

# Traditional Chinese medicine Yiqi Huoxue recipe attenuates hepatic fibrosis via YAP/TAZ signaling

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**Summary.** Background/Aims. The Yiqi Huoxue (YQHX) recipe has been shown to attenuate liver fibrosis, but precise mechanisms have not yet been elucidated. Recently, Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) signaling has been implicated in liver fibrogenesis. This study investigated whether the YAP/TAZ signaling is involved in the therapeutic effect of YQHX on hepatic fibrosis.

**Materials and Methods.** Wistar rats were used to generate a model of carbon tetrachloride (CCl<sub>4</sub>)-induced liver fibrosis. Chronic hepatitis B (CHB) patients with liver fibrosis were enrolled and assigned to receive either nucleoside/nucleotide analogues (NAs) or NAs plus YQHX. Histology, immunohistochemistry, qRT-PCR, and western blotting were conducted to mechanistically assess the therapeutic effects of YQHX on liver fibrosis.

**Results.** YQHX markedly alleviated morphological alterations in CCl<sub>4</sub>-induced liver fibrosis and decreased markers of hepatic fibrosis in rats. Furthermore, YQHX significantly suppressed CCl<sub>4</sub>-mediated activation of the transforming growth factor-beta (TGF-β)/Smad signaling pathway. Notably, CCl<sub>4</sub> induced up-regulation of YAP, TAZ, and connective tissue growth factor (CTGF), which were significantly abrogated by YQHX. Consistent with the above major findings in rats, CHB patients treated with NAs plus YQHX had greater improvement in liver fibrosis than those given NAs alone (71.4% vs. 28.6%; P=0.057). In addition, hepatic and plasma levels of YAP were significantly decreased after YQHX treatment in CHB patients with liver fibrosis.

**Conclusion.** YAP/TAZ signaling plays a role, at least in part, in the anti-fibrotic activity of YQHX. The findings may help to better understand the mechanisms

of YQHX in the treatment of liver fibrosis.

**Key words:** Liver fibrosis, Therapy strategy, Traditional Chinese medicine, Yes-associated protein, TCM mechanism research

## Introduction

Liver fibrosis is a pathological process involving persistent hepatic injuries and subsequent wound healing responses in chronic liver diseases. It has been well recognized that the activation of hepatic stellate cells (HSCs), transformation of HSCs into myofibroblasts, and excessive accumulation and deposition of extracellular matrix (ECM) components represent characteristic cellular changes during liver fibrogenesis. Notably, liver fibrosis is associated with poor prognosis and clinical outcomes of affected patients with chronic

**Abbreviations.** YQHX, Yiqi Huoxue; YAP, Yes-associated protein; TAZ, transcriptional co-activator with PDZ-binding motif; HSCs, hepatic stellate cells; ECM, extracellular matrix; TGF-β, transforming growth factor-beta; TCM, traditional Chinese medicine; CHB, chronic hepatitis B; CCl<sub>4</sub>, carbon tetrachloride; H&E, hematoxylin and eosin; α-SMA, alpha-smooth muscle actin; Col I, collagen I; IHC, immunohistochemistry; PBS, phosphate-buffered saline; HRP, horse radish peroxidase; RT, room temperature; DAB, 3,3'-diaminobenzidine; IOD, integral optical density; RIPA, radioimmunoprecipitation assay; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; CTGF, connective tissue growth factor; TBST, tris-buffered saline with Tween; cDNA, complementary DNA; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; NCBI, National Center of Biotechnology Information; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NAs, nucleoside/nucleotide analogues; LSM, liver stiffness measurement; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; α-SMA, alpha-smooth muscle actin; SD, standard deviations; ANOVA, analysis of variance; LSD, least significant difference; FZHY, Fuzheng Huayu decoction; TEAD, transcriptional factor TEA domain.

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liver diseases (Duseja, 2013). If left untreated, liver fibrosis can progress to cirrhosis or even life-threatening hepatocellular carcinoma. Although a number of drugs, including antisense transforming growth factor-beta (TGF- $\beta$ ) receptor, cytokines, antioxidants, and chemically synthesized drugs, have been showed to be effective for the treatment of experimental hepatic fibrosis (Rockey, 2005), unfortunately their efficacies in human studies remain unsatisfactory. Until now, there is no medication specifically approved to treat liver fibrosis, and current treatment mainly relies on treating the underlying causes.

Yiqi Huoxue (YQHX) recipe, a traditional Chinese medicine (TCM), has been demonstrated to have anti-fibrotic efficacy for its effects of “nourishing the liver and kidney, invigorating qi and blood, and nourishing the spleen and stomach” (Cui et al., 2020). The YQHX recipe includes: Radix Astragali, which reinforces qi and benefits detumescence; *Salvia miltiorrhiza*, which promotes blood circulation and alleviates blood stasis; Radix Curcumae, which invigorates qi, promotes blood circulation, detoxifies liver, and relieves depression; and other Chinese herbs. In previous studies, YQHX has been shown to inhibit the transformation of HSC to myofibroblasts, prevent the formation of intercellular matrix components, and regulate cell autophagy (Wang et al., 2017; Niu et al., 2018). Although, YQHX treatment has been shown to attenuate liver fibrosis in animal and human studies, the underlying molecular mechanisms have not been fully elucidated.

Recently, studies of differential gene expression patterns in relation to the activation of HSCs revealed that Yes-associated protein (YAP), as a downstream effector of the Hippo pathway, is a critical regulator involved in activation of HSCs (Liu et al., 2015; Mannaerts et al., 2015). It has been shown that YAP is translocated to the nucleus early during the activation of HSC, which drives the expression of its target genes, such as transcriptional co-activator with PDZ-binding motif (TAZ). Importantly, YAP/TAZ signaling has been implicated in the pathogenesis of liver fibrogenesis. However, it is unknown whether YAP/TAZ signaling could play a role in the anti-fibrotic effect of YQHX.

In the present study, the effects of YQHX treatment on hepatic fibrosis were assessed and the involvement of YAP/TAZ signaling in its anti-fibrotic activity was investigated in both rats and patients with chronic hepatitis B (CHB)-associated liver fibrosis. The insights gained from this study may lead to the better understanding of the molecular mechanisms whereby YQHX is able to combat hepatic fibrosis.

## Materials and methods

### Yiqi Huoxue recipe

The Chinese herbal medicine formula YQHX is a new drug authorized by the Third Hospital of Hebei Medical University (Grant No. Z20113160). YQHX

recipe is composed of medicinal herbs, including Radix Astragali, *Salvia miltiorrhiza*, Radix Curcumae, Radix Paeoniae Rubra, *Poria cocos*, endothelium corneum, cardamom and yam. The YQHX granules were purchased from Tianjiang Pharmaceutical Co. Ltd. (Jiangyin, Jiangsu, China).

### Experimental animals

A total of 18 Wistar male rats (200 $\pm$ 10 g) were provided by the Laboratory Animal Center of Hebei Medical University (Shijiazhuang, Hebei, China). Rats were housed with access to water and rodent chow *ad libitum* in a room maintained at 20-22°C with a 12 h light-dark cycle. The rats were randomly divided into three groups: normal control (n=6), carbon tetrachloride (CCl<sub>4</sub>) model (n=6), and YQHX (n=6) groups. The rats in the CCl<sub>4</sub> model and YQHX groups were subcutaneously injected with CCl<sub>4</sub> dissolved in olive oil (CCl<sub>4</sub> to olive oil ratio of 3:7, v/v), at a standard dose (0.2 mL/100 g body weight), twice weekly for eight weeks to induce liver fibrosis. To examine the effects of YQHX on CCl<sub>4</sub>-induced liver fibrosis, the rats in the YQHX group were treated with YQHX (3.4 g/10 mL/kg body weight) twice weekly via gavage for eight weeks, while the rats in the control and CCl<sub>4</sub> model groups were administered with a gavage of distilled water (10 mL/kg body weight). At the end of the eight weeks, liver tissues were removed from each experimental rat and separated into two sections: one section was fixed in 10% formaldehyde for histological and immunohistochemical (IHC) examinations, and the other section was stored at -80°C for future analysis of tissue homogenates.

The study protocol involving the use of experimental rats was reviewed and approved by the Research Ethics Committee of Hebei Medical University and was in accordance with the Animal Management Rules of the Ministry of Health of the People's Republic of China.

### Histological examination

The liver tissues of experimental rats were fixed in 4% paraformaldehyde overnight, embedded in paraffin, and subsequently sliced into sections (4  $\mu$ m thickness). After deparaffinization with xylene, the liver sections were rehydrated with 100% ethanol for 5 min, then 80% ethanol for 5 min. The liver sections were then stained with hematoxylin and eosin (H&E), or Masson's trichrome staining for the histological examination of connective tissue.

### IHC examinations

Liver tissues sections were deparaffinized and rehydrated in graded ethanol. Citrate buffer was used for antigen retrieval on paraffin-embedded tissue sections. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. Liver tissue sections were

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blocked with 10% goat serum albumin and incubated at 4°C overnight with a primary antibody targeting alpha-smooth muscle actin ( $\alpha$ -SMA) (1:200; Abcam, Cambridge, UK), YAP (1:50; Proteintech, Wuhan, China), TGF- $\beta$ 1 (1:100; Wanleibio, China), Smad2 (1:100; Proteintech, Wuhan, China), or Smad3 (1:100; Proteintech, Wuhan, China). Next, the liver sections were washed with phosphate-buffered saline (PBS) three times, followed by incubation with the appropriated horse radish peroxidase (HRP)-conjugated secondary antibody at room temperature (RT) for 30 min. Then, liver sections were incubated with 3,3'-diaminobenzidine (DAB) for visualization, and counterstained with hematoxylin. Integral optical density (IOD) was determined using Image-Pro Plus 6.0 software at 400 $\times$  magnification from ten nonoverlapping fields per specimen. YAP-positive cells were counted.

### Western blot analysis

Liver tissues were homogenized in radioimmuno-precipitation assay (RIPA) buffer supplemented with phenylmethylsulfonyl fluoride (PMSF; 1 mM) and phosphatase inhibitors to extract total protein. Equal amounts (100  $\mu$ g) of total protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Mass, USA). The resulting membranes were blocked in 5% non-fat milk for 1 h at RT, and incubated overnight at 4°C with primary antibodies, including  $\alpha$ -SMA (1:2000; Abcam, Cambridge, UK), collagen I (Col I; 1:1000; Proteintech, Wuhan, China), TGF- $\beta$ 1 (1:1000; abcam, Cambridge, UK), Smad2 (1:1000; Proteintech, Wuhan, China), Smad3 (1:500; Proteintech, Wuhan, China), Smad7 (1:500; Wanleibio, Shenyang, China), YAP (1:500; Affinity Biosciences, Cincinnati, USA), TAZ (1:2000; Proteintech, Wuhan, China), or connective tissue growth factor (CTGF; 1:500; Proteintech, Wuhan, China). The blots were rinsed with tris buffered saline with Tween

(TBST) and incubated with a secondary antibody (anti-rabbit-IR680 conjugates; 1:10000; LI-COR, USA) for 1 h. The fluorescence protein bands were visualized and analyzed using the Odyssey (LI-COR) infrared imaging system. The intensity of each protein band was quantified using Image J software.

### Quantitative reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from liver tissue using TRIzol reagent (Invitrogen, California, USA). Complementary DNA (cDNA) was synthesized using a high-capacity cDNA reverse transcription kit (Takara, Hokkaido, Japan). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed in an ABI 7500 real-time PCR system (Applied Biosystems, California, USA) using TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus; Takara, Japan). For the primer design, the target genes were searched using the National Center of Biotechnology Information (NCBI), and the coding region of mRNA sequences were found. Then Primer Premier 5 was used to design the primers, which were evaluated by Oligo software and Primer-BLAST in NCBI. Primer sequences for qRT-PCR analysis are summarized in Table 1. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The  $2^{-\Delta\Delta Ct}$  method was used to calculate the relative expression and each experiment was performed in triplicate.

### Chronic hepatitis B patients with significant liver fibrosis

A total of 170 CHB patients with significant liver fibrosis and 60 age- and gender-matched healthy subjects were enrolled from the Third Hospital of Hebei Medical University (Shijiazhuang, Hebei, China) for this study. Of the 170 CHB patients, 60 were treated with nucleotide analogues (NAs) alone [entecavir (0.5 mg) or tenofovir (300 mg) per day], and 110 were treated with NAs and YQHX (10.6 g twice daily) in combination.

**Table 1.** Primer sequences.

Gene	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')
$\alpha$ -SMA	NM_031004	GCTCCATCCTGGCTTCTCTATC	GGGCCAGCTTCGTCACTACTC
Collagen I	NM_053304	ATCAGCCCAAACCCCAAGGAG	CGCAGGAAGGTCAGCTGGATAG
TGF- $\beta$	NM_021578	TGAGTGGCTGTCTTTTGACG	TGGGACTGATCCCATGATT
Smad2	XM_006254946	TACCACTCTCTCCCCTGTCAAT	GCAAACCTAAGCAGAACCCTCTC
Smad3	NM_013095	CTGGCTACCTGAGTGAAGATG	TGTGAAGCGTGAATGTCTC
Smad7	XM_006254959	CCCTGGGGGGCTTTTCAGATT	CGGACTTGATGAAGATGGGG
YAP	NM_001394328	CTGGAGCAGGATGGAGGGA	CACGCTGTTTCAGGAAGTCGTC
TAZ	XM_039099924	ATTGGACGGCTGATTGCTGAGTG	GTAGGGTGGACTGTTAGGGAGGAC
CTGF	NM_022266	CGCCAACCGCAAGATTG	ACACGGACCCACCGAAGAC
GADPH	NM_017008	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAAGCTT

$\alpha$ -SMA, alpha-smooth muscle actin; TGF- $\beta$ , transforming growth factor-beta; Smad2, SMAD family member 2; Smad3, SMAD family member 3; Smad7, SMAD family member 7; YAP, Yes-associated protein; TAZ, transcriptional activator with PDZ-binding motif; CTGF, connective tissue growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Liver biopsies and pathological examinations were performed in 28 patients who underwent paired liver biopsy at baseline and after 48 weeks of treatment, including 14 cases treated with NAs alone and 14 cases treated with NAs plus YQHX (Table 2). The stages of fibrosis in both groups at baseline and after 48 weeks of treatment were determined according to liver histopathology or a liver stiffness measurement (LSM).

#### Measurement of YAP and $\alpha$ -SMA levels in plasma and liver tissue samples

Blood samples were collected from healthy subjects and each patient at baseline, 24 and between 48 to 96 weeks of treatment in tubes containing ethylenediamine tetraacetic acid (EDTA). Plasma levels of YAP and  $\alpha$ -SMA were determined using enzyme-linked immunosorbent assay (ELISA) kits (ZC-32143, Shanghai Zcibio Technology Co, Ltd, Shanghai, China), according to manufacturer's instructions. All liver biopsy specimens were processed for H&E staining, Masson's trichrome staining and IHC staining of alpha-smooth muscle actin ( $\alpha$ -SMA; 1:200; Abcam, Cambridge, UK) and YAP (1:100; Proteintech, Wuhan, China). Histological staging of liver fibrosis was carried out by three specialists according to METAVIR and Ishak scoring systems, as well as a semiquantitative scoring system (Wang et al., 1998).

This study was performed in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of the Third hospital of Hebei Medical University. Written informed consent was obtained from all participants.

#### Statistical analysis

Statistical analysis was performed using SPSS version 24.0 (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as mean $\pm$ standard deviation (SD) or median (interquartile range). One-way analysis of variance (ANOVA) and least significant difference (LSD) test were used to evaluate differences between groups. Frequency data were expressed as numbers with percentages and were compared using the Chi-squared

test or Fisher's exact test. Spearman's correlation analysis was performed to evaluate the relationship between plasma YAP and  $\alpha$ -SMA. All reported P-values were two-sided, and P-values<0.05 were considered statistically significant.

## Results

### YQHX treatment alleviated liver fibrosis in rats

The effects of YQHX on CCl<sub>4</sub>-induced liver fibrosis were initially examined in rats. Histological analysis with H&E staining revealed morphological changes of liver tissues following CCl<sub>4</sub> treatment in the model group compared with the control group, including hepatic steatosis and cell enlargement, accompanied by cytolysis, necrosis, inflammatory cell infiltration, fibrosis, and pseudolobules (Fig. 1A). Notably, the histological features were markedly altered in the YQHX treatment group compared with the model group, including a significant reduction in fat vacuoles, reduced amount of fibrous hyperplasia around veins and occasional lymphocytic infiltration. In addition, Masson staining of rat liver tissues revealed that CCl<sub>4</sub>-induced hepatic fibrosis in the model group was characterized by obvious deposition of collagen fibers in the central vein and portal area of liver tissues, and the shape and structure of liver lobules were altered due to the formation of pseudolobules. It was noted that the collagen fiber deposition was less in the YQHX treatment group versus the model group (Fig. 1A). According to the IHC assay,  $\alpha$ -SMA- and Col I-positive regions were considerably increased following injection of CCl<sub>4</sub> compared with the normal control group, while reduced in rats treated with YQHX (Fig. 1A-C). The mRNA and protein expression of  $\alpha$ -SMA and Collagen I in liver tissue were consistent with the above results (Fig. 1D-F).

### YQHX treatment suppressed activation of TGF- $\beta$ /Smad signaling pathway in rats

Considering that the TGF- $\beta$ /Smad signaling pathway is recognized to play a crucial role in the initiation, development, and resolution of liver fibrosis, the effects of YQHX treatment on the expression levels of the key genes in the pathway (TGF- $\beta$ 1, Smad2, Smad3, Smad7) were assessed. As shown in Figure 2A-D, IHC analysis revealed that TGF- $\beta$ 1, Smad2, and Smad3 protein levels were significantly elevated following CCl<sub>4</sub> treatment in the model group compared with the control group, which were significantly reduced with YQHX treatment. In parallel, qRT-PCR and western blot analyses showed that CCl<sub>4</sub> significantly up-regulated the mRNA and protein expression of TGF- $\beta$ 1, Smad2, and Smad3, but significantly down-regulated the mRNA and protein expression of Smad7 (Fig. 2E-G). Notably, YQHX treatment significantly abrogated the CCl<sub>4</sub>-mediated effects on TGF- $\beta$ 1, Smad2, Smad3, and Smad7. These

**Table 2.** Baseline characteristics of CHB patients treated with NAs or NAs+YQHX who underwent paired liver biopsies.

Characteristics	NAs	NAs + YQHX	T/ $\chi$ <sup>2</sup>	P
Gender (male: female)	9: 5	10: 4	0.164	1.000
Age (years; mean $\pm$ SD)	39 $\pm$ 10	41 $\pm$ 9	0.522	0.607
METAVIR score				
F4/F2-3	4 (28.6)	6 (42.9)	0.622	0.695
G3-4/G1-2	6 (42.9)	6 (42.9)	0.000	1.000
Interval time (years; mean $\pm$ SD)	1.1 $\pm$ 0.4	1.1 $\pm$ 0.4	-1.462	0.397

CHB, chronic hepatitis B; YQHX, Yiqi Huoxue; NAs, nucleoside/nucleotide analogues; SD, standard deviation.

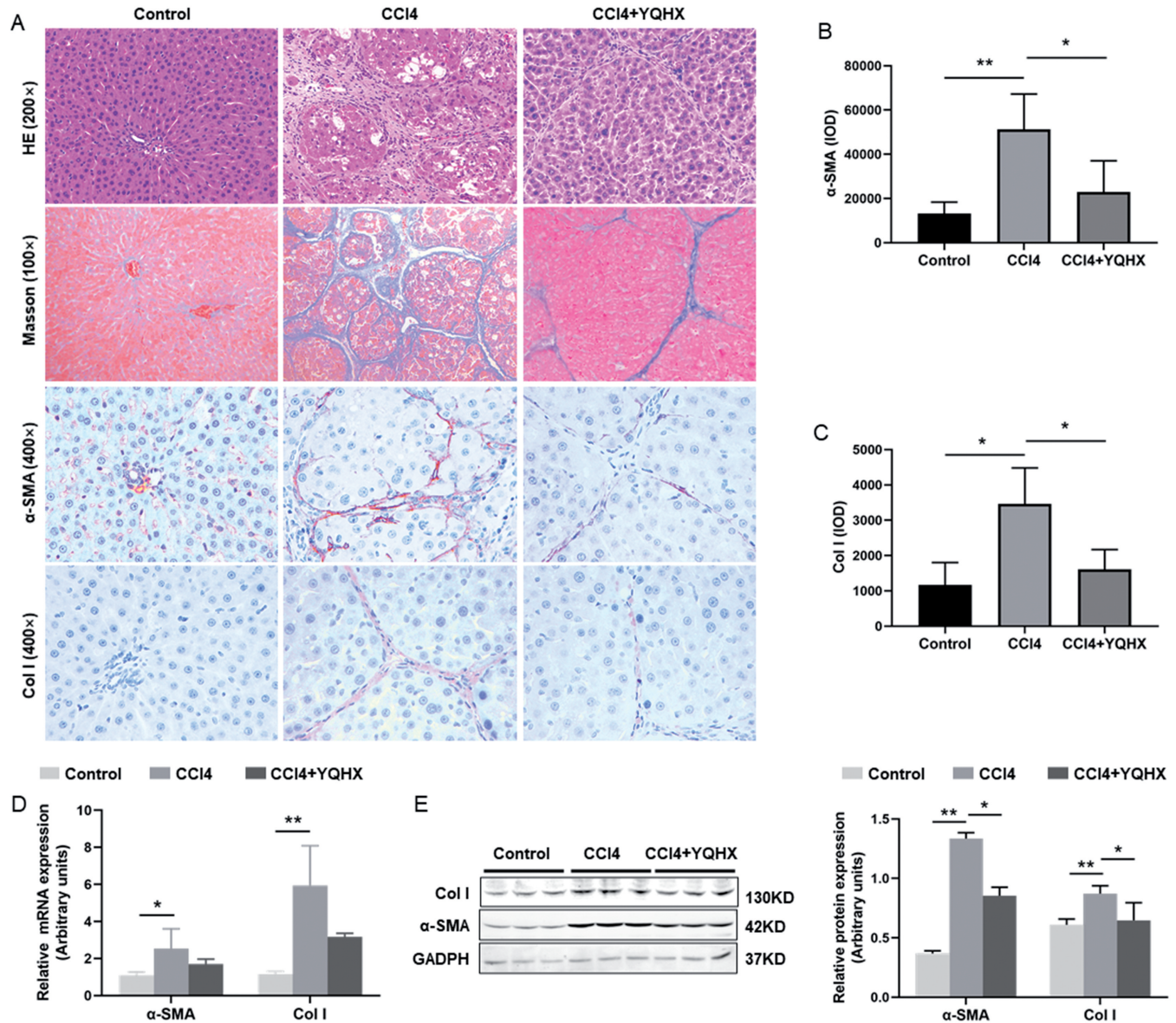
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data suggested that YQHX treatment suppressed the CCl<sub>4</sub>-mediated activation of the TGF-β/Smad signaling pathway in rats.

*Effect of YQHX on YAP/TAZ signaling pathway*

Intrigued by the anti-fibrotic effects of YQHX,

further studies were performed to elucidate the underlying molecular mechanisms of these effects. Recently, YAP has been shown to drive the activation of HSCs, thereby influencing liver fibrogenesis. Therefore, it was examined whether the YAP/TAZ signaling pathway could be involved in the anti-fibrotic activity of YQHX. IHC analysis showed that hepatic YAP was



**Fig. 1.** YQHX treatment inhibited HSC activation and collagen deposition in CCl<sub>4</sub>-induced liver fibrosis in rats. **A.** H&E staining (magnification, ×200), Masson's trichrome staining (×100), representative images of α-SMA and Col I immunohistochemical staining (×400). **B, C.** The quantitative results of IHC for α-SMA and Col I in liver tissues. **D.** qRT-PCR analysis of mRNA levels of hepatic α-SMA and Col I in rat. **E.** Western blot analysis of protein levels of hepatic α-SMA and Col I in rats. **F.** Quantification of α-SMA and Col I protein expression. Statistical analysis was performed by one-way ANOVA. \*P<0.05, \*\*P<0.01. Control group, normal liver tissues; CCl<sub>4</sub> model group, CCl<sub>4</sub>-induced liver fibrosis; YQHX group, CCl<sub>4</sub>-induced fibrosis plus YQHX treatment. CCl<sub>4</sub>, carbon tetrachloride; YQHX, Yiqi Huoxue; IHC, immunohistochemistry; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; mRNA, messenger RNA; H&E, hematoxylin and eosin; α-SMA, alpha-smooth muscle actin; Col I, collagen I; ANOVA, analysis of variance.

localized to round nuclei of biliary epithelial cells in portal tracts in the normal control group (Fig. 3A). As expected, hepatic YAP was markedly increased in perisinusoidal cells in the liver lobules and myofibroblast cells located in the fibrotic areas after  $\text{CCl}_4$  treatment in the model group compared with the control group, and these changes were significantly mitigated with YQHX treatment (Fig. 3A,B). Furthermore, qRT-PCR and western blot analyses showed that  $\text{CCl}_4$  injection significantly increased the mRNA and protein expression of YAP, TAZ, and CTGF. Notably, YQHX treatment significantly abrogated the  $\text{CCl}_4$ -mediated up-regulation of YAP, TAZ, and CTGF (Fig. 3C-E). The results implicated that YQHX may exert its anti-fibrotic activity through the inhibition of the YAP/TAZ signaling pathway.

#### YQHX treatment alleviated liver fibrosis in CHB patients

With these interesting findings in rats, the effects of YQHX on liver fibrosis and the potential molecular mechanisms were further examined in CHB patients. A total of 28 CHB patients underwent liver biopsies, including 14 paired samples in the NAs group and 14 paired samples in the NAs+YQHX group at baseline and after the 48-week treatment. As shown in Fig. 4 and Table 2, there was no significant difference in the stage of fibrosis or necroinflammation between the two groups at baseline. Fibrosis and inflammation were improved after 48 weeks of treatment in both groups, but NAs+YQHX had a significantly better therapeutic effect compared to NAs alone (Fig. 4A,B). According to the METAVIR scoring system, improvement in fibrosis was achieved in 10/14 patients (71.4%) of the NAs+YQHX group, but only 4/14 patients (28.6%) in the control NAs group, though this difference did not quite reach statistical significance ( $P=0.057$ ; Fig. 4C). According to the Ishak fibrosis scoring system, the NAs group reported 14.3% with a one-point decrease and 14.3% with a two-point decrease, whereas the NAs+YQHX group showed 35.7% with a one-point reduction and 35.7% with a two-point reduction when the first and second biopsies were compared over the entire paired

study period (Fig. 4E). Among patients with liver cirrhosis, 66.7% (4/6) of patients in the NAs+YQHX group achieved reversal of cirrhosis after the 48-week treatment, which was higher than the 25% (1/4) found in the NAs group. Notably, there were three patients (21.4%) who had worsened Ishak fibrosis score in the NAs group and one patient (7.1%) in the NAs+YQHX group. Based on the semi-quantitative fibrosis scoring system, the mean change from baseline was a 4.6-point reduction after 48 weeks of treatment with NAs+YQHX versus a mean 2.7-point reduction with NAs alone ( $P=0.028$ ; Fig. 4G). There was a significant decrease in both groups regarding the semi-quantitative necroinflammatory score during treatment ( $P$  values all  $<0.05$ , Fig. 4H). Furthermore, 9/14 patients (64.3%) in the NAs+YQHX group and 11/14 patients (78.6%) in the NAs group had improved METAVIR necroinflammatory grades at week 48 compared to the baseline biopsy ( $P=0.678$ , Fig. 4D). There was also no significant difference regarding the decrease of inflammatory scores between the NAs and combination groups based on the Ishak scoring system (Fig. 4F).

#### Effects of YQHX treatment on hepatic and plasma levels of YAP and $\alpha$ -SMA in CHB patients with liver fibrosis

Next, the effects of YQHX treatment on hepatic and plasma levels of YAP and  $\alpha$ -SMA were investigated in CHB patients with liver fibrosis. As shown in Figure 5, the IHC assay of  $\alpha$ -SMA and YAP in liver tissues was performed in 28 CHB patients who underwent paired liver biopsies. The results showed that  $\alpha$ -SMA-stained areas and YAP-positive cells in the liver tissues were markedly decreased after 48 weeks of treatment in both groups compared to baseline levels ( $P$  values all  $<0.001$ , Fig. 5C,D,F,G). Notably, the NAs+YQHX group exhibited significantly greater decreases in  $\alpha$ -SMA expression and the number of YAP-positive cells compared to the NAs group ( $P<0.001$ , Fig. 5E,H).

Furthermore, there was no significant difference at baseline between the two groups in terms of plasma  $\alpha$ -SMA and YAP levels (Table 3). The plasma levels of YAP were significantly elevated in CHB patients when

**Table 3.** Baseline characteristics of CHB patients treated with NAs alone and NAs+YQHX.

Parameter	NAs n=60	NAs+YQHX n=110	T/Z/ $\chi^2$	P
Gender (male), n (%)	37 (61.7)	74 (67.3)	0.538	0.463
Age (years; mean $\pm$ SD)	46 $\pm$ 10	47 $\pm$ 13	-0.247	0.805
HBV DNA (10g10 copies/mL; mean $\pm$ SD)	5.3 $\pm$ 1.1	5.1 $\pm$ 0.9	-0.345	0.579
ALT (U/L; median, P25, P75)	45 (34, 69)	42 (31, 57)	-0.056	0.378
AST (U/L; median, P25, P75)	40 (29, 58)	38 (32, 55)	-1.234	0.597
LSM (kPa; median, P25, P75)	14.3 (9.4, 20.3)	15.1 (10.8, 22.1)	-1.340	0.380
$\alpha$ -SMA (pg/mL; mean $\pm$ SD)	271.8 $\pm$ 36.9	278.2 $\pm$ 39.5	-0.983	0.327
YAP (ng/mL; mean $\pm$ SD)	7.22 $\pm$ 0.68	7.31 $\pm$ 0.87	-0.628	0.531

CHB, chronic hepatitis B; NAs, nucleoside/nucleotide analogues; YQHX, Yiqi Huoxue; HBV, hepatitis B virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LSM, liver stiffness measure;  $\alpha$ -SMA, alpha-smooth muscle actin; YAP, Yes-associated protein.

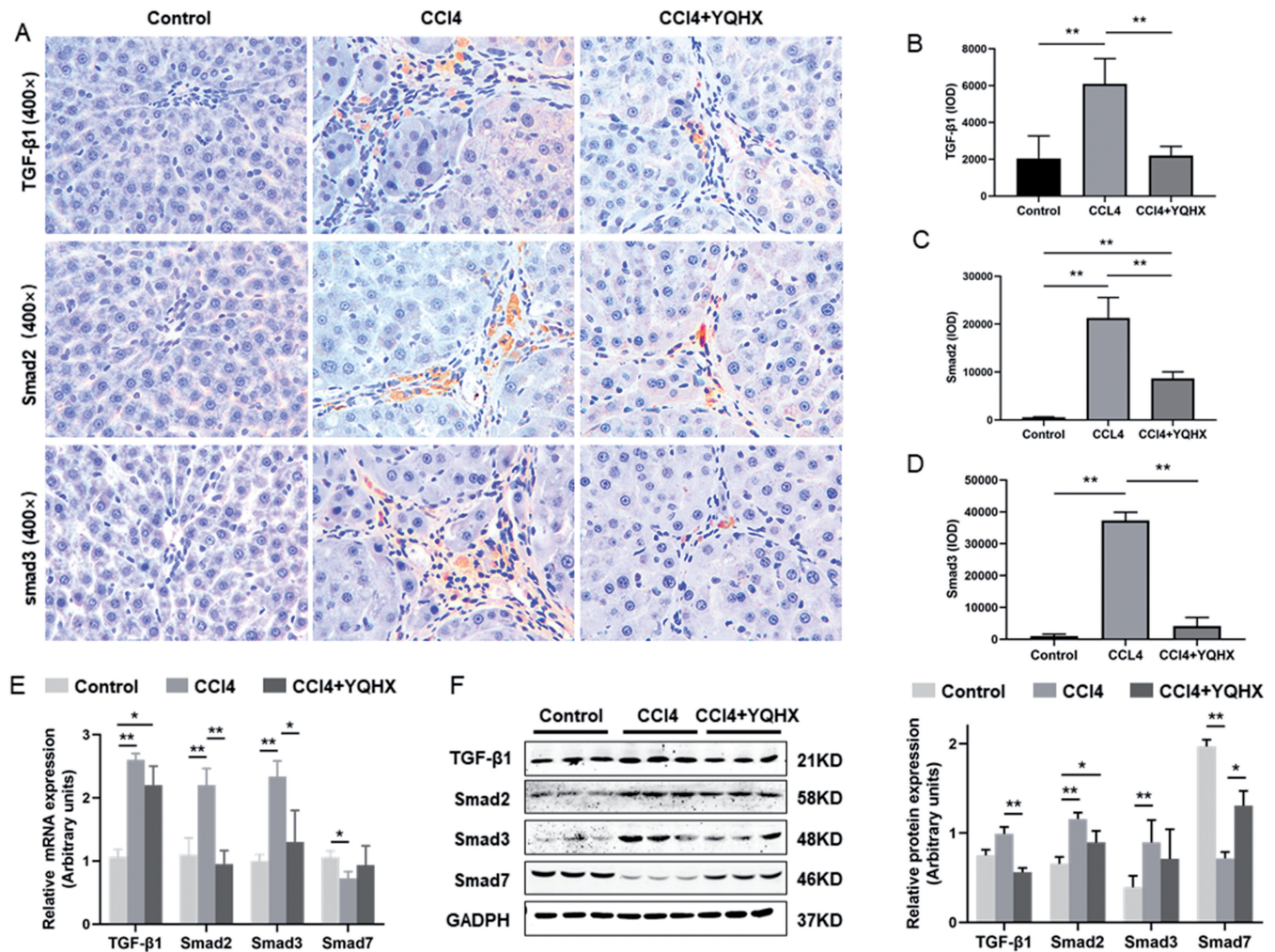
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compared to healthy controls ( $P$  values all  $<0.05$ ), and higher levels were associated with the progression of liver fibrosis (Fig. 6A). Correlation analysis revealed that plasma YAP levels were positively correlated with the levels of  $\alpha$ -SMA ( $r=0.735$ ,  $P<0.001$ , Fig. 6B). NAs and NAs+YQHX treatments for 24 and 48-96 weeks significantly decreased  $\alpha$ -SMA levels ( $P$  values all  $<0.01$ , Fig. 6C,D). Plasma YAP levels were reduced after 24 and 48-96 weeks of NAs+YQHX treatment and 48-96 weeks of NAs treatment alone (Fig. 6E,F). A greater decrease in plasma YAP levels was observed in patients treated with NAs+YQHX for 24 and 48-96 weeks, and the decrease in  $\alpha$ -SMA levels was greater in the NAs+YQHX group at 24 weeks of treatment ( $P$  values

all  $<0.05$ , Fig. 6G,H). These results in humans provided further evidence to support that YQHX may exert its anti-fibrotic effect through the mediation of the YAP/TAZ signaling pathway.

## Discussion

The major novel findings of this study are summarized as follows: (1) YQHX attenuated  $\text{CCl}_4$ -induced liver fibrosis in rats and CHB-related liver fibrosis in human patients; (2) YQHX exerted inhibitory effects on the activation of the TGF- $\beta$ /Smad pathway in  $\text{CCl}_4$ -induced liver fibrosis in rats; (3) YQHX abrogated the  $\text{CCl}_4$ -mediated up-regulation of key molecules



**Fig. 2.** YQHX treatment suppressed the activation of TGF- $\beta$ /Smad signaling in the  $\text{CCl}_4$ -induced rat model of liver fibrosis. **A.** IHC staining of TGF- $\beta$ 1, Smad2 and Smad3 in liver sections from Wistar rats ( $\times 400$ ). The quantitative results of IHC for TGF- $\beta$ 1 (**B**), Smad2 (**C**), Smad3 (**D**) in the rat liver tissues. **E.** qRT-PCR analysis of mRNA levels of hepatic TGF- $\beta$ 1, Smad2, Smad3, and Smad7. **F.** Western blot analysis of hepatic TGF- $\beta$ 1, Smad2, Smad3, and Smad7 protein expression in rats. **G.** Quantification of TGF- $\beta$ 1, Smad2, Smad3, and Smad7 protein expression. Statistical analysis was performed by one-way ANOVA. \* $P<0.05$ , \*\* $P<0.01$ . Control group, normal liver tissues;  $\text{CCl}_4$  model group,  $\text{CCl}_4$ -induced liver fibrosis; YQHX group,  $\text{CCl}_4$ -induced fibrosis plus YQHX treatment.  $\text{CCl}_4$ , carbon tetrachloride; YQHX, Yiqi Huoxue; IHC, immunohistochemistry; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; mRNA, messenger RNA; TGF- $\beta$ , transforming growth factor beta; ANOVA, analysis of variance.

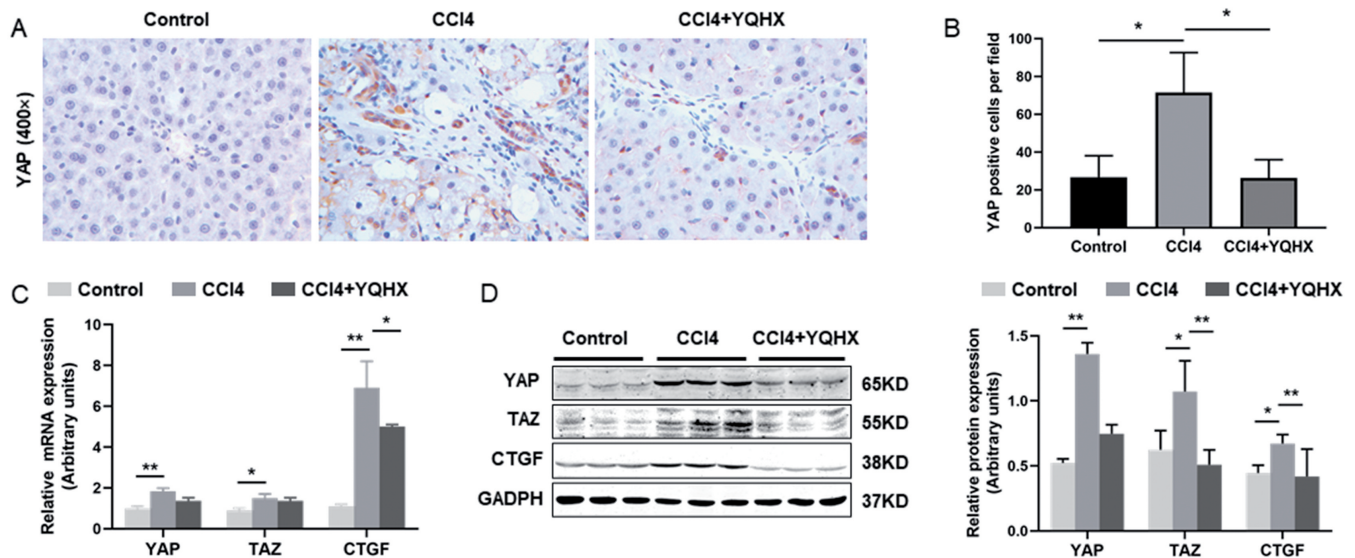
involved in YAP/TAZ signaling in rats with liver fibrosis; and (4) hepatic and plasma YAP levels were decreased in response to YQHX in CHB patients with liver fibrosis. These major findings suggested that YQHX attenuated hepatic fibrosis via YAP/TAZ signaling.

TCM has long been used to treat liver diseases, including liver fibrosis. At present, numerous TCM compounds have achieved promising therapeutic effects in clinical practice for the treatment of liver fibrosis (Liu et al., 2018; Song et al., 2018; Wang et al., 2018; Xia et al., 2018). The YQHX recipe contains extracts of Chinese herbs, including *Radix Astragali*, *Salviae miltiorrhizae*, *Radix Curcumae*, *Radix Paeoniae Rubra*, and some other herbs. Previous studies have shown that Huangqi Tang significantly enhanced the expression of hepatocyte growth factor in the liver tissues, and inhibited TGF- $\beta$ /Smad signal transduction (Du et al., 2012; Liu et al., 2012). A 1:1 mixture of *Radix Astragali* extracts and *Salviae miltiorrhizae* extract was shown to attenuate liver fibrosis through the regulation of TGF- $\beta$ 1 and cyclin D1 expression (Cao et al., 2020). In addition, *Astragalus* and *Radix Paeoniae Rubra* extract exerted inhibitory effects on cell proliferation and activation of HSCs, and the underlying mechanisms may involve the TGF- $\beta$ /Smad signaling pathway (Huang et al., 2015). In animal studies, YQHX has been shown to have anti-fibrotic effects in the liver. Moreover, our previous study compared the anti-fibrotic effects of YQHX and Fuzheng Huayu decoction (FZHY), indicating that YQHX was similar to FZHY in terms of aspartate

aminotransferase recovery rates after a 48-week treatment (100% vs 93.8%,  $P > 0.05$ ) and was superior to FZHY in terms of improving liver fibrosis as graded by LSM (80.0% vs 63.6%,  $P = 0.046$ ) (Cui et al., 2020). However, the mechanisms whereby YQHX exerts its anti-fibrotic activity remain largely unknown. To date, the potential role of YAP/TAZ signaling has not been investigated regarding the anti-fibrotic effect of YQHX. This is the first study, to the best of our knowledge, showing that YAP/TAZ signaling may play a role, at least in part, in the anti-fibrotic effects of YQHX.

In this study, the effects of YQHX on liver fibrosis and potential mechanisms were investigated in a well-validated model of CCl<sub>4</sub>-induced liver fibrosis in rats as well as in human patients with CHB-associated liver fibrosis. The rats were subcutaneously injected with olive oil containing 30% CCl<sub>4</sub> for modeling. After eight weeks, characteristic morphological changes of liver fibrosis, including collagen fiber deposition and inflammatory cell infiltration were detected in the liver, which indicated that the liver fibrosis model induced by CCl<sub>4</sub> was successfully established. Compared with the CCl<sub>4</sub> model group, the accumulation of collagen fibers and inflammatory cells were decreased in response to YQHX treatment, suggesting that YQHX alleviated liver fibrosis. In addition, the mRNA and protein expression of  $\alpha$ -SMA and Col I, markers of the development of liver fibrosis, were elevated in the model group and decreased with YQHX treatment.

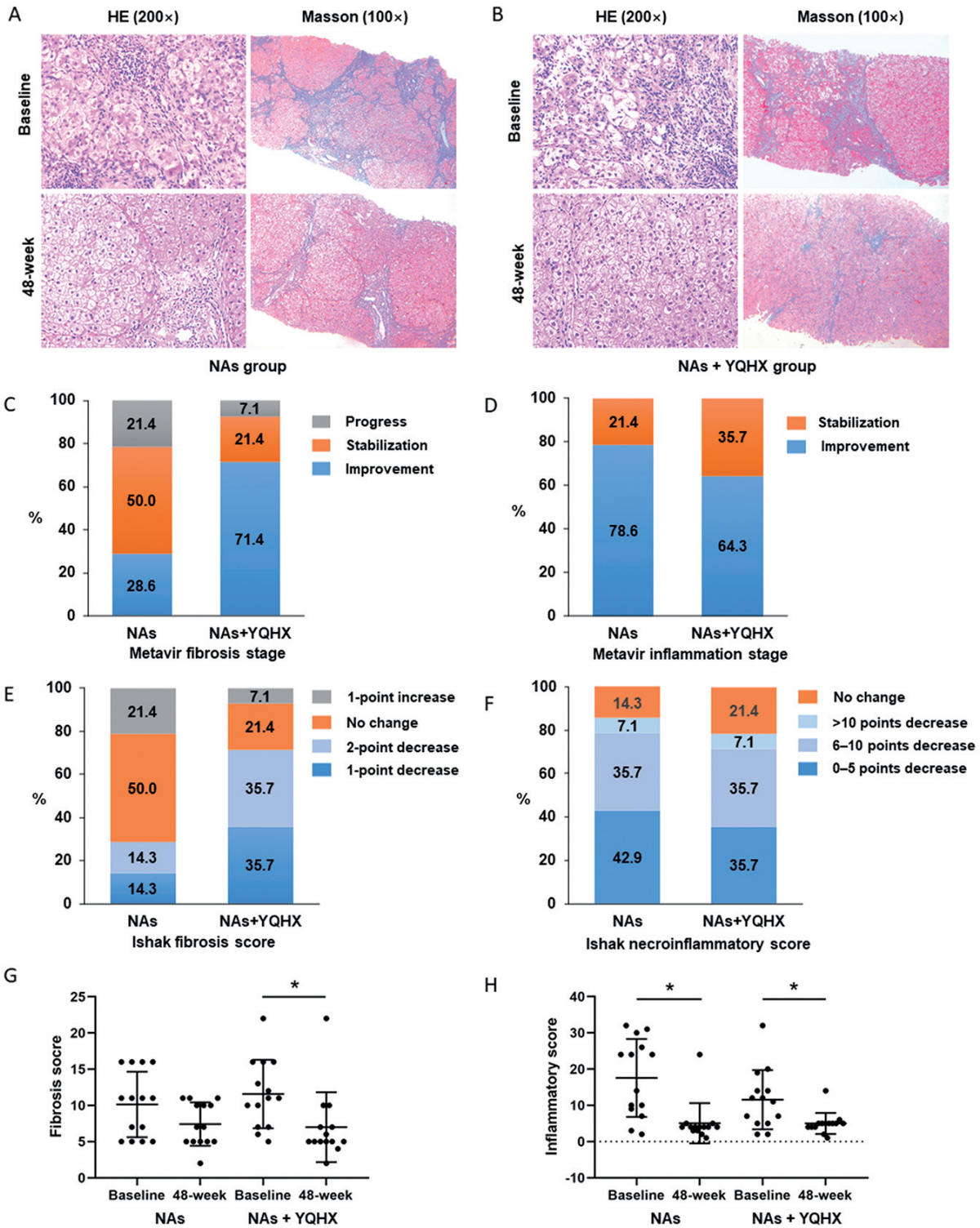
The TGF- $\beta$ /Smad signaling pathway plays a critical role in the pathogenesis of liver fibrosis, in which TGF-



**Fig. 3.** YQHX treatment abrogated the CCl<sub>4</sub>-mediated increases in YAP and TAZ expression in the rat model of liver fibrosis. **A.** IHC staining of YAP in the liver sections from the experimental rats ( $\times 400$ ). **B.** The quantitative results of IHC for YAP. **C.** qRT-PCR analysis of mRNA levels for hepatic YAP, TAZ and CTGF. **D.** Western blot analysis of hepatic YAP, TAZ and CTGF protein expression in rats. **E.** Quantification of YAP, TAZ, and CTGF protein levels. Statistical analysis was performed by one-way ANOVA. \* $P < 0.05$ , \*\* $P < 0.01$ . Control group, normal liver tissues; CCl<sub>4</sub> model group, CCl<sub>4</sub>-induced liver fibrosis; YQHX group, CCl<sub>4</sub>-induced fibrosis plus YQHX treatment; CCl<sub>4</sub>, carbon tetrachloride; YQHX, Yiqi Huoxue recipe; IHC: immunohistochemistry; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; mRNA, messenger RNA; YAP, Yes-associated Protein; TAZ, transcriptional coactivator with PDZ-binding motif; CTGF, connective tissue growth factor; ANOVA, analysis of variance.



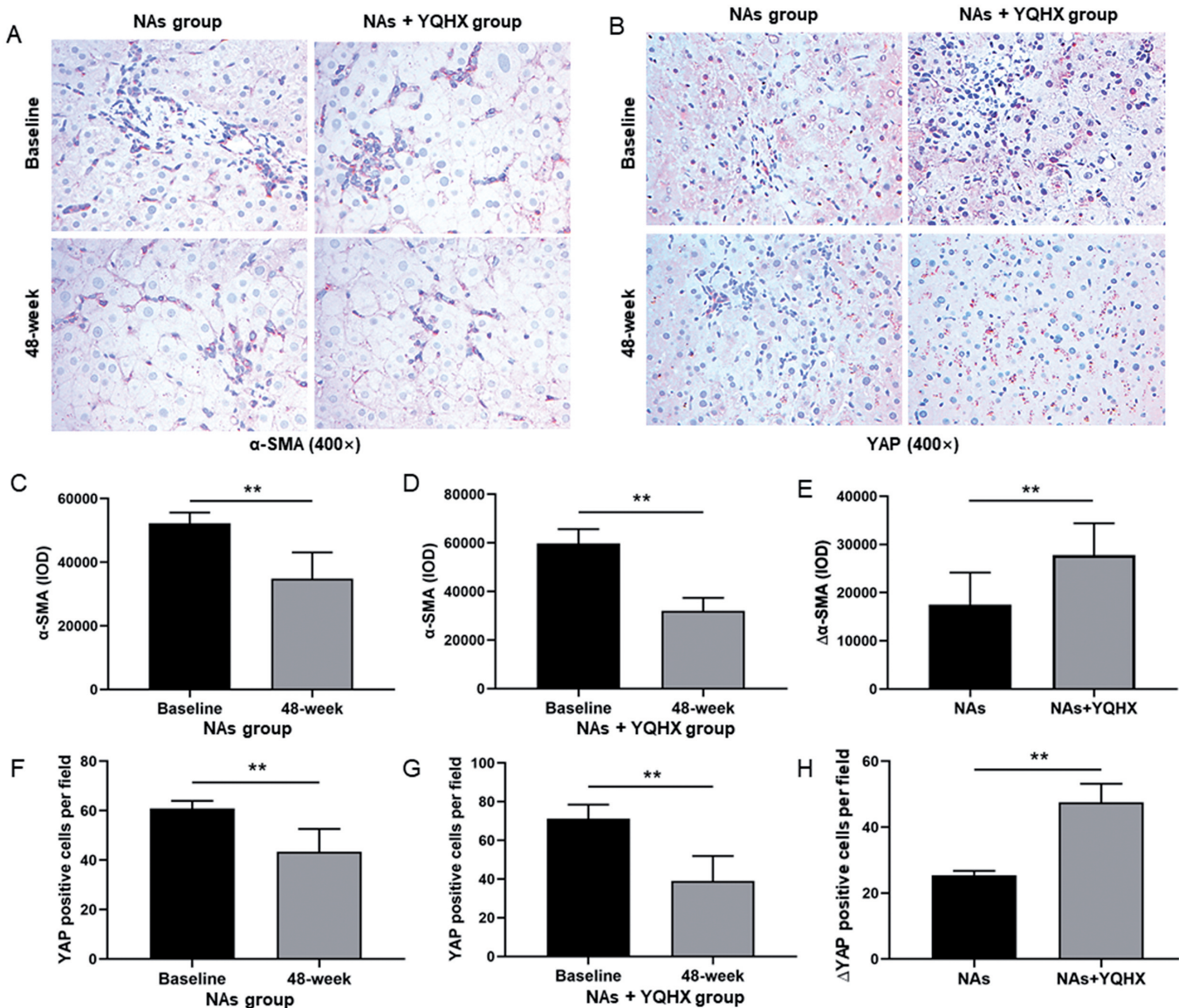
Yiqi Huoxue recipe attenuates liver fibrosis



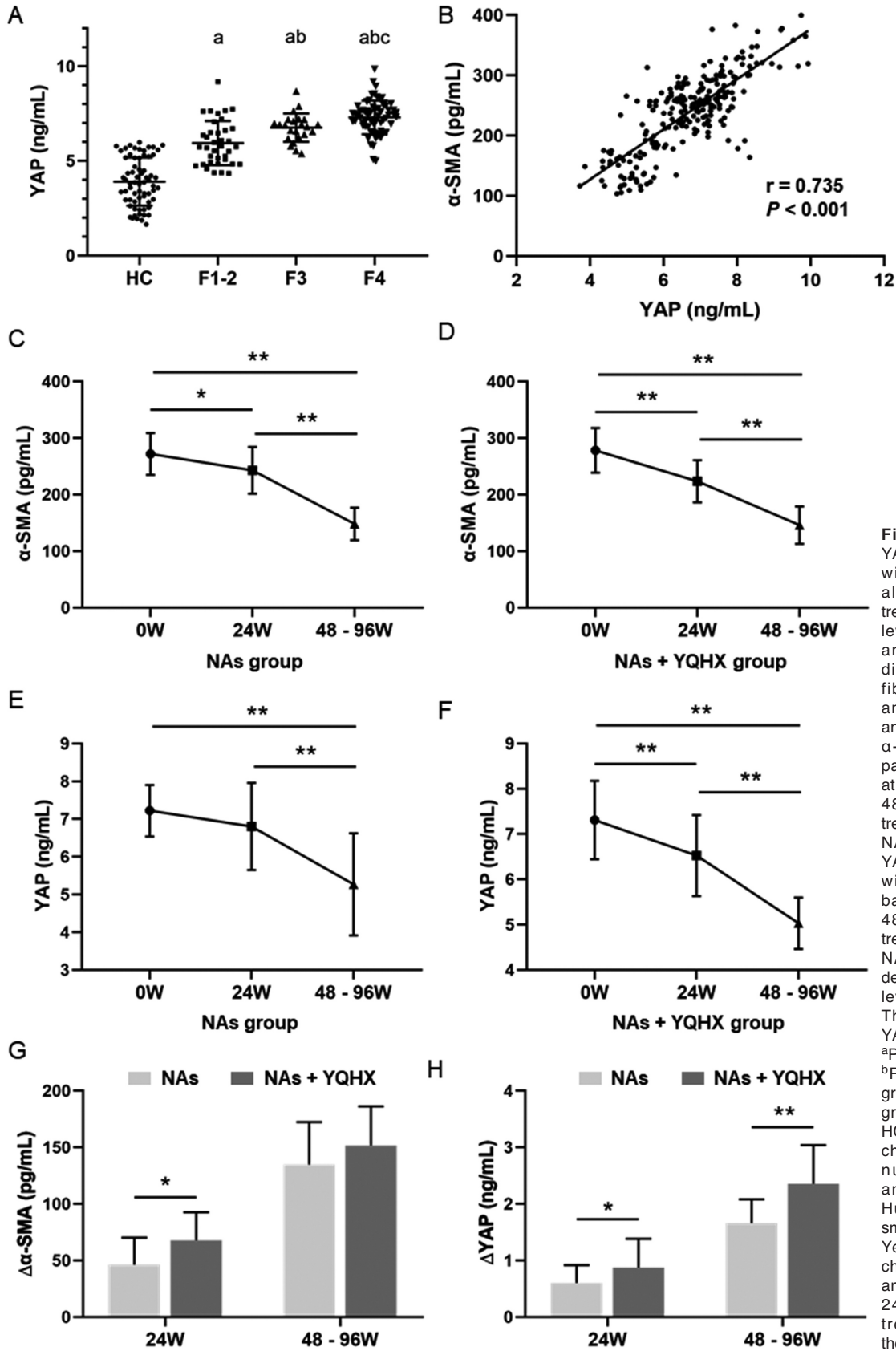
**Fig. 4.** Histological analysis of the liver tissues from CHB patients treated with NAs alone or NAs+YQHX. **A.** Representative images of H&E (× 200) and Masson's staining (× 100) of liver tissues in patients treated with NAs alone at baseline (upper panels) and after the 48-week treatment (lower panels). **B.** Representative images of H&E and Masson's staining of liver tissues in patients treated with NAs+YQHX at baseline (upper panels) and after the 48-week treatment (lower panels). Fibrosis and inflammation in the CHB patients treated with NAs alone or NAs+YQHX at baseline and after the 48-week treatment were assessed according to various scoring systems: METAVIR (**C-D**) scoring system; Ishak scoring system (**E-F**); and semi-quantitative histological scoring system (**G-H**). \*P<0.05. NAs group, CHB patients treated with NAs alone; NAs+YQHX group, CHB patients treated with NAs plus YQHX recipe. H&E: hematoxylin and eosin; NAs, nucleoside/nucleotide analogues; YQHX, Yiqi Huoxue; CHB, chronic hepatitis B.

$\beta 1$  directly activates HSCs. When TGF- $\beta 1$  binds to receptors on the cell membrane, Smad2 and Smad3 are activated. Phosphorylation of Smad2 and Smad3 encourages binding to Smad4 to form complexes that subsequently enter the nucleus to regulate the transcription of target genes (Xu et al., 2016). In the Smad family, Smad7 serves as the negative feedback regulator for TGF  $\beta$  signaling, acting to antagonize the

activity of the receptor regulated Smad proteins. In this study, the results showed that CCl<sub>4</sub> activated the TGF- $\beta$ /Smad signaling pathway by up-regulating the expression of TGF- $\beta 1$ , Smad2, and Smad3, and down-regulating the expression of Smad7. These findings were consistent with previous studies (Wu et al., 2017; Xia et al., 2018). Importantly, YQHX treatment inhibited the TGF- $\beta$ /Smad signaling pathway as evidenced by



**Fig. 5.** IHC of hepatic  $\alpha$ -SMA and YAP from CHB patients treated with NAs alone or NAs+YQHX. **A.** Representative images of IHC for hepatic  $\alpha$ -SMA (magnification,  $\times 400$ ) in patients treated with NAs (left) and NAs+YQHX (right) at baseline (upper panels) and after the 48-week treatment (lower panels). **B.** Representative images of IHC for hepatic YAP ( $\times 400$ ) in patients treated with NAs (left) and NAs+YQHX (right) at baseline (upper panels) and after the 48-week treatment (lower panels). The quantification of  $\alpha$ -SMA staining in the NAs group (**C**) and the NAs+YQHX group (**D**). **E.** The decrease of  $\alpha$ -SMA expression in both groups after the 48-week treatment; the number of YAP-positive cells in the NAs group (**F**) and the NAs+YQHX group (**G**). **H.** The reduction of YAP-positive cells in both groups after the 48-week treatment. \*\* $P < 0.01$ . NAs group, CHB patients treated with NAs alone; NAs+YQHX group, CHB patients treated with NAs and YQHX recipe. IHC, immunohistochemistry;  $\alpha$ -SMA, alpha-smooth muscle actin; YAP, Yes-associated protein; NAs, nucleoside/nucleotide analogues; YQHX, Yiqi Huoxue; CHB, chronic hepatitis B.  $\Delta$ : change of  $\alpha$ -SMA and YAP expression after the 48-week treatment compared to those at baseline.



**Fig. 6.** Elevated plasma YAP levels in CHB patients with liver fibrosis was alleviated by YQHX treatment. **A.** Plasma YAP levels in healthy controls and CHB patients with different stages of liver fibrosis. **B.** Correlation analysis of plasma YAP and  $\alpha$ -SMA levels; plasma  $\alpha$ -SMA levels of CHB patients with liver fibrosis at baseline, 24 weeks, and 48-96 weeks following treatment with NAs and **(C)** NAs+YQHX **(D)**. Plasma YAP levels of CHB patients with liver fibrosis at baseline, 24 weeks, and 48-96 weeks after treatment with NAs **(E)** and NAs+YQHX **(F)**. **G.** The decrease of plasma  $\alpha$ -SMA levels in both groups. **H.** The reduction of plasma YAP levels in both groups. <sup>a</sup>P<0.05 vs. the HC group; <sup>b</sup>P<0.05 vs. the F1-2 group; <sup>c</sup>P<0.05 vs. the F3 group. \*P<0.05, \*\*P<0.01. HC, healthy controls; CHB, chronic hepatitis B; NAs, nucleoside/nucleotide analogues; YQHX, Yiqi Huoxue;  $\alpha$ -SMA, alpha-smooth muscle actin; YAP, Yes-associated protein;  $\Delta$ : change of plasma  $\alpha$ -SMA and YAP expression after 24 or 48-96 weeks of treatment compared to those at baseline.

decreased levels of TGF- $\beta$ 1, Smad2, and Smad3, and increased Smad7 protein expression. These results proved that YQHX inhibited the activation of TGF- $\beta$ /Smad signaling induced by CCl<sub>4</sub>, thereby leading to anti-fibrotic effects in the liver.

Recently, YAP/TAZ signaling has been implicated in the pathogenesis of liver fibrogenesis, in which YAP serves as a mechano-transducer to promote the formation and deposition of ECM components (Dupont et al., 2011; Mannaerts et al., 2015). Activation of the Hippo signaling pathway results in phosphorylation of YAP, which causes it to remain in the cytoplasm. Unphosphorylated YAP controls gene expression by binding to transcriptional factor TEA domain (TEAD) in the nucleus, where it promotes the transcription of genes such as CTGF, secreted phosphoprotein 1, and TGF- $\beta$  (Zhao et al., 2008; Perumal et al., 2017). YAP/TAZ of the Hippo signaling pathway interacts with TGF- $\beta$ -induced Smad2/3 in the nucleus, resulting in the formation of the YAP/TAZ•TEAD•Smad2/3 complex, which regulates several transcription processes induced by TGF- $\beta$  (Liu et al., 2015; Szeto et al., 2016; Toyama et al., 2018). CTGF, a YAP target gene, is up-regulated in the fibrotic liver and in activated HSCs. A previous study has shown that CTGF induces the synthesis and secretion of ECM proteins, notably fibrillar collagens, which are a major component of fibrous deposits (Williams et al., 2000). In this study, the mRNA and protein expression of YAP, TAZ, and CTGF was found to be markedly increased following the eight-week CCl<sub>4</sub> exposure, and these effects were significantly abrogated by YQHX treatment in rats. IHC staining for YAP revealed that YAP-positive cells were mainly found in the lobules and fibrotic areas of the liver, and that positive regions were reduced with YQHX treatment. These data suggested that YQHX can protect against CCl<sub>4</sub>-induced liver fibrosis in rats via alleviation of HSC activation and degradation of ECM accumulation, and mechanistically this may be due to its ability to inhibit YAP/TAZ signaling pathways (Grannas et al., 2015; Nishio et al., 2016).

We extended our findings in rats to humans by studying CHB patients with liver fibrosis, in which 28 patients underwent liver biopsies at baseline and after the 48-week treatment. Notably, 71.4% of patients taking YQHX achieved improvement of liver fibrosis, which was higher than 28.6% in the control group ( $P=0.057$ ). Additionally, reversal of liver cirrhosis was achieved in up to 66.7% of patients in the NAs+YQHX group, considerably higher than the 25% in the NAs group. It was previously reported that, among the CHB patients treated with NAs for five years, 51-88% of patients showed improvement in liver fibrosis and 40-74% achieved cirrhosis reversal (Lok, 2013; Marcellin et al., 2013). However, the progression of HBV infection from chronic hepatitis to cirrhosis and even liver cancer is influenced by many factors, not only virus-specific characteristics but also host-related and other exogenous factors (Liaw, 2009). Although serum HBV DNA is

undetectable following long-term antiviral treatment, the disease still persists in some CHB patients (Sun et al., 2017). Hence, anti-fibrosis therapy is necessary. It may merit attention that NAs and YQHX in combination attenuated the condition in CHB patients with liver fibrosis. Furthermore, hepatic and plasma levels of YAP were significantly elevated in CHB patients with liver fibrosis, but significantly abrogated with YQHX treatment. Moreover, YAP expression in the serum and liver positively correlated with  $\alpha$ -SMA levels.

In conclusion, the findings have demonstrated that YQHX treatment attenuates liver fibrosis in rats and CHB patients. Furthermore, these data suggest that YQHX may exert its anti-fibrotic activity through inhibiting YAP/TAZ signaling. As such, the findings may contribute to a better understanding of the mechanisms by which YQHX exerts an anti-fibrotic effect during the treatment of liver fibrosis.

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*Author's contributions.* WZ was involved in study design, performed the experiments, and wrote the manuscript; XXZ, MMH, YGZ, and YHT performed the experiments and collected data for this study; LL, SMD, and LDL provided technical support; DDZ and WCL participated in revision of the manuscript; YMN designed and supervised the study, and participated in the revision of the manuscript.

*Data Availability.* The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

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*Conflicts of Interest.* The authors disclose no conflicts of interest in this research.

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