Berberine inhibits liver damage in rats with non-alcoholic fatty liver disease by regulating TLR4/MyD88/NF-κB pathway

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ABSTRACT

Background/Aims: *This study aimed to explore the therapeutic effects and underlying mechanism of berberine (BBR) on the non-alcoholic fatty liver disease (NAFLD) induced by high-fat diet (HFD).*

Materials and Methods: *Rats were randomly divided into the following 4 groups: control (normal diet), model (HFD), polyene phosphatidylcholine HFD+PPC, and BBR (HFD+BBR) group. The NAFLD models were prepared by feeding with HFD for 12 weeks. The liver tissues were observed by oil red O staining. H&E staining was used to detect pathological changes in the liver tissues. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were detected by an automatic biochemical analyzer. ELISA was performed to observe the inflammatory cytokines (TNF-α, IL-6, and IL-1β) expressions. The levels of TLR4, MyD88, and NF-κB p65 were analyzed using western blot and qRT-PCR, respectively. The nuclear translocation levels of NF-κB in the primary liver cells were measured using flow cytometry.*

Results: *BBR could significantly alleviate the liver tissue steatosis and inflammatory cell infiltration; reduce the NAFLD activity scores and serum levels of ALT, AST, TC, and LDL-C; decrease the levels of TNF-α, IL-6, and IL-1β, and reduce the expression of TLR4, MyD88, and NF-κB in the liver tissues. BBR could also reverse the nuclear translocation of NF-κB in the primary liver cells.*

Conclusion: *BBR alleviated the progress of NAFLD and liver damage, which might contribute to inhibit the nuclear translocation of NF-κB via the TLR4/MyD88/NF-κB pathway.*

Keywords: *Berberine, non-alcoholic fatty liver disease, TLR4, MyD88, NF-κB*

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a pathological condition characterized by the accumulation of triglycerides in the liver without a history of excessive alcohol consumption or other hepatic diseases, i.e., hepatitis B and hepatitis C virus infection (1, 2). It has a widely histologic type of disease, which can be further categorized into the non-alcoholic fatty liver and non-alcoholic steatohepatitis (NASH). NASH can develop to fibrosis, cirrhosis, and hepatocellular carcinoma, which brings a significant personal and societal burden. Currently, NAFLD is increasing worldwide. It is estimated that NAFLD has a global prevalence of 25% (3) and affects approximately 75-100 million Americans (4). A recent meta-analysis of 86 studies, including 8,515,431 samples from 22 countries, showed a prevalence rate of 31% and 32% in South America and the Middle East, respectively (5). To date, researchers have suggested that insulin resistance (6), inflammatory imbalance (7), and lipid metabolism disorder (8) play important roles in the pathogenesis of NAFLD. However, the underlying pathogenesis of NAFLD has not been entirely elucidated. Various studies have focused on efficient therapies for preventing NAFLD, for example, lifestyle change with weight loss, exercise, or specific drugs to improve insulin sensitivity. The common medicines include antioxidants, insulin sensitizers, or lipid-lowering drugs to control NAFLD comorbidities. However, none of these agents can alleviate liver fibrosis (9).

Berberine (BBR, $C_{20}H_{18}NO_4$, Figure 1) is one of the main active ingredients of the Chinese herbs, goldenseal (*Hydrastis canadensis*), golden thread (*Coptis chinensis*), and Oregon grape (*Berberis aquifolium*). Previous studies have reported that BBR was used to treat NAFLD in China and had the advantages of low price, minor side effects and diverse sources (10-13). It's reported that BBR could modulate the inflammatory reaction (14-15) and abnormal lipid metabolism (16, 17). Hepatic inflammation

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may play an important role in the pathogenesis of NAFLD. NF-κB is a vital transcription factor of the inflammatory response involved in regulating the expression of multiple genes, i.e., TNF-α and IL-6, which play an essential role in the progression of NAFLD (18, 19).

In this study, we focused on the potential mechanisms of BBR in the treatment of NAFLD. We tried to elucidate the critical roles of BBR on the nuclear translocation of NF-κB levels and the response of hepatic inflammation, so as to provide ideas and basis for the clinical treatment of NAFLD.

MATERIALS AND METHODS

Animal Model

A total of 60 adult male Sprague-Dawley rats weighing 180-220 g were purchased from the Qinglongshan experimental animal breeding farm (Nanjing, China). The rats were housed under standard conditions of controlled temperature (22°C±2°C) on reverse 12/12 hours light/dark cycle (lights off at 06:00 a.m.), and they had free access to tap water and food. All procedures were compliant with the guidelines for the accommodation and care of animals laid down by the Chinese convention for the protection of vertebrate animals used for experimental and other scientific purposes. All efforts were made to minimize animal suffering and reduce the number of animals used for the experiments.

Chemicals and Reagents

BBR (Lot. 180304) was obtained from Nanjing Bai Jing Yu Pharmaceutical Co., Ltd., and polyene phosphatidylcholine (PPC) capsules (Lot. 8BJD056B) were purchased from Beijing Sanofi Pharmaceutical Co., Ltd. Open Source Diets™ (D12492, Table 1) was provided by the Nanjing Jin Yi Bai Biological Technology Co., Ltd. The oil red O staining kit was purchased from Beijing Leagene Biotechnology Co., Ltd., and the hematoxylin-eosin staining kit was provided by Zhuhai Baso Biotechnology Co., Ltd. Total RNA extraction and reverse transcription kits were purchased from CWBIO Century Biotechnology Co., Ltd. Primers were supplied by Nanjing Kingsy Biotechnology Co., Ltd. The anti-TLR4, anti-MyD88, anti-NF-κB p65, and anti-phosphorylation NF-κB p65 (p-NF-κB p65) antibodies were purchased from Santa Cruz Biotechnology Co., Ltd. The anti-β-actin antibody was bought from CWBIO Biotechnology Co., Ltd. ECL luminescent kit (Cell Signaling Technology Co., Ltd., USA), TNF-α, IL-6, and IL-1β enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jin Yi Bai Biological Technology Co., Ltd., China), and 7-amino-actinomycin D (7-AAD) (Thermo Fisher Scientific, Rockford, IL, USA) were also used in our study.

Table 1. Energy proportions of protein, fat, and carbohydrate between normal diet and high-fat diet.

Energy proportions	Normal diet (kcal %)	High-fat diet (kcal %)
Fat	10	60
Protein	20	20
Carbohydrate	70	20

Model Preparation and Experimental Design

After 1 week of adaptive feeding, the rats were randomly assigned into 2 groups: the normal control group (n=18) and the model group (n=42). The controls were given a normal diet (ND) with free water supplement, and the models were given a high-fat diet (HFD) with free water for 12 weeks to prepare the NAFLD models. After the models were successfully established, 6 rats from each group were randomly selected and analyzed to confirm the establishment of the NAFLD model in the HFD group. Other rats in the HFD group were then randomly divided into 3 groups with 12 rats in each group: model (HFD), PPC (HFD+PPC), and BBR groups (HFD+BBR) group. The ND and HFD groups were given distilled water (10 $mLkg^{-1}day^{-1}$) by gavage; 200 $mgkg^{-1}day^{-1}$ PPC (dissolved in distilled water) was administered to the HFD+PPC group, and 200 $mgkg^{-1}day^{-1}$ BBR (dissolved in distilled water) was given to the HFD+BBR group by gavage. Each drug in each group was administered once a day for 12 weeks. A total of 6 rats from each group were used for hematoxylin and eosin (H&E) staining after perfusion by paraformaldehyde, and the other 7 rats from each group were used for collecting primary liver cells, oil red O staining, enzyme linked immunosorbent assay (ELISA), quantitative reverse transcription-polymerase chain reaction (qRT-PCR), and western blot.

MAIN POINTS

- *Berberine can alleviate the liver tissue steatosis and inflammatory cell infiltration.*
- *Berberine can reduce the NAFLD activity scores and serum levels of ALT, AST, TC, and LDL-C.*
- *Berberine can decrease the levels of TNF-α, IL-6, and IL-1β, and reduce the expression of TLR4, MyD88, and NF-κB in the liver tissues.*
- *Berberine can reverse the nuclear translocation of NF-κB in the primary liver cells.*

Isolation of Primary Rat Hepatocytes

As described previously, the primary hepatocytes were isolated by 2-step collagenase perfusion (20). 1 mL (1×10^6 cells) of the freshly isolated cells was suspended in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum and 1% penicillin and streptomycin in a temperature-controlled incubator. The 4th to 6th generations of the hepatocytes were used for further analysis.

Oil Red O Staining

The liver tissues were fixed in 4% paraformaldehyde solution overnight, dehydrated in 30% sucrose solution for 48 hours, and then embedded in optimal cutting temperature compound. The slicer was sliced at 10 μm and stained with red oil to evaluate the fat mass of the hepatocytes.

H&E Staining

The liver tissues were fixed with 4% paraformaldehyde solution overnight and stained with H&E in 4-μm slice after embedding in paraffin to observe the morphological changes of the liver tissue. Pathological analysis of H&E-stained sections of the liver tissues was performed, and the NAFLD activity scores (NAS) (21) were statistically evaluated (Table 2).

Biochemical Analysis

After 12 weeks of administration, the rats were sacrificed. The blood was collected from the eye sockets of the rats and centrifuged to separate the serum. The serum levels of triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were detected and used for biochemical analysis.

ELISA

The rat liver tissue homogenate was prepared with phosphate-buffered saline. The levels of inflammatory factors (TNF-α, IL-6, and IL-1β) were detected according to the instructions of the ELISA test kit.

qRT-PCR Analysis

Total RNA was extracted according to the manufacturer's instructions of the Trizol reagent (Invitrogen Life Technologies, MA, USA). The purity was determined using nanodrop, and the cDNA was synthesized by reverse transcription. The relevant reaction system was configured, and the reaction conditions were set according to the kit instructions. The primer sequences are shown in Table 3.

Table 2. Non-alcoholic fatty liver disease activity score evaluation system.

Index	Score
Hepatocyte steatosis	0 (<5%)
	1 (5%-33%)
	2 (34%-66%)
	3 (>66%)
	0 (none)
Infiltration of hepatic lobular cells	1 (<2)
	2 (2-4)
	3 (>4)
Hepatocyte ballooning	0 (none)
	1 (few)
	2 (many)
Total score	0-8

Table 3. Primer sequence.

Primer	Sequence (5′-3′)
TLR4	Forward: CGCTCTGGCATCATCTTCAT
	Reverse: CTCCTCAGGTCAAAGTTGTTGC
MyD88	Forward: GAGATCCGCGAGTTTGAGAC
	Reverse: TTGTCTGTGGGACACTGCTC
NF-κB p65	Forward: GGCAGCACTCCTTATCAAC
	Reverse: GGTGTCGTCCCATCGTAG
GAPDH	Forward: GCCAGCCTCGTCTCATAGACA
	Reverse: AGAGAAGGCAGCCCTGGTAAC

Western Blot

Appropriate amount of the liver tissues was weighed, and radio immunoprecipitation assay lysate was added to extract the protein. After quantified by nanodrop, the protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the polyvinylidene fluoride membrane. The membranes were blocked with 5% skim milk for 1 hour and washed with tris buffered saline (TBS) containing 0.1% Tween 20 (Sigma-Aldrich, MO, USA) (TBST) 3 times for 10 minutes each time. The membranes were then incubated with anti-TLR4, anti-MyD88, and anti-NF-κB p65 (1:200 dilution) overnight at 4°C. After washing 3 times with TBST

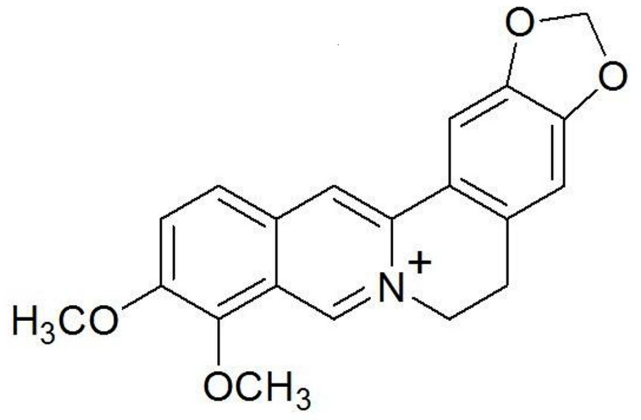

Figure 1. Chemical structure of berberine.

for 10 minutes each time, the membranes were incubated with the second antibodies (1:2,000 dilution) for 2 hours at 37°C. The membranes were then washed 3 times with TBST for 10 minutes each time. Finally, the signals were detected using enhanced chemiluminescence (Bio-Rad XRS+) and analyzed by Image Lab 5.0 (Bio-Rad Laboratories, USA).

Flow Cytometry Analysis

The nuclear translocation of NF-κB p65 was measured using flow cytometry (22). The primary liver cells were harvested and incubated with anti-p-NF-κB p65 for 1 hour at 37°C and then incubated with fluorescein isothiocyanate (FITC) fluorescent second antibody for another 1 hour at the room temperature. The nuclei were then stained with 7-AAD for 3 minutes. Finally, the nuclear translocation of NF-κB p65 was determined by IDEAS software (Merck Millipore, MA, USA).

Statistical Analysis

Data were expressed as mean ± standard error, and the Statistical Package for Social Sciences (SPSS) software (SPSS Inc.; Chicago, IL, USA) was used to perform all statistical analyses. Comparisons between multiple groups were performed using the one-way analysis of variance and the least significant difference test or Dunnett's T3 test. $p<0.05$ was set for all statistical comparisons.

RESULTS

Effect of BBR on the Clinicopathology of the Liver Tissues

The results of the oil red O staining showed successful model preparation. The intracellular lipid droplets in the liver cells were mainly large fat vacuote, and the fat droplets were markedly distributed in the slices. After sealing, the lipid droplets often gathered on the surface of the slices. The above pathological changes were significantly decreased in the HFD+BBR group than that in the HFD group (Figure 2a). At high magnification, the liver cells in the ND group were arranged neatly, the structure of the hepatic lobule was clear, the cells were free of lipidation, and there was no inflammatory cell infiltration (Figure 2b). The hepatocytes in the HFD group were swollen, lipid droplets were observed in the cells, the nuclei were marginalized, and inflammatory cell infiltration was observed. However, fatty degeneration was significantly relieved, and inflammatory cells decreased or even disappeared in the HFD+PPC and HFD+BBR groups. Moreover, the NAS scores in both the HFD+PPC and HFD+BBR groups significantly decreased compared with those in the HFD group ($p<0.05$). The scores in the HFD+BBR group were markedly decreased than those in the HFD+PPC group ($p<0.05$) (Figure 2c).

Effect of BBR on Serum Biochemical Parameters in Liver Damage

Compared with the ND group, the serum levels of ALT, AST, TG, TC, and LDL-C in the HFD group were significantly increased ($p<0.05$), and HDL-C levels were markedly decreased ($p<0.05$). The expression levels of ALT, AST, TC, and LDL-C were significantly lower in the HFD+PPC and the HFD+BBR groups than those in the HFD group ($p<0.05$) (Figure 3a, b). It indicated that BBR could alleviate liver injury in NAFLD induced by HFD.

Effect of BBR on Inflammatory Cytokines in Liver Damage

Compared with the ND group, the levels of inflammatory cytokines (TNF-α, IL-6, and IL-1β) in the liver tissues of the HFD group showed a significant increase ($p<0.05$). The expression levels of TNF-α, IL-6, and IL-1β in the liver tissues of the HFD+PPC and HFD+BBR groups were markedly lower ($p<0.05$) than those in the HFD group. The levels of inflammatory cytokines in the HFD+BBR group were significantly lower than those in the HFD+PPC group ($p<0.05$) (Figure 4).

Effect of BBR on TLR4/MyD88/NF-κB Pathway

A recent study (23) reported that TLR4/MyD88/NF-κB pathway was activated in NAFLD models, which indicated the functional role of the TLR4/MyD88/NF-κB pathway in liver damage. To elucidate the mechanism of the NF-κB axis, the protein levels and mRNA expressions of TLR4, MyD88, and NF-κB in the liver tissues were evaluated using western blot and qRT-PCR. It demonstrat-

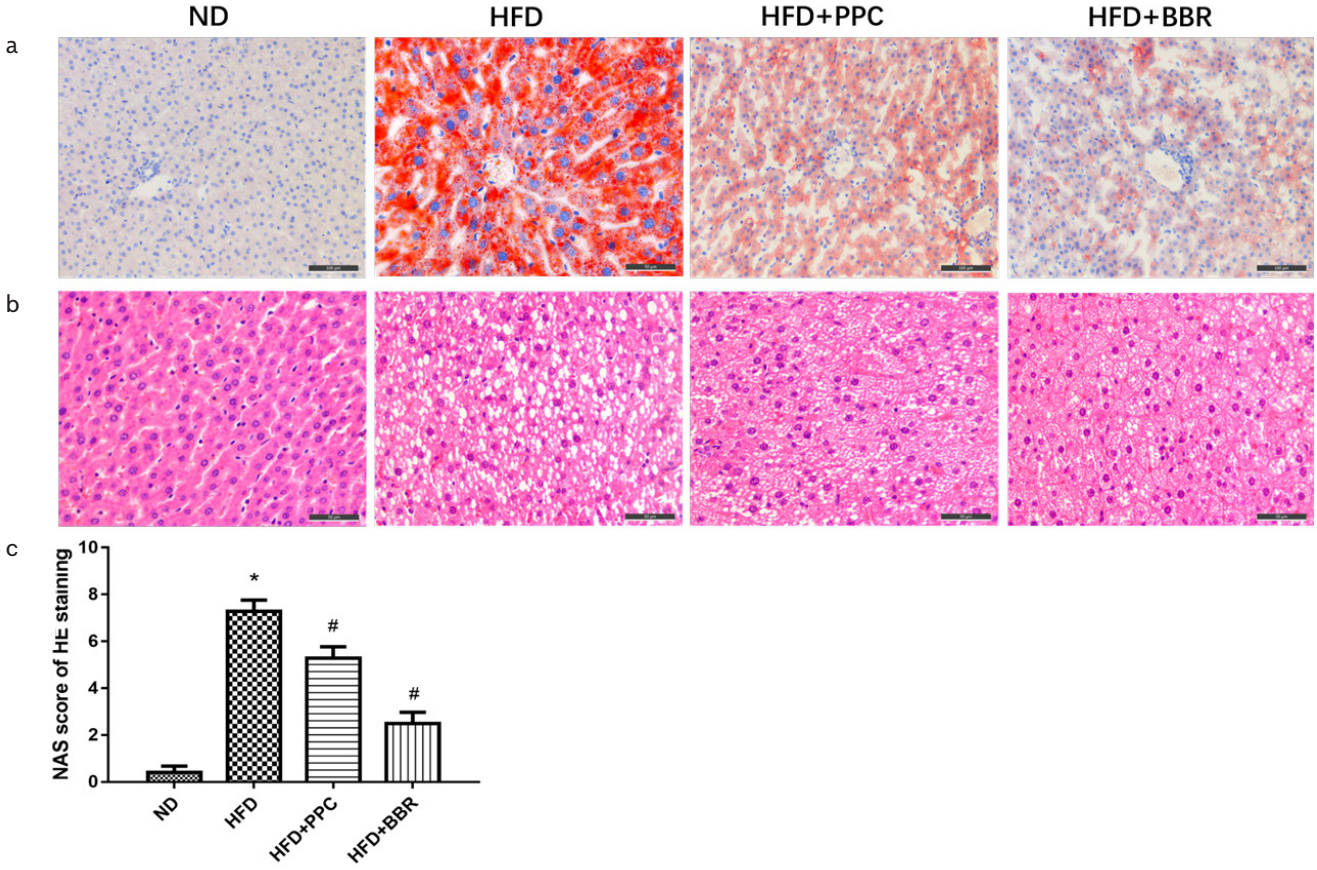

Figure 2. a-c. Berberine affects the clinicopathology in the liver tissues. (a) Representative images of oil red O staining (400x magnification). (b) Representative images of H&E staining (400x magnification). (c) Non-alcoholic fatty liver disease activity score in the liver tissue ($\bar{x}$±s, n=6). Compared with the ND group (*p<0.05); Compared with the HFD group (#p<0.05)
H&E: hematoxylin and eosin; ND: normal diet; HFD: high-fat diet

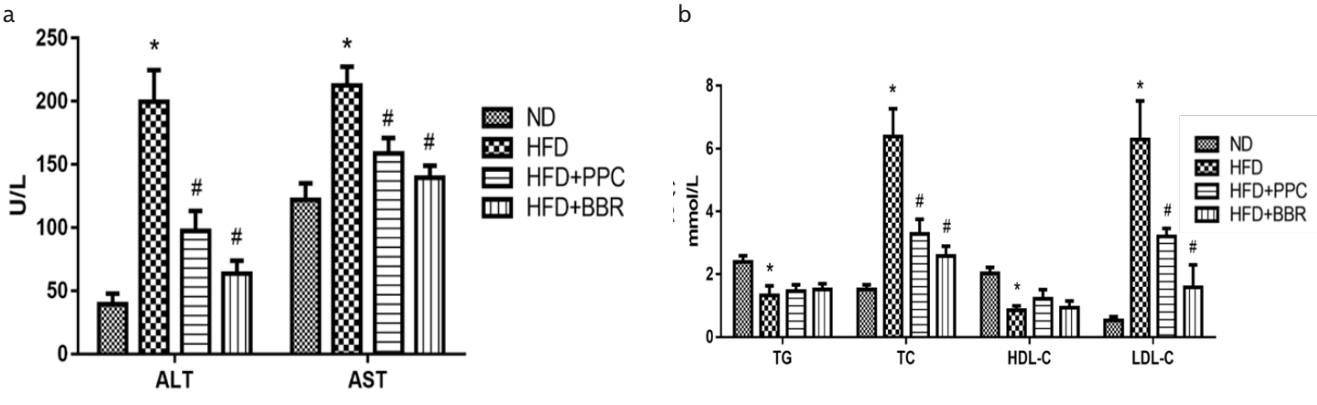

Figure 3. a, b. Berberine decreases the serum biochemical parameters in liver damage. (a) Expression levels of ALT and AST in serum ($\bar{x}$±s, n=6). (b) Expression levels of TG, TC, HDL-C, and LDL-C in serum ($\bar{x}$±s, n=6).
ALT: alanine aminotransferase; AST, aspartate aminotransferase; HDL-C: high-density lipoprotein cholesterol; ND: normal diet; HFD: high-fat diet; LDL-C; low-density lipoprotein cholesterol; TC: total cholesterol; TG: triglyceride

ed that the protein levels of TLR4, MyD88, and NF-κB in the HFD group were markedly increased (p<0.01) compared with those in the ND group. The protein levels in the HFD+PPC and HFD+BBR groups showed a significant reduction (p<0.05) in comparison with those in the HFD group, and the protein expression in the HFD+BBR group was significantly lower than that in the HFD+PPC group (p<0.05) (Figure 5a). Moreover, the mRNA expression levels by qRT-PCR (Figure 5b) were consistent with the results of the western blot.

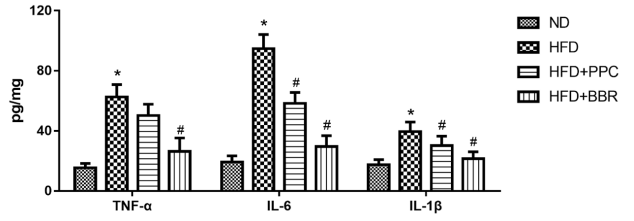

Figure 4. Berberine reduces the expression levels of hepatic inflammatory cytokines in the liver tissue ($\bar{x}\pm s$, n=6). Compared with the ND group, *p<0.05, Compared with the HFD group ,#p<0.05.
ND: normal diet; HFD: high-fat diet

Effect of BBR on p-NF-κB Nuclear Translocation in Primary Liver Cells

To determine the critical role of p-NF-κB, the primary liver cells of the 4 groups were harvested for analysis using flow cytometry. Nuclear localization of NF-κB was measured using the similarity feature in the final graph presented in IDEAS. The cell stained with FITC-labeled antibody (green) and 7-AAD (red) was measured for the nuclear localization of the NF-κB. We found that the nuclear translocation levels of NF-κB in the HFD group were markedly upregulated compared with those in the ND group. The nuclear translocation levels in the HFD+PPC and HFD+BBR groups showed a significant reduction (p<0.05) compared with those in the HFD group. The

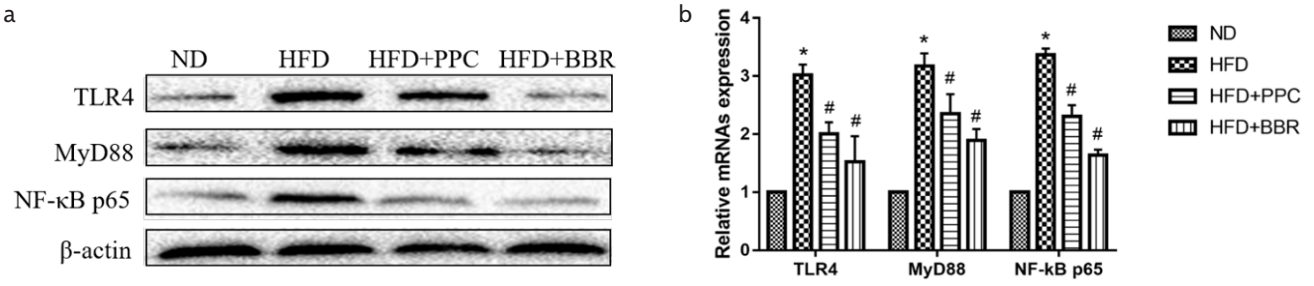

Figure 5. a, b. Berberine attenuates TLR4/MyD88/NF-κB pathway activation. (a) Protein levels of TLR4, MyD88, and NF-κB in the liver tissues analyzed by western blot. (b) mRNA expressions of TLR4, MyD88, and NF-κB in the liver tissues analyzed by quantitative reverse transcription-polymerase chain reaction. Compared with the ND group, *p<0.05, Compared with the HFD group, #p<0.05.
ND: normal diet; HFD: high-fat diet; mRNA: messenger RNA

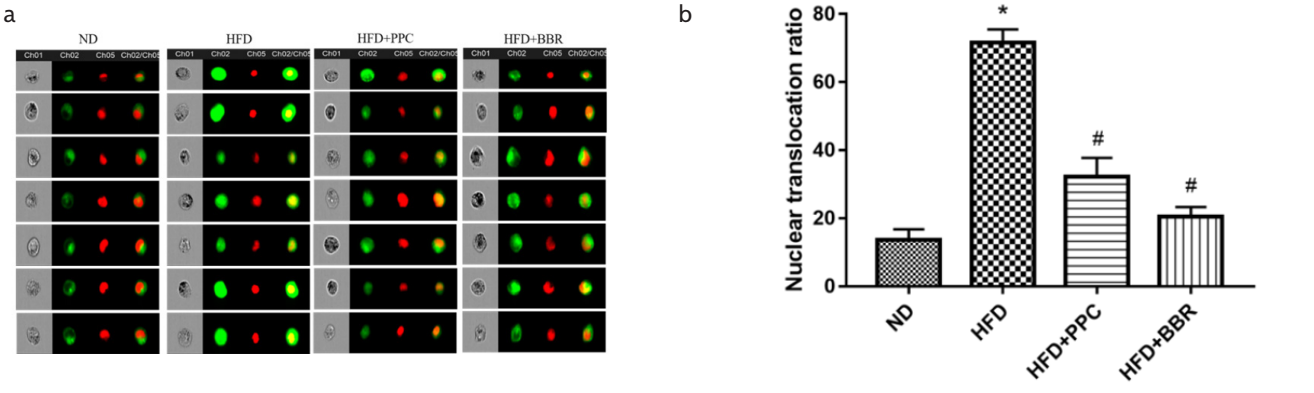

Figure 6. a, b. Berberine attenuates NF-κB nuclear translocation in primary liver cells. Compared with the ND group, *p<0.05; Compared with the HFD group, #p<0.05.
ND: normal diet; HFD: high-fat diet

nuclear translocation levels in the HFD+BBR group were significantly lower than those in the HFD+PPC group ($p<0.05$), as presented in Figure 6.

DISCUSSION

NAFLD is becoming one of the most common liver diseases, and its incidence rate shows an increasing trend, especially among obese people. BBR is effective in reducing lipid metabolism and inflammation (24), but its mechanism still needs to be further explored. The results of this study showed that the lipid content in the liver tissues of the models was increased, the arrangement of hepatocytes was irregular, and the lipid droplets in the cells were mainly large fat vacuote. The hepatocytes showed diffuse fatty lesions, which indicated that NAFLD models were successfully prepared. At the same time, we found that BBR could significantly inhibit inflammation, fat accumulation and reduce NAS scores, indicating that BBR could effectively alleviate the development of NAFLD.

Our results suggested that the fatty content in the liver tissues of the model rats induced by HFD was significantly higher than that in the ND group. The hepatic histopathological results showed obvious fatty degeneration in the model rats. However, the fatty content was reduced, and fatty degeneration of hepatocytes was reversed by BBR. Besides, the serum levels of ALT, AST, TC, and LDL-C were significantly decreased, which was consistent with the effects of BBR on db/db and ob/ob mice (25). It is worth noting that the level of TC increased, and the level of LDL-C decreased after the rats were fed with HFD. After 12 weeks of BBR treatment, the levels of TC and LDL-C decreased, which indicated that BBR could effectively alleviate the main clinical biomarkers of NAFLD, but there was no effect on the level of HDL-C. In addition, the serum TG level did not increase significantly after long-term feeding with HFD. However, it was lower than that in the ND group. It might be associated with the deposition of large amounts of TG in the hepatocytes of rats fed with HFD. Lipid metabolism function gradually declined after liver damage, and the liver TG could not be discharged into the blood for decomposition; thus, the serum levels decline rather than increase, which was consistent with a previous study (26).

NF-κB can regulate the expression of various genes in the pathological process of NAFLD. Free fatty acids can activate the transcription factor NF-κB; increase the expression of inflammatory cytokines, such as TNF-α, IL-6, and IL-1β; and cause liver damage. Long-term HFD and obesity induced by HFD can induce inflammatory reactions, leading to elevated levels of inflammatory factors in the liver and ultimately leading to NAFLD (23). In this study, the expression of TLR4, MyD88, and NF-κB-p65 proteins in the liver tissues of the HFD group was significantly higher than that in the ND group; the levels of TNF-a, IL-6, and IL-1β were also markedly increased, which suggested the activation of the NF-κB axis. The results were consistent with a recent study (27).

BBR could significantly reduce the expression of p-NF-κB p65, which has been proved by other studies (28-30). The translocation of NF-κB in the primary liver cells of different groups was measured using flow cytometry. We found that BBR could markedly reduce the nuclear translocation of NF-κB. In our study, we also found that BBR could significantly reduce the expression levels of TNF-a, IL-6, and IL-1β in the liver tissues of NAFLD rats, alleviate hepatocyte inflammation, and alleviate the development of NAFLD. It is speculated that BBR may play an anti-inflammatory role in NAFLD by inhibiting the NF-κB signaling pathway activation and reducing the expression of inflammatory cytokines, such as TNF-a, IL-6, and IL-1β.

BBR is isolated from natural herbs, which have many biological functions. Because of its advantages in treating and preventing various diseases, the anti-inflammatory effects of BBR have been well studied. In our study, we revealed that BBR had a prominent anti-inflammatory effect on NAFLD. The potential mechanism might be relate to the activation of the TLR4/MyD88/NF-κB pathway, reduction of NF-κB nuclear translocation and inhibition of inflammatory cytokines expression.

In conclusion, our study suggested that BBR had a beneficial effect on NAFLD. It could not only alleviate hepatic steatosis, inflammatory cell infiltration and reduce the NAS scores, but also decrease serum levels of inflammatory cytokines, and downregulate the expression of TLR4, MyD88, and NF-κB in rat liver tissues. Furthermore, BBR could inhibit the activation of nuclear translocation of NF-κB in the primary liver cells, all of which highlight the critical role of BBR in the treatment of NAFLD.

Ethics Committee Approval: The ethics committee approval was received for this study from the Ethics Committee of Nanjing University of Chinese Medicine.

Informed Consent: N/A.

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Conflict of Interest: The authors have no conflict of interest to declare.

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