Mechanic study of Qushi Kaiyu decoction on non-alcoholic fatty liver disease model rats based on the inhibition of TLR4/NF-κB pathway

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Author contributions
Yu-Long Shi carried out the experiments and manuscript writing. Xiao-Yang Yan, Li-Ya Zhu and Min Lin provided experimental help, and performed data analysis and result interpretation. De-Ke Lyu and Jia-Bao Liao was responsible for reviewing, editing, and supervision; Feng Chen provided ideas and technical guidance for the whole work.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
NAFLD, non-alcoholic fatty liver disease; QSKY, Qushi Kaiyu decoction; TCM, traditional Chinese medicine; TC, total cholesterol; TG, triglyceride; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SOD, superoxide dismutase; MDA, malondiadehyde; ELISA, enzyme-linked immunosorbent assay; HE, hematoxylin-eosin.

Background: The objective of this research was to examine the impact of the Chinese herbal formula Qushi Kaiyu (QSKY) on rats with non-alcoholic fatty liver disease (NAFLD) and its inhibitory effect on the TLR4/NF-κB pathway. Methods: NAFLD model rats was constructed through high-fat diet. Meanwhile, rats were treated with QSKY (6.4 g/kg) by gavage. The therapeutic effect of QSKY on NAFLD was assessed by testing body weight change, the liver index, lipid concentrations in blood, and antioxidant and inflammatory levels; assessing liver function; and performing pathological staining including hematoxylin-eosin and Oil Red O. The protein levels of key factors in the TLR4/NF-κB pathway (TLR4, MyD88, p65 and IκB) in rat liver tissue were determined using western blotting in order to explore the mechanism responsible for the therapeutic effects of QSKY in rats with NAFLD. Results: QSKY significantly reduced the liver index and body weight value; reduced triglyceride, cholesterol, alanine aminotransferase, and aspartate aminotransferase levels in NAFLD rats; improved the pathological changes, such as ballooning degeneration, fat accumulation, necrosis, and inflammation; elevated GSH-Px and superoxide dismutase activities and lowered malondialdehyde levels, indicating that QSKY enhanced the antioxidant capacity; and reduced inflammatory cytokine (IL-6, IL-1β, and TNF-α) levels. Western blotting results showed that QSKY significantly reduced TLR4, MyD88, and decreased the phosphorylation of IκB and p65 protein levels in the livers of rats with NAFLD. Conclusions: QSKY showed therapeutic effects on NAFLD and can alleviate oxidative stress and inflammation. This mechanism may be related to an improvement in TLR4/NF-κB pathway.

Keywords: Qushi Kaiyu decoction; non-alcoholic fatty liver disease; oxidative stress; TLR4/MyD88/NF-κB

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**Introduction**

Non-alcoholic fatty liver disease (NAFLD), characterized by diffuse hepatic steatosis and excessive triglyceride in liver cells, can be caused by factors other than alcohol and other known liver injury factor [1]. The worldwide prevalence of NAFLD is over 25.24%, whereas in China, the morbidity is more than 29.2% [2]. NAFLD’s pathogenesis is not fully understood and unclear; however, previous researches have showed that it is closely associated with diabetes and hyperlipidemia [3]. There is short of specific agents against NAFLD in clinical practice, and the treatment mainly focuses on regulating metabolic disorders, reducing inflammation, and improving hepatic fibrosis [4]. Although these methods have achieved some efficacy clinically, they do not delay disease progression in some of the NAFLD patients [5]. Furthermore, the use of certain drugs leads to adverse reactions [6]. Thus, a detailed exploration of the pathogenesis of NAFLD and developing related drugs are crucial topics currently [7].

Traditional Chinese medicine (TCM) has demonstrated its advantages in targeting multiple pathways and causing fewer toxic side-effects and it is effective on NAFLD. In recent decades, accumulated studies have been conducted regarding the treatment of NAFLD using TCM [8]. In one study, Ruangan decoction was found to protect liver cells by reducing blood lipid levels and lowering transaminase levels, thereby improving the clinical symptoms of NAFLD patients. Another research study has corroborated the beneficial effects of Lingui Zhugan decoction in ameliorating liver lipid accumulation in mice with NAFLD. This improvement is attributed to the inhibition of the STING/TLR1/2-NF-κB pathway specifically in hepatic macrophages [9]. Clinical studies have shown that the combination of Shugan Jianpi decoction and Ciliao Zexie therapy reduces blood lipid levels, alleviates liver fibrosis, and improves liver function in patients with NAFLD [10].

The Qushi Kaiyu decoction (QSKY), consisting of 12 g of *Bupleuri Radix*, 9 g of *Paonieae Alba Radix*, 9 g of *Aurantii Immaturus Fructus*, 9 g of *Pinelliae Rhizoma*, 6 g of *Rhei Radix et Rhizoma*, 9 g of *Menthae Herba*, 9 g of *Nelumbinis Folium*, and 9 g of *Eupatori Herba*, is derived from the TCM formula, Dachaihu decoction [11]. Previous study showed that QSKY has a curative effect on NAFLD in rats and reduces inflammation in the liver. To further explore the specific mechanism responsible for the QSKY’s effect on NAFLD, we established a NAFLD rat model induced by a high-fat diet and treated the rats with QSKY. We first demonstrated the QSKY’s therapeutic effect on NAFLD; then investigated its effects on inflammation and oxidative stress in rats with NAFLD, and finally explored its mechanism of action by examining the effects of QSKY on TLR4/ NF-κB pathway.

**Methodology**

*Drugs and reagents*

A high-fat diet (17.7% sucrose, 17.7% fructose, 19.4% protein, and 40% fat) was obtained from Huafukang Biotechnology Co., Ltd. (Beijing, China). Aspartate aminotransferase (AST), superoxide dismutase (SOD), total cholesterol (TC), alanine aminotransferase (ALT), GSH-Px, triglyceride (TG), and malondialdehyde (MDA) assay kits were bought from Nanjing Jiancheng Biological Engineering Research Institute (Nanjing, China). Antibodies against TLR4, MYD88, p65 antibody, IκB, and p-IκB were obtained from Proteintech (Rosemont, IL, USA). p-P65 was bought from Cell Signaling Technology (Danvers, MA, USA).

*Preparation of QSKY decoction*

QSKY consists of *Bupleuri Radix* (12 g), Banxia (9 g), *Paonieae Alba Radix* (9 g), *Aurantii Immaturus Fructus* (9 g), *Rhei Radix et Rhizoma* (6 g), *Menthae Herba* (9 g), *Eupatori Herba* (9 g) and *Nelumbinis Folium* (9 g). The herbal ingredients were soaked in eight times the amount of purified water for 1 h and then boiled twice, for 30 min each time. After boiling, the herbal residue was filtered, and the two decoction batches were combined and concentrated to obtain a concentration of 2 g of crude herbs per mL. The solution was refrigerated at 4 °C for subsequent use.

**Establishment and grouping**

Thirty male Sprague Dawley rats, (average age of 8 weeks and a weight range of 200 ± 20 g) were purchased from Huafukang Biotechnology (Beijing, China). The animals were housed in a room with 25 ± 2 °C temperatures, 50 ± 15% humidity, and a 12-hour light-dark cycle, and were free accessed to food and water. The animal experiment was permitted by the Animal Ethics Committee of Jiaxing Hospital of TCM, whose batch number is SL-2021-0012. The research was conducted in accordance with internationally recognized principles for the use and care of laboratory animals, Guide for the Care and Use of Laboratory Animals: Eighth Edition (Washington, DC: The National Academies Press).

After an one-week adaptive feeding, the rats were divided into three groups: control/model/QSKY groups (n = 10 per group). The control group of rats was fed a normal diet, whereas the other two groups were fed a high-fat diet. The QSKY group was administered QSKY at a dose of 6.4 g/kg per day by gavage, whereas the control and model groups were administered the same volume of physiological saline as a vehicle control [12]. The generation of the model and the administration of QSKY or saline were started simultaneously and continued for 12 weeks. The dose of QSKY was determined based on twice the equivalent human dose. The condition of the rats was monitored daily, and their body weights were measured and recorded weekly.

**Tissue sample preparation**

Twelve weeks following QSKY administration, rats were anesthetized with pentobarbital sodium and euthanized. The blood was obtained from the heart, and the serum was subsequently separated. Livers were quickly harvested and the liver weight was recorded. Tissues used for pathological sectioning were cut from the same area on the tissue lobes for each rat and were fixed in 4% paraformaldehyde solution or stored after snap freezing. The remaining tissue was deposited at –80 °C after snap freezing.

**Biochemical measurements**

Following the instructions provided in the TC, TG, ALT, and AST assay kits, the optical density values of the samples were determined using a fully automatic enzyme-linked immunosorbent assay (ELISA) plate reader and were entered into the appropriate formulae to analyze the TG and TC levels and the ALT and AST activity in the sera of each group of rats.

The frozen liver samples were mixed with physiological saline at a weight: volume ratio of 1:9 and completely disrupted by ultrasonication in an ice bath. After centrifugation (2500 rpm, 10 min), the supernatants were collected as a 10% liver homogenate. SOD, MDA, and GSH-Px levels in the liver tissue were tested using commercial assay kits.

**Histopathological staining**

The liver tissues were fixed, embedded in paraffin, and then sectioned. Following hematoxylin-eosin (HE) staining, the liver tissues were examined under a microscope to assess for gross pathological changes and inflammation. Fresh frozen liver tissues stored at –80 °C were cut into sections and received Oil Red O staining to observe the accumulation of fat under a light microscope.

**ELISA**

ELISAs were used to determine the expression levels of IL-1β, TNF-α, and IL-6 in the liver tissues of rats in each group. Specific assays were performed according to the manufacturer’s protocol.

**Western blotting analyses of relevant protein levels**

Western blotting analysis was conducted to determine the protein levels of key factors in the TLR4/ NF-κB pathway in liver tissue. Rat liver tissue was homogenized and centrifuged, and total protein was...
extracted. The protein samples were separated by SDS-PAGE and transferred onto a PVDF membrane Anti-rat TLR4 (1:1000); MyD88 (1:4000); inhibitor of NF-κB (1:4000); p-IκB (1:4000); NF-κB p65 (1:8000); p-P65 (1:2000), and β-actin (1:10000), were added and the membranes were incubated overnight at 4 °C. Then the relative secondary antibody (goat anti-rabbit IgG, 1:20000) was added. Protein expression levels were determined using actin as an internal reference, and the average optical density values were quantified using ImageJ.

**Statistics**

Statistical analysis was conducted using Statistical Product and Service Solutions Statistics 20.0. The measurement data were expressed as mean ± standard deviation and analyzed using Student’s t-test. For comparisons among multiple groups, analysis of variance was employed. Statistical significance was defined as P < 0.05.

**Results**

Liver index and body weight values of rats following model generation and drug administration

After the generation of the model and administration of the drug, the liver index and body weight values of rats were significantly increased in the model group compared to the control group. However, in the QSKY group, the liver index and body weight values of rats were significantly reduced (Figure 1).

**Biochemical indices**

The serum activity of ALT, TG, AST, and TC was significantly elevated in the model group compared to the normal group. However, in the QSKY group, the serum activity of AST, ALT, TG, and TC was significantly lower compared to the model group (Figure 2).

**Pathology**

HE staining revealed that the liver morphology in control rats exhibited normal and clear structure, characterized by evenly sized liver cells, centrally located nuclei, abundant cytoplasm, and well-defined sinusoid arrangement. In contrast, the liver tissues of the model group displayed notable pathological alterations, including pronounced hepatocyte steatosis, balloon-like changes, and inflammation, when compared to the control group. However, in comparison to the model group, the QSKY group exhibited varying degrees of improvement in steatosis, balloon-like changes, and lobular inflammation (Figure 3).

**Oil Red O staining**

Oil Red O staining revealed that the liver tissues of rats in the control group exhibited a regular pattern. In contrast, liver tissue sections of rats in the model group showed the presence of diffuse granular lipid droplets. However, in the QSKY group, the accumulation of lipid droplets in liver cells was significantly reduced when compared to rats with NAFLD (Figure 4).

Figure 1 Body weight and liver index of rats after modeling and administration. **”, in comparison to control group, P < 0.01; “”, in comparison to the model group, P < 0.01. QSKY, Qushi Kaiyu decoction.

Figure 2 Serum biochemical indexes of rats after modeling and administration. **”, in comparison to control group, P < 0.01; “”, in comparison to the model group, P < 0.01. QSKY, Qushi Kaiyu decoction; TC, total cholesterol; TG, triglyceride; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
Anti-oxidative effects of QSKY on NAFLD

We investigated the impact of QSKY on oxidative stress in rats with NAFLD by assessing the activities of GSH-Px and SOD, as well as the level of MDA in the liver. The activities of GSH-Px and SOD were observed to decrease, while the level of MDA increased in the model group compared to the control group. However, treatment with QSKY significantly elevated the activities of GSH-Px and SOD, and reduced the level of MDA in the liver of rats with NAFLD ($P < 0.01$) (Figure 5).

Inflammatory cytokine levels

Following the establishment of the NAFLD model and administration of the drug, the levels of inflammatory cytokines TNF-α, IL-6, and IL-1β in the liver were significantly elevated in the model group compared to the control group. However, in the QSKY group, the levels of these cytokines were significantly reduced in comparison to the model group (Figure 6).

Effects of QSKY on the protein levels of key factors in the TLR4/NF-κB pathway

The protein levels of TLR4, MyD88, and the phosphorylation of IκB and p65 in the liver were assessed through western blotting. The results demonstrated a significant increase in the levels of TLR4, MyD88, and the phosphorylation of IκB and p65 in the liver of the model group when compared to the control group (Figure 7A). However, following QSKY treatment, there was a notable reduction in the levels of TLR4, MyD88, and the phosphorylation of IκB and p65 (Figure 7B).

Discussion

NAFLD is a complex, multifactorial disease closely related to genetic, environmental, and other factors. However, the exact mechanisms underlying the progression of NAFLD are not totally understood. The “two-hit theory of liver injury” suggests that insulin resistance is the central link of the first hit, and it leads to elevated free fatty acid levels and lipid deposition, which promote steatosis. The “second hit” involves oxidative stress, with lipid accumulation in the liver leading to lipid peroxidation, which induces inflammation and fibrosis, and promotes liver disease progression [13]. The “multiple-hit theory of liver injury” suggests that oxidative stress is the main factor leading to liver injury and disease progression in NAFLD [14].

Dachaihu decoction has been found to have potentials of clearing and benefiting the liver and gallbladder, promoting intestinal movement, eliminating turbidity, and regulating and strengthening the spleen [15, 16]. Previous clinical studies and animal experiments have indicated that Dachaihu decoction can improve liver fat levels in patients with NAFLD. QSKY is a modification of Dachaihu decoction. It includes the addition of Nelumbinis Folium, Perilla Folium, and Menthae Herba, while excluding Zingiberis Rhizoma and Jujubae Fructus. Nelumbinis Folium and Perilla Folium are widely used to treat alcoholic liver disease [17]. Preliminary studies have indicated that QSKY effectively improves blood lipid levels, inflammation, and hepatic histopathological changes in rats with NAFLD [11], demonstrating its therapeutic effects on NAFLD. To elucidate the specific mechanism of QSKY in NAFLD, we further investigated the effects of QSKY on TLR4/MyD88/NF-κB pathway, based on previous research findings.

After twelve weeks of consuming a high-fat diet, rats in the model group exhibited obviously elevated liver index, body weight values, blood lipid levels, and liver-function-related biochemical indicators, as well as obvious hepatic cell steatosis. QSKY lowered the liver index values in the rats with NAFLD, improved the blood lipid levels, and alleviated liver cell steatosis, indicating a good therapeutic effect against NAFLD. Furthermore, our findings demonstrated that QSKY administration resulted in increased activity of SOD and GSH-Px, as well as decreased levels of MDA, IL-1β, TNF-α, and IL-6 in the liver of rats with NAFLD. These observations indicate that QSKY enhances the antioxidant capacity and ameliorates inflammation.

TLR4 is a key signal transduction receptor involved in inflammatory responses. When hepatic cells undergo steatosis, a series of cascading signal transduction pathways activate TLR4, triggering the aggregation of MyD88 and activation of the MyD88-dependent signaling pathway [18, 19]. MyD88, as a signal adapter molecule,
Figure 5 Effect of QSKY on oxidative stress in NAFLD rats. **, in comparison to control group, $P < 0.01$; †, in comparison to the model group, $P < 0.01$. QSKY, Qushi Kaiyu decoction; NAFLD, non-alcoholic fatty liver disease; SOD, superoxide dismutase; MDA, malondialdehyde.

Figure 6 Levels of inflammatory factors in liver tissue of rats in each group after modeling and administration. **, in comparison to control group, $P < 0.01$; †, in comparison to the model group, $P < 0.01$. QSKY, Qushi Kaiyu decoction.

Figure 7 Influence of TLR4/ NF-κB pathway key factors protein levels in rat liver. **, in comparison to control group, $P < 0.01$; †, in comparison to the model group, $P < 0.01$; *, in comparison to the model group, $P < 0.05$. QSKY, Qushi Kaiyu decoction.

causes the dissociation of NF-κB dimers and the inhibitory factor IκB in downstream signaling pathways. The dissociation of IκB can lead to rapid phosphorylation, facilitating the translocation of NF-κB to the κB-binding site within the nucleus. This, in turn, activates the transcription of inflammatory factors, thereby influencing the synthesis and secretion of various inflammatory mediators and aggravating liver injury [20, 21]. Our study observed a significant increase in the protein levels of TLR4, MyD88, p65, and p-IκB in the liver of rats in the model group. After QSKY intervention, the above indicators significantly improved, indicating that QSKY may effectively treat NAFLD in rats by modulating the inflammatory pathway.

In summary, our study examined the therapeutic effects of QSKY in rats with NAFLD and identified a potential mechanism involving the reduction of inflammatory factors in the liver.

References

1. Lazarus JV, Mark HE, Villota-Rivas M, et al. The global NAFLD policy review and preparedness index: Are countries ready to


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