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OBM Integrative and Complementary Medicine

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Original Research

YNCRG Alleviated Hepatic Steatosis in Metabolic Syndrome Model Rats by Improving Lipid Metabolism and Chronic Inflammatory Reaction

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Academic Editor: Rafat A. Siddiqui

Special Issue: Metabolic Syndrome

OBM Integrative and Complementary Medicine
2022, volume 7, issue 2
doi:10.21926/obm.icm.2202023

Received: October 25, 2021
Accepted: June 20, 2022
Published: June 30, 2022

Abstract

YNCRG is a combination of five traditional Chinese ingredients with medicinal properties. We studied its effects and mechanism of action on a rat model of non-alcoholic fatty liver with metabolic syndrome. Eight-week-old male SHRcp rat(s) and Wister Kyoto (WKY) rats were adaptively fed for one week, after which they were randomly divided into three groups, including the control group (n = 9), the YNCRG group (n = 8), and the WKY group (n = 8). The WKY group could freely consume water, the control group was provided water every day, and

the YNCRG group was gavaged with an aqueous solution of YNCRG (3.6 g/kg body weight/day) for eight weeks. After eight weeks of intervention, YNCRG effectively reduced the weight of rats and significantly reduced the serum levels of AST, ALT, CHO, and TG compared to the weight of the rats in the control group. The RT-PCR results showed that mRNA levels of NF-κB, TLR4, TNF-α, and IL-1β increased, and the levels of MCP-1 and ICAM-1 also increased, but YNCRG effectively reduced the mRNA levels. The results of the Western blotting assay showed that YNCRG did not affect the AMPK/ACC signaling pathway, but it strongly promoted the phosphorylation of PKA, ERK, and C/EBPβ in rat livers. YNCRG also significantly promoted the phosphorylation of IRS-1 (Tyr465) but inhibited IRS-1 (Ser1101); both are upstream factors of ERK. We found that YNCRG can reduce the accumulation of lipids in the liver of rats with metabolic syndrome, probably by affecting lipid metabolism and the signaling pathway associated with inflammatory reactions.

Keywords
YNCRG; lipid metabolism; NAFLD; TLR4/NF-κB signaling pathway

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most frequent cause of liver disease and often coexists with metabolic syndrome, obesity, and type 2 diabetes. The pathological feature of NAFLD is hepatic steatosis, which occurs mainly due to the excessive accumulation of triglycerides in the hepatic cells. With the development of chronic inflammation of NAFLD, steatohepatitis, fibrosis, cirrhosis, and even hepatocellular carcinoma might occur [1-3]. Approximately 24% of people around the world suffer from NAFLD. Approximately 100 million people in the United States will suffer from NAFLD by 2030 [4]. For Asians, this proportion is between 2.04% and 52%, based on large population surveys. A follow-up study found that 40-50% of NAFLD patients might develop non-alcoholic steatohepatitis (NASH) within 13 years, and 25-50% of NASH patients might develop cirrhosis within 13 years [5]. Lifestyle intervention is important for the treatment of NAFLD. Many pharmacological interventions have been tested for limiting the development of NAFLD, such as pioglitazone, vitamin E, obeticholic acid (OCA), and elastin. Although these agents can ameliorate hepatic steatosis and inflammation and even improve liver insulin sensitivity, they have various side effects [6, 7].

Traditional Chinese medicine has few side effects and multiple targets. Therefore, pharmacodynamic research has commercial value. In another study, we found that YNCRG can improve the symptoms of metabolic syndrome such as obesity, hypertension, and insulin resistance in rats. YNCRG can effectively reduce total cholesterol levels and AST and ALT levels in SHR. In Cg-Lepr^{cp}/NDmcr rats ((SHRcp rat(s)), YNCRG can inhibit lipogenesis and promote lipolysis in the adipose tissue [8]. YNCRG is a herbal formula prepared by our team and consists of five ingredients, including Tochu leaf extract, Red koji rice (*Monascus purpureus*), Noni (*Morinda citrifolia*), L-citrulline, and gamma-aminobutyric acid. The pharmacological activities of these five ingredients were discussed in our previous study [8]. The SHRCP rat is a common animal model used for studying metabolic syndrome, and there is a close relationship between metabolic syndrome and NAFLD [9,

10]. Based on the beneficial effects of lipid metabolism, in this study, we elucidated the effects and possible mechanism of action of YNCRG on hepatic steatosis in rats with metabolic syndrome.

2. Materials and Methods

2.1 Preparation of YNCRG

Tochu leaf extract was purchased from Maruzen Pharmaceutical Co., Ltd., Hiroshima, Japan, number: 01101047; Red koji rice was purchased from Kobayashi Pharmaceutical Co., Ltd., Osaka, Japan, number: 320-180801; Noni was purchased from Earth Ship Co., Ltd, Osaka, Japan, number: 40004; L-citrulline was purchased from Protein Chemical Co., Ltd., Tokyo, Japan, number: 75A05CT; gamma-aminobutyric acid was purchased from Coach Boueki Co., Ltd, Osaka, Japan, number: 20180104. These five ingredients were dissolved in water at a ratio of 4:2:2:1:1, respectively.

2.2 Animals and Treatment

Eight-week-old male SHRcp rat(s) and Wister Kyoto (WKY) rats (Japan SLC, Inc., Shizuoka, Japan) were adaptively fed for one week, after which they were randomly placed in one of three groups, including the normal (WKY) group (Wister Kyoto (WKY) rats, n = 8), the control group (SHRcp rat(s), n = 9), and the YNCRG group (SHRcp rat(s), n = 8). The WKY group could drink water freely, the control group was provided water every day via gavage, and the YNCRG group was gavaged with an aqueous solution of YNCRG (3.6 g/kg body weight/day). As mentioned previously, we administered multiple doses of YNCRG, and we selected the experimental period for which YNCRG had the strongest effect on the metabolic syndrome of rats [8]. We weighed the rats daily. After eight weeks of intervention, all rats were anesthetized with 5% isoflurane at an oxygen flow rate of 2 L/min and killed by bleeding the abdominal aorta with a large-bore needle. Then, the organs, including the liver, heart, kidneys, pancreas, and brain, were excised, immediately placed in liquid nitrogen, and stored at -80° for subsequent analysis. All procedures were performed following the guidelines established by the Japanese Physiological Society. This study was approved by the Experimental Animal Ethics Committee of the Mukogawa Women's University in Japan (Number: P-06-2018-01-A).

2.3 Biochemical Analysis of Serum

Biochemical assay kits (all from Wako, Japan) were used to determine the levels of total cholesterol (CHO), triglycerides (TG), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) from the serum of rats at 0, 4, and 8 weeks. Serum was collected from the tail artery on the 0^{th} and 4^{th} week, and the abdominal aorta on the 8^{th} week.

2.4 Hematoxylin and Eosin Staining

The liver was dehydrated with different concentrations of ethanol gradient. Then, the liver was embedded and sliced, following the manufacturer's instructions for hematoxylin and eosin staining (H&E staining) [11]. Finally, H&E staining was performed to identify the morphological changes in liver tissue by examining them under a light microscope (Olympus, Japan) (original magnification, 200×).

2.5 RNA Extraction and Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was extracted from the liver using the Sepasol-RNA I Super G (Nacalai Tesque, JAPAN) kit. Then, the adjusted concentration of RNA (0.5 μg) was reverse-transcribed to cDNA. We used the THUNDERBIRD SYBR qPCR Mix to quantify the level of target gene expression (TOYOBO, Tokyo, Japan) (Table 1) and set the following thermocycling conditions: 95°C, 30 s, 1 cycle; 95°C, 5 s, 60°C, 30 s, 40 cycles; at 95°C, 15 s, 60°C, 30 s, 1 cycle (Takara Bio Inc., Japan). The results were analyzed using the $2^{-\Delta\Delta Ct}$ method, and the expression of mRNA was evaluated by relative quantification using GAPDH as an internal reference.

Table 1 The list of the primer sequences that were used in real-time PCR.

Genes	Sense primer (5'-3')	Antisense primer (5'-3')
Icam1	GGCGTCCATTTACACCTATTA	TTCCTTTTCTTCTCTTGCTTG
Ccl2	TTGGCTCAGCCAGATGC	CCAGCCTACTCATTGGGATCA
Tnfα	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC
Il10	GCCAAGCCTTGTCAGAAATGA	TTTCTGGGCCATGGTTCTCT
Il1β	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC
Nfκb	ACGATCTGTTTCCCCTCATC	TGCTTCTCTCCCCAGGAATA
Tlr4	TTGCCTTCATTACAGGGACTT	CAGAGCGGCTACTCAGAAACT
Gapdh	AGAACATCATCCCTGCATCCA	CCGTTCAGCTCTGGGATGAC

2.6 Western Blotting Analysis

Total liver protein was extracted using a homogenization buffer (50 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1% Nonidet-P40, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM B extracted protein and acid [EDTA], 50 mM NaF, 2 mM Na_3VO_4, 30 mM sodium pyrophosphate, 2 mM phenylmethanesulfonyl fluoride [PMSF], 1 mM benzoidine, 0.02 mg/mL trypsin inhibitor, 0.02 mg/mL leupeptin, and 0.02 mg/mL aprotinin). The protein concentration was determined using the BSA protein determination kit (Bio-Rad), by performing SDS-PAGE (10–12.5%) to isolate the target protein. Then, the target protein was transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham Life Science, Inc., Buckinghamshire, UK). The membrane was blocked after 30 min with Blocking-one when the above procedure was completed. Next, the membrane was incubated with different primary antibodies at 4°C overnight. After washing three times with TTBS, the membrane was incubated for 1 h at room temperature with the secondary antibody (anti-rabbit or mouse IgG-horseradish peroxidase) for blocking. The PVDF membrane was rinsed thrice (10 min/wash) with TTBS, and all protein bands were analyzed using the Image J software (National Institutes of Health).

2.7 Statistical Analysis

The data are expressed as the mean ±SD (standard deviation). The Student's *t*-test and the one-way analysis of variance, followed by Dunnett's test or Tukey's test, were performed to determine the differences between groups. The GraphPad Prism V6.0 software was used to draw charts. All differences among and between groups were considered to be statistically significant at $P < 0.05$.

3. Results

3.1 Effects of YNCRG on Weight and Metabolic Parameters

First, we determined the effect of YNCRG on the weight of the rats and found that YNCRG can inhibit weight gain, which was significant after week 8, compared to the changes in the weight of the rats in the control group (Figure 1A). The effect of YNCRG on the metabolic parameters in rats is shown in Figure 1. The levels of serum TG, CHO, AST, and ALT increased significantly in the control group compared to those in the WKY group on the eighth week. YNCRG effectively reduced serum TG, CHO, AST, and ALT levels after eight weeks of intervention, indicating that YNCRG can improve lipid metabolism and liver function (Figures 1B-E). These results were similar to those of our previous study [8].

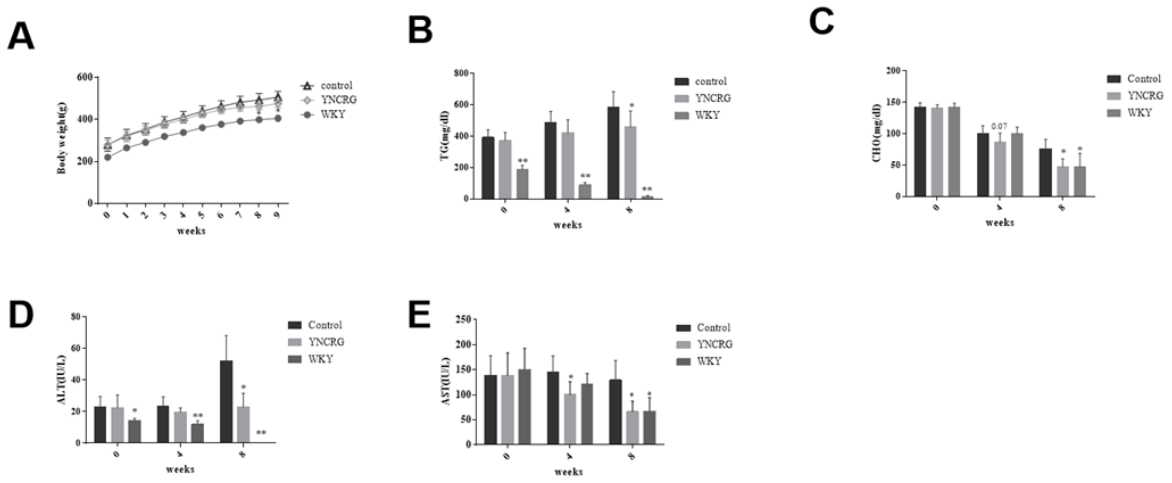

Figure 1 A. The weight change trend in the control, YNCRG group, and WKY groups after eight weeks of experimental intervention in eight-week-old male SHRCP rats. The levels of B. TG, C. CHO, D. ALT, and E. AST in the control, YNCRG, and WKY groups at 0, 4, and 8 weeks; **P* < *0.05* and ***P* < *0.01* compared to the control group. Data are expressed as the mean ±SD (control group [n = 9], YNCRG group [n = 8], and WKY group [n = 8]).

3.2 Effects of YNCRG on Liver Morphology

To further evaluate the effect of YNCRG on rat liver morphology, we performed H&E staining and observed the morphological changes in the liver. The results showed that the control group showed macrovesicular steatosis in hepatic tissue compared to the WKY group, while the application of YNCRG reversed the pathological symptoms (Figure 2).

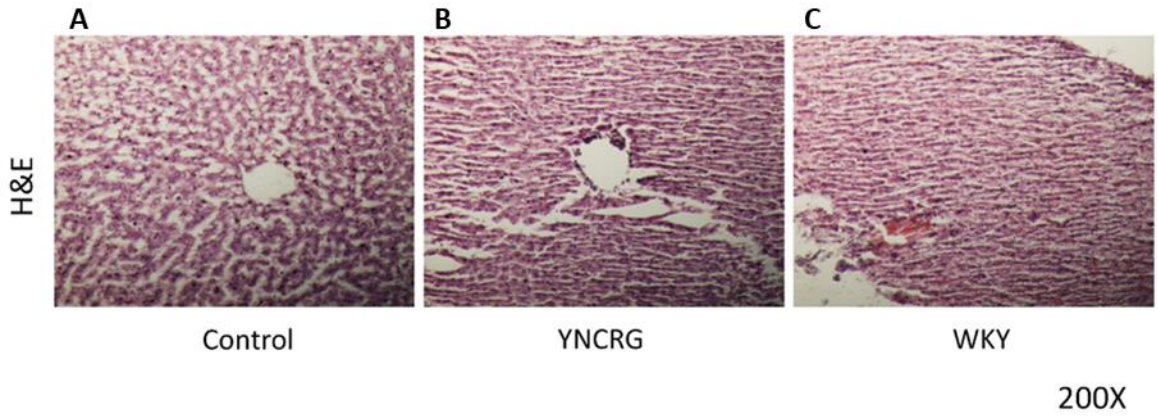

Figure 2 Eight weeks of experimental intervention in eight-week-old male SHRCP rats. A. The changes in the liver morphology of the control group. B. The changes in the liver morphology of the YNCRG group. C. The changes in the liver morphology of the WKY group. Magnification: 200×.

3.3 No Effect of YNCRG on the SIRT1/AMPK Signaling Pathway

The SIRT1/AMPK signaling pathway can ameliorate the non-alcoholic fatty liver [12-14]. Therefore, we evaluated the factors related to the SIRT1/AMPK signaling pathway and found that YNCRG does not affect this signaling pathway (Figure 3).

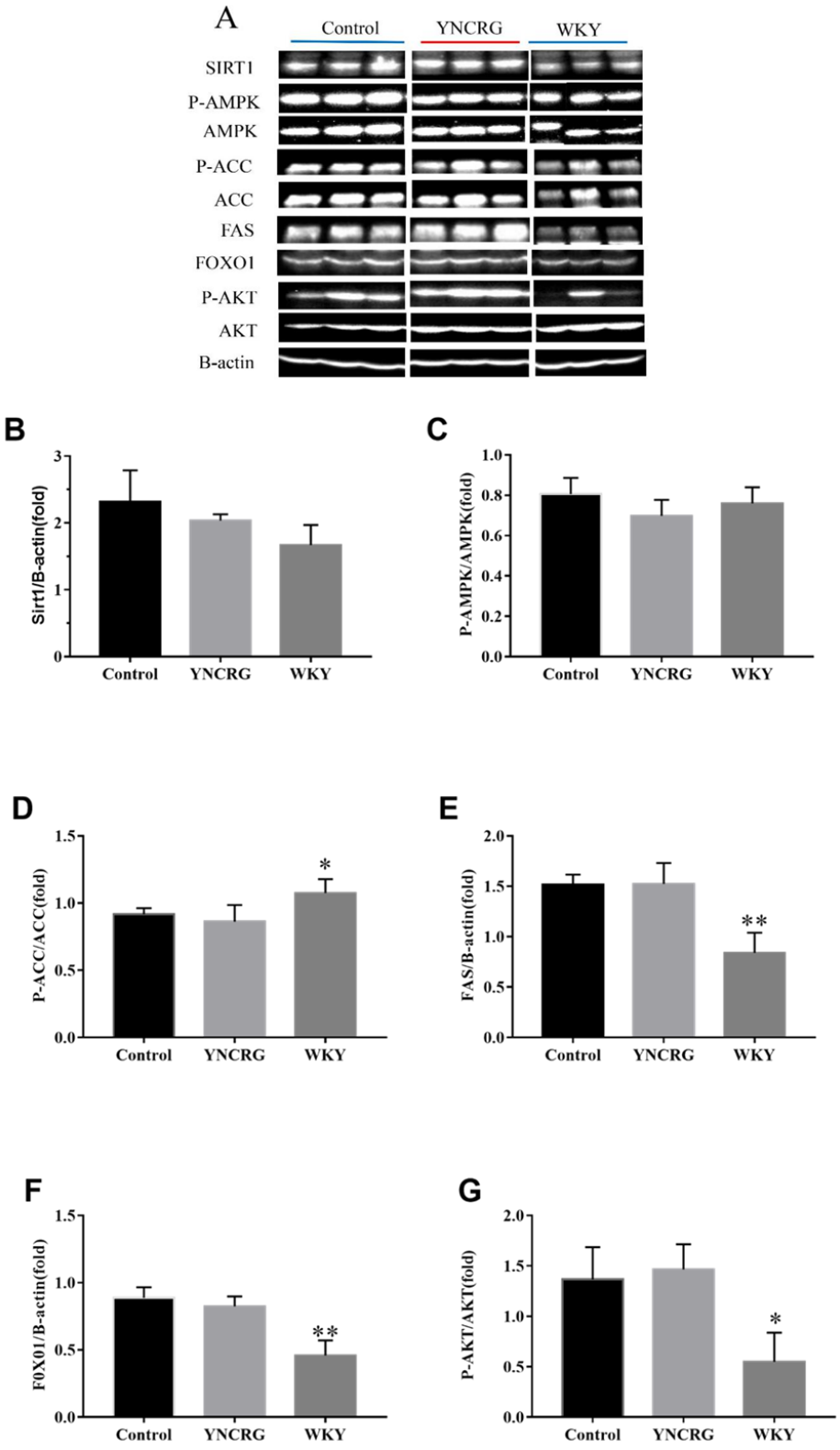

Figure 3 The effects of YNCRG on the SIRT1/AMPK signaling pathway of the liver after eight weeks of intervention. (A) The protein expression levels of SIRT1, P-AMPK, AMPK, P-ACC, ACC, FAS, FOXO1, P-AKT, and AKT of the control group (n = 9), the YNCRG group (n = 8), and the WKY group (n = 8). (B-G) The bar graphs indicate the average levels of SIRT1, P-AMPK, AMPK, P-ACC, ACC, FAS, FOXO1, P-AKT, and AKT. The data are expressed as the mean ±SD; **P < 0.05* and ***P < 0.01* compared to the control group.

3.4 Effect of YNCRG on the PKA-C/EBPβ Signaling Pathway

YNCRG treatment significantly increased the expression of PKA compared to its expression in the rats of the control group (Figure 4B). AMPK, a downstream factor of PKA, was unaffected. However, ERK, another downstream factor of PKA, was phosphorylated. The results revealed that YNCRG effectively promoted ERK activation in the YNCRG group compared to that in the control group. (Figure 4C). Additionally, YNCRG treatment also significantly promoted the phosphorylation of IRS1 (Tyr465) and inhibited the phosphorylation of IRS1 (Ser1101) (Figures 4D-E). We also determined the expression of the factors downstream of ERK and found that YNCRG could significantly promote the expression of C/EBPβ (Figure 4F). Although the expression of C/EBPα and PPARγ showed a decreasing trend, their levels among the groups of rats did not differ significantly (Figures 4G-H).

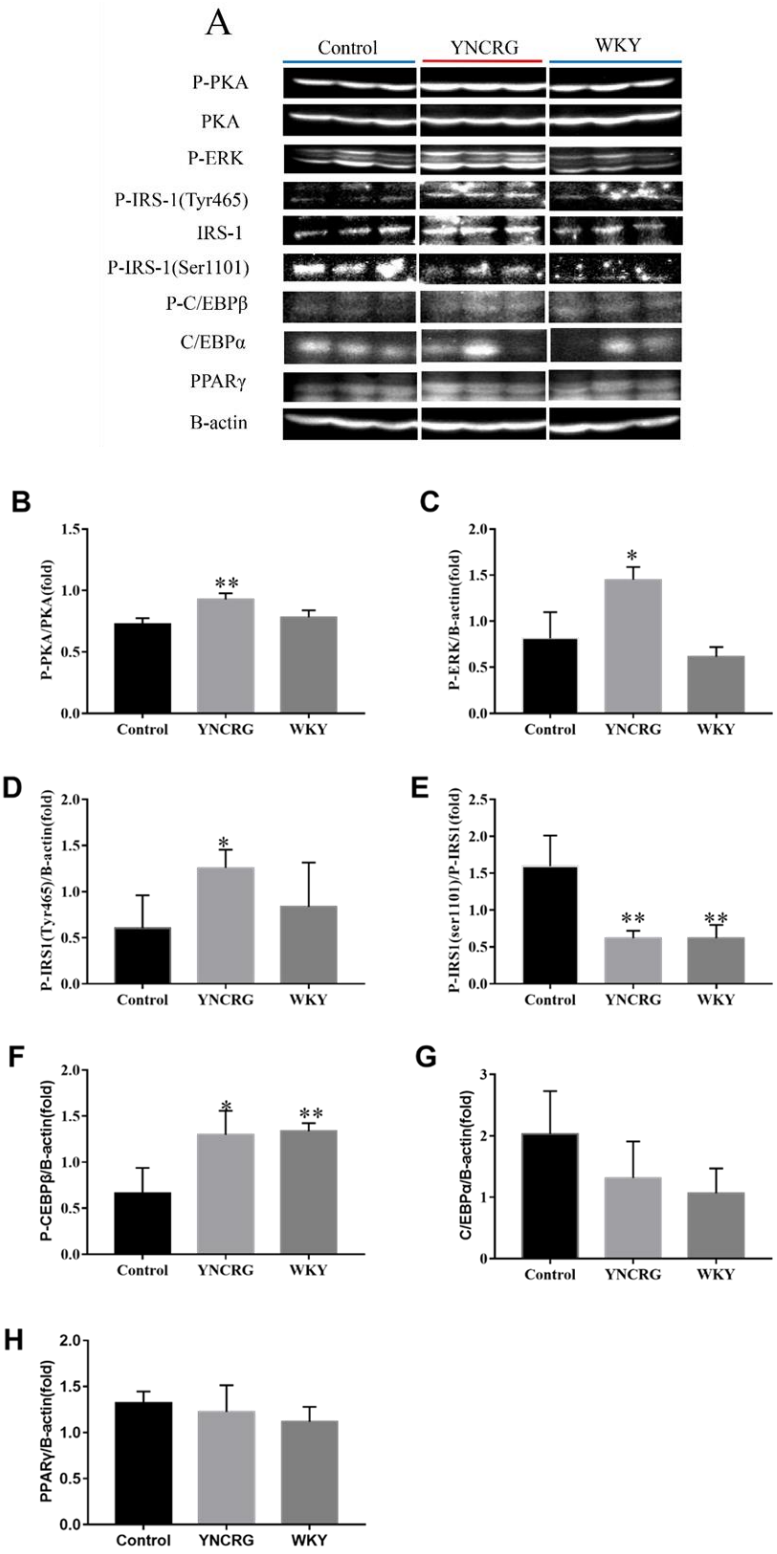

Figure 4 The effects of YNCRG on the PKA-C/EBPβ signaling pathway in the liver after eight weeks of intervention. (A) The protein expression levels of P-PKA, PKA, P-ERK, P-IRS1 (Tyr465), IRS-1, P-IRS1 (Ser1101), P-C/EBPβ, C/EBPα, and PPARγ in the control group (n = 9), the YNCRG group (n = 8), and the WKY group (n = 8). (B-H) The bar graphs indicate the average levels of P-PKA, PKA, P-ERK, P-IRS1 (Tyr465), IRS-1, P-IRS1 (Ser1101), P-C/EBPβ, C/EBPα, and PPARγ. The data are expressed as the mean ±SD; *$P < 0.05$ and **$P < 0.01$ compared to the control group.

3.5 Effect of YNCRG on the Expression of Inflammation-Related Genes

To determine whether YNCRG affects inflammation in the liver of rats, the expression of factors related to inflammation in rats were detected. The results indicated that eight weeks of YNCRG intervention significantly reduced the mRNA of *Nfκb* and *Tlr4* in the liver. *Tnfα* and *Il1β* were also inhibited, but the difference in their levels between the YNCRG and control groups was not significant. Moreover, YNCRG had an inhibitory effect on the mRNA expression of inflammatory adhesion factor *Icam1* and significantly influenced the mRNA expression of chemokine *Ccl2*. Additionally, it promoted the expression of the *Il10* mRNA, although there was no significant difference in its level between the YNCRG and control groups (Figure 5).

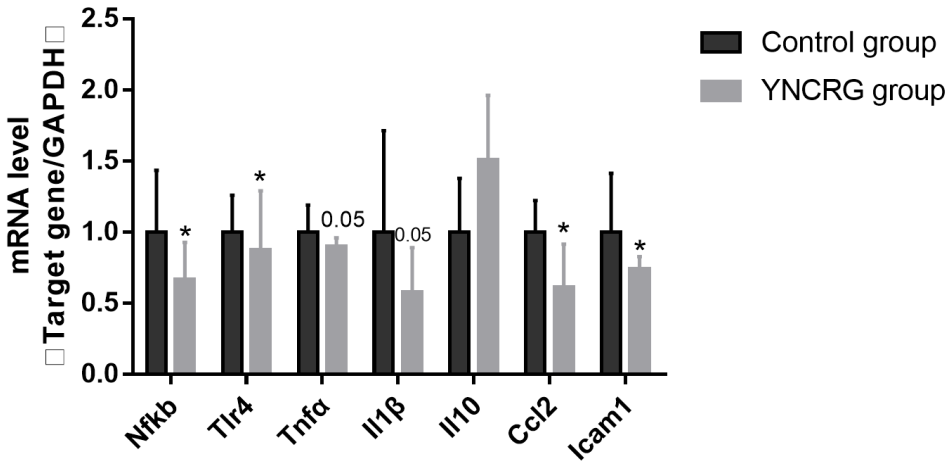

Figure 5 The effects of YNCRG on the mRNA expression of inflammation-related genes in the liver after eight weeks of intervention. The data are expressed as the mean ±SD; YNCRG group (n = 8), and control group (n = 9); **P < 0.05* and ***P < 0.01* compared to the control group.

4. Discussion

SHR_{CP} is a rat model of metabolic syndrome that spontaneously develops obesity, hypertension, hyperlipidemia, hyperglycemia, and hyperinsulinemia [15, 16]. Wistar Kyoto (WKY) rats were developed from an outbred Wistar stock, which was often used as a control for the SHR rat [17]. The abnormal lipid metabolism caused by metabolic syndrome is closely related to the occurrence of non-alcoholic fatty liver. Along with the accumulation of lipids in hepatocytes, it can lead to liver damage, inflammation, fibrosis, and even cirrhosis [18]. Therefore, we selected this rat model to study the effect and mechanism of action of YNCRG on NAFLD.

YNCRG is a combination of five traditional Chinese medicinal ingredients and was formulated by our research team based on previous studies. The results of the LC-MS analysis showed that Tochu leaves, red koji rice, and Noni mainly contain chlorogenic acid, scandoside methyl ester, (+)-pinoresinol, genistin acetate, mangiferin, dehydroconiferyl alcohol, and 6α-hydroxyadoxoside [8]. Tochu leaf extract can inhibit liver fatty acid and cholesterol biosynthesis [19], red koji rice can prevent obesity [20], Noni can prevent oxidative stress and obesity in mice administered a high-fat diet [21], L-citrulline can improve metabolic syndrome primarily by enhancing glucolipid metabolism [22], and gamma-aminobutyric acid can help adipose tissue to selectively suppress inflammation

and improve insulin resistance in obesity [23]. Hepatic steatosis is promoted by the abnormal deposition of saturated FAs, such as CHO and TG, and the constant accumulation of saturated FAs promotes the progression of liver inflammation and NAFLD. ALT and AST are important markers for evaluating liver damage, and ALT is a more specific marker of hepatic injury than AST [24-26]. We found that TG and ALT levels in the control group were significantly higher than that in the WKY group at the beginning of the experiment. After eight weeks of intervention, YNCRG significantly inhibited the weight gain of rats and reduced the level of serum TG, CHO, AST, and ALT levels. Moreover, these results indicated that YNCRG probably has a positive effect on liver function.

The progression of non-alcoholic fatty liver disease is closely related to mitochondrial dysfunction caused by oxidative stress and the release of inflammatory cytokines [27, 28]. The signals generated by TLRs are transduced through the NF-κB pathway to recruit pro-inflammatory cytokines [29], hence, the activation of the NF-κB pathway can promote the production of TNF-α, IL-1β, IL-6, and other pro-inflammatory factors, which are closely related to the development of NAFLD. Inhibiting the NF-κB signaling pathway and related inflammatory factors can significantly improve the symptoms of NAFLD in rats [30, 31]. Furthermore, the progression of NAFLD to NASH requires the recruitment of activated macrophages, and CCL2 and ICAM-1 promote the activity of macrophages [32]. The mRNA expression level of NF-κB in liver tissue was significantly reduced after eight weeks of YNCRG intervention (Figure 5), and inflammatory factors such as TNF-α and IL-1β were also reduced, which is consistent with previous studies. The expression of the CCL2 and ICAM-1 mRNA in the control group increased significantly, but YNCRG could reverse this phenomenon after intervention for eight weeks (Figure 5).

Additionally, the pathogenesis of NAFLD is related to the disorder of liver lipid metabolism caused by IR, which might occur due to various cytotoxic events such as hepatocyte inflammation, oxidative stress, and liver injury [33]. The activation of ERK might decrease energy expenditure in obesity [34]. However, the constitutive activation of ERK can regulate adipocyte lipolysis [35]. Liu et al. found that the activation of ERK and PKA are associated with lipolysis [36]. In this study, we found that PKA and phosphorylation of ERK were significantly upregulated. Thus, we speculated that the upregulation of the PKA and ERK pathways might be associated with the regulation of lipolysis in the liver.

The activation of C/EBPβ can enhance the differentiation and lipolysis of adipocytes [37]. Moreover, inhibiting the expression of PPARγ and C/EBPα in the liver alleviates hepatic lipogenesis [38, 39]. In our previous study, we found that insulin resistance improves when the phosphorylation of IRS-1 is inhibited at serine 1101 [39]. Furthermore, enhancing the activation of IRS1 phosphorylation at tyrosine 465 can rescue the inhibited insulin signaling pathway [40]. These findings were consistent with our results. We further found that YNCRG significantly enhanced the expression of C/EBPβ, and it might also inhibit the downstream proteins like PPARγ and C/EBPα. Additionally, we found that YNCRG significantly promoted the phosphorylation of IRS-1 (Tyr465) while inhibiting IRS-1 (Ser1101), as both are upstream factors of ERK. Hence, these results indicated that the lipolytic effect of YNCRG might be mediated by the PKA/C/EBPβ cascade, and it also showed some positive effects on insulin signaling by affecting the IRS1/ERK signaling pathway.

To summarize, our results showed that after eight weeks of intervention with YNCRG, the weight, blood lipid level, and liver function of the rats improved significantly. The morphological characteristics of the liver also showed that YNCRG could reverse hepatic steatosis. We investigated different signaling pathways and found that YNCRG can improve non-alcoholic fatty liver disease by

activating the PKA/ERK/C/EBPβ signaling pathway and inhibiting the TLR4/NF-κB signaling pathway. Also, YNCRG might activate the IRS1/ERK signaling pathway. This study showed that YNCRG has a beneficial effect on NAFLD, considering that it can activate the lipid metabolism signaling pathway and insulin signaling pathway in the liver to modulate lipid accumulation and inhibit the key transcription factors of the inflammatory signaling pathway. However, as the results of this study are based on experiments conducted using only one animal model, further experiments using different animal models should be conducted in future studies. Also, total ERK levels need to be determined to evaluate the strength of ERK-phosphorylation events to confirm the results of this study.

Abbreviations

TNF-α	Tumor necrosis factor-α
IL-1β	Interleukin-1 beta
IL-10	Interleukin-10
TLR4	Toll-Like Receptor 4
NF-κB	Nuclear factor kappa B
ICAM-1	Intercellular Adhesion Molecule-1
MCP-1	Monocyte chemoattractant protein-1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Sirt1	Sirtuin 1
FOXO1	Forkhead box O1
PKA	Protein kinase A
ERK	Extracellular-signal-regulated kinase
C/EBP	CCAAT/enhancer-binding protein
PPARγ	peroxisome proliferator-activated receptor γ
IRS1	insulin receptor substrate 1
AKT	protein kinase B
AMPK	AMP-activated protein kinase
ACC	acetyl-coenzyme A carboxylase
FAS	fatty acid synthase
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
CHO	cholesterol
TG	Triglycerides

Author Contributions

BS and MK designed the study. BS, MH, MK and LQ performed the experiments and wrote the manuscript. BS, LW MG, and TL revised the manuscript. All authors approved the study for publication. BS and MK contributed equally to this study and are co-first authors.

Funding

Mukogawa Women's University, Traditional Chinese Medicine of Beijing Key Laboratory (No. BZ0259), Beijing International Scientific and Technological Cooperation Base for the Prevention and Treatment of Diabetes with Traditional Chinese Medicine.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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