Original Article

Protective Effects of Berberine on Nonalcoholic Fatty Liver Disease in *db/db* Mice via AMPK/SIRT1 Pathway Activation*

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[Abstract] Objective: Berberine (BBR) has emerged as a promising therapeutic agent for nonalcoholic fatty liver disease (NAFLD). This study aims to elucidate the underlying molecular mechanisms. **Methods:** In this study, *db/db* mice were chosen as an animal model for NAFLD. A total of 10 healthy C57BL/6J mice and 30 *db/db* mice were randomly allocated to one of 4 groups: the normal control (NC) group, the diabetic control (DC) group, the Metformin (MET) therapy group, and the BBR therapy group. The total cholesterol (TC), triacylglycerol (TG), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the serum were measured. The glutathione peroxidase (GSH-Px), glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), interleukin (IL)-1β, tumor necrosis factor (TNF)-α and monocyte chemotactic protein 1 (MCP-1) levels in liver tissue were measured. Hematoxylin and eosin (H&E), acid-Schiff (PAS) and TUNEL stanning was performed for histopathological analysis. Western blotting and immunohistochemistry were conducted to detect the expression levels of key proteins in the AMPK/SIRT1 pathway. **Results:** BBR could improve lipid metabolism, attenuate hepatic steatosis and alleviate liver injury significantly. The excessive oxidative stress, high levels of inflammation and abnormal apoptosis in *db/db* mice were reversed after BBR intervention. BBR clearly changed the expression of AMP-activated protein kinase (AMPK)/Sirtuin 1 (SIRT1), and their downstream proteins. **Conclusion:** BBR could reverse NAFLD-related liver injury, likely by activating the AMPK/SIRT1 signaling pathway to inhibit oxidative stress, inflammation and apoptosis in hepatic tissue.

Keywords: nonalcoholic fatty liver disease; berberine; inflammation; oxidative stress; apoptosis

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Nonalcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver conditions, ranging from simple hepatic steatosis to nonalcoholic steatohepatitis (NASH), cirrhosis, and even hepatocellular carcinoma (HCC)[1]. NAFLD is swiftly becoming the leading cause of chronic liver disease worldwide[2, 3], largely attributed to the global increase in obesity and diabetes rates, as well as the aging population[4]. Recent studies indicate that the prevalence of NAFLD in China is approximately 30%[5]. The exact pathogenesis of NAFLD remains elusive, but it is widely accepted to involve complex interactions among obesity, abnormal lipid metabolism, insulin resistance, inflammation, and oxidative stress[6, 7]. Physical exercise and diet control are currently believed to be efficient intervention methods for NAFLD[8]. However, there are currently no approved medications for NAFLD treatment. Given the increasing incidence of NAFLD worldwide, there is an urgent need for safe and efficacious drugs for NAFLD treatment.

Sirtuin 1 (SIRT1), an NAD^+-dependent deacetylase, is a soluble protein predominantly found in the mitochondrial matrix and is highly expressed in liver tissue[9]. SIRT1 exhibits broad substrate specificity and deacetylates various targets, such as NF-κB, FOXO transcription factors, PPAR-γ, and PGC-1α, thereby modulating cellular processes ranging from energy metabolism to cell survival[10]. Previous studies have indicated a link between SIRT1 and abnormal lipid metabolism, with downregulated SIRT1 expression observed in mice fed with a high-fat diet. Notably, modulation of SIRT1 expression has been shown to reduce hepatic fat accumulation[11–13]. Furthermore, specific knockout of SIRT1 in the livers of mice has been demonstrated to induce liver steatosis, insulin resistance, hyperlipidemia, and inflammation[14].

AMP-activated protein kinase (AMPK) is a pivotal regulator of biological energy metabolism. Its activation stimulates catabolic pathways, inhibits various anabolic pathways, and enhances ATP production[15, 16]. AMPK boosts SIRT1 activity by augmenting cellular NAD^+ levels, thereby influencing the regulation of downstream targets of SIRT1[17]. Multiple studies have underscored

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the close relationship between AMPK and SIRT1 in lipid metabolism, with reciprocal regulation occurring within a finely tuned network[18–20]. Moreover, AMPK can directly phosphorylate and thereby inhibit acetyl-CoA carboxylase (ACC), the rate-limiting enzyme in fatty acid synthesis[21, 22]. Additionally, the AMPK/SIRT1 axis has been implicated in the regulation of fatty acid β-oxidation[23]. In essence, the AMPK/SIRT1 axis plays a pivotal role in lipid metabolism, and the activation of AMPK/SIRT1 represents a promising therapeutic strategy for the treatment of NAFLD.

Berberine (BBR), an isoquinoline quaternary alkaloid isolated from Chinese *Coptis chinensis*, has long been utilized in clinical practice for treating gastrointestinal infections and diarrhea[24, 25]. In recent decades, a considerable number of additional pharmacological effects of BBR, such as weight loss, hypoglycemic effects, lipid-lowering effects, antidepressant effects, antihypertensive activity and anticancer activity, have been reported[26–30]. Furthermore, BBR has emerged as a promising therapeutic agent for NAFLD due to its ability to treat metabolic disorders[31]. Numerous animal experiments and clinical trials have validated the therapeutic efficacy of BBR in NAFLD treatment[32, 33]. The possible mechanisms of BBR in the treatment of NAFLD include improving insulin resistance, reducing lipid accumulation via the regulation of AMPK, alleviating oxidative stress, and regulating the gut microenvironment[34–36]. Although extensive efforts have been made to elucidate the mechanisms of BBR in NAFLD treatment, conclusive evidence remains elusive. BBR serves as an activator of AMPK, and previous studies have indicated that the ability of BBR to improve mitochondrial function was impaired in SIRT1-knockdown cells, suggesting a potential association between the mitochondrial effects of SIRT1 and BBR[37, 38]. Thus, BBR may improve hepatic lipid metabolism by regulating the AMPK/SIRT1 pathway to ameliorate NAFLD. The present study aimed to investigate the impact of BBR on oxidative stress, inflammation, and apoptosis in the liver tissues of *db/db* mice, as well as its effects on the AMPK/SIRT1 pathway. This research may offer novel insights into the underlying mechanism of BBR in NAFLD treatment.

1 MATERIALS AND METHODS

1.1 Materials

Metformin (MET) hydrochloride tablets (H20023-370, Sino-US Shanghai Squibb Pharmaceutical Co., Ltd., China) and BBR hydsrochloride tablets (H21022453, Northeast Pharmaceutical Group Co., Ltd., China) were used as therapeutic agents for subsequent animal experiments.

1.2 Animals

In *db/db* mice, a mutation in the leptin receptor gene leads to obesity, insulin resistance, and diabetes, all of which are risk factors for developing NAFLD. Thus, they serve as a relevant and useful model for studying the pathophysiology of NAFLD and testing potential therapeutic interventions. In this study, *db/db* mice were chosen as an animal model for NAFLD.

The ethical approval for all animal experiments was granted by the Institutional Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology. All animal care and experimental procedures were conducted in strict accordance with the Guidelines of the Institutional Animal Care and Use Committee of Tongji Medical College and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Male mice aged 8 weeks with a C57BL/6JNju background were procured from the Hubei Provincial Center for Food and Drug Safety, while male mice with a C57BL/BKS-Lepr$^{em2Cd479/Nju}$ [C57BL/6JNju-*db/db*, genotyping is (Lepr^{db}) mut/mut] background of the same age were obtained from the Model Animal Research Center of Nanjing University. The mice were housed in a specific-pathogen-free (SPF) room, with standard cages accommodating 4 mice per cage, and were maintained under controlled conditions: 22 ± 1°C and 12-h light-dark cycle. The rats were provided *ad libitum* access to water and standard chow with 6% kcal from fat (14.3 MJ/kg) obtained from the Hubei Provincial Center for Disease Control and Prevention.

1.3 Animal Experimental Design

After one week of acclimatization, 10 healthy C57BL/6J mice and 30 *db/db* mice were randomly allocated to one of 4 groups: the normal control (NC) group, which included healthy mice treated with distilled water; the diabetic control (DC) group, which included *db/db* mice treated with distilled water; the MET therapy group, which included *db/db* mice treated with MET (300 mg/kg body weight, daily); and the BBR therapy group, which included *db/db* mice treated with BBR (300 mg/kg body weight, daily). The drugs were administered for a duration of 8 weeks.

Upon completion of the experiment, the mice were fasted overnight and subsequently anesthetized with sodium pentobarbital (150 mg/kg body weight, intraperitoneal injection). Blood samples were collected from each mouse and promptly centrifuged at 1200 × g for 15 min at 4°C to obtain serum. Liver tissues were harvested and transferred to microtubes and then snap-frozen in liquid nitrogen. All the samples were stored at –80°C until further analysis.

1.4 Body Weight, Blood Glucose Level and Liver Index

Throughout the experimental period, body weight and blood glucose level were monitored weekly. The liver index was determined using the following formula: liver index = (liver weight/final body weight)×100%.

1.5 Biochemical Analysis

The total cholesterol (TC), triacylglycerol (TG), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the serum were measured by assay kits (#A111-1-1, #A110-1-1, #A113-1-1, #A112-1-1, #C010-2-1, #C009-2-1) from Nanjing Jiancheng

Bioengineering Institute. The glutathione peroxidase (GSH-Px), glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD) and catalase (CAT) levels in the liver tissue were measured with assay kits (#A005-1-2, #A006-2-1, #A003-1-2, #A001-2-2, and #A007-1-1, respectively) from Nanjing Jiancheng Bioengineering Institute. The interleukin (IL)-1β, tumor necrosis factor (TNF)-α and monocyte chemotactic protein 1 (MCP-1) levels in liver tissue were measured with ELISA kits (#JL18442, #JL10484, #JL20304) from Shanghai Jianglai Industrial Limited by Share Ltd., China). All the experimental procedures were conducted in accordance with the manufacturer's instructions.

1.6 Histological Assessment

The liver tissues were fixed in 10% formalin solution and subsequently embedded in paraffin wax. Sections were prepared and stained with hematoxylin and eosin (H&E) as well as periodic acid-Schiff (PAS). The stained sections were then examined under a light microscope for histopathological analysis.

1.7 Western Blotting

The protein expression levels were examined by Western blotting as described elsewhere[39]. Total protein was extracted from liver tissue using RIPA buffer (25 mmol/L NaCl, 25 mmol/L Tris-HCl, 0.5 mmol/L EDTA, 1% Triton X-100, and 0.1% SDS) supplemented with protease and phosphatase inhibitors (1%). Following centrifugation at 8000 r/min and 4°C for 15 min, the supernatants were collected for subsequent assays. The protein concentration was quantified using a BCA protein assay kit (Beyotime Institute of Biotechnology, China), and equal amounts of proteins were loaded onto 8%–15% SDS-PAGE gels before being transferred to nitrocellulose membranes. After blocking with 5% nonfat milk for 2.5 h, the membranes were incubated with primary antibodies overnight at 4°C. After 3 washes with TBST, the membranes were incubated with secondary antibodies for 1 h at room temperature. After an additional 3 washes with TBST, protein bands were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific, USA), and digital images of the blots were captured using an automated imaging system (Gene Gnome5, Synoptics Ltd., UK). The primary antibodies used were anti-p-AMPK antibody (ab32047, Abcam, UK), anti-SIRT1 antibody (#8469, Cell Signaling Technology, USA), anti-p-ACC antibody (ab68191, Abcam, UK), anti-NF-κB antibody (ab16502, Abcam, UK), anti-Bax antibody (ab182733, Abcam, UK), anti-Bcl-2 antibody (ab182858, Abcam, UK), anti-cleaved caspase-3 antibody (#9661, Cell Signaling Technology, USA), and anti-GAPDH antibody (#2118, Cell Signaling Technology, USA).

1.8 TUNEL Staining

The liver sections were initially fixed with distilled protease K (20 mg/mL). Next, the slides were treated with a balanced buffer and endoxynucleotide transferase. An anti-digoxin-peroxidase conjugate was subsequently added to each liver tissue section to detect peroxidase activity immobilized within the liver. 3,3′-Diaminobenzidine (DAB) was used to stain the sections, which were then subjected to restaining. Histopathological changes were assessed utilizing a Nikon TE 2000 fluorescence microscope.

1.9 Immunohistochemistry

The paraffin-embedded liver tissue sections were stained using a polymer horseradish peroxidase (HRP) detection system. Initially, the slides were incubated with a primary antibody in a 5% BSA solution at 4°C overnight, followed by incubation with a secondary antibody at 37°C for 30 min. The primary antibodies utilized were anti-p-AMPK antibody (ab32047, Abcam, UK) and anti-SIRT1 antibody (#8469, Cell Signaling Technology, USA). Subsequently, the sections were stained with DAB, counterstained with Mayer's hematoxylin, and visualized using a highly sensitive inverted microscope (LV200, Olympus, Japan).

1.10 Statistical Analysis

Statistical analysis was conducted using SPSS 19.0. All results are presented as mean ± standard deviation (SD). The data were analyzed using one-way ANOVA followed by Dunnett's *T* test. *P* values less than 0.05 were considered to indicate statistical significance.

2 RESULTS

2.1 Effect of BBR on BGL, Body Weight, Liver Weight and Liver Index

Obesity is closely related to the development of NAFLD, so we measured the body weight of each group. As shown in table 1, the body weight of the mice in the DC group was clearly greater than that of the mice in the NC group ($P<0.001$). However, after 8 weeks of drug treatment, the body weights of the mice in the MET and BBR groups were significantly lower than those in the DC group ($P<0.001$), which indicated that MET and BBR could attenuate obesity in *db/db* mice. In addition, the liver weight and liver index were also greater in the *db/db* group than in the NC group ($P<0.01$). The liver weight and liver index were obviously lower in the MET+BBR group than in the DC group ($P<0.01$). Moreover, compared with those of the DC group, the BGL levels of the MET and BBR groups were significantly decreased ($P<0.001$). MET and BBR showed good hypoglycemic activity, as

Table 1 The blood glucose level (BGL), body weight, liver weight and liver index of each group

Groups	BGL (mmol/L)	Body weight (g)	Liver weight (g)	Liver index (%)
NC	6.8±0.44	22.5±0.82	0.89±0.05	3.94±0.21
DC	$31.3\pm1.92^{\#\#\#}$	$57.18\pm5.82^{\#\#\#}$	$2.97\pm0.16^{\#\#\#}$	$5.24\pm0.58^{\#\#}$
MET	$15.8\pm4.93^{***}$	$44.98\pm5.16^{**}$	$1.93\pm0.39^{***}$	$4.28\pm0.57^{*}$
BBR	$13.0\pm3.57^{***}$	$35.36\pm4.18^{***}$	$1.70\pm0.30^{***}$	$4.81\pm0.50^{*}$

$\bar{x}\pm s$, $n=10$. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs*. NC group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. DC group

previously reported.

2.2 BBR Improves Serum Lipid Profiles in *db/db* Mice

Since lipid metabolism abnormalities in NAFLD manifest as hyperlipidemia, we measured the typical biochemical parameters to clarify the protective effect of BBR. As shown in fig. 1, as compared with those in the NC group, the serum levels of TC, TG and LDL-C in the *db/db* group were significantly increased, while the HDL-C levels were significantly decreased (P<0.001). After administration of MET and BBR, the changes in the serum lipid profiles of *db/db* mice were markedly reversed. The serum levels of TC, TG and LDL-C in the MET and BBR groups were significantly lower (P<0.001), while the HDL-C level was obviously greater (P<0.05). These data suggest that BBR could improve lipid metabolism in *db/db* mice to a comparable extent to MET.

2.3 BBR Attenuates Hepatic Steatosis in *db/db* Mice

H&E staining and PAS staining were conducted to assess the protective effect of BBR on hepatic histology (fig. 2A). In the normal mice in the NC group, liver histology revealed a clear, typical liver lobular architecture with a single layer of hepatocytes surrounding the central vein and no noticeable glycogen accumulation. In contrast, liver tissues from *db/db* mice exhibited hepatocyte degeneration, vacuolation, necrosis, and glycogen accumulation. However, both MET and BBR significantly ameliorated these histopathological changes, including microvesicular fatty acid changes and glycogen accumulation, in *db/db* mice, indicating that BBR could protect the liver from injury at the histological level.

2.4 BBR Ameliorates Liver Injury in *db/db* Mice

Serum transaminase levels, including AST and ALT, were measured to confirm whether BBR could ameliorate liver cell injury. AST and ALT are considered markers of liver injury, and the data are displayed in fig. 2B. The serum levels of AST and ALT were significantly greater in *db/db* mice than in NC mice (P<0.001). However, following MET and BBR treatment, the serum levels of AST and ALT in *db/db* mice showed a notable decrease (P<0.05). These results demonstrate that the hepatoprotective effect of BBR is similar to that of MET.

2.5 BBR Improves Hepatic Antioxidant Capacity in *db/db* Mice

The levels of GSH-Px, GSH, MDA, SOD, CAT and reactive oxygen species (ROS) in the liver were measured to investigate the effects of BBR on oxidative stress. As shown in fig. 3, compared to those in the DC group, MET and BBR significantly increased the levels of GSH-Px, GSH, SOD and CAT (P<0.05) and significantly decreased the level of MDA in *db/db* mice. DHE staining revealed that both MET and BBR significantly inhibited ROS production in the livers of *db/db* mice. These findings suggest that both MET and BBR can alleviate oxidative stress in the liver.

2.6 BBR Alleviates Hepatic Inflammatory Stress in *db/db* Mice

The levels of inflammatory markers, including IL-1β, IL-6, TNF-α, and MCP-1, in the livers of the mice were measured to assess the effect of BBR on inflammatory stress. As shown in fig. 4, the levels of IL-1β, IL-6, TNF-α, and MCP-1 were increased in the livers of the mice in the DC group, indicating an inflammatory reaction in the liver. However, after MET and BBR treatment, the levels of IL-1β, IL-6, TNF-α, and MCP-1 were significantly decreased, demonstrating that BBR could alleviate

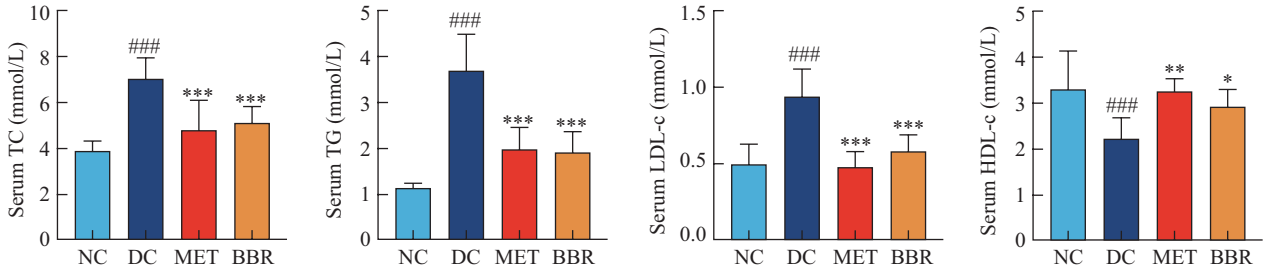

Fig. 1 Berberine (BBR) improves serum lipid profiles in *db/db* mice
The data are presented as the mean±standard deviation. $^{###}P$<0.001 *vs*. NC group; $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs*. DC group

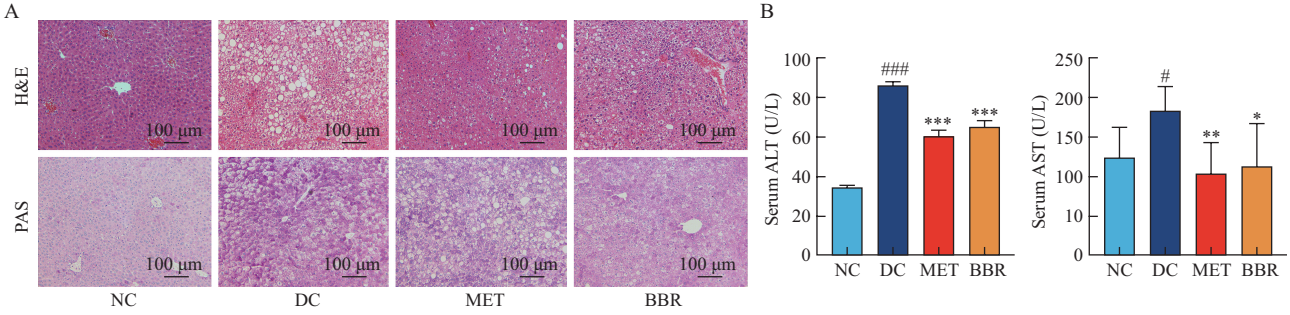

Fig. 2 Berberine (BBR) attenuates hepatic steatosis and liver injury in *db/db* mice
A: histological examination of liver slices with H&E staining and PAS staining; B: the serum ALT and AST levels. The data are presented as mean±standard deviation. $^{#}P$<0.05, $^{###}P$<0.001 *vs*. NC group; $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs*. DC group

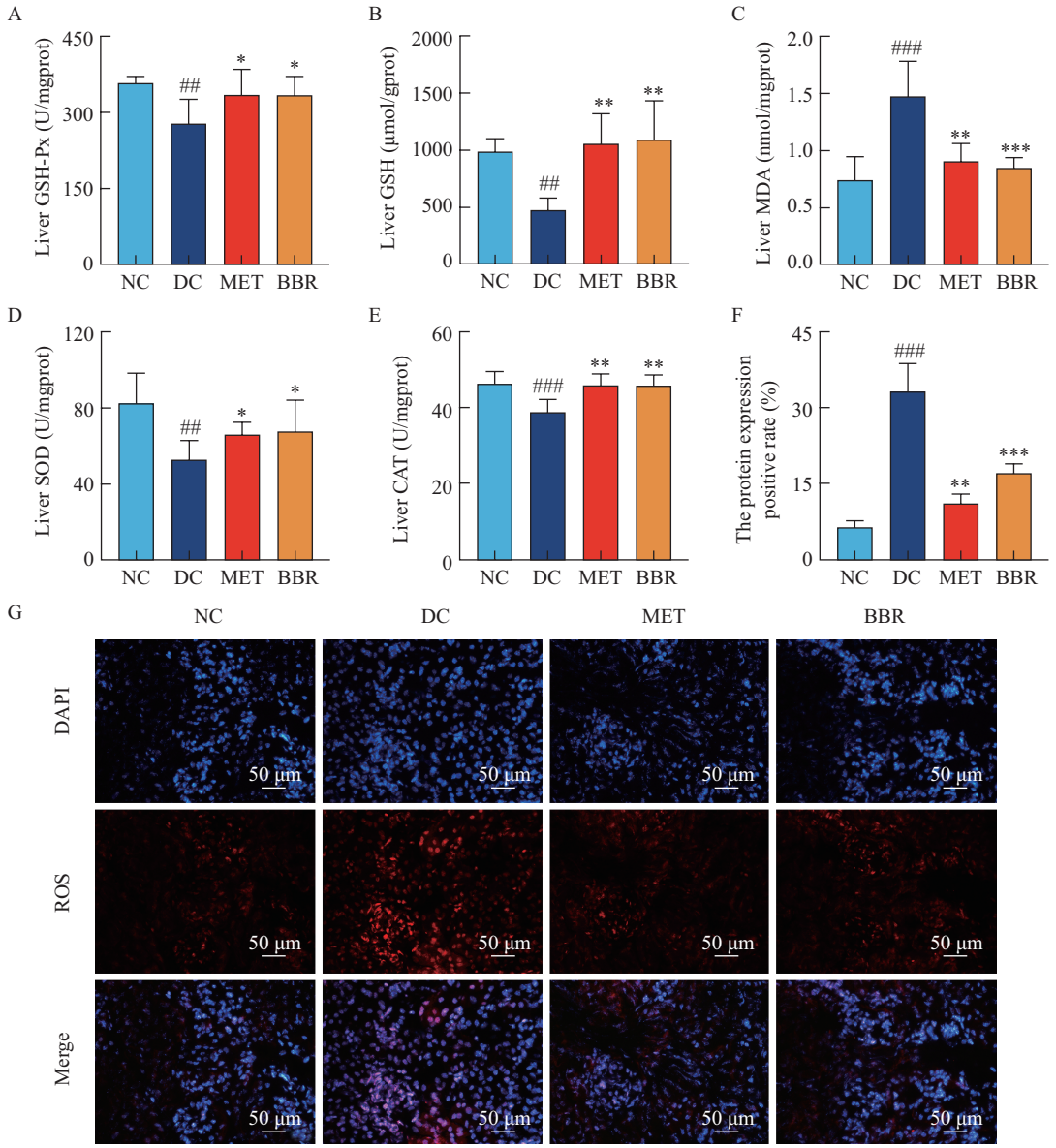

Fig. 3 Berberine (BBR) improves hepatic antioxidant capacity in *db/db* mice
The markers of hepatic oxidative stress included GSH-Px (A), GSH (B), MDA (C), SOD (D) and CAT (E). The ROS level was determined by DHE staining (G), DHE fluorescence intensity was measured by IPP, and the results are expressed as the ratio of the fluorescence intensity of the DHE-positive area to that of the DAPI-positive area (F). The data are presented as mean±standard deviation. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs*. NC group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. DC group

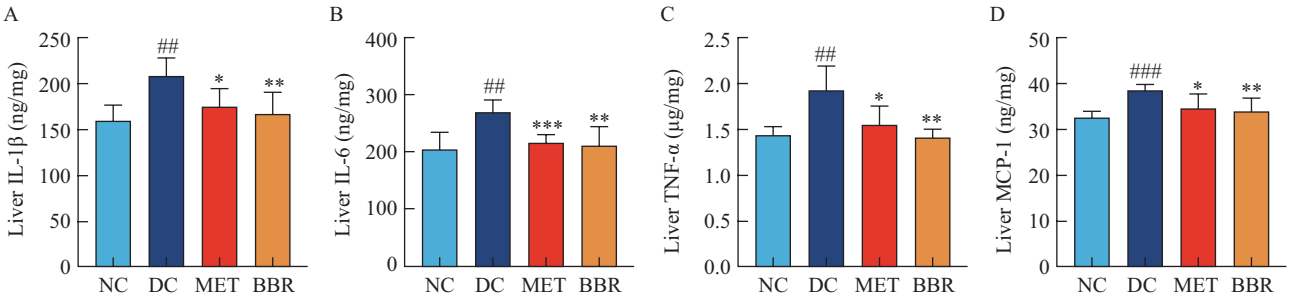

Fig. 4 Berberine alleviates hepatic inflammatory stress in *db/db* mice
The levels of IL-1β (A), IL-6 (B), TNF-α (C) and MCP-1 (D) in liver tissue were measured enzymatically. The data are presented as mean±standard deviation. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs*. NC group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. DC group

inflammatory stress in the NAFLD model.

2.7 Effect of BBR on Apoptotic Proteins in Livers of *db/db* Mice

The protein expression levels of Bax, Bcl-2, and cleaved caspase 3, which play key roles in apoptosis, were detected in the mouse liver using Western blot assays. The results showed that the expression of Bax and cleaved caspase 3 increased, while the expression of

Bcl-2 decreased in livers from the DC group, indicating that apoptosis occurred in diabetic livers. However, the changes in the expression of these proteins were significantly reversed after MET and BBR treatment. To further analyze liver cell apoptosis more clearly, TUNEL staining was performed. Almost no apoptotic cells were observed in the NC group, while a large number of apoptotic cells were observed in the DC group (fig. 5). However, the number of apoptotic cells was reduced in the MET and BBR groups. Taken together, these data suggest that BBR could effectively improve liver cell apoptosis in the NAFLD model.

2.8 Effect of BBR on the AMPK/SIRT1 Signaling Pathway

We further investigated the underlying molecular mechanism explaining the protective effect of BBR on NAFLD by analyzing its effect on the AMPK/SIRT1 pathway (fig. 6). The expression of AMPK/SIRT1 and its downstream proteins, such as ACC, NF-κB, and FOXO1, which are related to the regulation of inflammation, oxidative stress, and apoptosis, was detected using Western blot assays. BBR significantly activated the AMPK/SIRT1 pathway, as evidenced by the increased expression of p-AMPK and SIRT1. Moreover, the changes in the expression levels of p-ACC, NF-κB, and FOXO1 were reversed in the NAFLD model group after MET and BBR treatment.

3 DISCUSSION

NAFLD, a hepatic manifestation of metabolic syndrome, is characterized by fat accumulation in the hepatocyte cytoplasm without apparent alcohol consumption or other liver injury factors[40]. NAFLD is becoming more common around the world, especially in Middle Eastern, Western and Asian nations, as the number of people with obesity increases, affecting approximately 2% of the world's population[5]. However, the development of NAFLD is a complex process and is not completely understood[7]. The process of NAFLD is widely accepted to be divided into two steps. In the first step, hepatic fat accumulation increases insulin levels. The second step is cellular and molecular changes involving oxidative stress, inflammation and apoptosis in the liver due to a variety of factors, such as hyperinsulinemia, cytokine injury and changes in immune system function[41].

BBR, which is extracted from various botanical sources, including *Coptis chinensis*, *Rhizoma coptidis*, and *Hydrastis canadensis*, has a long history of use in Ayurvedic and Chinese medicine[29]. Given its multifaceted effects, including increasing insulin sensitivity, lipid-lowering properties, and improvements in glycometabolism, BBR has emerged as a promising natural therapeutic agent for the treatment of NAFLD[31]. This study demonstrated that BBR significantly improved insulin resistance and serum lipid profiles, decreased ALT/AST levels in serum, and alleviated histopathological changes, including microvesicular fatty acid changes and glycogen accumulation, in *db/db* mice. All these data verified the liver-protective effect of BBR, which was consistent with previous reports.

To further understand the mechanism of BBR in the treatment of NAFLD, we measured the effect of BBR on 3 important factors, oxidative stress, inflammation and apoptosis, which are related to the progression of NAFLD. Oxidative stress arises from an imbalance between the production of ROS and the antioxidant defense system, with a predisposition toward ROS accumulation[42]. Excessive hepatic lipid accumulation can trigger the overproduction of oxidants, resulting in oxidative modifications to cellular macromolecules. This process leads to the accumulation of damaged macromolecules,

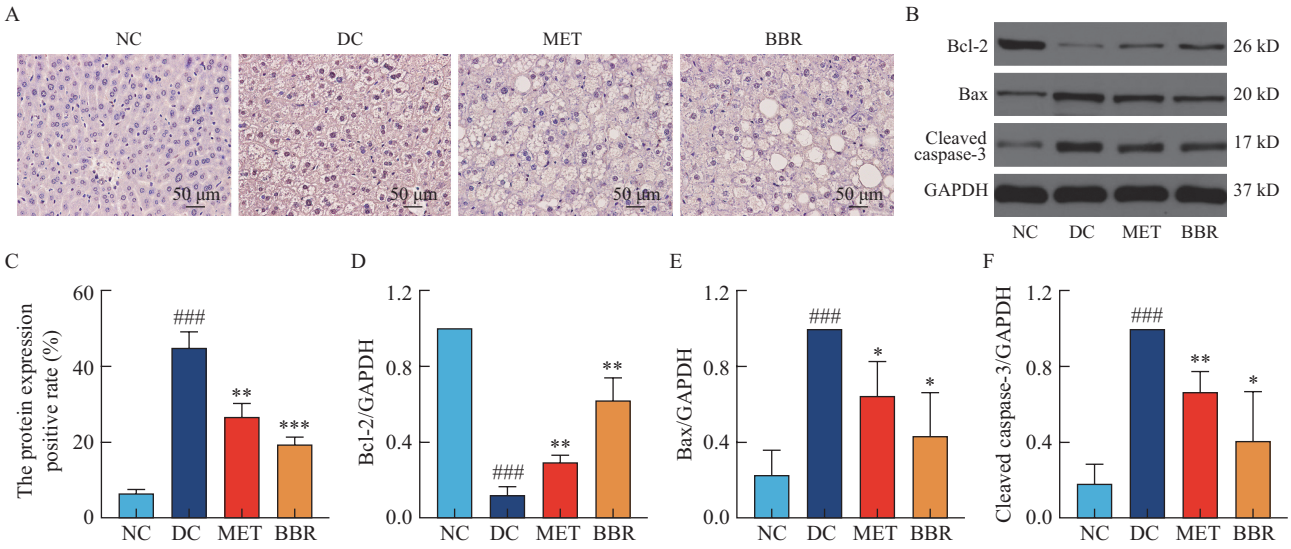

Fig. 5 Effect of berberine (BBR) on the expression of apoptotic proteins in the livers of *db/db* mice
The liver slices subjected to TUNEL staining (A) were analyzed using ImageJ (C). The protein expression of bcl-2, bax and cleaved caspase-3 in liver tissue from Western blotting (B) was analyzed by ImageJ (D, E, F). The expression of each protein was normalized to that of GAPDH as the loading control. The data are presented as mean ± standard deviation. $^{###}P<0.001$ *vs*. NC group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. DC group

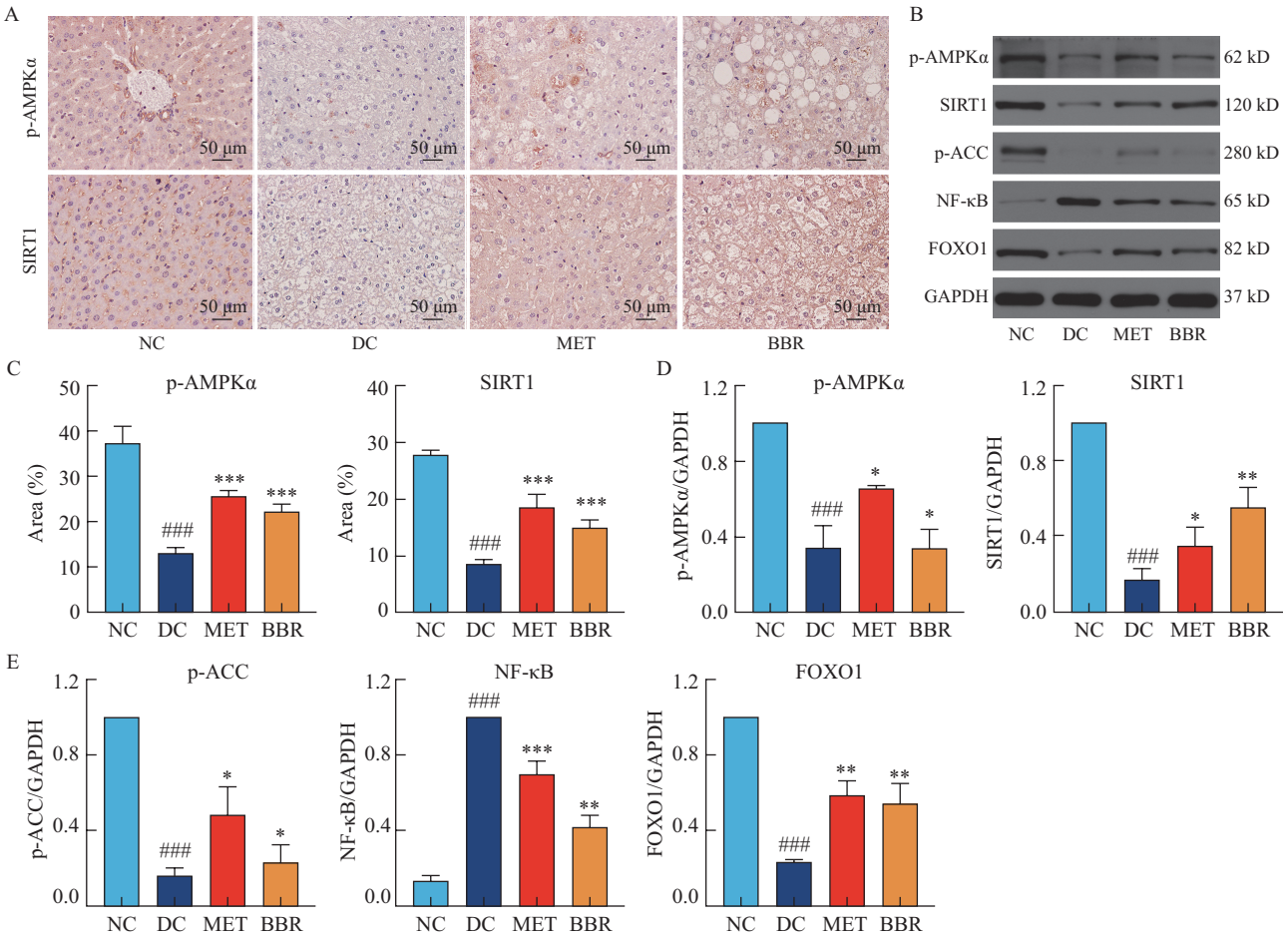

Fig. 6 Effect of berberine (BBR) on the AMPK/SIRT1 signaling pathway

The protein expression of p-AMPKα and SIRT1 determined by immunohistochemistry (A) was analyzed by ImageJ (C). The protein expression of p-AMPKα, SIRT1, p-ACC, NF-κB and FOXO1 in liver tissue from Western blotting (B) was analyzed by ImageJ (D, E). The expression of each protein was normalized to that of GAPDH as the loading control. The data are presented as mean ± standard deviation. $^{###}P<0.001$ *vs*. NC group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. DC group

ultimately inducing liver injury[43]. The findings in the present study revealed that the levels of GSH-Px, GSH, SOD, and CAT were significantly decreased in *db/db* mice, while the levels of MDA and ROS were increased. After BBR treatment, these changes were reversed, and the production of ROS was distinctly suppressed.

In addition to oxidative stress, inflammation is crucial for NASH development and progression. Several studies have shown that NF-κB is abnormally activated in *db/db* mice[44, 45] and can further promote the production of proinflammatory mediators such as IL-1β, IL-6, TNF-α and MCP-1. These cytokines play a role in the recruitment and activation of Kupffer cells, which are resident hepatic macrophages that mediate inflammation in NASH. Our results demonstrated that BBR significantly decreased the levels of IL-1β, IL-6, TNF-α, and MCP-1, thereby alleviating the inflammatory reaction in the livers of *db/db* mice.

Cell death, including apoptosis, plays a significant role in the progression of NASH to NAFLD. Several clinical studies have demonstrated that terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive hepatocytes are significantly increased in the livers of NASH patients[46]. The expression of proapoptotic proteins such as Bax and cleaved caspase 3 is increased in the livers of NASH patients. These key proteins can be activated by oxidative stress and inflammation, thereby promoting the induction of apoptotic pathways[47, 48]. Our results demonstrated that BBR could alleviate liver cell apoptosis in an NAFLD model by decreasing the expression of Bax and cleaved caspase 3 and increasing the expression of Bcl-2. Taken together, these data indicate that BBR may alleviate liver injury by suppressing inflammation, oxidative stress, and apoptosis.

Sirtuins are a group of highly conserved NAD^+-dependent histone and protein deacetylases and/or ADP-ribosyl transferases. Accumulating evidence indicates that sirtuins play important roles in regulating metabolic processes related to fatty liver diseases[49]. SIRT1 is the most extensively studied member and plays a pivotal role in both NAFLD and alcoholic fatty liver disease (AFLD)[50]. Previous studies have shown that SIRT1 deficiency in hepatic SIRT1 knockout ($SIRT1^{LKO}$) mice leads to increased lipid deposition and notably increased TG and free fatty acid (FFA) levels in the liver and plasma. Additionally, $SIRT1^{LKO}$ mice exhibit hyperglycemia and insulin resistance, potentially attributed to increased intracellular ROS accumulation in the liver[51]. These

findings suggest that SIRT1 deficiency not only induces hepatic steatosis but also facilitates the progression to advanced stages of metabolic disorders. Consistent with these observations, our results revealed the downregulation of SIRT1 expression in the NAFLD model, corroborating previous research findings.

AMPK can activate SIRT1 by increasing NAD^+, which is a compulsory cosubstrate in the SIRT1 activation process, because AMPK can boost NAD^+ synthesis via induction of the enzyme nicotinamide ribosyl phosphotransferase (NAMPT), the rate limiting the conversion of nicotinamide to NAD^+[17]. AMPK can also phosphorylate and inactivate acetyl-CoA carboxylase (ACC) directly, thus inhibiting fatty acid biosynthesis[21]. MET and BBR are natural activators of AMPK. As we can see from the data, after MET and BBR treatment, the expression of SIRT1 and p-ACC in *db/db* mice was elevated. This indicated that the activation of AMPK/SIRT1 might be an important pathway for the protective effect of BBR on liver injury.

SIRT1 deacetylates a broad spectrum of substrates, such as FOXO transcription factors and NF-κB, which play pivotal roles in oxidative stress, inflammation and apoptosis, respectively[10]. Studies have shown that the activation of SIRT1 by the NAD^+ precursor nicotinamide riboside can deacetylate and activate FOXO, resulting in the increased expression of target antioxidant genes such as SOD, CAT, and GPX. This process leads to a reduction in MDA and ROS levels, thereby offering protection against hepatic oxidative stress in fatty liver[52]. In the context of inflammation, NF-κB and its signaling pathway play central roles in hepatic inflammation. Upon activation, NF-κB enhances the expression of downstream genes involved in inflammation, such as IL-1β, IL-6, TNF-α, and MCP-1[53]. However, SIRT1 has been shown to interact with RelA/P65, a subunit of NF-κB, and inhibit its transcriptional activity by deacetylating RelA/P65 at lysine 310[54]. A previous study demonstrated that SIRT1 deficiency in macrophages led to NF-κB hyperacetylation and increased NF-κB transcriptional activation in the liver, consequently promoting hepatic inflammation. Conversely, the overexpression of SIRT1 in transgenic mice exhibited beneficial effects on fatty liver and decreased the activation of proinflammatory cytokines by downregulating NF-κB[55]. In addition, SIRT1 has been demonstrated to protect cells against apoptosis. According to previous studies, SIRT1 can regulate the expression level of Bcl-2/Bax and suppress the activation of caspase 3. Furthermore, Bcl-2 inhibits the mitochondrial pathway of apoptosis through localization at the membranes of the mitochondria and endoplasmic reticulum, while inactivated caspase 3 has no ability to cut different substrates and to induce the expansion of the protease cascade and eventually cell death[56]. The data in this study showed that the expression of the FOXO transcription factors NF-κB Bcl-2/Bax and cleaved caspase 3 were changed in a positive way in the MET/BBR treatment group through the AMPK/SIRT1 pathway, which indicated that BBR regulated oxidative stress, inflammation and apoptosis in the liver possibly via the AMPK/SIRT1 pathway.

To sum up, this study investigated the protective effect of BBR against NAFLD in *db/db* mice and explored its potential mechanism. Our findings demonstrated that BBR effectively improved insulin resistance, serum lipid profiles, hepatic steatosis, and liver injury in these mice. Moreover, BBR treatment reversed oxidative stress, inflammation, and apoptosis, suggesting that BBR has hepatoprotective effects through the suppression of these pathways. Further mechanistic analysis revealed that BBR activated the AMPK/SIRT1 pathway and subsequently modulated the expression of downstream proteins, such as FOXO transcription factors, NF-κB, Bcl-2/Bax, and cleaved caspase 3, all of which are implicated in oxidative stress, inflammation, and apoptosis. Therefore, our study suggested that BBR attenuates NAFLD in *db/db* mice by inhibiting inflammation, oxidative stress, and apoptosis through activation of the AMPK/SIRT1 pathway.

Conflict of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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