Anluohuaxianwan Alleviates Carbon Tetrachloride-Induced Hepatic Fibrosis in Rats through Upregulation of Peroxisome Proliferator-Activated Receptor-Gamma and Downregulation of Nuclear Factor-Kappa B/IκBα Signaling Pathway

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Abstract

Objective: The aim of this study was to investigate the effects of traditional Chinese medicine Anluohuaxianwan (ALHXW) on peroxisome proliferator-activated receptor-gamma (PPARγ) and nuclear factor-kappa B (NF-κB) signaling pathways using a rat model of carbon tetrachloride (CCL₄)-induced liver fibrosis. Methods: Thirty-six male Wistar rats were randomly assigned into three groups: control, model, and treatment. The model and treatment groups were injected intraperitoneally with 40% CCL₄ (2 ml/kg), and the control group was given saline (2 ml/kg) twice a week for 6 weeks. In parallel, the treatment group was gavaged with ALHXW solution daily, while the control and model groups were gavaged with saline with 6 weeks. Liver function was measured, and liver fibrosis and necroinflammation were assessed. Protein and messenger RNA expression levels of PPARγ, NF-κB, and Inhibitor α of NF-κB (IκBα) were analyzed by Western blot and reverse transcription–quantitative polymerase chain reaction. Results: ALHXW markedly alleviated liver injury compared with the model group, as indicated by the improvements in disease status, the morphology of liver and spleen, the liver and spleen indexes, and liver function. The extent of liver fibrosis was improved, hepatic stellate cell activation was inhibited, the expression of PPARγ and IκBα was significantly higher, and the expression of NF-κB was significantly lower in the treatment group as compared with the model group. Conclusions: ALHXW treatment can alleviate CCL₄-induced liver fibrosis in rats, and the potential antifibrogenic mechanisms may occur through the upregulation of PPARγ expression and downregulation of NF-κB/IκBα signaling pathway.

Keywords: Anluohuaxianwan, hepatic fibrosis, mechanism, nuclear factor-kappa B/IκBα, peroxisome proliferator-activated receptor-gamma

INTRODUCTION

A variety of etiologies, such as viral hepatitis, nonalcoholic steatohepatitis, alcoholic liver disease, toxin-induced insults, cholestatic disorders, and autoimmune hepatitis, can contribute to liver injury and induce liver fibrosis.[1] During chronic injury, hepatic stellate cells (HSCs) go through a transdifferentiation from quiescent cells to proliferative myofibroblasts (MF Bs) that produce superfluous extracellular matrix (ECM); meanwhile, tissue inhibitor of metalloproteinases block the matrix-degrading action performed by matrix metalloproteinases, thereby leading to extra-deposition of ECM, finally resulting in liver fibrotic formation.[2] The activation of HSC is the major precursor...
of liver fibrosis. Many studies have indicated that cytokine peroxisome proliferator-activated receptor-gamma (PPARγ) and nuclear factor-kappa B (NF-κB) all play a vital role in HSC activation and fibrogenesis.\(^3,4\)

PPARγ is predominantly presented in the liver and adipose tissue and belongs to the superfamily of nuclear receptors controlling the transcription of a subset of genes.\(^4\) PPARγ plays an important role in many pathological processes, such as inflammation, tumorigenesis, obesity, and ECM remodeling. PPARγ is mainly expressed in quiescent Vitamin A-storing HSC, and the expression of PPARγ is inhibited in activated HSC. Upregulation of PPARγ can suppress the α-smooth muscle actin (α-SMA) production and ECM deposition by modulating HSC adipogenic phenotype, inhibiting HSC activation, inducing the senescence and apoptosis of activated HSC, thereby alleviating liver fibrosis.\(^5\) The NF-κB family, defined to transcription factors, is a key regulator of inflammation, cell growth, apoptosis, and cancer. NF-κB not only participates in liver cell apoptosis and proliferation but also promotes persistence of HSC activation and a chronic wound healing response.\(^6\) Inhibition of the NF-κB pathway is sufficient to suppress HSC activation and promote HSC apoptosis in vitro and in vivo.\(^3\) At present, NF-κB is a potential target for antifibrotic strategies in liver fibrotic therapy.

Liver fibrosis is a complex process involving a large number of cytokines which act through multiple signaling pathways. Antifibrotic drugs acting on a single target or pathway have not been shown to have efficacy. Traditional Chinese medicine (TCM), with its advantages of multi-ingredient, multi-target, and low adverse effects, has a potential for more effective antifibrotic treatment.\(^7\) Anluohuaxianwan (ALHXW) is a TCM, which invigorates the spleen, nourishes the liver, cools and activates blood circulation, and dissipates heat, cooling blood, and mechanisms associated with ALHXW activity were investigated using CCl\(_4\)-induced hepatic fibrotic models.

### Methods

#### The preparation and quality control of Anluohuaxianwan

ALHXW was composed of 14 ingredients [Supplement Table 1] and provided by Sunlon Pharmaceutical Co., Ltd. To evaluate the quality of ALHXW, a total of six different batches (1753800, 1753900, 1754000, 1754100, 1754200, and 1754300) of ALHXW samples were tested. The results of thin-layer chromatography (TLC) analyses demonstrated that the contents of ginsenoside R1, rhein, and paenol all could be detected at the same locations like the corresponding spots of the reference substances. The TLC quantitative analyses showed that the contents of ginsenoside Rg1 were all 1.5 mg/g in ALHXW using dual wavelength linear scanning (λ\(\alpha\) = 530 nm and λ\(\gamma\) = 700 nm). The relative standard deviation (SD) values of measurements of the moisture content and dissolution time limit for six batches of ALHXW were 3.76% and 2.98%, respectively.

#### Study design

Thirty-six male Wistar rats with weight 180 ± 10 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China. This study was conducted according to the recommendations of the guidelines for the Care and Use of Experimental Animals at Peking University Health Science Center. All rats were housed in the clean and air condition room (25°C ± 1°C, relative humidity 55% ±5%, 12-h light/dark cycle) at the Department of Laboratory Animal Science, Peking University Health Science Center. Adequate feed and drinking water were provided.

A total of 36 male rats were randomly divided into control, model, and treatment groups. Rats in the model and treatment groups were injected intraperitoneally with 40% CCl\(_4\) (2 ml/kg, mixed 2:3 in olive oil) (Sinopharm Chemical Reagent Co., Ltd.), and rats in the control group were administered intraperitoneally with saline (2 ml/kg) twice a week for 6 weeks.\(^12,14\) Meanwhile, rats in the treatment group were gavaged with ALHXW solution (concentration 0.15 g/ml). Rats in the control and model groups were given saline daily for 6 weeks. At the end of 3 weeks, half of the rats were randomly selected for blood sample collection. At the end of 6 weeks, all of the rats were chosen for blood sample collection and then sacrificed. Subsequently, the liver and spleen tissues were obtained and weighed. Then, a part of liver tissue was fixed in 10% formalin solution for the following histopathologic examination. The remaining liver tissue was immediately stored at −80°C for future analysis. In this study, the dosage of ALHXW given was determined in a
previous study.[12] Using the daily dose for clinical patients as a reference, we calculated the final dose for rats by the dose conversion formula.

**Alanine aminotransferase and aspartate aminotransferase measurement**

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were measured using commercially available kits (Shenzhen Mindray Bio-Medical Electronics Co., Ltd.) at the Department of Laboratory Animal Science, Peking University Health Science Center.

**Hydroxyproline, hyaluronic acid, and laminin measurement**

Hydroxyproline (Hyp), hyaluronic acid (HA), and laminin (LN) concentrations in serum were measured by enzyme-linked immunosorbent assay using a commercial kit (Winter Song Boye Biotechnology Co., Ltd., Beijing, China).

**HE, Masson, and α-smooth muscle actin staining and pathology assessment**

Liver tissue was fixed in 10% formalin and embedded in paraffin and sliced at 4 μm by microtome (Shanghai, China). The hematoxylin-eosin staining, Masson’s trichrome staining, and α-SMA immunohistochemical staining were performed at the Department of Pathology, Peking University Health Science Center. The criteria (scoring system) used to determine fibrosis severity are as follows: score 0 – no fibrosis; score 1 – the portal area expanded and mild fibrotic formation; score 2 – moderate fibrosis presents in the portal area and surrounding areas, without bridges between portals; score 3 – formation of bridges between portals; and score 4 – fibrous septa extend to form bridges between portal to central and numerous bridges or septa formation (cirrhosis). The criteria used to assess the inflammation are as follows: score 0 – no inflammation; score 1 – slight inflammation in the portal area and no hepatocyte necrosis; score 2 – mild inflammation in the portal area (<50%) and single or focal hepatocyte necrosis; score 3 – moderate inflammation in the portal area (>50%) and multifocal hepatocyte necrosis; and score 4 – severe inflammation in the portal area (nearly 100%) and hepatocyte bridging necrosis. The criteria used to evaluate the extent of HSC activation are as follows: score 0 – no abnormality, no activated HSC; score 1 – slight abnormality, 0 <n (activated HSC) <5; score 2 – mild abnormality, 5 ≤n (activated HSC) <10; score 3 – moderate abnormality, 10 ≤n (activated HSC) ≤15; and score 4 – severe abnormality, n (activated HSC) >15. There are principles of counting activated HSC; ten high-power microscopic fields (×400) were randomly chosen per slide; the number of activated HSC per filed was counted in the hepatic lobule interior instead of fibrous septa.

**Reverse transcription–quantitative polymerase chain reaction for peroxisome proliferator-activated receptor-gamma and nuclear factor-kappa B p65 messenger RNA analysis**

Total RNA was extracted from liver tissues using Trizol reagent (Invitrogen, USA). RNA concentration was quantitated by 260/280 nm measurement using NanoDrop One (Thermo Fisher Scientific, USA). The complementary DNA was produced from the reverse transcription of RNA using a reverse transcription kit (Thermo Fisher Scientific, USA).

The quantitative polymerase chain reaction (qPCR) primers used in this study were synthesized by Sangon Biotech Co., Ltd., Shanghai, China. The sequences of the primers were as follows: GAPDH forward: 5’ GCC CTC AAC TAT GAT GAC ATC AAG A 3’ and reverse: 5’ GTA GCC CAG CAG GCC CTT TAG T 3’; PPARγ forward: 5’ GCC TGG CAA AGC ATT TGT AT 3’ and reverse: 5’ ACT GCC ACC CTT GAA AAA TG 3’; and NF-κB p65 forward: 5’ AAC ACT GCC GAG CTC AAG AT 3’ and reverse: 5’ CAT CCG TTG CAG AAA AGG AG 3’. The qPCR was performed according to the protocol with the initial step holding temperature at 95°C for 30 s, followed by 40 cycles between 95°C for 5 s and 60°C for 30 s using the ABI StepOnePlus (Applied Biosystems, USA). In addition, GAPDH was chosen as the housekeeping gene, and the relative expressions of target genes were presented according to the 2⁻ΔΔCt method.

**Western blot analysis for peroxisome proliferator-activated receptor-gamma, nuclear factor-kappa B p65, and IκBα protein detection**

The protein samples were extracted from the frozen liver tissues using RIPA lysis buffer (Beyotime Biotech, Shanghai, China). The concentration of protein was detected using BCA protein detection kit (Shenzhen Mindray Bio-Medical Electronics Co., Ltd.). Further, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) (Biodragon, Beijing, China), overnight at 4°C. The PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) (Biodragon, Beijing, China), overnight at 4°C. Further, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) (Biodragon, Beijing, China), overnight at 4°C. Further, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) (Biodragon, Beijing, China), overnight at 4°C. Further, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) (Biodragon, Beijing, China), overnight at 4°C. Further, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) (Biodragon, Beijing, China), overnight at 4°C. Further, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) (Biodragon, Beijing, China), overnight at 4°C. Further, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000) (Biodragon, Beijing, China), overnight at 4°C.
RESULTS

Effect of Anluohuaxianwan on the morphology of liver and spleen, the body weight, and liver and spleen indexes in rats

At the end of the experiment, most of the rats in the model group had disease status with an abdominal bulge. Compared with the model group, the rats in the treatment group had improved significantly. No death of rats was observed in the control and treatment groups except one died in the model group (death rate: 8.33%).

The typical liver and spleen specimens of the rats in three groups are shown in Figure 1. The anatomy of the rat’s liver demonstrated that a smooth liver surface and soft texture in the control group; a rough, granular liver surface and hard texture in the model group; and a smoother liver surface and softer texture in the treatment group as compared with the model group. The anatomy of the rat’s spleen presented the same morphology changes like the liver.

The weight of the body, the liver, and the spleen of rats were measured at the end of 6 weeks. The liver and spleen indexes were calculated according to the formula: (liver or spleen weight/body weight) × 100%. As shown in Table 1, the mean body weight of rats in each group was similar. At the end of 6 weeks, the body weights of rats in the model and treatment groups were significantly lower than that of the control group (all \( P \leq 0.01 \)). The liver weight, spleen weight, and indexes of rats in the model group were significantly higher than those of the control group (\( P < 0.05 \)), while the declines in these parameters were observed in rats treated with ALHXW.

Effect of Anluohuaxianwan on serum alanine aminotransferase and aspartate aminotransferase

ALT and AST levels, reflecting liver function, were tested at the end of 3 and 6 weeks, as shown in Table 2. The significant increases of ALT and AST levels were observed in the model group at both two points as compared with those of the control group. After 3 weeks of ALHXW therapy, the ALT and AST levels in the treatment group declined by 23.46% and 19.96%, respectively, as compared with the model group. Similar results were observed for the ALT and AST levels after 6 weeks of ALHXW treatment.

Effect of Anluohuaxianwan on serum hydroxyproline, hyaluronic acid, and laminin

Hyp, HA, and LN concentrations in serum were measured at the end of 6 weeks, as shown in Table 3. The concentrations of Hyp, HA, and LN in the model and treatment group were significantly higher than that of the control group (\( P < 0.01 \)). Compared with the model group, the concentrations of Hyp, HA, and LN in the treatment group were significantly lower (\( P < 0.01 \)).

Pathological assessment of liver tissue in rats

The typical pathology results of liver tissues demonstrated fibrotic staging and necroinflammation grading in three groups.

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Table 1: Effect of Anluohuaxianwan on body weight and liver and spleen indexes of rats (mean±standard deviation)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Spleen weight (g)</th>
<th>Liver index (%)</th>
<th>Spleen index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>Final (6 weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12</td>
<td>219.25±7.62</td>
<td>420.42±28.90</td>
<td>12.60±0.78</td>
<td>0.87±0.12</td>
<td>3.01±0.22</td>
</tr>
<tr>
<td>Model</td>
<td>11</td>
<td>219.33±7.27</td>
<td>341.90±30.02**</td>
<td>15.18±2.33**</td>
<td>1.07±0.16*</td>
<td>4.43±0.42**</td>
</tr>
<tr>
<td>Treatment</td>
<td>12</td>
<td>219.33±7.20</td>
<td>346.83±24.97**</td>
<td>14.87±1.61**</td>
<td>0.99±0.26</td>
<td>4.29±0.48**</td>
</tr>
</tbody>
</table>

*\( P < 0.05 \), **\( P < 0.01 \) as compared with control group

Table 2: Effect of Anluohuaxianwan on serum alanine aminotransferase and aspartate aminotransferase of rats (mean±standard deviation)

<table>
<thead>
<tr>
<th>Time-point</th>
<th>Enzymes</th>
<th>Control group</th>
<th>Model group</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td>At end of 3 weeks</td>
<td>ALT (U/L)</td>
<td>45.40±5.13</td>
<td>889.98±328.32**</td>
<td>681.15±375.97**</td>
</tr>
<tr>
<td></td>
<td>AST (U/L)</td>
<td>126.50±16.43</td>
<td>1405.53±516.95**</td>
<td>1124.98±400.70**</td>
</tr>
<tr>
<td>At end of 6 weeks</td>
<td>ALT (U/L)</td>
<td>55.81±15.87</td>
<td>1205.62±653.91**</td>
<td>1198.43±763.21**</td>
</tr>
<tr>
<td></td>
<td>AST (U/L)</td>
<td>171.63±42.13</td>
<td>2470.26±2082.38**</td>
<td>1775.94±1053.88**</td>
</tr>
</tbody>
</table>

At the end of 3 weeks, \( n=6 \) per group, at the end of 6 weeks, control group \( n=12 \), model group \( n=11 \), treatment group \( n=12 \). **\( P < 0.01 \) as compared with control group. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase

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Figure 1: The typical liver and spleen specimens of the rats in three groups (control group, \( n = 12 \); model group, \( n = 11 \); treatment group, \( n = 12 \)
are shown in Figure 2 and summarized in Table 4. The liver tissue in the control group had normal lobular architecture, without fatty degeneration or fibrotic development. The lobular architectures of the liver tissue were destructed, and the infiltration of inflammation cells and deposition of collagen were observed in the liver of the majority of rats in the model group. The extents of liver inflammation and fibrosis in the model and treatment groups were significantly higher than that of the control group (all $P \leq 0.01$). Notably, the liver fibrosis improved significantly in the treatment group as compared with the model group ($P < 0.05$). At the same time, a less liver inflammation was also observed in the treatment group.

The degree of HSC activation and the distribution of activated HSC in the liver tissue were reflected by $\alpha$-SMA immunohistochemical staining of hepatic tissue. The results are shown in Figure 2 and Table 4. In the control group, the positive area of $\alpha$-SMA staining was confined to the portal vessels and central vein. In the model group, the expression of $\alpha$-SMA increased significantly compared with the control group ($P \leq 0.01$), and the positive area of $\alpha$-SMA staining distributed mainly in liver sinusoids and fibrous septa. After the treatment with ALHXW, the $\alpha$-SMA expression decreased compared with the model group ($P = 0.058$), and the positive area located mainly in liver sinusoids.

The quantitation of peroxisome proliferator-activated receptor-gamma and nuclear factor-kappa B p65 messenger RNA

The messenger RNA (mRNA) expression levels of PPAR$\gamma$ and NF-κB p65 in rat liver tissue were quantitated by reverse transcription quantitative polymerase chain reaction. The relative expression levels of PPAR$\gamma$ mRNA in liver tissues among three groups are shown in Figure 3, demonstrating that the expression levels of PPAR$\gamma$ mRNA in the liver of rats in the model, and treatment groups were significantly lower than that of the control group (all $P < 0.01$), whereas the enhanced expression of PPAR$\gamma$ mRNA was observed in liver tissue in ALHXW-treated rats, significantly higher than that of model rats ($P < 0.05$). In addition, the relative expression levels of NF-κB p65 mRNA in liver tissues among three groups are also shown in Figure 3, reflecting that the expression levels of NF-κB p65 mRNA in liver tissue of the model and treatment groups were significantly higher than that of the control group (all $P < 0.05$). The downregulated expression level of NF-κB p65 mRNA in liver tissue of the treatment group was presented, significantly lower than that in the model group ($P < 0.05$).

The protein expression of peroxisome proliferator-activated receptor-gamma, nuclear factor-kappa B p65, and IκBα

Western blotting was used to test the protein expression of PPAR$\gamma$, NF-κB p65, and IκBα in liver tissues. The significantly downregulated protein expression levels of PPAR$\gamma$ in the model and treatment groups were found as compared with the control group (all $P < 0.05$). Through 6-week therapy with ALHXW, the protein expression level of PPAR$\gamma$ in the treatment group was upregulated, significantly higher than that of the model group ($P < 0.05$) [Figure 4].

As shown in Figure 4, the protein expression levels of NF-κB p65 and p-NF-κB p65 in the model and treatment groups were significantly higher than that in the control group (all $P < 0.05$). After the treatment of ALHXW, the protein expression of NF-κB p65 and p-NF-κB p65 in the treatment group was significantly suppressed compared with the model group ($P \leq 0.01$). Conversely, the protein expression levels of IκBα in the liver tissues in the model and treatment groups were significantly lower than that in the control group (all $P < 0.05$) [Figure 4]. The treatment with ALHXW significantly increased the protein expression of IκBα as compared with the model group ($P \leq 0.01$) [Figure 4].

<table>
<thead>
<tr>
<th>Staining</th>
<th>Evaluation index</th>
<th>Group</th>
<th>$n$</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Score (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HE</td>
<td>Liver inflammation</td>
<td>Control</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Model</td>
<td>11</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>2.91±1.04**</td>
</tr>
<tr>
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<td>Treatment</td>
<td>12</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>2.66±0.89**</td>
</tr>
<tr>
<td>Masson</td>
<td>Liver fibrosis</td>
<td>Control</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Model</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>4.00±0.00**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treatment</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3.00±0.89**</td>
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<tr>
<td>$\alpha$-SMA</td>
<td>HSCs activation</td>
<td>Control</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Model</td>
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<td>0</td>
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<td>1</td>
<td>5</td>
<td>3.83±0.41**</td>
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<td>1</td>
<td>2</td>
<td>2.83±0.98**</td>
</tr>
</tbody>
</table>

**$P$$\leq$0.01 as compared with control group, $^*$$P$$<$$0.05$ as compared with model group. HSCs: Hepatic stellate cells, SD: Standard deviation, $\alpha$-SMA: $\alpha$-smooth muscle actin, HE: Hematoxylin eosin.
In this study, CCl₄, a classical chemical agent, was used to induce rat liver fibrosis. A single dose of CCl₄ causes acute hepatocellular injury with centrilobular necrosis and steatosis, while prolonged administration may lead to liver fibrosis, cirrhosis, and hepatocellular carcinoma. CCl₄-induced liver fibrosis is a useful model for drug screening for liver fibrosis because it shares many characteristics with human fibrosis induced by different etiologies. In our study, we used the rat model with CCl₄-induced hepatic fibrosis to investigate the antifibrotic efficacy and mechanisms associated with ALHXW. Clinical studies have demonstrated that ALHXW has significant effects on alleviating liver fibrosis for patients. However, the antifibrogenic mechanism of ALHXW remains to be investigated. ALHXW exerts an antifibrotic effect by suppressing the TGF-β1/Smad signaling pathway that was published by Dr. Lu from our laboratory. Here, we present a novel finding that the antifibrotic mechanism of ALHXW may target the PPARγ and NF-κB/IκBα signaling pathways. Our data show that most of the rats in the model group had disease status with an abdominal bulge, while the rats in the treatment group had been improved significantly at the end of 6 weeks. The liver and the spleen in the treatment group showed a smoother surface and softer texture compared with the model group. The extent of liver fibrosis in most of the rats in the model group had progressed into phase 3–4, while there is a significant reduction in the treatment group (P < 0.05), most of rats only stayed at Phase 2–3. The Hyp, HA, and LN are serum markers of liver fibrosis. The concentrations of Hyp, HA, and LN in the treatment group were significantly lower than that of the model group (P < 0.01), the results of which were consistent with the extent of liver fibrosis. CCl₄ can impair liver, spleen, and other organs and affect food digestion and absorption in rats, likely accounting for the lower body weight in the model and treatment rats we observed. Further, the liver weight and indexes in the model and treatment groups were significantly higher than that of the control group. Compared with the model group, the body weight of rats in the treatment group increased, while the liver weight, spleen weight, and respective indexes decreased. Our findings demonstrate that ALHXW can protect the rats against CCl₄-induced liver fibrosis.
At the end of the 3 and 6 weeks in the experiment, the levels of ALT and AST in the model and treatment groups were significantly higher than those in the control group ($P \leq 0.01$), indicating that CCl$_4$ caused serious injuries to the liver. After 3 weeks of ALHXW treatment, the ALT and AST levels in the treatment group decreased by 23.46% and 19.96%, respectively, as compared with the model group, demonstrating that ALHXW can protect liver cell and improve liver function. After 6 weeks of ALHXW therapy, the ALT and AST levels in the treatment group were lower than that in the model group, but the difference was not significant. The results might relate to the experimental protocol in which rats were sacrificed, and blood and liver tissue samples were taken on the 2nd day after stopping CCl$_4$ injection of the last time. CCl$_4$ causes an acute liver injury once given, and the heaviest injury occurs within the 2–3 days, and then, acute injuries are gradually repaired following the metabolism of CCl$_4$. The current study just ended at the point of heaviest liver injury caused by CCl$_4$, blood samples were detected, and there was no significant difference in liver function between the treatment and model groups, indicating that the protective effect of ALHXW against the severe acute liver injury is limited.

Activation of HSC is the major precursor of liver fibrosis, and $\alpha$-SMA is an important marker of HSC activation. Activated HSC will have a phenotypic transformation into MFB, which can secrete a large amount of ECM. In this study, $\alpha$-SMA immunohistochemical staining of liver tissue was used to reflect the extent of HSC activation and their distribution. In the model and treatment groups, $\alpha$-SMA expressions in liver tissues were significantly higher as compared with the control group ($P \leq 0.01$). After ALHXW treatment, $\alpha$-SMA expression in liver tissue was lower than that of the model group ($P = 0.058$), suggesting that ALHXW can inhibit the activation of HSC. Mechanisms underlying the effect of ALHXW on HSC inactivation may be related to the modulations of PPAR$\gamma$ and NF-$\kappa$B/I$\kappa$B$\alpha$ signaling pathways.

PPAR$\gamma$ belongs to a superfamily of nuclear receptors and has four major isoforms, namely $\gamma_1, \gamma_2, \gamma_3,$ and $\gamma_4$, which have similar transcriptional activities, and the difference among them is their N-terminal sequence. In a healthy liver, HSC contains lipid droplets which store hepatic retinoid and Vitamin A, and PPAR-$\gamma$ expressed in HSCs plays a crucial role in the maintenance of quiescent HSCs. On fibrogenic injury, quiescent HSC is activated transforming into MFB, the lipid droplets declined quickly, and the adipogenic phenotype no longer existed. PPAR$\gamma$ plays an important role in adipocyte differentiation and lipid metabolism. In addition, the antifibrotic effect of PPAR$\gamma$ can be played by modulating the adipogenic phenotype of HSC. A series of studies demonstrated that PPAR$\gamma$ performed the protective effects in the development of hepatic fibrosis. In vitro experiment, the vector-mediated expression of PPAR$\gamma$ itself is sufficient to reverse the morphology of activated HSC to the quiescent phenotype. In vivo experiment, curcumin promoted the expression of senescence marker Hmga1 in rat fibrotic liver, the mechanism of which might occur through the activation of PPAR$\gamma$/P53 signaling pathway. In our study, the interesting finding was presented that the mRNA and protein expression levels of PPAR$\gamma$ in liver of the ALHXW treatment rats were significantly higher than those in the model rats ($P < 0.05$),
indicating that the ALHXW can attenuate HSC activation and ameliorate liver fibrosis by upregulating the expression of PPARγ. A similar phenomenon was also observed in the study concerning TCM Dan-shao-hua-xian capsules and Litsea coreana LevL. Dan-shao-hua-xian capsules can reverse the process of CCl₄-induced liver fibrosis in rats, and the immunohistochemical and Western blotting analyses showed that the antifibrotic effect exerts by upregulating the expression of PPAR-γ in the liver tissues.[23] Total flavonoids from Litsea coreana LevL. are able to lessen liver injury and protect rats from liver fibrosis by increasing the mRNA and protein expression of PPARγ in the liver.[24]

NF-κB is a family of transcription factors, including NF-κB1 p50, NF-κB2 p52, RELA (also called p65), RELB, and c-REL.[25] In normal liver, NF-κB binds to IκBζ, exists as inactive complexes in cytoplasm. On liver injury, IκBζ will undergo ubiquitination and proteasomal degradation, and then, free NF-κB is able to translocate to the nucleus to play a role.[25,26] NF-κB acts as a key regulator of inflammation, thus playing a major role in liver fibrosis. NF-κB transcriptional activity increases dramatically when HSC is activated, and NF-κB activity maintains stable at significantly higher level.[27] NF-κB exerts a profibrotic effect by maintaining the activity of activated HSC and inhibiting apoptosis of activated HSC.[28] Therefore, NF-κB inhibition is a potential mechanism for the suppression of the liver inflammation and the attenuation of HSC activity. In the current study, the expression levels of NF-κB p65 and p-NF-κB p65 in rats’ liver tissues of the treatment group were significantly lower than that of the model group (P < 0.05), while the expression level of IκBζ was vice versa in the corresponding group (P ≤ 0.01), demonstrating that the ALHXW inhibited CCl₄-induced HSC activation by suppressing NF-κB expression and the subsequent inflammatory response. In a recent study with respect to TCM Liwuweiulining tablets, the same changes in NF-κB p65/IκBζ levels in bile duct ligation-induced rat’s fibrotic liver were observed, revealing that Liwuweiulining tablets attenuate fibrosis also through inhibiting the activation of NF-κB p65 and increasing the expression of IκBζ.[29] In addition, Fuzheng Huayu recipe (FZHY), a TCM, exerts protective effects against nutritional steatohepatitis and fibrosis in mice by modulating the expression of IKK-β/NF-κB.[30] Compared to control mice, mice fed MCD diet exhibited upregulation of hepatic IKK-β and NF-κB p65 (P ≤ 0.01), while administration of FZHY decreased the expression of IKK-β and NF-κB p65 mRNAs (P ≤ 0.01).[30]

Conclusions

ALHXW treatment can inhibit CCl₄-induced liver fibrosis in rats, as confirmed by the improvements in disease status, the morphology of liver and spleen, histological findings, and liver function. The potential antifibrogenic mechanisms of ALHXW may occur through the upregulation of PPARγ expression and the downregulation of NF-κB/IκBζ signaling pathway. ALHXW also exerts an antifibrotic effect by suppressing the TGF-β1/Smad signaling pathway.[12] It is anticipated that our findings on the antifibrogenic mechanisms of ALHXW will contribute to an evidence-based use of ALHXW in clinical applications and benefit the patients.

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Conflicts of interest

There are no conflicts of interest.

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