Antiliver Fibrosis Formula of Fuzheng Huayu Alleviates Inflammatory Response

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1.Introduction

Hepatic fibrosis is a chronic wound-healing response characterized by inflammation [1]. Genetic changes, hepatitis virus infections, excessive alcohol consumption, lipid metabolic disorders, and autoimmune diseases trigger continuous liver injury and subsequent chronic inflammation that lead to liver fibrosis [2, 3]. Understanding the underlined inflammatory mechanisms is critical to developing strategies to control fibrosis [4].

Hepatic macrophages are the primary immune cells, consisting of liver-resident macrophages, monocyte-derived macrophages, and Kupffer cells that trigger liver inflammatory response and also play a crucial role in the pathologic progress of fibrosis [5]. Traditionally, macrophages can be polarized into proinflammatory/M1 macrophages or anti-inflammatory/M2 macrophages upon different microenvironmental stimulations. M1 macrophages predominantly express inducible nitric oxide synthase (iNOS) and secrete classical proinflammatory cytokines such as interleukin (IL)-6 and IL-1β to exaggerate the inflammatory response induced by T helper (Th)-1 signals such as lipopolysaccharide (LPS) and interferon (IFN)-γ. Accumulating evidence strongly implies that iNOS-derived nitric oxide (NO) has been associated with the pathogenesis of liver diseases during inflammatory conditions. These inflammatory mediators also emerge as vital profibrotic hubs. IL-1β promotes liver fibrosis partially in an IL-17-dependent manner [6]. Thus, targeting macrophages and inhibition of M1 macrophage-led...
inflammation development are approaches to interfere with fibrosis [7].

MicroRNAs (miRNAs) are small, noncoding RNAs that negatively control target gene expressions by promoting degradation or translational inhibition. miR-155 exerts a proinflammatory effect during the progression of hepatic fibrosis in immune cells [8]. PPAR-γ is involved in the progression of liver fibrosis as one of the target genes of miR-155 [9]. CCL4 administration decreases the expression of PPAR-γ in liver tissue and the antifibrotic effect of crocin partly via enhancing PPAR-γ to mediate inflammatory response and fibrogenic events [10].

Fuzheng Huayu’s formula (FZHY) was approved by the Chinese State Food and Drug Administration (SFDA) (No: Z20050546) as an antifibrotic medicine in 2002. FZHY is composed of six Chinese medicines, including Radix Salvia Miltiorrhize (Danshen), Cordyceps (Chong Cao), Semen Persicae (Taoren), Pollen Pin (Song Huafen), Gynostemma Pentaphyllamn (Jiaogulan), and Fructus Schisandrae chinensis (Wuweizi), which is used to invigorate blood circulation, remove blood stasis, tonify essence, and nourish liver according to the theory of TCM. FZHY’s antifibrotic effect has been confirmed in accumulating experimental and clinical evidence [11–20]. Current data showed FZHY alleviated inflammatory cytokines TNF-α and IL-6 and pro-fibrotic genes VEGF and TGF-β1 in the liver fibrotic rat model [21]. However, its anti-inflammatory mechanisms remain to be elucidated.

Thus, in this present study, we evaluated the anti-inflammatory effect and relevant mechanisms of FZHY.

2. Materials and Methods

2.1. Materials. FZHY was obtained from Huanghai Pharmaceutical Co. (Shanghai, China). RPMI 1640 medium, fetal bovine serum (FBS), and 0.25% trypsin were purchased from Gibco (Rockville, MD, USA). BCA protein assay kit and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA). Special antibodies including p-JNK(#9251; 1:1000), CD40 (ab252428; 1:1000), IL-6 (ab259341; 1:1000), and horseradish peroxidase-labeled goat antirabbit IgG were purchased from Abcam (Cambridge, UK). CD86 (13395-1-AP; 1:500) was purchased from ProteinTech Group (Chicago, IL, USA). Polyvinylidene difluoride (PVDF) membranes, western blotting detection reagent ECL, and murine recombinant IFN-γ were purchased from Millipore (Bedford, MA, USA). LPS, 1400W, Griess reagent, and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). MicroRNA primers, transcription kits, and universal PCR master mix were obtained from GenePharma Company (Shanghai, China). Standard compounds sodium danshensu, salvianolic acid B, and adenosine were purchased from the National Institutes for Food and Drug Control (Beijing, China).

2.2. High-Performance Liquid Chromatography (HPLC) Assay. FZHY standardization was performed using HPLC fingerprinting with chemical standard compounds such as sodium danshensu, salvianolic acid B (two compounds isolated from Radix Salvia Miltiorrhize), and adenosine (a compound isolated from Cordyceps) according to Chinese Pharmacopoeia (2015 edition).

2.3. Cell Culture. RAW 264.7 cells from the American Type Culture Collection (ATTC, Rockville, MD) were cultured in RPMI 1640 medium supplemented with 10% FBS. The cell passages below 10 were used, and three replicates were performed in each experiment.

2.4. Cell Viability. The effect of FZHY on the proliferation of RAW 264.7 cells was detected by the MTT assay. RAW 264.7 cells were incubated into 96-well plates at a density of 10,000 cells/well and were allowed to adhere overnight. Cells were treated with FZHY in different concentrations (0, 12.5, 25, 50, 100, 200, 400, 800, and 1000 μg/mL) and under 1400W (50 μM), respectively. After 24 hours, 0.5 mg/ml MTT was added to each group and was incubated for additional 4 hours. The supernatant of each group was discarded and then the absorbance at 490 nm with formazan-DSMO dissolution was read. The cell availability of each group was calculated compared to the cell availability of the control group as 100%.

2.5. Nitrite Assay. RAW 264.7 cells were seeded into 96-well plates at a density of 100,000 cells/well and were divided into the control group, the model group, FZHY treatment groups (25, 50, 100, and 200 μg/mL), and the positive drug group 1400W (50 μM, a selective inhibitor of iNOS), respectively. After serum-free treatment for 24 h, the control group was treated with serum-free 1640 medium, and the model group was stimulated with LPS (100 ng/mL)/IFN-γ (100 U/mL), while the FZHY administration groups were treated with 25, 50, 100, and 200 μg/mL and stimulators LPS (100 ng/mL)/IFN-γ (100 U/mL). After 24 hours, 100 μL of the supernatant of each group was collected and added to 100 μL of Griess reaction reagent for 10 mins and then was read at 540 nm using a microplate reader. Then, the nitrite content in the cell supernatant was calculated using the nitrate standard curve.

2.6. Animal Experiment. The murine liver fibrosis model induced by CCl4 and administration with or without FZHY was proceeded in a previous study [20], and preserved livers were used for further experiments to detect the inflammation-relevant mediators.

2.7. qPCR Analysis. RAW 264.7 cells were cultured and divided into the control, model, and FZHY treatment groups (100 and 200 μg/mL), respectively. After being allowed to adhere overnight, cells were treated with serum-free 1640 medium for 24 h. The samples of cells and liver tissues were collected, and the total RNA was extracted by the Trizol
method, and miR-155, iNOS, CD86, CD40, IL-6, PPAR-γ, and HO-1 were detected. The primers of qRT-PCR for iNOS, CD86, CD40, IL-6, PPAR-γ, and HO-1 are listed in Table 1.

2.8. Western Blot Analysis. RAW 264.7 cells were cultured in 30 mm culture dishes with 1 × 10⁶ cells. The protein samples of each group were collected, and western blotting was performed after protein denaturation. The protein expressions of iNOS, HO-1, CD40, CD86, and IL-6 compared with β-actin and the phosphorylation levels of p38, JNK, ERK, and STAT1 compared with total p38, JNK, ERK, and STAT1 were determined.

2.9. Statistical Evaluation. Data were presented as the mean ± SD of results obtained from at least three experiments. Data were assessed by ANOVA analysis and the t-test. P < 0.05 was considered as statistically significant.

3. Results

3.1. FZHY Suppressed the Expression of Inducible Inflammatory Synthase iNOS In Vitro and In Vivo. To examine the cell viability, RAW 264.7 cells were incubated with FZHY at increasing concentrations (0, 12.5, 25, 50, 100, 200, 400, 800, and 1000 μg/mL) and with 1400W (50 μM) for 24 h. FZHY presented no significant influence on cell viability up to 200 μg/mL by the MTT assay as well as 1400w at the dosage of 50 μM (Figure 1(a)).

iNOS has been used as the major biomarker for the definition of M1 proinflammatory macrophages, so we further examined the effect of FZHY at dosages of 25, 50, 100, and 200 μg/mL on nitrite accumulation, the stable oxidative metabolite of nitric oxide, and in the supernatant from differently treated cells. FZHY inhibited nitrite accumulation induced by LPS plus IFN-γ in a concentration-dependent manner (Figure 1(b)). As expected, FZHY inhibited iNOS expressions at gene and protein levels in a concentration-dependent manner on inflammatory macrophages (Figures 1(c) and 1(d)). 1400W, a well-known inducible nitric oxide synthase (iNOS) selective inhibitor, reduced nitrite production by 97.45% at 50 μM compared to the model group induced by LPS plus IFN-γ.

Compared with the normal group, the infiltration of inflammatory cells and collagen deposition in the portal area were significantly increased in CCl₄-induced liver fibrosis mice, while FZHY presented the prevention and curing effects on CCl₄-induced liver inflammation and fibrosis [20]. iNOS also altered its expression in liver fibrosis. iNOS deficiency improved liver inflammation and genes encoding collagen, leading to decrease fibrosis [22]. We continued to test iNOS in the liver tissues from CCl₄-induced liver fibrosis mice with and without FZHY administration. Results demonstrated that FZHY administration did reduce iNOS expression in liver tissues (Figures 1(e) and 1(f)). Based on these results, FZHY strongly attenuated iNOS in inflammatory macrophages and liver tissues from CCl₄-induced liver fibrosis mice.

3.2. FZHY Enhanced Expression of Anti-Inflammatory Enzyme HO-1. HO-1 is the inducible and rate-limiting enzyme in heme catabolism and exhibits anti-inflammatory functions to resolve cellular oxidative stress and inflammatory cascade reaction. LPS failed to induce iNOS production in HO-1-overexpressing cells suggesting that HO-1 protected RAW 264.7 cells from inflammation damage [23]. Therefore, we examined the effect of FZHY on HO-1 using qPCR and western blot analysis on macrophages. Results showed that FZHY strikingly increased expressions of HO-1 at gene and protein levels (Figure 2).

3.3. FZHY Reduced Other Inflammatory Mediators. iNOS, CD86, CD40, and IL-6 are typical proinflammatory mediators in M1 macrophages [7]. Results showed CD86, CD40, and IL-6 mRNA and protein levels were augmented in LPS plus IFN-γ stimulated macrophages compared with the control group. The expressions of inflammatory mediator concentration dependently reduced after FZHY treatment. These findings suggested that FZHY restrained expressions of these proinflammatory mediators (Figure 3).

3.4. STAT1/MAPK Signaling Pathways Were Involved in the FZHY-Led Effect. The signal transducer and activator of transcription 1 (STAT1) were involved in the mediation of IFN-γ intracellular signaling [24]. Mitogen-activated protein kinases (MAPKs), including p38, extracellular signal-regulated kinase (ERK), and c-JunN-terminal kinases (JNK), played pivotal roles in inflammatory responses [25]. Activated STAT1/MAPK signal pathways led to the induction of iNOS and other proinflammatory cytokines.

Thus, we next investigated whether FZHY inhibited iNOS expression by regulating STAT1/MAPK pathways. LPS plus IFN-γ increased the phosphorylation levels of MAPK and STAT-1. FZHY treatment concentration dependently abrogated phosphorylation of STAT1 and MAPK (Figure 4).

These data suggested that STAT1/MAPK pathways were involved in the FZHY-led effect.

3.5. FZHY Modulated miR-155/PPAR-γ Axis. miR-155/PPAR-γ axis regulated the progress of inflammation and liver fibrosis [9]. Under the inflammation condition, the level of miR-155 was notably boosted. However, FZHY dramatically struck the elevated level of miR-155 and upregulated expression of its target gene PPAR-γ on macrophages and liver tissues from CCl₄-induced liver fibrosis mice (Figure 5).

3.6. Chemical Quality Control of FZHY by HPLC Analysis. We identified three compounds as the chemical quality control of FZHY by HPLC analysis (Figure 6). The content of sodium danshensu (8.3%), salvianolic acid B (13.25%), and adenosine (3.95%) in FZHY met the requirements of Chinese Pharmacopoeia.
Table 1: Primer sequences used for qRT-PCR amplification.

| Gene      | Sense sequence                  | Antisense sequence                          |
|-----------|--------------------------------|---------------------------------------------|
| iNOS      | 5'-GGAGCCGATGTGGATTGTC-3'       | 5'-GTGAGGGCCTGTCAGTGAAG-3'                  |
| CD86      | 5'-GACCGGACTTGAACACCCAG-3'      | 5'-GTGAGGGCCTGTCAGTGAAG-3'                  |
| CD40      | 5'-ATTGTGCCGAGGAAACCG-3'        | 5'-CCATCCGAGGTAAACCACAGA-3'                 |
| IL-6      | 5'-CCATCCGAGGTAAACCACAGA-3'     | 5'-CCATCCGAGGTAAACCACAGA-3'                 |
| PPAR-γ    | 5'-AGACGACTCGATTCCTTGAG-3'      | 5'-CCATCCGAGGTAAACCACAGA-3'                 |
| HO-1      | 5'-CAGAGATGGCGTACCTGTC-3'       | 5'-CCATCCGAGGTAAACCACAGA-3'                 |
| GAPDH     | 5'-AACGATTGTTGCTATGTCG-3'       | 5'-CCATCCGAGGTAAACCACAGA-3'                 |

4. Discussion

Inflammation is a key component and a contributor to profibrogenic progress. Increasing evidence showed that anti-inflammatory therapy exerted its effect in the treatment of liver fibrosis [26]. Targeting chronic inflammation in the context of fibrogenesis might lead to potential antifibrotic therapies. Macrophages play a central role in the progression of liver inflammation and fibrosis progression [27]. M1 macrophages induced enzymes and secreted cytokines to regulate fibrogenesis [28]. Thus, controlling M1 macrophage polarization during fibrosis provides a crucial strategy.

LPS plus IFN-γ can activate M1 macrophages that exert a proinflammatory phenotype. iNOS is a significant marker of M1 macrophages. Excessive NO, a gas signal molecule with high reactive properties, produced by iNOS results in oxidative and nitroxidative stress under inflammatory conditions. These findings demonstrated that FZHY suppressed the expression of iNOS at gene and protein levels in a concentration-dependent manner. The accumulation of nitrite and the steady production of NO are also reduced by FZHY (Figure 1). Thus, FZHY's anti-inflammatory activity depends on its inhibition of NO and iNOS. Other M1 markers, including CD86, CD40, and IL-6, were also diminished upon FZHY administration (Figure 3).

Current evidence demonstrated that HO-1 presented a crucial role in anti-inflammatory response and antioxidant progress [29]. Induction of HO-1 in LPS stimulated...
macrophages and suppressed the production of proinflammatory mediators, while deficiency of HO-1 presented an inflammatory phenotype [30]. Induction of HO-1 caused by the NO produced from iNOS attenuated iNOS expression and NO production [31]. There is a negative feedback loop between HO-1 and iNOS. A previous study showed that FZHY upregulated the antioxidative gene HO-1 in ameliorating nutritional fibrosing steatohepatitis [32]. Notably, FZHY also simultaneously induced antioxidative enzyme HO-1 expression to resolve inflammatory damage triggering a cellular protective mechanism (Figure 2). These data implied that the anti-inflammatory action of FZHY was partially attributed to the induction of HO-1.

The STAT1/MAPK signaling pathways were involved in the progression of inflammation [33]. Results suggested that the activation of STAT1/MAPK pathways was remarkably abolished by FZHY (Figure 4).

microRNAs (miRNAs) are crucial for the progression of inflammation and fibrosis. Accumulating studies suggested that proinflammatory miR-155 promoted liver fibrosis.

Figure 2: FZHY induced anti-inflammatory HO-1. (a) HO-1 mRNA in each group was assessed by qPCR. (b) HO-1 protein in each group was assessed by western blot. Values are presented as the mean ± SD of at least three replicates. ###P < 0.01 versus C group; **P < 0.01 versus M group; *P < 0.05 versus M group.

Figure 3: FZHY regulated the proinflammatory mediators. The mRNA and protein levels of markers including CD86 (a, b), CD 40 (c, d), and IL-6 (e, f) were detected by qPCR and western blotting. Values are presented as the mean ± SD of at least three replicates. ###P < 0.01 versus C group; **P < 0.01 versus M group; *P < 0.05 versus M group.
Figure 4: Effects of FZHY on STAT1/MAPKs signaling pathways. (a) p-STAT1 and STAT1, (b) p-JNK and JNK, (c) p-ERK and ERK, and (d) p-p38 and p38 protein expressions were assessed by western blotting and calculated using an imaging system. Values are presented as the mean ± SD of at least three replicates. ##P < 0.01 versus C group; #P < 0.05 versus C group; ∗P < 0.05 versus M group; ∗∗P < 0.01 versus M group.

Figure 5: FZHY regulated miR-155/PPAR-γ axis. mir-155 and PPAR-γ mRNA in each group from macrophages (a, b) and liver tissues (c, d) were assessed by qPCR. Values are presented as the mean ± SD of at least three replicates. ##P < 0.01 versus C group; #P < 0.05 versus C group; ∗P < 0.05 versus M group; ∗∗P < 0.01 versus M group.
Elevated miR-155 was observed in a mouse model of liver fibrosis and cirrhotic livers of alcoholic patients. Profibrotic genes by alcohol diet or CCl4 treatment were reduced in miR-155 KO mice [9]. miR-155 targeted PPAR-γ, SMAD2/5, Snail1, and STAT3 to regulate fibrosis phenotype [34]. miR-155 inhibitor increased the expression of PPAR-γ in alcohol-treated macrophages. Activation of PPAR-γ suppressed iNOS expression in M1 macrophages and displayed anti-inflammatory properties. The tyrosine nitration of PPAR-γ by iNOS impaired its transcriptional activity and stability [35].

A network pharmacology approach and a cell-based assay revealed that schisandrin B, salvianolic acid A, and...
kaempferol from FZHY could bind to PPAR-γ [19]. We found that proinflammatory miR-155 increased while its target gene PPAR-γ decreased upon stimulation by LPS in IFN-γ or CCL4-induced liver fibrosis mice, while FZHY reduced the level of miR-155 and upregulated the expression of PPAR-γ (Figure 5).

5. Conclusions

Collectively, our findings demonstrated that FZHY exerted an anti-inflammatory effect on LPS plus IFN-γ-induced inflammation with modulation of proinflammatory and anti-inflammatory mediators via MAPK/STAT-1 signaling pathways and miR-155/PPAR-γ axis (Figure 7). Future studies are needed to elucidate active compounds from FZHY and core targets and pathways in depth underlying the relationship between inflammation and fibrosis.

Data Availability

The data used to support the findings of this study are included in the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

Qing-Qi Chang, Yi-Feng Pan, and Jia-Yi Yang contributed equally to this work.

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