Original Article

Modulation of Gut Microbiota by Berberine Improves Steatohepatitis in High-Fat Diet-Fed BALB/C Mice

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Abstract

Background: Dysbiosis of the gut microbiota underlies non-alcoholic steatohepatitis (NASH). Ingredient of Chinese herbal medicine, berberine, has been proved to regulate the gut microbiota without systemic side effects. Therefore, we explored its effects on NASH induced by high-fat diet (HFD).

Methods: BALB/c mice were randomized into three groups, including: control, model, and berberine treatment. With the exception of the control group with the standard diet, the model, and the treatment groups were treated by HFD. Mice from treatment group were further subjected to berberine (200 mg/kg/d) gavage since the 5th week. At the end of the 13th week, gut bacteria, liver endotoxin receptor, and inflammation cytokines were assessed by real-time PCR. NASH and its predisposing factors were evaluated biochemically and pathologically.

Results: Compared to their decreases in the model group, berberine administration restored the relative level of *Bifidobacteria* (2.16 ± 0.63 *vs.* 0.50 ± 0.08, $P < 0.01$) and the ratio of *Bacteroidetes*/ *Firmicutes* (0.76 ± 0.26 *vs.* 0.39 ± 0.11, $P < 0.01$), respectively, in the treatment groups. Microbiota restoration led to significant reductions in body weight, serum levels of lipids, glucose, insulin, and homeostasis model assessment of insulin resistance. Improvements were also observed in the serum transaminase activity and nonalcoholic fatty liver disease activity score, which demonstrated the attenuation of NASH. Mechanically, expression levels of CD14, IL-1, IL-6 and TNF-α were statistically down-regulated (treatment group *vs* model group, $P < 0.01$).

Conclusions: Berberine alleviates NASH and its predisposing factors. Normalization of gut microbiota might underlie its effect.

Keywords: Berberine, gut, inflammation, microbiota, nonalcoholic steatohepatitis

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Introduction

Non-alcoholic fatty liver disease (NAFLD) reflects a spectrum of clinical pathological syndromes that are related to insulin resistance (IR), primarily simple fatty liver and non-alcoholic steatohepatitis (NASH).[1] Despite the benign cause of simple fatty liver, NASH has been associated with pathological progression and can progress to liver cirrhosis and hepatocellular carcinoma (HCC).[1–2] Excluding metabolic causes, such as obesity and type 2 diabetes, a growing body of evidence has demonstrated the relationship between abnormal gut microbiota and the occurrence of NASH.[3] In brief, high-fat diets (HFDs) often induce abnormalities in the gut microbiota in mammals.[4–7] Host-microbe interactions based on microbial disorders further contribute to overweight, obesity, IR and inflammatory responses, and most of these conditions are predisposing factors for NASH.[4–7] Thus, the gut microbiota has been suggested to play a critical role in the development and progression of NASH.

Due to its potential causative role, the gut microbiota qualifies as a therapeutic target for NASH. Antibiotics have been employed to modulate the gut microbiota.[8–9] To avoid systemic side effects, antibiotics that are not absorbed by the intestines are preferable for microbial regulation. Berberine, an over-the-counter (OTC) drug in China, is a medicinal alkaloid isolated from *Coptis chinensis*, and has been widely used to inhibit diarrhea-related dysbacteriosis with a low dose and good safety. Pharmacokinetic studies show the maximum concentration (Cmax) of berberine no more than 30 ng/mL (mostly average at 15 ng/mL) in serum/ plasma of rat, mouse, and pig.[10] The Cmax of berberine in human plasma is evaluated to be 0.4 ng/mL after a single oral dose of 400 mg.[11] *In vitro* studies also suggest an effective level of berberine ranging from 2500 to 25000 ng/mL.[10] Moreover, the monomer and metabolites of berberine are minimally absorbed by the gastrointestinal tract.[12]

Therefore, we established an HFD-induced model of NASH and administered berberine. The relative proportions of *Bacteroidetes, Firmicutes, Bifidobacteria* and *Lactobacillus* species were tested in the cecal content to reveal the effects of berberine on the gut microbiota. Indexes related to IR, including body weight, serum lipids, fasting blood glucose (FBG), insulin, and homeostasis model assessment of insulin resistance (HOMA-IR), as well as indices related to NASH, including liver function test and hepatic pathology, were further utilized to demonstrate the results of microbial regulation. Moreover, the expression levels of endotoxin receptors and inflammatory cytokines were examined to elucidate the mechanisms underlying the effects of berberine treatment.

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Materials and Methods

Animals and Grouping

Adult male BALB/c mice (18 – 22 g, SLAC Laboratory Animal Company, Shanghai, China) were randomized into three groups, including: control group (n = 9), model group (n = 10) and the treatment group (n = 11). With the exception of those in the control group (100% normal diet; overall calories: 3.6 kcal/g), the mice were subjected to a HFD (10% lard, 2% cholesterol, and 88% normal diet; overall calories: 4.8 kcal/g[13]) for 13 weeks. Berberine (Sigma-Aldrich, USA) was freshly suspended in distilled water and orally administered to the treatment group at a dose of 200 mg/kg/d by gavage for 8 weeks. It was administered intragastrically to the treatment group beginning in the 5th week, and an equal volume of vehicle was given intragastrically to both the control and model groups. Body weights were recorded every week, and dietary consumption (calorie intake) of each mouse was recorded every day. The mice were group-housed in a controlled environment (inverted 12 hours daylight cycle, lights on at 8:00 A.M.). At the end of the 13th week, the mice were fasted for 12 hours and killed by cervical dislocation. The serum, hepatic, and cecal samples were collected from all groups. The epididymal fat index was calculated as the ratio of the bilateral epididymal fat to the body weight.

The mice received human care, according to the American Physiological Society (APS)'s guiding principles regarding the care and use of animals. The study was approved by the ethical committee of Shanghai Jiao Tong University School of Medicine.

Serum lipids, IR and liver function test

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides (TG), total cholesterol (TC), FBG, and insulin, were biochemically analyzed. The HMOA-IR was calculated according to the following formula: HMOA-IR = [fasting plasma insulin (μU/mL) × FBG (mmol/L)]/22.5.

Liver pathology

Liver samples from each mouse were subjected to hematoxylin-eosin (H&E) staining and subsequently assessed according to the NAFLD activity score (NAS), which includes hepatic steatosis (0 – 3), inflammation (0 – 3) and hepatocyte ballooning (0 – 2).[14] NASH was defined by a NAS score of 5 or more in combination with a score of at least 1 score for ballooning degeneration. One experienced pathologist, who was blinded to the purposes of the study, independently interpreted the results.

Hepatic endotoxin receptor and inflammatory cytokines

Total RNAs were extracted from the frozen hepatic tissue. cDNAs were synthesized using PrimeScript RT-PCR Kit (Takara, Japan) from 2 μL of the total RNAs. The PCR conditions were as follows: 15 min at 37°C, and 5 sec at 85°C. The hepatic expressions of the endotoxin receptor, cluster of differentiation 14 (CD14) and inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 and IL-6, were measured using real-time PCR (Table 1) using a SYBR Green Real-Time PCR Kit (Takara, Japan).[15–16] Relative expression levels were evaluated against that of ribosomal protein L19 (RPL19). The PCR was conducted in a 20 μL system with the following specifications: 30 sec at 95°C; as well as 40 circles of 5 sec at 95°C and 34 sec at 60°C.

Gut microbiota

Microbiota DNA was extracted from the cecal contents using a QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. The relative proportions of *Bacteroidetes*, *Firmicutes*, *Lactobacillus* and *Bifidobacterium* within the gut microbiota were then detected by real-time PCR (Table 1)[17–19] using a SYBR Green Real-Time PCR Kit (Takara, Japan) as described above. The relative expression levels were evaluated against universal bacterial primers.

Statistical analyses

The data are expressed as the means ± the standard deviation (SD). The obtained results were compiled and analyzed by Statistical Package for Social Science (SPSS 17.0) using one-way ANOVA followed by post hoc Tukey's test. Differences with $P < 0.05$ were considered statistically significant.

Table 1. The sequences of primers for the quantitative real-time PCR

Gene	Primer sequence (5'-3')
CD14	Forward: 5′- GAGTTGTGACTGGCCCAGTCAGC -3′ Reverse: 5′- GCAAAAGCCAGAGTTCCTGAC -3′
TNF-α	Forward: 5′- TGGGACAGTGACCTGGACTGT -3′ Reverse: 5′- TTCGGAAAGCCCATTTGAGT -3′
IL-1	Forward: 5′- TCGCTCAGGGTCACAAGAAA -3′ Reverse: 5′- CATCAGAGGCAAGGAGGAAAAC -3′
IL-6	Forward: 5′- CCGGAGAGGAGACTTCAC -3′ Reverse: 5′- TCCACGATTTCCCAGAGA -3′
RPL19	Forward: 5′- GAAGGTCAAAGGGAATGTGTTCA -3′ Reverse: 5′- CCTTGTCTGCCTTCAGCTTGT -3′
Lactobacillus	Forward: 5′- CATCCAGTGCAAACCTAAGAG -3′ Reverse: 5′- GATCCGCTTGCCTTCGCA -3′
Bifidobacterium	Forward: 5′- GGGTGGTAATGCCGGATG -3′ Reverse: 5′-TAAGCCATGGACTTTCACACC -3′
Bacteroidetes	Forward: 5′- GGARCATGTGGTTTAATTCGATGAT -3′ Reverse: 5′-AGCTGACGACAACCATGCAG -3′
Firmicutes	Forward: 5′- GGAGYATGTGGTTTAATTCGAAGCA -3′ Reverse: 5′-AGCTGACGACAACCATGCAC -3′
Universal bacterial	Forward: 5′-CGTGCCAGCCGCGGCGTGCCAG CCGCGGTAATACG -3′ Reverse: 5′-GGGTTGCGCTCGTTGCGGGACTT AACCCAACAT -3′

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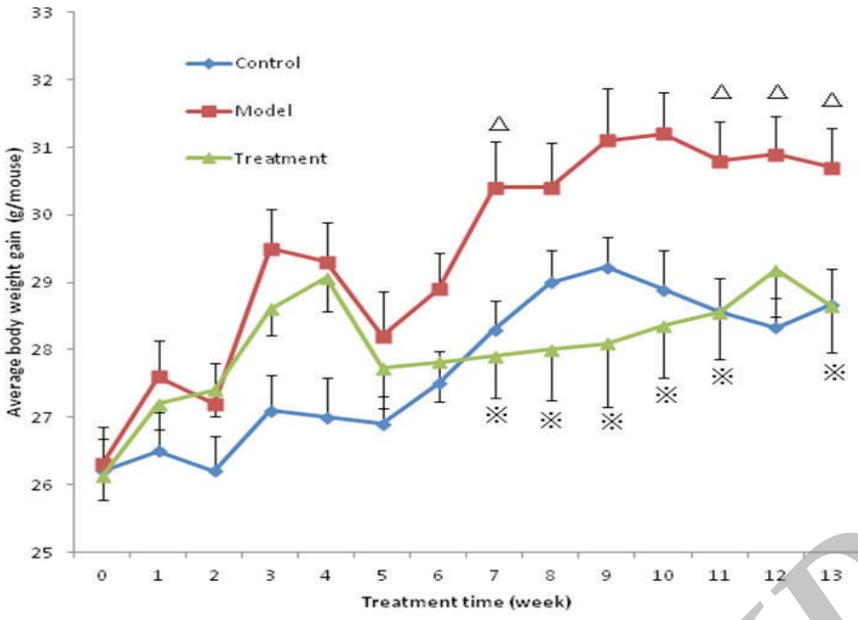

Figure 1. Mouse body weights during the experiment. After the gavage at the end of 5th week, all the groups lost weight initially and gained weight after their adaption. Compared with the control group, the model group demonstrated weight gaining much faster. In contrast, there was no little difference in the weight gaining between the control and treatment groups; $^{\triangle}P < 0.05$ versus the control group, $^{*}P < 0.05$ versus the model group.

Results

Berberine restored the gut microbiota in mice with HFD

Compared to those of the control group, the *Bacteroidete*, *Lactobacillus* and *Bifidobacterium* levels exhibited significant decreases in the model group after the HFD ($P < 0.01 – 0.05$, Figure 2). In contrast, berberine administration restored these relative proportions. The *Bifidobacterium* content was much greater than that of the control group ($P < 0.01$, Figure 2). *Lactobacillus* showed an increasing trend, with no statistical differences (Figure 2). The levels of *Firmicutes* across the three groups were slightly changed with no significant differences.

Berberine improved the metabolic parameters associated with HFD

The dietary calorie intakes were similar between the model group and the treatment group. The body weight and epididymal fat indices were significantly increased in the model group (*vs.* control group, $P < 0.01 – 0.05$; Table 2). However, the therapeutic interference by berberine led to reductions in the body weight to levels that were approximately equal to those of the control group, with the decline trend of the epididymal fat index in the treatment group.

The serum indices of metabolism, *i.e.*, TG, TC, FBG, plasma insulin and HOMA-IR, of the model group were also greatly elevated compared to those of the control group ($P < 0.01 – 0.05$, Table 2). The serum TC level exhibited a trend toward a decline, and all other abnormal indexes exhibited significant improvements after treatment with berberine for 9 weeks ($P < 0.01 – 0.05$, Table 2).

Berberine treatment resulted in the attenuation of NASH

The mice with long-term HFD suffered from increased serum aminotransferase activities ($P < 0.01 – 0.05$, Table 2). The simultaneous administration of berberine prevented the abnormalities of both ALT and AST (Table 2), which suggested a protective effect against hepatocyte injury.

There were no obvious histopathological changes in the control group based on H&E staining (Figure 3A). Following HFD exposure for 13 weeks, the model group was characterized by micro- and macrovesicular steatosis, hepatocyte ballooning and extensive infiltration of inflammatory cells in the hepatic lobule, with focal inflammatory necrosis (Figure 3B). In contrast, the treatment group exhibited an amelioration of hepatic steatosis and necro-inflammation. However, the ballooning degeneration remained the same in the treatment group (Figure 3C). Thus, berberine treatment resulted in a prominent decrease in NAS ($P < 0.01$, Table 2) and protected the HFD-treated mice from NASH.

The regulations of the endotoxin receptor and inflammatory cytokines contributed to the effects of berberine on NASH

The hepatic expression of endotoxin receptor (CD14), which exhibited a trend toward an increase in the model group, was statistically normalized in the treatment group ($P < 0.01$, Figure 4). Several important cytokines, including IL-6, IL-1 and TNF-α, were closely associated with the endotoxin-stimulated hepatic inflammation. The significant up-regulations of these cytokines in the model group and their down-regulation in the treatment group were consistent with the expression pattern of the endotoxin receptor ($P < 0.01 – 0.05$, Figure 4).

Discussion

In our experiments, the model group exhibited metabolic disorders after 13 weeks of a high-fat, and high-calorie diet. Compared with those in the control group, the body weights and epididymal fat indices of the model group were significantly higher. The blood lipids, FBG, plasma insulin and HOMA-IR, which are serum indexes of metabolism, were also greatly increased. In addition to these abnormal metabolic parameters, liver pathology of the model group reflected steatohepatitis with increased activities of ALT and AST. Mechanically, the hepatic endotoxin receptor CD14 and its downstream cytokines, including

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Table 2. Effects of berberine on dietary intake, nutrition status, serum metabolic indexes, and NAFLD activity score (mean ± SD)

Group	N	Diet intake (kcal/mouse/d)	Body weight (g)	Epididymal fat index	ALT (U/L)	AST (U/L)	TG (mmol/L)	TC (mmol/L)	FBG (mmol/L)	Insulin (pmol/L)	HOMA-IR	NAS	Steatosis score	Degree of inflammation			
														-	+	++	+++
Control	9	18.31 ± 2.29	28.67 ± 1.58	1.02 ± 0.12	120.0 ± 10.22	65.0 ± 3.63	0.44 ± 0.19	2.34 ± 0.10	4.69 ± 0.75	0.45 ± 0.36	13.47 ± 11.18	0	0	9	0	0	0
Model	10	23.22 ± 2.00[b]	30.70 ± 1.83[a]	1.49 ± 0.37[b]	346.0 ± 142.01[b]	137.5 ± 88.50[a]	1.04 ± 0.11[b]	4.75 ± 0.53[b]	7.56 ± 1.38[b]	1.67 ± 0.95[a]	76.67 ± 38.12[b]	5.60 ± 1.90[b]	2.10 ± 0.74[b]	0	4	4	2
Treatment	11	22.86 ± 2.72[b]	28.64 ± 2.25[c]	1.28 ± 0.34	175.0 ± 20.14[d]	88.50 ± 11.18	0.70 ± 0.20[c]	4.17 ± 0.49[b]	6.13 ± 0.89[ac]	0.46 ± 0.33[c]	18.34 ± 12.45[d]	2.73 ± 0.90[bd]	0.82 ± 0.75[ad]	8	3	0	0

NAFLD: non-alcoholic fatty liver disease; ALT: alanine transaminase; AST: Aspertate Aminotransferase; TG: triglyceride; TC: cholesterol; FBG: fasting blood-glucose; HOMA-IR: homeostasis model assessment-insulin resistance index; NAS: non-alcoholic fatty liver disease activity score; Compared to control group; a: P < 0.05; b: P < 0.01; compared to model group; c: P < 0.05; d: *P* < 0.01.

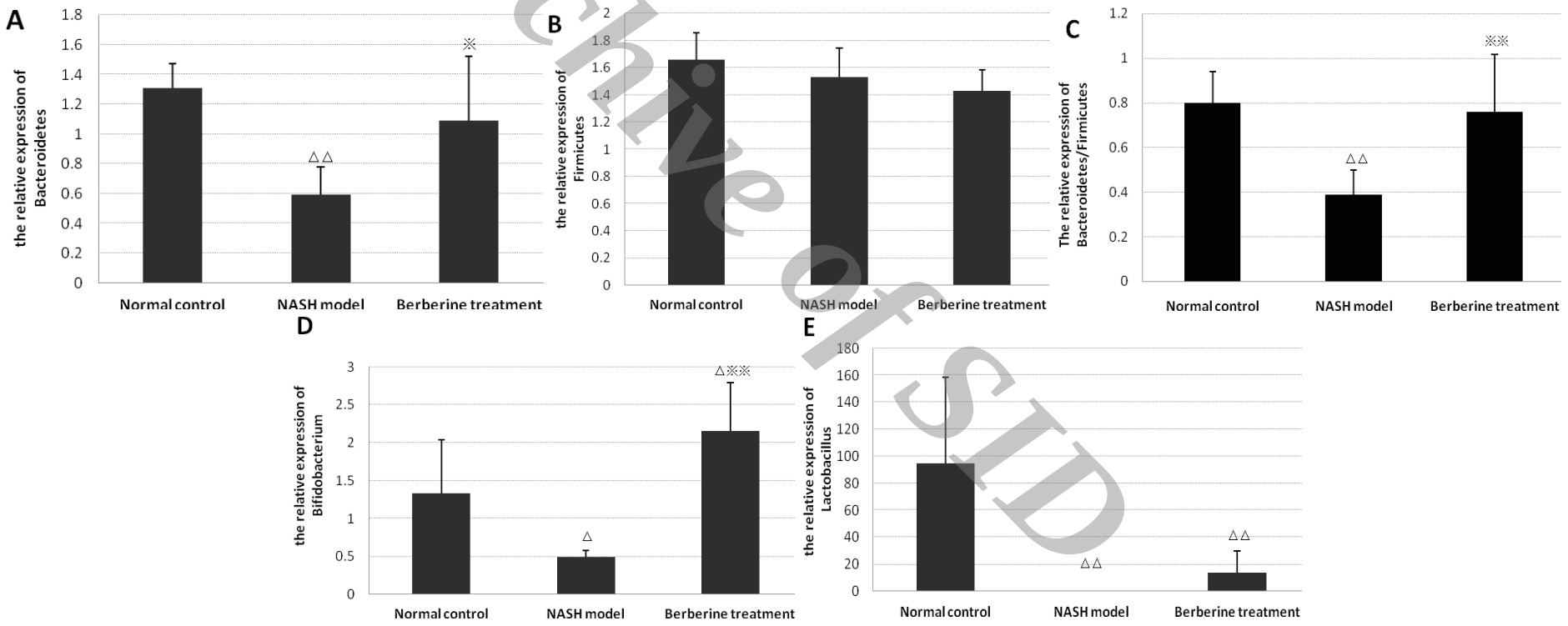

Figure 2. Berberine treatment regulates the gut microbiota. The relative expressions of **A)** *Bacteroidetes;* **B)** *Firmicutes*; **C)** *Bacteroidetes/ Firmicutes*; **D)** *Bifidobacterium*; and **E)** *Lactobacillus*; $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$, versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, versus the model group.

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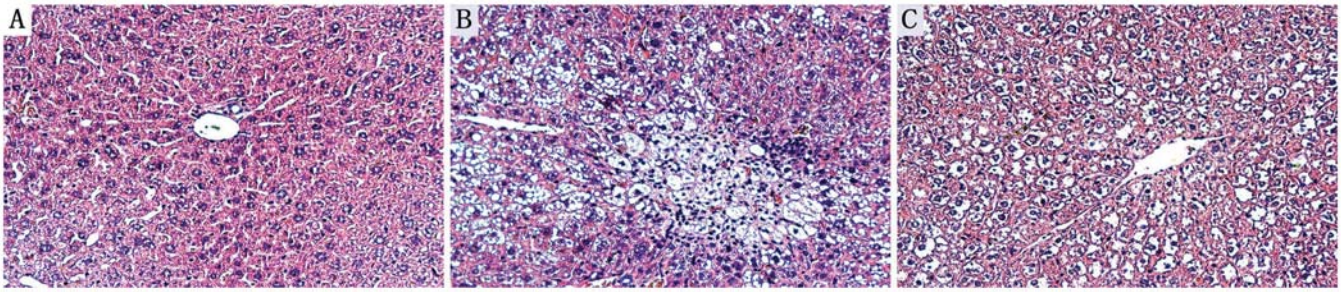

Figure 3. Berberine treatment attenuates high-fat-diet induced NASH in mice; HE staining of the **A)** control group; **B)** model group; **C)** and treatment group (×200).

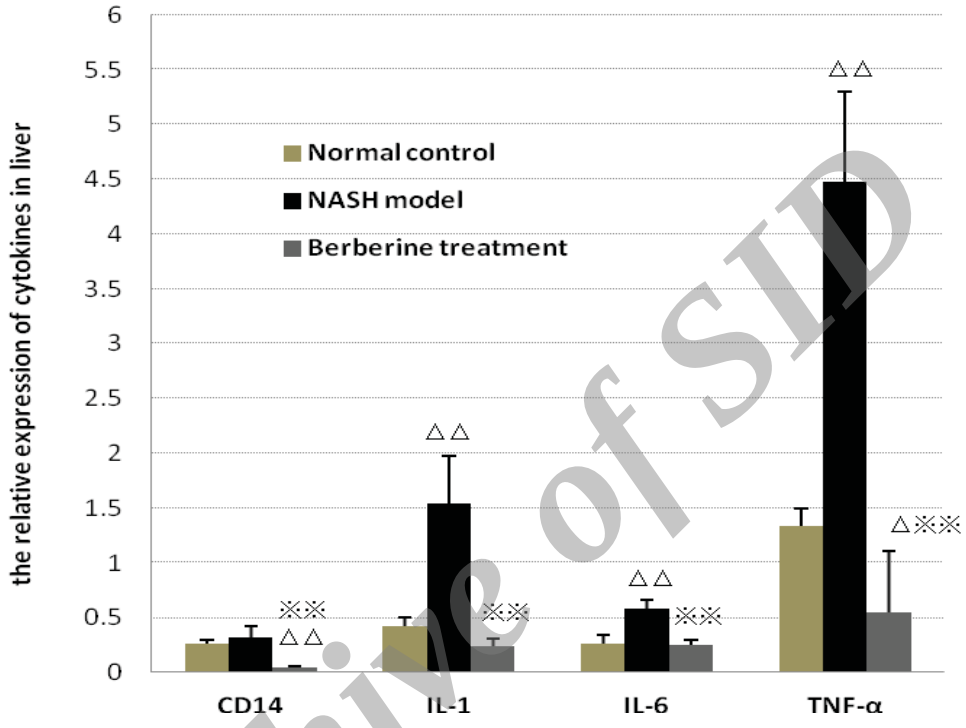

Figure 4. Berberine treatment down-regulates endotoxin receptor and inflammatory cytokines in the liver. Results are expressed as the means ± the SDs. $P < 0.05$ was considered statistically significant. $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$, versus the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, versus the model group.

IL-6, IL-1 and TNF-α, were found to be expressed at much higher levels than those of the control group. It is well known that toll-like receptor 4 (TLR-4)/MyD88 signaling is activated *via* the endotoxin receptor (CD14) in Kupffer cells.[20–22] Secretion of downstream cytokines, primarily IL-6, IL-1 and TNF-α, is up-regulated by the TLR-4/MyD88 signaling. Following cytokine overproduction, inflammatory cell recruitment occurs in the liver and induces steatohepatitis.[23–25] Thus, the innate immune response mediated by TLR-4/MyD88 signaling is suggested in the model group.

Bacteroidetes, one of dominant bacteria phyla in the gut that belongs to the gram-negative intestinal microbiota, were significantly decreased in the model group after its exposure to HFD. Consistently, European children with high-calorie diet exhibit less abundance of *Bacteroidetes* than that of the children from rural Africa.[26] Despite of the uncertain reason, death of *Bacteroidetes* may be responsible for their decrease.[27] Endogenous lipopolysaccharide (LPS), the active component of endotoxins, is continuously produced upon the death of intestinal gram-negative bacteria.[28] Thus, HFD leads to 2 – 3 fold increase in the plasma concentration of LPS.[29] LPS has been proved to impair the integrity of gastrointestinal mucosa.[30] Dietary fat ingestion also increases the translocation of intestinal LPS.[31] LPS-induced activation of intestinal macrophages, by the stimulation of proinflammatory cytokines, reflects another mechanism by which luminal bacteria might disrupt intestinal barrier function.[32] LPS endotoxemia is then occurred by transmucosal permeability. As a result, LPS-related injury of hepatocyte membrane, mainly on the basis of innate immune response, occurred in the model group with the elevated levels of serum AST and ALT.

In contrast to the actions of *Bacteroidetes*, *Lactobacillus* and *Bifidobacterium* prevent the component redistribution of tight junctions, and maintain the integrity of the mucous membrane barrier of small intestine.[33–35] Mechanically, *Bifidobacterium* and *Lactobacillus* inhibit the epithelial barrier disruption by TNF-α, and promote wound repair in TNF-α-stimulated enterocytes.[36] Besides, *Bifidobacterium* increases the endogenous GLP-2, which improves the function of gut barrier.[33] The intact mucosal barrier prevents mice from metabolic endotoxemia, and consequently the endotoxin-stimulated immune response in the liver. However, these geniuses decreased upon HFD exposure in the model group. Both the increase of mucosal injury and the lack of protective factors facilitate the LPS endotoxemia and subsequently, steatohepatitis.

Although the *Firmicutes* levels among three groups were

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similar, the ratio of *Bacteroidetes* to *Firmicutes* decreased in the model group due to the declines in *Bacteroidetes*. Organisms in the *Bacteroidetes* and *Firmicutes* phyla represent more than 90% of the microbiota in both mice and humans.[37] Interestingly, recent studies have revealed the prominent difference in *Bacteroidetes*: *Firmicutes* ratio between lean and obese people.[38] This ratio is further confirmed to be associated with intestinal energy absorption. A 20% decrease in *Bacteroidetes* and a corresponding increase in *Firmicutes* is related to an increased energy harvest of ≈ 150 kcal.[39] Another study shows the high abundance of *Bacteroidetes*, and an enrichment of genes related to carbohydrate metabolism in microbiomes of lean individuals. On the contrary, *Firmicutes* dominate the obese-related microbiome, which was enriched with genes related to nutrient transporters.[40] With the greater abundance of *Bacteroidetes* and depletion of *Firmicutes*, high Bacteroidetes: Firmicutes ratio in African children improves energy intake, while protect them from inflammations.[26] By these reasons, the model group with lowered *Bacteroidetes*: *Firmicutes* ratio exhibited a series of metabolic disorders, and finally resulted in NASH.

Dramatically, the gut microbiota, including *Bacteroidates*, *Lactobacillus* and *Bifidobacterium*, obviously rebounded in the treatment group after berberine administration, while *Firmicutes* exhibited no obvious change. In detail, the relative level of *Lactobacillus* was elevated in the treatment group. Its level of *Bifidobacterium* was even higher than that of the control group. Compared to the increase of body weight, epididymal fat index and hyperlipidemia in the model group, restoration of Bacteroidetes: Firmicutes ratio by berberine treatment led to significant weight loss and improvement of lipid metabolism, even though dietary caloric intake in the treatment group was similar to the model group. Improved IR indicators (FBG, plasma insulin and HOMA-IR) also characterized the berberine-treated mice (treatment group) with restored gut microbiota. Correspondingly, the normalization of *Bacteroidetes*, *Lactobacillus* and *Bifidobacterium* in the treatment group was accompanied by the down-regulated levels of endotoxin receptor (CD14) and inflammatory cytokines (IL-6, IL-1, and TNF-α), probably by the inactivation of innate immune response depending on the LPS-stimulated TLR-4/MyD88 signaling.

These microbiota-based effects of berberine suggest it an important role in the therapy of NASH. Upon pathological evaluation, the mice that were subjected to berberine gavage (treatment group) exhibited a milder degree of hepatic steatosis and inflammatory infiltration than those observed in the model group. According to the aspect of inflammation, limited inflammation and necrotic foci were detected in the lobules or portal area of the treatment group. In agree with its pathological observations, the NAS scoring of treatment group was lower than the diagnostic criteria of NASH. Another striking result obtained from the berberine-treated mice was the significantly reduced activity of serum aminotransferases, which indicated the alleviation of hepatic inflammatory injuries. Both the serological and pathological alternations confirmed that berberine administration could be a promising solution for NASH.

In the present study, BALB/c instead of C57BL/6 mice were employed to establish the experimental NASH, though NASH-related pathological characteristics may be induced in both species.[41] BALB/c mice have been described to develop obesity and NASH much more sever than those in the C57BL/6 mice under the similar amount of calorie intake. Thus, the association between microbiota and steatohepatitis may be highlighted on the basis of NASH model using BALB/c mice. Additionally, we assessed gut microbiota by real-time PCR, which is widely used for the quantification of target-specific bacteria.[42] But it is difficult to detect novel species by this method. Therefore, a combination of real-time PCR and other techniques will be needed for further exploration, and may provide us with more information about the diversity and abundance of NASH-specific gut microbiota.

In conclusion, berberine is an effective agent that restores HFD-induced dysbiosis of gut microbiota. The normalization of gut microbiota alleviates the predisposing factors for liver steatosis and consequently, protects the host from NASH. Decreased levels of endotoxin receptor and inflammatory cytokines are likely to reflect the mechanisms of berberine treatment.

Conflicts of interest: *None.*

Author's Contribution

Yi Cao and Qin Pan contributed equally to this work and share the co-first authorships.

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