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Modulation of IKKβ/NF-κB and TGF-β1/Smad via Fuzheng Huayu recipe involves in prevention of nutritional steatohepatitis and fibrosis in mice

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ABSTRACT

Objective(s): Fuzheng Huayu recipe (FZHY) exerts significant protective effects against liver fibrosis by strengthening the body's resistance and removing blood stasis. However, the molecular mechanisms through which FZHY affects liver fibrosis are still unclear. In this study, we examined the expression levels of factors involved in the inhibitor κB kinase-β (IKK-β)/nuclear factor-κB (NF-κB) and transforming growth factor beta 1 (TGF-β1)/Smad signaling pathways to elucidate whether FZHY could attenuate nutritional steatohepatitis and fibrosis in mice.

Materials and Methods: C57BL/6J mice were fed with methionine-choline deficient (MCD) diet for 8 weeks to induce fibrotic steatohepatitis. FZHY and/or heme oxygenase-1 (HO-1) chemical inducer (hemin) were administered to mice. The effects of FZHY alone and in combination with hemin were assessed by comparing the severity of hepatic injury, activation of hepatic stellate cells (HSCs), and the expression of oxidative stress, inflammation and fibrogenesis related genes.

Results: Administration of FZHY, hemin and FZHY plus hemin significantly ameliorated liver injury. Additionally, our analysis indicated that administration of these agents significantly attenuated oxidative stress, downregulated the expression of pro-inflammatory and pro-fibrotic genes, including IKK-β, NF-κB, monocyte chemoattractant protein-1 (MCP-1), α-smooth muscle actin (α-SMA), TGF-β1, Smad3 and Smad4, and upregulated the expression of the antifibrogenic gene Smad7 ($P< 0.001$).

Conclusion: FZHY-containing therapies prevented nutritional steatohepatitis and fibrosis through modulating the expression of factors associated with the IKKβ/NF-κB and TGF-β1/Smad signaling pathways and oxidative stress related genes.

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Introduction

Non-alcoholic steatohepatitis (NASH) is now recognized as the most common cause of liver dysfunction worldwide. Active hepatic fibrogenesis is a crucial pathological process involved in the development of NASH due to its high prevalence and potential for severe hepatic outcomes, such as liver cirrhosis, liver failure, and hepatocellular carcinoma (1). From 2000 to 2010, the percentage of orthotopic liver transplants performed for NASH in the United States increased by 6.2% (2). Future projections estimate that in the next decade, NASH and related fibrosis will surpass viral hepatitis as the major cause of end-stage liver disease, becoming the primary indication for liver transplantation (3). According to the "two hit" hypothesis proposed by Day and James (4), the appearances of oxidative stress, overexpression of key pathogenic factors involved in the development of necro-inflammation and fibrosis, such as nuclear factor kappa B (NF-κB), tumor necrosis factor alpha (TNF α), interleukin 6 (IL-6), α-smooth muscle actin (α-SMA) and transforming growth factor beta 1 (TGF-β1) are potentially major therapeutic targets in steatohepatitis and advanced fibrosis. However, the precise cellular and biochemical mechanisms of NASH are not fully understood, and specific antifibrotic drug targets have not yet been identified. Thus, it is very important to explore the mechanisms of NASH pathogenesis, especially in order to identify novel effective and curative therapies for NASH and related fibrosis.

Fuzheng Huayu recipe (FZHY) is comprised of 6 traditional Chinese medicines: *Fructus Schisandrae chinensis* (Wuweizi), *Semen persicae* (Taoren), *Radix Salvia miltiorrhizae* (Danshen), *Fermentation Mycelium Powder* (Dongchongxiacao), *Pollen Pini* (Songhuafen), and *Gynostemma pentaphyllammak* (Jiaogulan) (5). This concoction functions to treat imbalanced bodily functions and is used to nourish the

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liver, dissolve blood stasis, and tonify the spirit, etc. (6). Previous studies have shown that FZHY exerts protective effects against liver fibrosis in animal models through inhibiting collagen synthesis and restoring mitochondrial dysfunction (7). However, the molecular mechanisms mediating the therapeutic effects of FZHY are still unclear. Oxidative stress is an essential "second hit" in the pathogenesis of NASH, because increased free radicals can cause lipid peroxidation, which is a significant aspect of oxidative damage and oxidative modification of phospholipid polyunsaturated fatty acid residues in membrane structure and then induces the inflammatory response and activation of stellate cells further promoting collagen synthesis and fibrogenesis (8, 9). Heme oxygenase-1 (HO-1) is a stress-responsive protein induced by various oxidative agents, and its induction is thought to be an adaptive cellular response to survive from exposure to environmental stresses. Our previous study demonstrated that upregulation of HO-1 provided a beneficial role in modulating oxidative stress, inflammatory insults and hepatic stellate cells (HSC) activation in livers with fibrosing steatohepatitis (10).

In the present study, we used the HO-1 chemical inducer hemin as a control drug to explore (1) whether FZHY plays a role in attenuating liver fibrosis *in vivo*, (2) the effects of FZHY on the expression of genes involved in the inhibitor-κB kinase-β (IKK-β)/NF-κB and TGF-β1/Smad signaling pathways, which also play critical roles in oxidative stress-induced steatohepatitis and fibrosis.

Materials and Methods

Animals and treatments

Eight-week-old male C57BL/6J mice (body weights, 20 to 25 g) were obtained from the Experimental Animal Center of the Chinese Academy of Medical Sciences and were bred in a temperature-controlled animal facility with a 12 hr light-dark cycle. They had free access to water and were allowed to adapt to their food and environment for 1 week before the start of the experiment. The mice were randomly divided into 5 groups (n = 6 mice per group): 1) MCD group, mice fed methionine-choline deficient diet (ICN, Aurora, Ohio); 2) control group, mice fed MCD diet supplemented with choline bitartate (2 g/kg) and DL-methionine (3 g/kg; ICN); 3) MCD + hemin group, mice fed MCD diet administered with hemin (30 μmol/kg) by intraperitoneal (IP) injection 3 times per week; 4) MCD + FZHY group, mice fed MCD diet supplemented with FZHY (15 g/kg/d, Huanghai Pharmaceutical Company Limited, Shanghai, China); 5) MCD + FZHY + hemin group, mice fed MCD diet combined with FZHY and hemin treatments, as described above. The experiment lasted up to 8 weeks (10, 11). During the experiment, body weights and rate of food intake were recorded. All animals were killed after overnight fasting at the end of the experiment. Livers were weighed and fixed in 10% formalin for histological analysis or snap-frozen in lipid nitrogen followed by storage at -80 ˚C until use. All protocols and procedures were performed following the guidelines of the Hebei Committee for the Care and Use of Laboratory Animals and were approved by the Animal Experimentation Ethics Committee of Hebei Medical University.

Histological analysis

Formalin-fixed livers were embedded in paraffin, and 4-μm sections were stained with hematoxylin and eosin and Masson-trichrome stains. Two experienced hepatopathologists independently evaluated the slides and assigned scores for hepatic steatosis, inflammation, and fibrosis. The stage of fibrosis was assessed using a 4-point scale (1, mild/moderate zone 3 perisinusoidal fibrosis or portal fibrosis only; 2, zone 3 and portal/periportal fibrosis; 3, bridging fibrosis; and 4, cirrhosis).

Determination of the mRNA expression levels of hepatic inflammation and fibrogenesis related genes

Total RNA was isolated from frozen liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The mRNA expression levels of HO-1, IKK-β, NF-κB, monocyte chemoattractant protein-1 (MCP-1), Smad3, Smad4, and Smad7 were determined by reverse transcription semiquantitative polymerase chain reaction (RT-PCR) in a 25 μl reaction volume using Promega Green Master Mix (Promega, San Luis Obispo, CA, USA) on an ABI Prism 2720 instrument (Applied Biosystems, Foster City, CA, USA). Expression levels of the target genes were normalized against the endogenous reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Specific primers for HO-1, IKK-β, NF-κB, MCP-1, Smad3, Smad4, and Smad7 were designed using Primer Express 2.0 (Table 1).

Table 1. Primers for real-time quantitative PCR analysis

Gene	Product length (bp)	Primer sequences
HO-1	427	F 5′-AACAAGCAGAACCCAGTCTATG-3′ R 5′-TGAGCAGGAAGGCGGTCTTA-3′
IKK-β	308	F 5′-GGACTTCTTCAAAACCAGCATC-3′ R 5′-CACCTTCTGTCCTTTGGTCTCT-3′
NF-κB (P65)	168	F 5′-GCGAGAGAAGCACAGATACCA-3′ R 5′-GGTCAGCCTCATAGTAGCCAT-3′
MCP-1	194	F 5′-AGGCTGGAGAGCTACAAGAGGA-3′ R 5′-GACCTTAGGGCAGATGCAGTTT-3′
Smad3	85	F 5′-CCTACTGTCCAATGTCAACCG -3′ R 5′-CTCTCCCAATGTGTCGCCT-3′
Smad4	99	F 5′-GTTGTGACTGTGGATGGCTATG-3′ R 5′-CGCTCTCTCAATCGCTTCTG-3′
Smad7	145	F 5′-TCCTCCTTACTCCAGATACCCA-3′ R 5′-CCCAGGCTCCAGAAGAAGTT-3′
GAPDH	120	F 5′-TGAACGGGAAGCTCACTGG-3′ R 5′-GCTTCACCACCTTCTTGATGTC-3′

Abbreviations: HO-1, heme oxygenase-1; IKK-β, inhibitor of nuclear factor κB kinase-β; NF-κB, nuclear factor kappa B; MCP-1, monocyte chemoattractant protein-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Immunohistochemical evaluation of α-SMA and TGF-β1 expression

Immunostaining for α-SMA and TGF-β1 was performed in paraffin-embedded liver sections using specific antibodies recognizing α-SMA and TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an avidin-biotin complex (ABC) immunoperoxidase. Briefly, endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide. After blocking with 10% non-immunized rabbit serum, primary antibodies targeting α-SMA (dilution 1:100) and TGF-β1 (dilution 1:200) were applied. Primary antibodies were omitted, and non-immunized goat serum was used as a negative control. After extensive rinsing, the biotinylated secondary antibody and ABC complex/horseradish peroxidase (HRP) were applied. Peroxidase activity was visualized with diaminobenzidine. The sections were then counterstained with hematoxylin. Quantitative analysis of α-SMA- and TGF-β1-stained liver sections was performed by morphometric analysis.

Western blotting analysis of hepatic protein expression

Total protein was extracted from liver tissues, and the protein concentrations were measured by the Bradford method (DC Protein Assay; Bio-Rad, Hercules, CA, USA) as previously described (12). Equal amounts of protein from each sample (100 μg/well) were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels, and proteins were then transferred onto equilibrated polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, UK) by electroblotting. The membranes were incubated with primary antibodies targeting Smad3, Smad4 or Smad7 (Santa Cruz Biotechnology) overnight at 4°C. Membranes were further incubated with secondary antibodies for 1 hr at room temperature. Proteins were detected by enhanced chemiluminescence (ECL; Amersham Corporation, Arlington Heights, CA, USA), and bands were quantified by scanning densitometry using a digital Kodak Gel Logic 200 system (Carestream Molecular Imaging, USA). β-actin was used as a loading control for protein normalization.

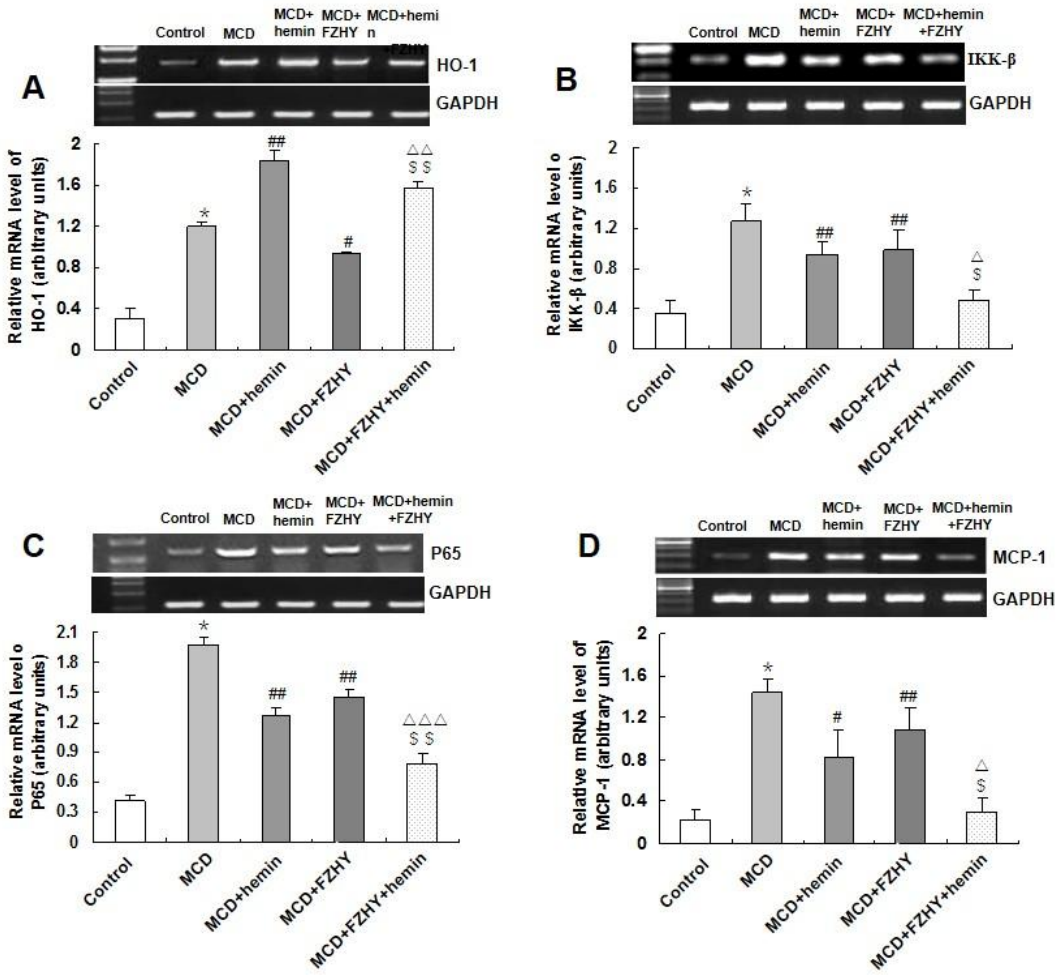

Figure 1. Effects of FZHY and/or hemin on HO-1 and hepatic inflammatory factors in MCD diet-induced fibrosing steatohepatitis. The expression levels of HO-1 (A), IKK-β (B), NF-κB p65 (C), and MCP-1 (D) mRNAs were determined by RT-PCR. Data are expressed as the mean±SD (n=6 per group). *P < 0.001, compared with the control group; $^{\#}P$<0.01, $^{\#\#}P$< 0.001, compared with the MCD group; $^{\$}P$<0.05, $^{\$\$}P$<0.001, compared with the MCD+FZHY group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01, $^{\triangle\triangle\triangle}P$<0.001, compared with the MCD+hemin group

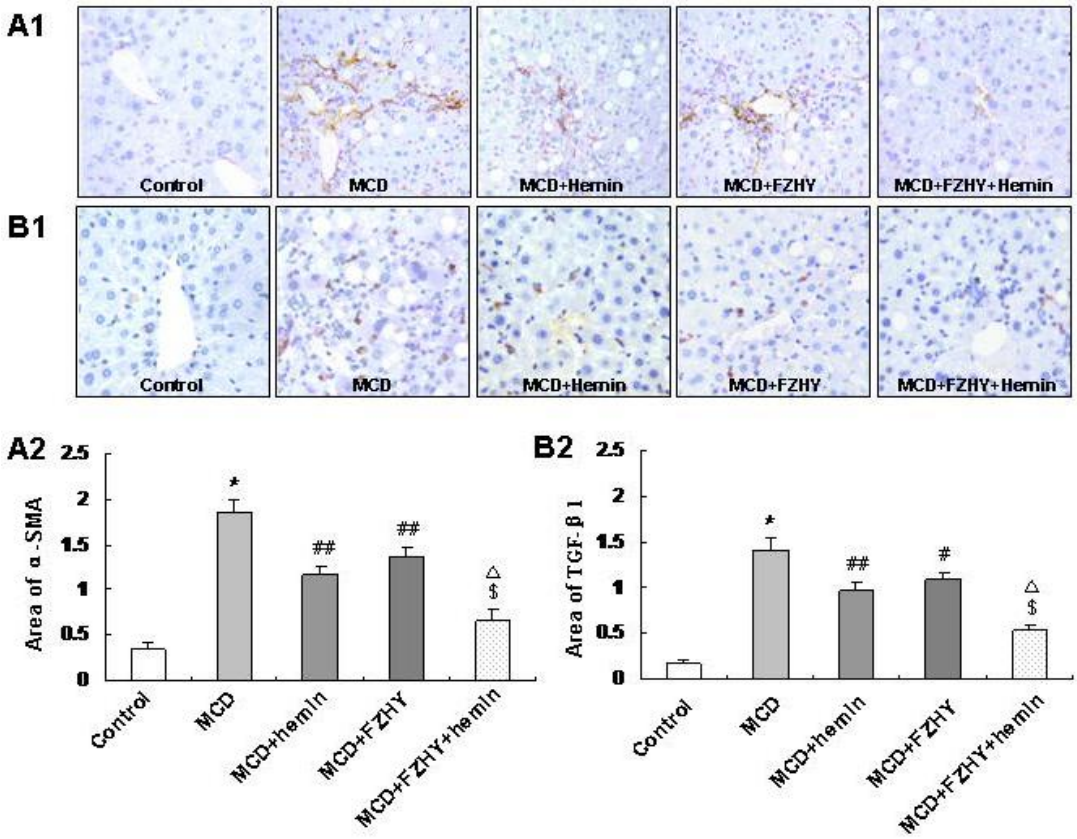

Figure 2. Effects of FZHY and/or hemin on hepatic α-SMA and TGF-β1 expression in MCD diet-induced fibrosing steatohepatitis. Protein levels of α-SMA (A) and TGF-β1 (B) were detected by immunohistochemistry staining. The expression levels of α-SMA and TGF-β1 were estimated by determining the average area density (area of positive cells/total area). Original magnification, 200×. Data are expressed as the mean±SD (n = 6 per group). **P*<0.001, compared with the control group; #*P*<0.01, ##*P*<0.001, compared with the MCD group; $*P*<0.001, compared with the MCD+FZHY group; △*P*<0.001, compared with the MCD+hemin group

Statistical analysis

All data are presented as means±standard deviations (SDs). Statistical analysis was performed by one-way analysis of variance (ANOVA) and Student-Newman-Keuls test for evaluating differences between groups using SPSS 13.0 (v. 13.0; SPSS Inc., Chicago, IL, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

Effect of FZHY on hepatic inflammation and fibrosis in mice fed with MCD diet

The liver sections from mice fed an MCD diet for 8 weeks exhibited severe macrosteatosis, spot or focal hepatocyte necrosis, inflammatory infiltration, portal fibrosis and fibrous septum. FZHY with or without hemin administration could significantly ameliorate the severity of liver injury, which have been demonstrated in our previous study (7).

FZHY attenuated hepatic oxidative stress and inhibited the expression of IKK-β/NF-κB signaling pathway genes in mice fed with MCD diet

To determine the mechanisms mediating the effects of FZHY on the liver, we investigated the mRNA expression levels of NF-κB-dependent inflammatory genes in the liver tissues, including IKK-β, NF-κB p65, MCP-1 and HO-1 (an anti-oxidative stress factor). Relative to control mice, mice fed MCD diet exhibited upregulation of hepatic IKK-β, NF-κB p65, MCP-1 and HO-1 genes (*P*< 0.001, Figure 1). Additionally, administration of FZHY decreased the expression of IKK-β, NF-κB p65 and MCP-1 mRNAs. Concomitant with the downregulation of inflammatory genes and improvement of liver histology, the expression of HO-1 was also reduced, indicating a reduction in oxidative stress-mediated liver injury.

FZHY suppressed the activation of hepatic stellate cells (HSCs) in MCD diet-induced fibrotic steatohepatitis

Next, we evaluated the role of FZHY in the development of liver fibrosis by assessing the hepatic expression of α-SMA, a well-known marker of activated HSCs. As shown in Figure 2A, increased α-SMA expression in activated HSCs, fibrotic areas, and vessel walls was observed in liver sections of MCD-treated mice, while little α-SMA was deposited in liver sections from control mice. FZHY and/or hemin treatment significantly suppressed α-SMA

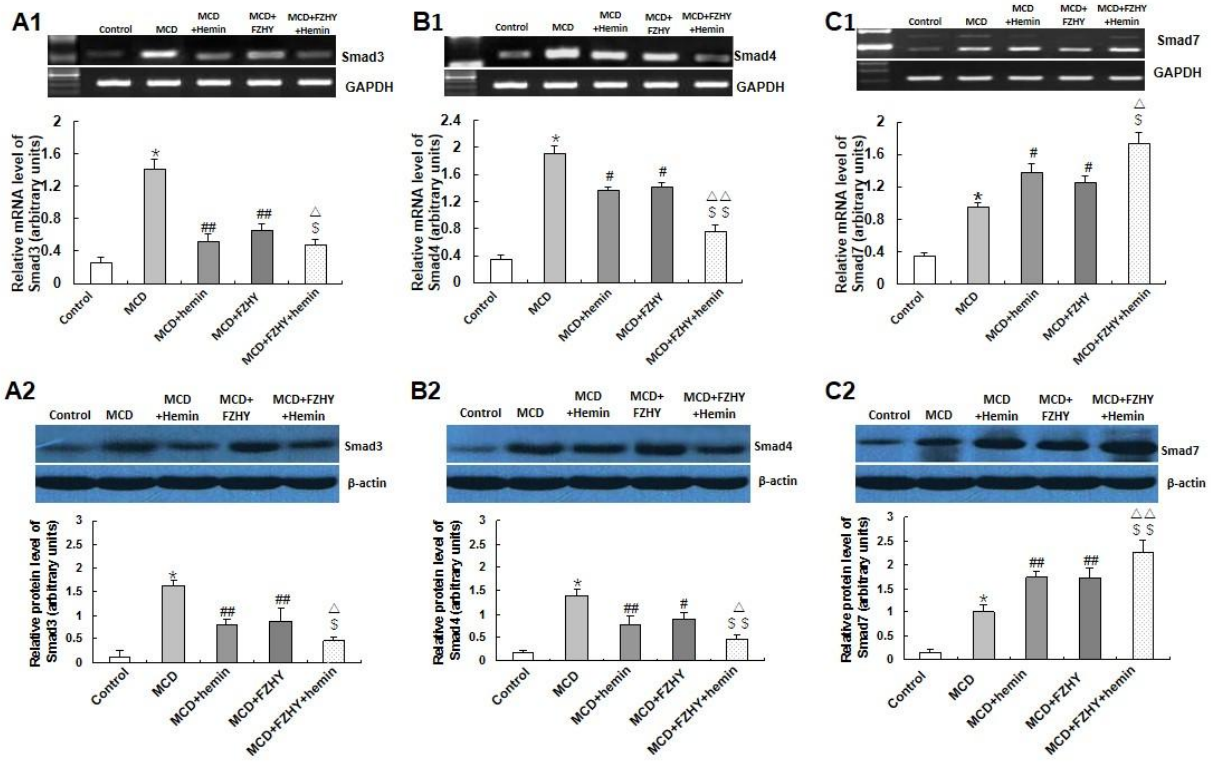

Figure 3. Effects of FZHY and/or hemin on hepatic Smad3, Smad4, and Smad7 expression in MCD diet-induced fibrosing steatohepatitis. The expression levels of *Smad3* (A1), *Smad4* (B1), and *Smad7* (C1) mRNAs were determined by RT-PCR. Protein levels of Smad3 (A2), Smad4 (B2), and Smad7 (C2) were detected by western blotting. Data are expressed as the mean±SD (n=6 per group). **P*<0.001, compared with the control group; #*P*<0.01, ##*P*<0.001, compared with the MCD group; $*P*<0.05, $$*P*<0.01 compared with the MCD+FZHY group; △*P*<0.05, △△*P*<0.01, compared with the MCD+hemin group

expression in MCD-treated mice.

Effects of FZHY on the activation of TGF-β1/Smad pathway genes in non-alcoholic fibrosing steatohepatitis induced by MCD diet

Immunohistochemistry analysis revealed increased expression of TGF-β1 in activated HSCs, inflammatory regions, and fibrotic areas in liver sections of MCD-treated mice compared with that of control mice. FZHY or hemin treatment significantly inhibited the upregulation of TGF-β1, and there was a further decrease in the expression of TGF-β1 in liver sections of mice treated with FZHY plus hemin (Figure 2B).

To evaluate the detailed mechanisms through which FZHY alleviates liver fibrosis, we investigated the hepatic expression levels of fibrosis-related genes. As shown in Figure 3, the hepatic mRNA and protein expression levels of Smad3, Smad4, and Smad7 were markedly elevated in mice fed an MCD diet (*P*<0.001). The expression levels of Smad3 and Smad4 were significantly downregulated, while the expression of Smad7 was further upregulated in the livers of mice treated with FZHY or hemin compared with mice consuming an MCD diet alone. These effects were exacerbated in mice treated with FZHY plus hemin. Thus, FZHY may alleviate liver fibrosis through modulating the expression of genes in the TGF-β1/Smad pathway.

Discussion

The pathogenesis of NASH is thought to involve a multi-step process, for which oxidative stress is the most popular proposed mechanism of hepatocellular injury (13). More advanced liver damage correlates with greater degrees of oxidative stress. Excessive production of reactive oxygen species (ROS) is a characteristic of oxidative stress and can activate inflammatory pathways, enhance the production of both type I collagen and TGF-β, and promote hepatocyte injury, necro-inflammation, and hepatic fibrogenesis (15-17). Additionally, oxidative stress has been shown to stimulate the activation of Kupffer cells and HSCs; thus, reducing oxidative stress may also provide anti-inflammatory and antifibrotic effects. Our previous studies revealed increased tissue levels of oxidative stress markers and lipid peroxidation products such as hepatic microsomal fatty acid oxidizing enzyme cytochrome P450 2E1 (CYP2E1) and malondialdehyde (MDA), and these effects were significantly reduced by FZHY pretreatment (7). Furthermore, the expression of HO-1, an inducible microsomal enzyme, is upregulated in response to cellular stress and by pro-oxidative stimuli, and HO-1 has been shown to exert protective effects against ROS-mediated liver fibrosis (17). In the current study, hepatic HO-1 mRNA expression was increased with the

progression of liver fibrosis, suggesting that there was enhanced oxidative stress in the livers of mice fed an MCD diet. FZHY administration for 8 weeks resulted in improved liver pathology, concomitant with a dramatic reduction in HO-1 expression. This effect may be due to the decreased expression of hepatic MDA and CYP2E1 following FZHY treatment.

Damaged hepatocytes and kupffer cells can also release inflammatory and fibrogenic mediators that recruit inflammatory cells and provoke HSC activation (15). Thus, inflammation is not merely associated with chronic liver disease, but also promotes disease progression (18). IKK-β is a pro-inflammatory kinase that phosphorylates the inhibitor of NF-κB, leading to NF-κB activation via its nuclear translocation (19). NF-κB is an inducible transcription factor and has critical roles in regulating the expression of multiple inflammatory genes, including TNF-α, IL-6 and MCP-1 (20, 21). Growing evidence indicates that NASH patients exhibit high levels of NF-κB induction (22). Additionally, NF-κB has been shown to be activated by TNF-α and ROS. Conversely, suppression of NF-κB activation prevents the induction of pro-inflammatory cytokines (23, 24). MCP-1 is a potent chemoattractant facilitating the recruitment of monocytes, lymphocytes, and activated HSC. Increased levels of MCP-1 can amplify inflammation in liver injury and are associated with the progression of fibrosis in chronic liver disease (25). Previous studies have shown that MCP-1 regulation is closely associated with the oxidative stress-sensitive expression of NF-κB (26). Indeed, in this study, we demonstrated that the mRNA levels of IKK-β, NF-κB p65 and MCP-1 were significantly elevated in MCD-treated mice when compared with control mice. Moreover, pre-administration of FZHY or hemin inhibited the expression of IKK-β, NF-κB p65 and MCP-1, and reduced inflammation and necrosis in liver sections. These data indicated that consumption of MCD diet induced liver injury that could be prevented by FZHY through modulating the NF-κB signaling pathway related genes. Additionally, the observed suppression of NF-κB expression was likely due to the restoration of antioxidant defenses and inhibition of IKK-β expression.

Growing evidence has shown that NF-κB activation can regulate not only inflammatory signals elicited in macrophages and inflammatory cells in the liver, but also fibrogenic responses in HSCs (27, 28). HSCs are the predominant cells in the liver and play a central role in the process of hepatic fibrosis. The persistence of oxidative stress and inflammatory stimuli accompanying sustained injury can lead to a perpetuation phase regulated by autocrine and paracrine stimulation (29) in which HSCs transform from a quiescent state to a myofibroblast-like phenotype characterized by increases in proliferation, extracellular matrix (ECM) accumulation, α-SMA expression, and retinoid loss (30-32). As shown in our study, the expression of α-SMA, a marker of activated HSCs, was very weak in liver sections of normal mice, but was considerably elevated upon MCD induction. Moreover, administration of FZHY or hemin resulted in a significant decrease in α-SMA expression in the livers of MCD-treated mice.

NF-κB activation can also enhance TGF-β signaling, which plays a major role in the process of HSC activation (33). Once activated, TGF-β1 binds to its cognate receptors on the cell surface, leading to phosphorylation of the intracellular mediator Smad3. TGF-β then recruits the common partner Smad4 to form a hetero-oligomer. The resulting complex finally translocates from the cytoplasm to the nucleus, further regulating specific TGF-β1 target genes and leading to collagen formation, HSC activation, and ECM synthesis (34-36). In this pathway, antagonistic Smad7 could combine with the Smad complex in the cytoplasm and prevent stimulating signals from being transmitted into the nucleus. Therefore, Smad7 functions in a negative feedback loop to terminate or reduce the strength of the signal (37). Disrupting TGF-β signaling pathways will reduce collagen synthesis, prevent scar formation, and accelerate matrix degradation, thereby inhibiting the progression of liver fibrosis (38). In this study, we observed a marked increase in the protein expression of TGF-β1 in mice fed MCD diet. Moreover, the mRNA and protein expression levels of Smad3, Smad4 and Smad7 were also dramatically increased in the livers of mice fed MCD diet compared with those in control mice. Treatment with FZHY or hemin significantly downregulated the expression of TGF-β1, Smad3 and Smad4, and further upregulated Smad7 expression. Therefore, FZHY might play a role in inhibiting the expression of factors involved in the IKK-β/NF-κB and TGF-β1/Smad pathways, hence interrupting the positive feedback loop in hepatic fibrogenesis. This effect was supported by observations that FZHY could significantly reduce collagen deposition, downregulate the protein expression of α-SMA and TGFβ1, and inhibit the nuclear translocation of Smad3 in fibrotic kidney tissue (39).

Conclusion

The present study provided evidence of the protective role of FZHY in ameliorating hepatic oxidative stress, inflammation and fibrosis in experimental nutritional steatohepatitis. Changes in the expression levels of pro-inflammatory and pro-fibrotic genes involved in the IKK-β/NF-κB and TGF-β1/Smad pathways may be responsible for the observed effects of FZHY on alleviating steatohepatitis and fibrosis.

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Conflict of interest

The authors declared no conflict of interest.

References

1. Basaranoglu M, Basaranoglu G, Sabuncu T, Sentürk H. Fructose as a key player in the development of fatty liver disease. World J Gastroenterol 2013; 19:1166-1172.
2. Afzali A, Berry K, Ioannou GN. Excellent posttransplant survival for patients with nonalcoholic steatohepatitis in the United States. Liver Transpl 2012; 18:29-37.
3. Friedman SL. Liver fibrosis in 2012: Convergent pathways that cause hepatic fibrosis in NASH. Nat Rev Gastroenterol Hepatol 2013; 10:71-72.
4. Day CP, James OF. Hepatic steatosis: innocent bystander or quity party? Hepatology 1998; 27:1463-1466.
5. Xie H, Tao Y, Lv J, Liu P, Liu C. Proteomic analysis of the effect of fuzheng huayu recipe on fibrotic liver in rats. Evid Based Complement Alternat Med 2013; 2013:972863.
6. Liu C, Hu Y, Xu L, Liu C, Liu P. Effect of Fuzheng Huayu formula and its actions against liver fibrosis. Chin Med 2009; 4:12.
7. Jia YH, Wang RQ, Mi HM, Kong LB, Ren WG, *et al*. Fuzheng Huayu recipe prevents nutritional fibrosing steatohepatitis in mice. Lipids Health Dis 2012; 11:45-55.
8. García-Galiano D, Sánchez-Garrido MA, Espejo I, Montero JL, Costán G, Marchal T, *et al*. IL-6 and IGF-1 are independent prognostic factors of liver steatosis and non-alcoholic steatohepatitis in morbidly obese patients. Obes Surg 2007; 17:493-503.
9. Yilmaz O, Ersan Y, Dilek Ozsahin A, Ihsan Ozturk A, Ozkan Y. Consequences of the combined α-tocopherol, ascorbic acid and α-lipoic acid on the glutathione, cholesterol and fatty acid composition in muscle and liver of diabetic rats. Iran J Basic Med Sci 2013; 16:165-172.
10. Wang RQ, Nan YM, Wu WJ, Kong LB, Han F, Zhao SX, *et al.* Induction of heme oxygenase-1 protects against nutritional fibrosing steatohepatitis in mice. Lipids Health Dis 2011; 10:31-41.
11. Yu J, Chu ES, Wang R, Wang S, Wu CW, Wong VW, *et al*. Heme oxygenase-1 protects against steatohepatitis in both cultured hepatocytes and mice. Gastroenterol 2010; 138:694-704.
12. Yu J, Ip E, Dela Peña A, Hou JY, Sesha J, Pera N, *et al*. COX-2 induction in mice with experimental nutritional steatohepatitis: role as pro-inflammatory mediator. Hepatol 2006; 43:826-836.
13. Basaranoglu M, Basaranoglu G, Sentürk H. From fatty liver to fibrosis: a tale of "second hit". World J Gastroenterol 2013; 19:1158-1165.
14. Tsochatzis EA, Papatheodoridis GV, Archimandritis AJ. Adipokines in nonalcoholic steatohepatitis: from pathogenesis to implications in diagnosis and therapy. Mediators Inflamm 2009; 2009:831670.
15. Cohen-Naftaly M, Friedman SL. Current status of novel antifibrotic therapies in patients with chronic liver disease. Therap Adv Gastroenterol 2011; 4:391-417.
16. Wang QM, Du JL, Duan ZJ, Guo SB, Sun XY, Liu Z. Inhibiting heme oxygenase-1 attenuates rat liver fibrosis by removing iron accumulation. World J Gastroenterol 2013; 19:2921-2934.
17. Grochot-Przeczek A, Dulak J, Jozkowicz A. Haem oxygenase-1: non-canonical roles in physiology and pathology. Clin Sci (Lond) 2012; 122:93-103.
18. Wasmuth HE, Tacke F, Trautwein C. Chemokines in liver inflammation and fibrosis. Semin Liver Dis 2010; 30:215-225.
19. Basaranoglu M, Basaranoglu G, Sabuncu T, Sentürk H. Fructose as a key player in the development of fatty liver disease. World J Gastroenterol 2013; 19:1166-1172.
20. Park HJ, Lee JY, Chung MY, Park YK, Bower AM, Koo SI, *et al.* Green tea extract suppresses NFκB activation and inflammatory responses in diet-induced obese rats with nonalcoholic steatohepatitis. J Nutr 2012; 142:57-63.
21. Liu C, Tao Q, Sun M, Wu JZ, Yang W, Jian P, *et al*. Kupffer cells are associated with apoptosis, inflammation and fibrotic effects in hepatic fibrosis in rats. Lab Invest 2010; 90:1805-1816.
22. Locatelli I, Sutti S, Vacchiano M, Bozzola C, Albano E. NF-κB1 deficiency stimulates the progression of non-alcoholic steatohepatitis (NASH) in mice by promoting NKT-cell-mediated responses. Clin Sci (Lond) 2013; 124:279-287.
23. Tuyama AC, Chang CY. Non-alcoholic fatty liver disease. J Diabetes 2012; 4:266-280.
24. Hansel B, Giral P, Nobecourt E, Chantepie S, Bruckert E, Chapman MJ, *et al*. Metabolic syndrome is associated with elevated oxidative stress and dysfunctional dense high-density lipoprotein particles displaying impaired antioxidative activity. J Clin Endocrinol Metab 2004; 89:4963-4971.
25. Saugspier M, Dorn C, Thasler WE, Gehrig M, Heilmann J, Hellerbrand C. Hop bitter acids exhibit anti-fibrogenic effects on hepatic stellate cells *in vitro*. Exp Mol Pathol 2012; 92:222-228.
26. Santos Rocha SW, Silva BS, Gomes FO, Soares e Silva AK, Raposo C, Barbosa KP, *et al.* Effect of diethylcarbamazine on chronic hepatic inflammation induced by alcohol in C57BL/6 mice. Eur J Pharmacol 2012; 689:194-203.
27. Oakley F, Teoh V, Ching-A-Sue G, Bataller R, Colmenero J, Jonsson JR, *et al.* Angiotensin II activates I kappaB kinase phosphorylation of RelA at Ser 536 to promote myofibroblast survival and liver fibrosis. Gastroenterol 2009; 136:2334-2344.
28. Luedde T, Schwabe RF. NF-κB in the liver--linking injury, fibrosis and hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol 2011; 8:108-118.
29. Ghiassi-Nejad Z, Friedman SL. Advances in antifibrotic therapy. Expert Rev Gastroenterol Hepatol 2008; 2:803-816.
30. Li S, Wang L, Yan X, Wang Q, Tao Y, Li J, *et al*. Salvianolic acid B attenuates rat hepatic fibrosis via downregulating angiotensin II signaling. Evid Based Complement Alternat Med 2012; 2012:160726.
31. Yoshida K, Matsuzaki K. Differential Regulation of TGF-β/Smad Signaling in Hepatic Stellate Cells

between Acute and Chronic Liver Injuries. Front Physiol 2012; 3:53.
32. Chai NL, Fu Q, Shi H, Cai CH, Wan J, Xu SP, *et al.* Oxymatrine liposome attenuates hepatic fibrosis via targeting hepatic stellate cells. World J Gastroenterol 2012; 18:4199-4206.
33. Seki E, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, *et al*. TLR4 enhances TGF-beta signaling and hepatic fibrosis. Nat Med 2007; 13:1324-1332.
34. Dong C, Li HJ, Chang S, Liao HJ, Zhang ZP, Huang P, *et al*. A disintegrin and metalloprotease with thrombospondin motif 2 may contribute to cirrhosis in humans through the transforming growth factor-β/SMAD pathway. Gut Liver 2013; 7:213-220.
35. Yang J, Zheng J, Wu L, Shi M, Zhang H, Wang X, *et al*. NDRG2 ameliorates hepatic fibrosis by inhibiting the TGF-β1/Smad pathway and altering the MMP2/TIMP2 ratio in rats. PLoS One 2011; 6:e27710.
36. Zong L, Qu Y, Xu MY, Dong YW, Lu LG. 18α-glycyrrhetinic acid down-regulates expression of type I and III collagen via TGF-B1/Smad signaling pathway in human and rat hepatic stellate cells. Int J Med Sci 2012; 9:370-379.
37. Yoshida K, Matsuzaki K. Differential regulation of TGF-β/Smad signaling in hepatic stellate cells between acute and chronic liver injuries. Front Physiol 2012; 3:53.
38. Szuster-Ciesielska A, Mizerska-Dudka M, Daniluk J, Kandefer-Szerszeń M. Butein inhibits ethanol-induced activation of liver stellate cells through TGF-β, NFκB, p38, and JNK signaling pathways and inhibition of oxidative stress. J Gastroenterol 2013; 48:222-237.
39. Wang QL, Yuan JL, Tao YY, Zhang Y, Liu P, Liu CH. Fuzheng Huayu recipe and vitamin E reverse renal interstitial fibrosis through counteracting TGF-beta1-induced epithelial-to-mesenchymal transition. J Ethnopharmacol 2010; 127:631-640.