of fibrosis indicators Coll-α_1 and α-SMA from these LX-2 cells was detected by western blot and real-time PCR. The values were represented by means ± SD at least 3 separate experimental. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to TGF-β_1 induced group. Wog: wogonin.

Coll-α_1 were little change in the wogonin alone group, while TGF-β_1 significantly promoted α-SMA and Coll-α_1 expression in HSCs, and wogonin largely inhibits their expression in TGF-β_1-induced HSCs (Fig. 4C, D, E and F). In addition, the effects of wogonin on the cell cycle and cell apoptosis of activated HSCs were detected by flow cytometry (Fig. 5A and B). The result suggested that wogonin had few effects on the cell cycle, but could effectively promote apoptosis of HSCs. Subsequently, the expression of cle-caspase3, cle-caspase9 protein were almost absent in the normal and TGF-β_1 stimulation group, while wogonin increased their expression (Fig. 5C). At the same time, the ratio of Bax/Bcl-2 in TGF-β_1 group was slightly decreased compared with the normal group, while significantly increased in wogonin group. Thus, wogonin could promote cell apoptosis of activated HSCs. These data further verified that wogonin could attenuate liver fibrosis by promoting cell apoptosis of activated HSCs.

4. Discussion

Liver fibrosis is a complex process with repeated healing of wounds caused by various injuries resulting in scar formation and leading to the final formation of liver fibrosis [35]. Worsening liver fibrosis can lead to irreversible cirrhosis and even liver cancer. The stage of liver fibrosis or even early cirrhosis can be reserved by suitable treatment [36]. But, effective therapy for liver fibrosis is crucial and lacking.

Wogonin, a kind of TCM ingredient with extensive pharmacological activity and has not been reported in liver fibrosis. Our team verified the protective effect of wogonin in liver fibrosis both *in vivo* and *in vitro*. Our results suggested that wogonin attenuates liver fibrosis in dose-dependent manner *in vivo*, and significantly inhibits the activation of TGF-β_1 induced HSCs *in vitro*. The expression of α-SMA and Coll-α_1 in HSCs isolated from liver showed a dose-dependent decrease in mice treated with wogonin group compared with CCl_4 stimulated group. And wogonin reduces the expression of α-SMA and Coll-α_1 in activated HSCs. In terms of mechanisms of action, excessive collagen deposition in liver and imbalance of extracellular matrix are the main feature of liver fibrosis. Related studies have showed that activated HSCs are the key factor to promote the synthesis and deposition of ECM and seriously affect liver injury [37,38]. Inhibiting proliferation, activation of HSCs and promoting the HSCs apoptosis may become a key strategy for liver fibrosis.

Apoptosis is a new programmed cell death mode and plays a decisive role in many diseases [14,19,39,40]. Inhibiting the activation of HSCs and promoting the apoptosis of HSCs have become a hot in studies on inhibiting fibrosis [40–42]. The levels of apoptosis mainly include caspase family activated and cytochrome released by mitochondria [15,43]. Caspase cleavage proteins on aspartic acid residues induce a series of complex cascade amplified apoptotic reactions [43]. Recently, it have been reported frequently that caspase9 and caspase3 play central roles in apoptotic responses [13,43,44]. Given the above apoptosis mechanism and roles of wogonin in apoptosis, we detected that whether wogonin could play an anti-liver fibrosis role *via* inhibiting activation and promoting apoptosis of HSCs.

Our results showed that wogonin does not affect the cycle of activated HSCs, but regulates cell apoptosis. The expression of cle-caspase9 and cle-caspase3 and the ratio of Bax/Bcl-2 were significantly increased in dose-dependent manner *in vivo*. Meanwhile, wogonin significantly promoted the activation of caspase9, caspase3 and increased the ratio of Bax/Bcl-2 in activated HSCs *in vitro*.

All above results prove that wogonin inhibits the progression of liver fibrosis by promoting the apoptosis of activated HSCs. Thus, wogonin has potential to become a new drug for the prevention and treatment of liver fibrosis in the future.

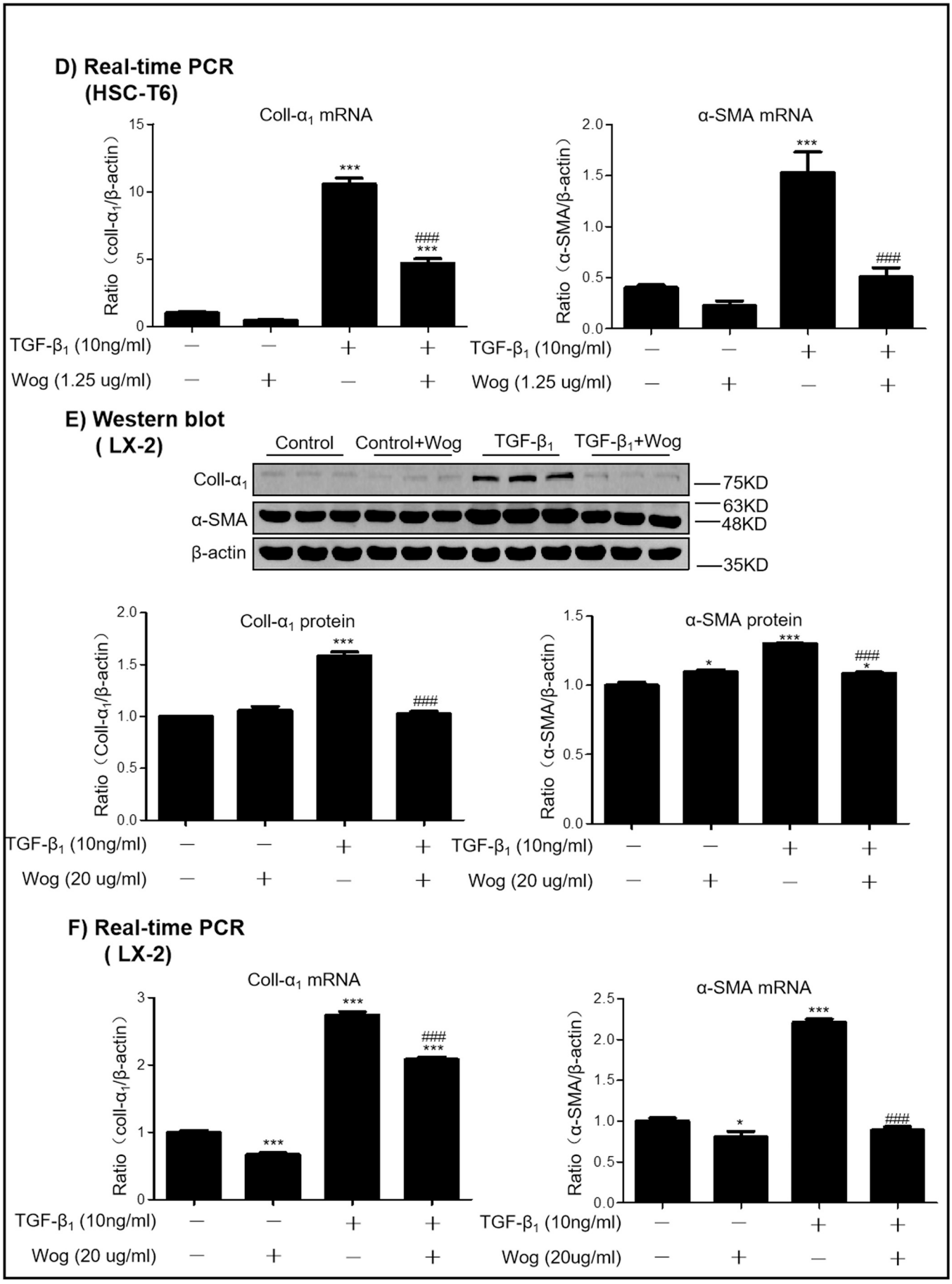

Fig. 4. (*continued*)

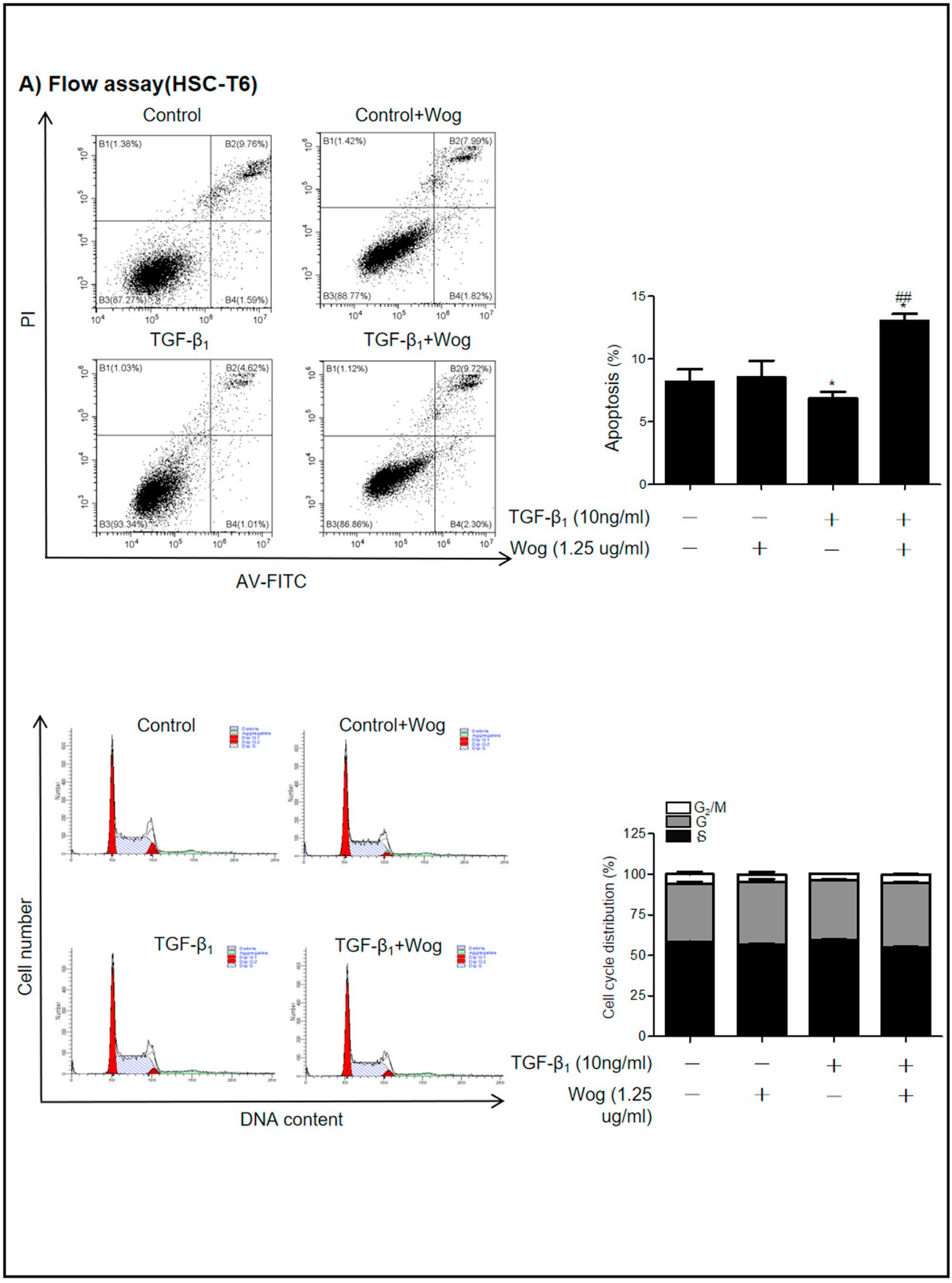

Fig. 5. Wogonin promotes apoptosis of TGF-β_1 induced HSCs *in vitro*. A: cell apoptosis and cycle were detected by flow cytometry of T6 cell in each group. B: cell apoptosis and cycle were detected by flow cytometry of LX-2 cell in each group. C: the expression of apoptosis indicators Cle-caspase3, Cle-caspase9 and the ratio of Bax/Bcl-2 of T6 cells was detected by western blot. The values were represented by means ± SD at least 3 separate experimental. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control mice. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 compared to TGF-β_1 induced group. Wog: wogonin.

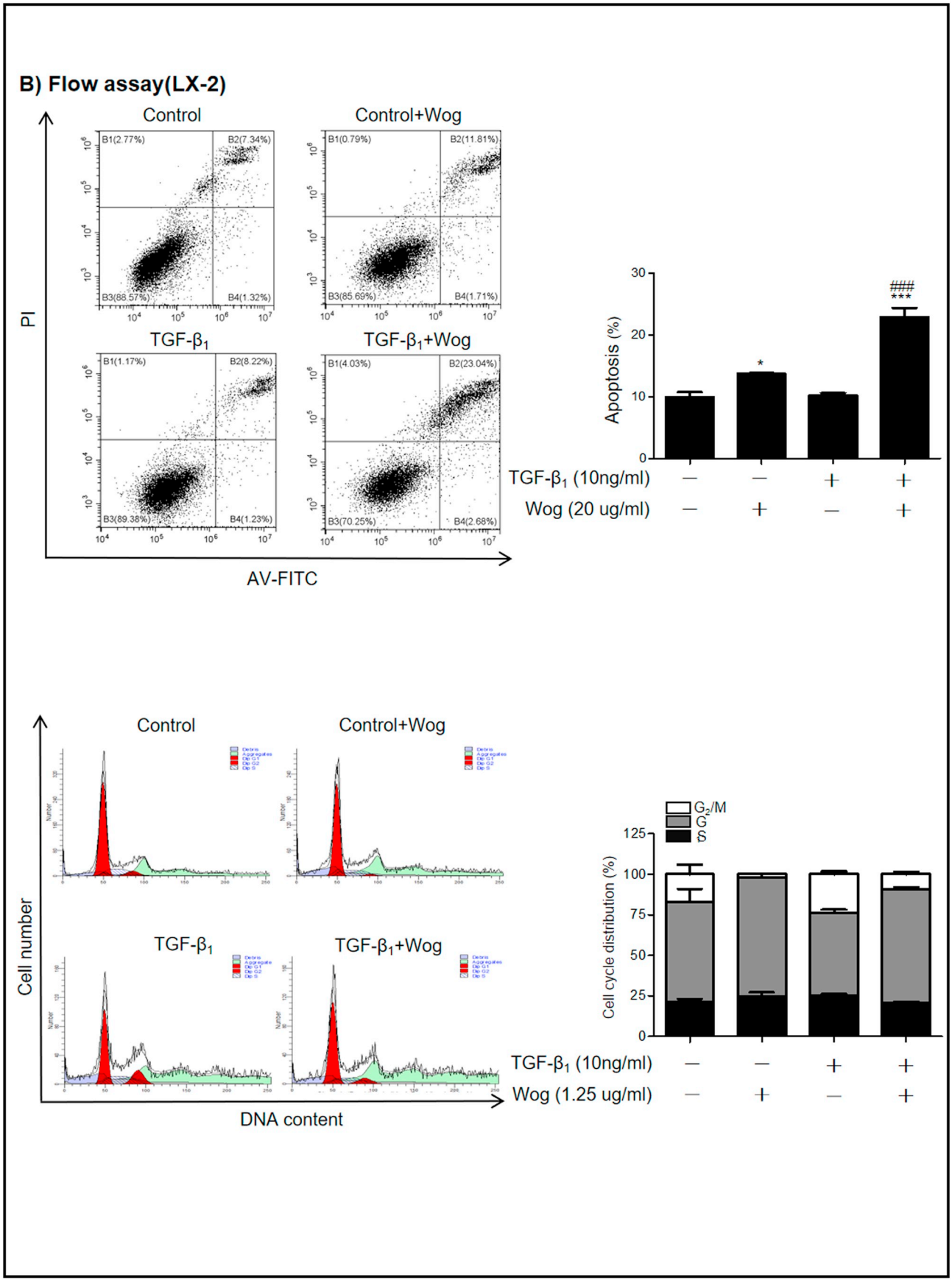

Fig. 5. (*continued*)

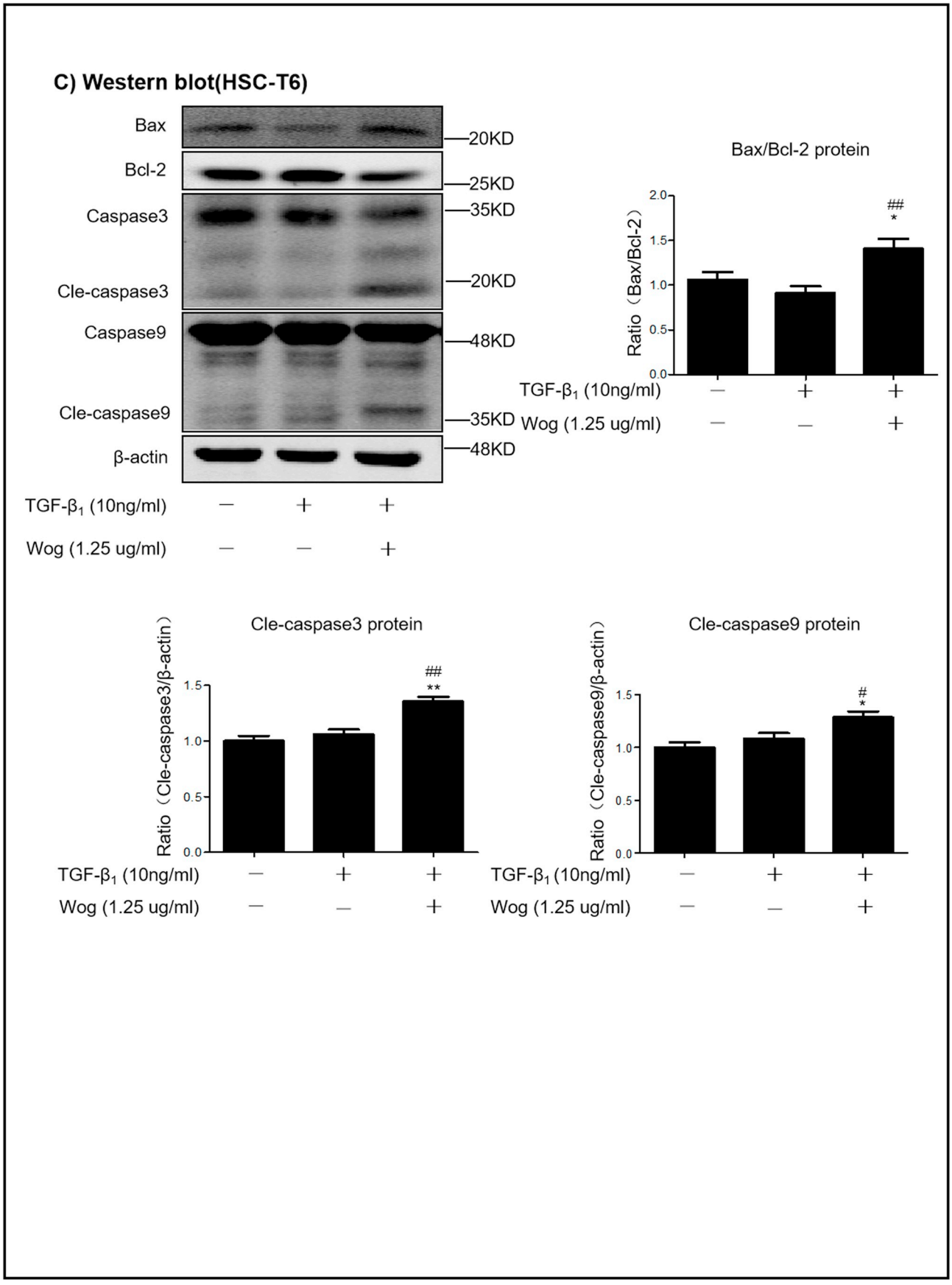

Fig. 5. (*continued*)

Declaration of Competing Interest

The authors disclose no conflict of interest with respect to this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.05.056.

References

[1] P. Fagone, K. Mangano, A. Pesce, T.R. Portale, S. Puleo, F. Nicoletti, Emerging therapeutic targets for the treatment of hepatic fibrosis, Drug Discov. Today 21 (2016) 369–375.
[2] L. Giannitrapani, M. Soresi, M.L. Bondi, G. Montalto, M. Cervello, Nanotechnology applications for the therapy of liver fibrosis, World J. Gastroenterol. 20 (2014) 7242–7251.
[3] Y. Pang, C. Kartsonaki, I. Turnbull, Y. Guo, R. Clarke, Y. Chen, F. Bragg, L. Yang, Z. Bian, I.Y. Millwood, J. Hao, X. Han, Y. Zang, J. Chen, L. Li, M.V. Holmes, Z. Chen, Diabetes, plasma glucose, and incidence of fatty liver, cirrhosis, and liver cancer: a prospective study of 0.5 million people, Hepatology 68 (2018) 1308–1318.
[4] T. Senoo, R. Sasaki, Y. Akazawa, T. Ichikawa, S. Miuma, H. Miyaaki, N. Taura, K. Nakao, Geranylgeranylacetone attenuates fibrogenic activity and induces apoptosis in cultured human hepatic stellate cells and reduces liver fibrosis in carbon tetrachloride-treated mice, BMC Gastroenterol. 18 (2018) 34.
[5] E. Cortes, D. Lachowski, A. Rice, A. Chronopoulos, B. Robinson, S. Thorpe, D.A. Lee, L.A. Possamai, H. Wang, D.J. Pinato, A.E. Del Rio Hernandez, Retinoic acid receptor-beta is downregulated in hepatocellular carcinoma and cirrhosis and its expression inhibits myosin-driven activation and durotaxis in hepatic stellate cells, Hepatology 69 (2) (2018) 785–802.
[6] Y.R. Wang, R.T. Hong, Y.Y. Xie, J.M. Xu, Melatonin ameliorates liver fibrosis induced by carbon tetrachloride in rats via inhibiting TGF-beta1/Smad signaling pathway, Curr. Med. Sci. 38 (2018) 236–244.
[7] M. Chen, J. Liu, W. Yang, W. Ling, Lipopolysaccharide mediates hepatic stellate cell activation by regulating autophagy and retinoic acid signaling, Autophagy 13 (2017) 1813–1827.
[8] J. Zhao, N. Tang, K. Wu, W. Dai, C. Ye, J. Shi, J. Zhang, B. Ning, X. Zeng, Y. Lin, MiR-21 simultaneously regulates ERK1 signaling in HSC activation and hepatocyte EMT in hepatic fibrosis, PLoS One 9 (2014) e108005.
[9] S.L.F. Takaaki Higashi, Yujin Hoshida, Hepatic stellate cells as key target in liver fibrosis, Adv. Drug Deliv. Rev. 1 (2017) 27–42.
[10] J.E. Puche, Y. Saiman, S.L. Friedman, Hepatic stellate cells and liver fibrosis, Compr. Physiol. 3 (2013) 1473–1492.
[11] F. Yu, S. Ji, L. Su, L. Wan, S. Zhang, C. Dai, Y. Wang, J. Fu, Q. Zhang, Adipose-derived mesenchymal stem cells inhibit activation of hepatic stellate cells in vitro and ameliorate rat liver fibrosis in vivo, J. Formos. Med. Assoc. 114 (2015) 130–138.
[12] S. Movassaghi, Z. Nadia Sharifi, F. Mohammadzadeh, M. Soleimani, Pentoxifylline protects the rat liver against fibrosis and apoptosis induced by acute administration of 3,4-methylenedioxymethamphetamine (MDMA or ecstasy), Iran J. Basic Med. Sci. 16 (2013) 922–927.
[13] L.M. Kuo, P.J. Chen, P.J. Sung, Y.C. Chang, C.T. Ho, Y.H. Wu, T.L. Hwang, The bioactive extract of Pinnigorgia sp. induces apoptosis of hepatic stellate cells via ROS-ERK/JNK-Caspase-3 signaling, Mar. Drugs 16 (2018).
[14] X. Dong, B. Ni, J. Fu, X. Yin, L. You, X. Leng, X. Liang, J. Ni, Emodin induces apoptosis in human hepatocellular carcinoma HepaRG cells via the mitochondrial caspasedependent pathway, Oncol. Rep. 40 (2018) 1985–1993.
[15] Y. Li, Y. Yan, F. Liu, L. Wang, F. Feng, Y. Xiao, Effects of calpain inhibitor on the apoptosis of hepatic stellate cells induced by calcium ionophore A23187, J. Cell. Biochem. 120 (2) (2018) 1–9.
[16] M. Li, S. Wang, X. Li, L. Jiang, X. Wang, R. Kou, Q. Wang, L. Xu, N. Zhao, K. Xie, Diallyl sulfide protects against lipopolysaccharide/d-galactosamine-induced acute liver injury by inhibiting oxidative stress, inflammation and apoptosis in mice, Food Chem. Toxicol. 120 (2018) 500–509.
[17] Y. Chen, Z. Wu, B. Yuan, Y. Dong, L. Zhang, Z. Zeng, MicroRNA-146a-5p attenuates irradiation-induced and LPS-induced hepatic stellate cell activation and hepatocyte apoptosis through inhibition of TLR4 pathway, Cell Death Dis. 9 (2018) 22.
[18] F.T. Bu, Y. Chen, H.X. Yu, X. Chen, Y. Yang, X.Y. Pan, Q. Wang, Y.T. Wu, C. Huang, X.M. Meng, J. Li, SENP2 alleviates CCl4-induced liver fibrosis by promoting activated hepatic stellate cell apoptosis and reversion, Toxicol. Lett. 289 (2018) 86–98.
[19] H. Moghtaderi, H. Sepehri, L. Delphi, F. Attari, Gallic acid and curcumin induce cytotoxicity and apoptosis in human breast cancer cell MDA-MB-231, Bioimpacts 8 (2018) 185–194.
[20] W. Wang, Q. Guo, Q. You, K. Zhang, Y. Yang, J. Yu, W. Liu, L. Zhao, H. Gu, Y. Hu, Z. Tan, X. Wang, Involvement of bax/bcl-2 in wogonin-induced apoptosis of human hepatoma cell line SMMC-7721, Anti-Cancer Drugs 17 (2006) 797–805.
[21] Q. Guo, L. Zhao, Q. You, Y. Yang, H. Gu, G. Song, N. Lu, J. Xin, Anti-hepatitis B virus activity of wogonin in vitro and in vivo, Antivir. Res. 74 (2007) 16–24.
[22] C.C. Lin, D.E. Shieh, The anti-inflammatory activity of Scutellaria rivularis extracts and its active components, baicalin, baicalein and wogonin, Am. J. Chin. Med. 24 (1996) 31–36.
[23] H.D. Li, X. Chen, Y. Yang, H.M. Huang, L. Zhang, X. Zhang, C. Huang, X.M. Meng, J. Li, Wogonin attenuates inflammation by activating PPAR-gamma in alcoholic liver disease, Int. Immunopharmacol. 50 (2017) 95–106.
[24] C.D. Lucas, D.A. Dorward, S. Sharma, J. Rennie, J.M. Felton, A.L. Alessandri, R. Duffin, J. Schwarze, C. Haslett, A.G. Rossi, Wogonin induces eosinophil apoptosis and attenuates allergic airway inflammation, Am. J. Respir. Crit. Care Med. 191 (2015) 626–636.
[25] K.A. Kim, J.H. Jung, Y.S. Choi, G. Kang, S.T. Kim, Anti-inflammatory effect of wogonin on allergic responses in ovalbumin-induced allergic rhinitis in the mouse, Allergy Rhinol (Providence) 9 (2018) (2152656718764145).
[26] C.M. Lin, Y.H. Chen, J.R. Ong, H.P. Ma, K.G. Shyu, K.J. Bai, Functional role of wogonin in anti-angiogenesis, Am. J. Chin. Med. 40 (2012) 415–427.
[27] R.K. Seong, J.A. Kim, O.S. Shin, Wogonin, a flavonoid isolated from Scutellaria baicalensis, has anti-viral activities against influenza infection via modulation of AMPK pathways, Acta Virol. 62 (2018) 78–85.
[28] X.M. Meng, G.L. Ren, L. Gao, H.D. Li, W.F. Wu, X.F. Li, T. Xu, X.F. Wang, T.T. Ma, Z. Li, C. Huang, Y. Huang, L. Zhang, X.W. Lv, J. Li, Anti-fibrotic effect of wogonin in renal tubular epithelial cells via Smad3-dependent mechanisms, Eur. J. Pharmacol. 789 (2016) 134–143.
[29] N.H. Ryu, J.M. Shin, J.Y. Um, I.H. Park, H.M. Lee, Wogonin inhibits transforming growth factor beta1-induced extracellular matrix production via the p38/activator protein 1 signaling pathway in nasal polyp-derived fibroblasts, Am. J. Rhinol. Allergy 30 (2016) 128–133.
[30] D.R. Li, H.X. Hou, W. Zhang, L. Li, Effects of wogonin on inducing apoptosis of human ovarian cancer A2780 cells and telomerase activity, Ai Zheng 22 (2003) 801–805.
[31] E. Lee, R. Enomoto, C. Koshiba, H. Hirano, Inhibition of P-glycoprotein by wogonin is involved with the potentiation of etoposide-induced apoptosis in cancer cells, Ann. N. Y. Acad. Sci. 1171 (2009) 132–136.
[32] E. Lee, R. Enomoto, C. Suzuki, M. Ohno, T. Ohashi, A. Miyauchi, E. Tanimoto, K. Maeda, H. Hirano, T. Yokoi, C. Sugahara, Wogonin, a plant flavone, potentiates etoposide-induced apoptosis in cancer cells, Ann. N. Y. Acad. Sci. 1095 (2007) 521–526.
[33] C.Y. Wu, Z.H. Tang, L. Jiang, X.F. Li, Z.S. Jiang, L.S. Liu, PCSK9 siRNA inhibits HUVEC apoptosis induced by ox-LDL via Bcl/Bax-caspase9-caspase3 pathway, Mol. Cell. Biochem. 359 (2012) 347–358.
[34] R. Wu, S. Tang, M. Wang, X. Xu, C. Yao, S. Wang, MicroRNA-497 induces apoptosis and suppresses proliferation via the Bcl-2/Bax-Caspase9-Caspase3 pathway and cyclin D2 protein in HUVECs, PLoS One 11 (2016) e0167052.
[35] M. Ekstedt, H. Hagstrom, P. Nasr, M. Fredrikson, P. Stal, S. Kechagias, R. Hultcrantz, Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up, Hepatology 61 (2015) 1547–1554.
[36] T.T. Chang, Y.F. Liaw, S.S. Wu, E. Schiff, K.H. Han, C.L. Lai, R. Safadi, S.S. Lee, W. Halota, Z. Goodman, Y.C. Chi, H. Zhang, R. Hindes, U. Iloeje, S. Beebe, B. Kreter, Long-term entecavir therapy results in the reversal of fibrosis/cirrhosis and continued histological improvement in patients with chronic hepatitis B, Hepatology 52 (2010) 886–893.
[37] R. Bataller, D.A. Brenner, Hepatic stellate cells as a target for the treatment of liver fibrosis, Semin. Liver Dis. 21 (2001) 437–451.
[38] A.M. Gressner, R. Weiskirchen, Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets, J. Cell. Mol. Med. 10 (2006) 76–99.
[39] R. Wang, H. Zhang, Y. Wang, F. Song, Y. Yuan, Inhibitory effects of quercetin on the progression of liver fibrosis through the regulation of NF-small ka, CyrillicB/Ismall ka, CyrillicBalpha, p38 MAPK, and Bcl-2/Bax signaling, Int. Immunopharmacol. 47 (2017) 126–133.
[40] L. Wei, Q. Chen, A. Guo, J. Fan, R. Wang, H. Zhang, Asiatic acid attenuates CCl4-

induced liver fibrosis in rats by regulating the PI3K/AKT/mTOR and Bcl-2/Bax signaling pathways, Int. Immunopharmacol. 60 (2018) 1–8.
[41] S.R. Dubash, S. Merchant, K. Heinzmann, F. Mauri, I. Lavdas, M. Inglese, K. Kozlowski, N. Rama, N. Masrour, J.F. Steel, A. Thornton, A.K. Lim, C. Lewanski, S. Cleator, R.C. Coombes, L. Kenny, E.O. Aboagye, Clinical translation of [(18)F] ICMT-11 for measuring chemotherapy-induced caspase 3/7 activation in breast and lung cancer, Eur. J. Nucl. Med. Mol. Imaging 45 (2018) 2285–2299.
[42] F. Bai, Q. Huang, J. Nie, S. Lu, C. Lu, X. Zhu, Y. Wang, L. Zhuo, Z. Lu, X. Lin, Trolline ameliorates liver fibrosis by inhibiting the NF-kappaB pathway, promoting HSC apoptosis and suppressing autophagy, Cell. Physiol. Biochem. 44 (2017) 436–446.
[43] P. De, J.H. Carlson, B. Leyland-Jones, C. Williams, N. Dey, Triple fluorescence staining to evaluate mechanism-based apoptosis following chemotherapeutic and targeted anti-cancer drugs in live tumor cells, Sci. Rep. 8 (2018) 13192.
[44] S.M. Hwang, H.J. Lee, J.H. Jung, D.Y. Sim, J. Hwang, J.E. Park, B.S. Shim, S.H. Kim, Inhibition of Wnt3a/FOXM1/beta-catenin axis and activation of GSK3beta and caspases are critically involved in apoptotic effect of moracin D in breast cancers, Int. J. Mol. Sci. 19 (2018).