Berberine Ameliorates High-Fat Diet-Induced Non-Alcoholic Fatty Liver Disease in Rats via Activation of SIRT3/AMPK/ACC Pathway*

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Summary: This study aimed to verify the effects of berberine (BBR) on the fat metabolism proteins involved in the sirtuin 3 (SIRT3)/adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK)/acetyl-CoA carboxylase (ACC) pathway in the liver tissues of rats with high-fat diet (HFD)-induced non-alcoholic fatty liver disease (NAFLD). Forty-eight rats were randomly divided into the normal control (NC) group, HFD group or BBR group, with 16 rats in each group. After 8 and 16 weeks of treatment, serum and liver samples were collected. Subsequently, body parameters, biochemical parameters and liver pathology were examined. The expression levels of proteins involved in the SIRT3/AMPK/ACC pathway in the liver were detected by Western blotting. After 8 and 16 weeks of a HFD, the successful establishment of rat models with different degrees of NAFLD was confirmed by hematoxylin and eosin (H&E) and Oil Red O staining. NAFLD rat models exhibited obesity and hyperlipidemia, and the protein expression levels of SIRT3, p-AMPK, p-ACC, and CPT-1A in the liver were significantly decreased compared to those in the NC group. The concurrent administration of BBR with the HFD effectively improved serum and liver lipid profiles and ameliorated liver injury. Furthermore, the protein expression levels of SIRT3, p-AMPK, p-ACC, and CPT-1A in the liver were significantly increased in the BBR group as compared with those in the HFD group. In conclusion, our data suggest that the mechanism by which BBR ameliorates HFD-induced hepatic steatosis may be related to the activation of the SIRT3/AMPK/ACC pathway in the liver.
Key words: berberine; non-alcoholic fatty liver disease; sirtuin 3; lipid metabolism

Non-alcoholic fatty liver disease (NAFLD) is a common liver disease, ranging from simple hepatic steatosis to an intermediate stage with non-alcoholic steatohepatitis (NASH) to liver fibrosis and cirrhosis[1, 2]. NAFLD has become a worldwide epidemic aggravated by the consistent increase in obesity. The prevalence of NAFLD and NASH is now the leading cause of chronic liver disease in Western countries, and is becoming increasingly common worldwide[3]. The median prevalence of ultrasonographic steatosis in Chinese populations is approximately 10% but varies from 1% to more than 30%[4]. NAFLD is considered to be a critical component of the metabolic syndrome, which is defined by the presence of central obesity, insulin resistance, hyperlipidemia, hyperglycemia and hypertension[5]. Despite the progress that has been made in understanding how fat accumulates in the liver, the exact mechanisms underlying the pathogenesis of NAFLD remain unclear.

Sirtuin 3 (SIRT3), a soluble protein located in the mitochondrial matrix, is one of the most studied sirtuins involved in the regulation of lipid metabolism[6, 7]. A previous study demonstrated that mice lacking SIRT3 show characteristics of fatty acid oxidation disorders, suggesting that SIRT3 plays a critical role in hepatic fatty acid oxidation[8]. Another study confirmed that compared to wild-type mice, mice lacking SIRT3 and fed on a high-fat diet (HFD) exhibit an accelerated

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development of the metabolic syndrome hallmarks such as obesity, insulin resistance, hyperlipidemia and steatohepatitis[9]. Furthermore, it has been proposed that SIRT3 stimulates adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) phosphorylation and then inactivates acetyl-CoA carboxylase (ACC), thereby reducing hepatic lipid deposition[10, 11]. Thus, it is possible that activation of the SIRT3/AMPK/ACC pathway might offer therapeutic potential for the treatment of NAFLD or hyperlipidemia.

Berberine (BBR) is a protoberberine isoquinoline alkaloid that is found in many plant species such as *Coptis chinensis* Franch. (Huanglian) used in traditional Chinese medicine[12]. Over the past decade, BBR has received increasing attention as many studies have demonstrated its anti-obesity, anti-hyperglycemia and anti-dyslipidemia effects[13–16]. Nevertheless, the precise mechanism underlying its protective effects remains unclear. Recently, it was demonstrated that the potent protective effects of BBR against the metabolic syndrome may rely on increasing mitochondrial SIRT3 activity, normalizing mitochondrial function and preventing a state of energetic deficiency from impaired oxidative phosphorylation[17]. These findings prompted us test whether BBR alters lipid metabolism by regulating the SIRT3/AMPK/ACC pathway in the liver to ameliorate the hepatic steatosis associated with obesity. Hence, in this work, we investigated the effects of BBR on the fat metabolism proteins involved in the SIRT3/AMPK/ACC pathway in the liver tissues of rats with HFD-induced NAFLD. We hope that our findings offer more information about the underlying mechanisms and pathways involved in BBR action in the regulation of NAFLD.

1 MATERIALS AND METHODS

1.1 Animals

Forty-eight specific pathogen-free male Sprague-Dawley rats, aged 6–7 weeks and weighing 180–220 g, were purchased from the Laboratory Animal Center of Guangzhou University of Chinese Medicine, China [Approval No. SYXK (Yue) 2013−0034] and were housed in a rat facility with free access to diet and water. The animals were maintained on a 12/12-h light/dark cycle under a controlled temperature of 22±2°C and humidity of 50%±10%. The animals were acclimatized for 1 week prior to the experiments.

1.2 Experimental Procedures

All rats were randomly divided into 3 groups (16 rats per group), namely, the normal control (NC) group, the HFD group and the BBR group. Rats in the NC group were given free access to regular rat chow supplied by the Laboratory Animal Center of Jinan University, while rats in the HFD group and BBR group were fed on a HFD (composed of 88% regular chow, 10% lard oil, 1.5% cholesterol, and 0.5% bile salt) which was purchased from Guangdong Medical Laboratory Animal Center, China. Rats in the BBR group were intragastrically administered 100 mg/kg BBR (Mysun Pharma, China) once a day[18]. Rats in the NC and HFD groups were given the corresponding dose of distilled water once a day. At the end of 8 and 16 weeks after the last administration, 8 rats in each group were fasted overnight and then anesthetized with a 3% pentobarbital intraperitoneal injection. Before being sacrificed, the body weight and length (naso-anal length) of the rats were measured to calculate Lee's index [body weight $(g)^{1/3}\times1000$/length (cm)]. Blood samples were collected from the abdominal aorta and centrifuged at 3000 r/min for 10 min at 4°C; then, the serum was collected and stored at –80°C for biochemical analyses. The livers were immediately removed and weighed to calculate the liver index (liver weight/body weight×100%). Liver samples were then stored at –80°C for following experiments. All experimental procedures were approved by the Laboratory Animal Ethics Committee of Jinan University.

1.3 Measurement of Serum Biochemistry

The concentration of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in the serum were measured using an automatic biochemical analyzer (Hitachi, Japan).

1.4 Measurement of Liver TG and TC

Liver tissues were placed in isopropanol and homogenized using a homogenizer (Qiagen, Germany). Then, the homogenates were centrifuged at 10 000 r/min for 15 min at 4°C, and the supernatants were collected to determine the TC and TG levels in the liver using an automatic biochemical analyzer (Hitachi, Japan).

1.5 Histopathological Evaluation

The paraffin-embedded liver sections were sliced at a thickness of 4 μm and then examined by hematoxylin and eosin (H&E) staining. Frozen liver sections embedded in optimum cutting temperature compound (Sakura Finetek, USA) were sliced at a thickness of 10 μm and then examined by Oil Red O staining (Nanjing Jiancheng Technology Co., Ltd., China).

1.6 Western Blot Analysis

Western blotting was used to determine the protein expression levels of liver SIRT3, AMPK, phosphor-AMPK (p-AMPK), ACC, phosphor-ACC (p-ACC), carnitine palmitoyltransferase 1A (CPT-1A) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH was used as an internal control. To extract total tissue protein, frozen liver samples were homogenized in RIPA lysis buffer containing protease and phosphatase inhibitors. After 30 min at 4°C, the

tissue lysate samples were centrifuged at 10 000 g for 5 min at 4°C, and the supernatants were collected. The protein concentration was measured using a BCA protein assay kit (Nanjing KeyGen Biotech. Co. Ltd., China). Equal amounts of protein were loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to polyvinylidene difluoride (PVDF) membranes. After being blocked with Tris-buffered saline with Tween 20 (TBST) containing 5% skim milk for 1 h at room temperature, membranes were incubated with a SIRT3 antibody (1:1000, Cell Signaling Technology, USA), AMPK antibody (1:1000, Cell Signaling Technology, USA), p-AMPK antibody (1:1000, Cell Signaling Technology, USA), ACC antibody (1:1000, Cell Signaling Technology, USA), CPT-1A antibody (1:1000, Abcam, UK), or GAPDH antibody (1:10 000, KangChen Bio-tech, China). After being washed, membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:20 000, Southern Biotech, USA) at 37°C for 1 h. After washing four times, membranes were incubated with enhanced chemiluminescence substrate and exposed to X-ray film.

1.7 Statistical Analysis

Data are expressed as the means±standard deviations (SD). One-way analysis of variance (ANOVA) and Bonferroni's post hoc test were used for the determination of statistical significance between groups. *P* values less than 0.05 were considered statistically significant. All data were analyzed with SPSS 13.0 statistical software (SPSS Software, USA).

2 RESULTS

2.1 BBR Improves HFD-Induced Obesity in NAFLD Rats

Since obesity is closely associated with the development of NAFLD, we measured the main body parameters of the animals. After 8 and 16 weeks of HFD feeding, the model rats fed on the HFD without BBR exhibited a heavier body weight and liver weight than the NC group ($P<0.01$). Additionally, the liver index and Lee's index were also increased in the HFD group as compared with those in the NC group ($P<0.01$). In contrast, the concurrent administration of BBR effectively reverted the changes caused by HFD feeding for 16 weeks ($P<0.05$). However, the liver index and Lee's index were not significantly different between the HFD group and BBR group after 8 weeks of HFD feeding ($P>0.05$). In addition, no significant differences in body length were observed among the groups (table 1).

2.2 BBR Attenuates Hepatic Steatosis in NAFLD Rats

Since hepatic steatosis is a characteristic of NAFLD, we performed liver morphological and histological examinations to verify the effect of BBR. The macroscopic images of the rat livers showed that the HFD feeding increased the liver size and changed the liver color from dark to pale (fig. 1A). The results of the H&E and Oil Red O staining indicated that the liver tissues of rats fed on the HFD for 8 weeks displayed mild hepatic steatosis, while the rats in the 16-week HFD group displayed more severe steatosis (fig. 1B and 1C). In contrast, the concurrent administration of BBR markedly attenuated the severity of hepatic steatosis induced by HFD feeding, as evidenced by the improvements in lobular architecture, hepatocyte swelling, macrovesicular lipid droplets and inflammatory infiltration.

2.3 BBR Improves Serum and Liver Lipid Profiles of NAFLD Rats

Since the lipid metabolism abnormalities on NALFD manifest as fatty liver and hyperlipidemia, we examined typical biochemical parameters to clarify the protective effect of BBR against this disease. After 8 and 16 weeks of HFD feeding, the serum levels of TC, TG and LDL-C in the HFD group were significantly increased, while the HDL-C levels were significantly reduced as compared with those in the NC group ($P<0.01$). The concurrent administration of BBR markedly reversed these serum lipid profile abnormalities caused by the HFD feeding, especially at 16th week ($P<0.05$). In accordance with the increased serum lipid levels and histological results, liver TC and TG levels were markedly increased in the HFD group as compared with those in the NC group ($P<0.01$). However, the concurrent administration of BBR significantly reduced these increases caused by

Table 1 The body parameters in all groups

Items	8-week groups			16-week groups		
	NC	HFD	BBR	NC	HFD	BBR
Body weight (g)	400.62±13.21	460.25±30.20**	403.12±34.87$^{\#}$	496.37±38.34	566.25±28.47**	519.12±24.56$^{\#}$
Liver weight (g)	12.01±0.87	20.79±1.27**	17.26±1.44$^{\#\#}$	15.04±2.75	24.92±2.53**	19.44±3.55$^{\#\#}$
Liver index (%)	2.99±0.15	4.53±0.38**	4.30±0.36	3.01±0.34	4.40±0.33**	3.75±0.69$^{\#}$
Body length (cm)	24.91±0.53	25.19±0.51	24.44±0.76	26.06±1.05	26.12±0.69	26.12±0.44
Lee's index	295.97±5.46	306.43±4.25**	302.10±2.10	303.86±8.70	316.71±6.74**	307.59±3.54$^{\#}$

NC: normal control group; HFD: high-fat diet group; BBR: berberine group. Data are expressed as means±standard deviations (n=8). $^{**}P<0.01$ *vs.* the corresponding NC group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* the corresponding HFD group

the HFD (P<0.05), indicating that BBR can ameliorate hepatic lipid accumulation (table 2).

2.4 BBR Ameliorates Liver Injury in NAFLD Rats

To investigate whether BBR could ameliorate the HFD-induced liver cell injury, we examined serum transaminase levels, which are considered to be markers of liver injury. At the end of 16th week, serum ALT levels were significantly increased in the HFD group as compared with those in the NC group (P<0.05), but no significant changes were observed among the groups at 8th week. Furthermore, at both 8th and 16th weeks of HFD feeding, serum AST levels were significantly increased in the HFD group as compared with those in the NC group (P<0.01). The concurrent administration of BBR mitigated the increased ALT and AST levels caused by the HFD feeding, although there were no significant changes in the ALT levels among the groups at 8th week of HFD feeding (P>0.05). These results demonstrate the hepatoprotective effect of BBR against HFD feeding (table 2).

2.5 BBR-Mediated Protection against NAFLD Involves Activation of SIRT3/AMPK/ACC Pathway

To assess the effect of BBR on the SIRT3/AMPK/ACC pathway in rats, we assessed several proteins in this pathway in the liver. In the HFD group, the protein levels of SIRT3 in the liver were significantly decreased as compared with those in the NC group (P<0.01). Apart from the SIRT3 expression, the HFD feeding also induced a decrease in the liver protein expression levels of p-AMPK, p-ACC and CPT-1A (P<0.05). At 8th week, there was no significant difference in the p-ACC protein expression between the HFD group and NC group (P>0.05). In contrast, the concurrent administration of BBR significantly reversed the protein levels of SIRT3, p-AMPK, p-ACC and CPT-1A as compared with the HFD group (P<0.01). No

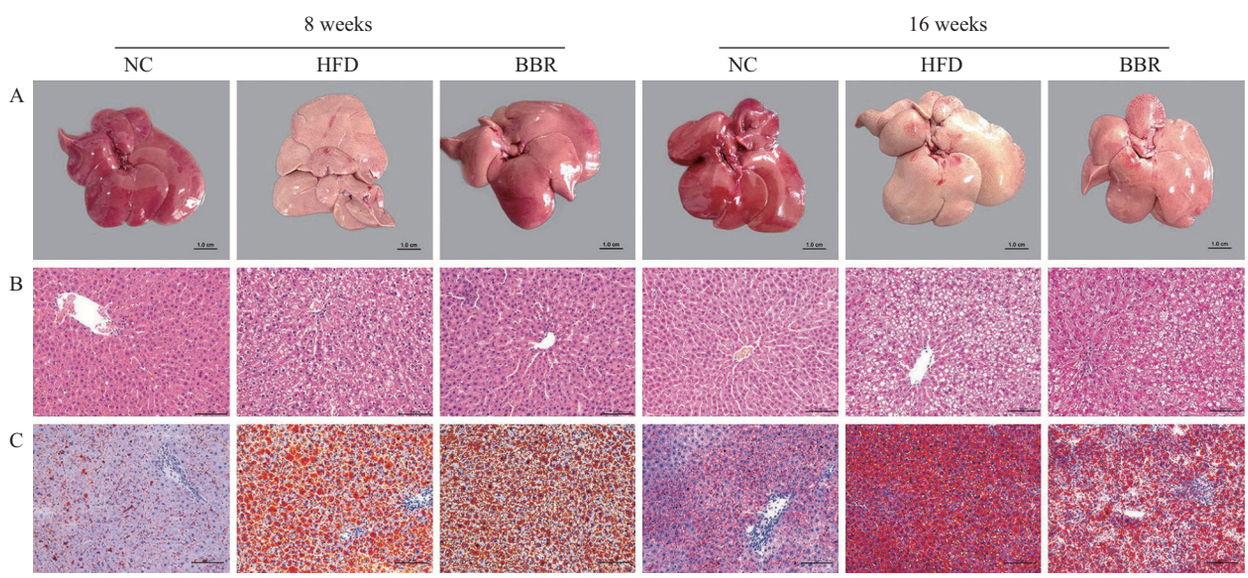

Fig. 1 Representative macroscopic and microscopic images of the liver from the study groups
A: representative macroscopic images of the liver from the study groups; B: representative images of liver sections stained with hematoxylin and eosin; C: representative images of liver sections stained with Oil Red O. Representative microscopic images were captured at 200× magnification.

Table 2 Serum and liver biochemical parameters in all groups

Items	8-week groups			16-week groups		
	NC	HFD	BBR	NC	HFD	BBR
Serum TC (mmol/L)	1.75±0.37	2.69±0.33**	2.15±0.28$^{\#}$	1.56±0.28	3.69±0.87**	2.55±0.81$^{\#}$
Serum TG (mmol/L)	0.65±0.11	1.27±0.18**	0.70±0.22$^{\#\#}$	0.66±0.20	1.34±0.35**	0.79±0.14$^{\#\#}$
Serum HDL-C (mmol/L)	1.07±0.22	0.78±0.17**	0.91±0.11	1.15±0.44	0.42±0.11**	0.70±0.23#
Serum LDL-C (mmol/L)	0.25±0.06	0.58±0.21**	0.34±0.09$^{\#}$	0.94±0.24	2.30±0.36**	1.66±0.33$^{\#\#}$
Serum ALT (U/L)	48.87±14.18	60.62±12.09	50.37±14.44	51.12±18.79	75.87±13.26*	53.75±16.22$^{\#}$
Serum AST (U/L)	76.75±13.34	109.62±21.55**	87.25±13.36$^{\#}$	69.50±5.95	201.25±44.22**	107.25±28.83$^{\#\#}$
Hepatic TC (mg/g)	0.72±0.13	4.97±0.55**	4.31±0.29$^{\#}$	1.87±0.47	5.50±0.82**	4.50±0.90$^{\#}$
Hepatic TG (mg/g)	1.87±0.70	6.77±0.52**	4.92±1.07$^{\#\#}$	2.53±1.39	7.95±0.77**	5.66±1.18$^{\#\#}$

NC: normal control group; HFD: high-fat diet group; BBR: berberine group. Data are expressed as means±standard deviations (n=8). $^{*}P$<0.05, $^{**}P$<0.01 *vs*. the corresponding NC group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs*. the corresponding HFD group. ALT: alanine aminotransferase; AST: aspartate aminotransferase; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TC: total cholesterol; TG: triglyceride

significant differences were observed in the AMPK and ACC expression (P>0.05). These results indicated that the concurrent administration of BBR activated SIRT1 and in turn promoted the phosphorylation of AMPK and ACC, ultimately activating CPT-1A (fig. 2).

3 DISCUSSION

BBR is an isoquinoline alkaloid found in the root of *Coptis chinensis* Franch. (Huanglian), a traditional Chinese herbal medicine that was initially recorded in the ancient Chinese herbal book "Shen Nong Ben Cao Jing". Over the past few years, there has been accumulating evidence demonstrating that BBR can improve key features of NAFLD, including hepatic steatosis, obesity and hyperlipidemia[19–21]. However, the precise mechanisms underlying the protective effects of BBR are not fully understood. Here, we focused on the mechanisms underlying the ability of BBR to ameliorate hepatic steatosis in NAFLD rats. A HFD is widely used to induce animal models of NAFLD, which mimic important pathogenic and histological features of human NAFLD[22]. We therefore used a HFD to induce NAFLD rat models in the present study. Our results showed that feeding rats a HFD for at least 8 weeks induced increases in body weight, liver weight and liver index. Meanwhile, Lee's index, often considered an indicator of obesity in rodents, was markedly increased in the HFD group, indicating the obesity of HFD-fed rats. The HFD also induced hyperlipidemia in the NAFLD rats, as evidenced by the abnormalities in serum and liver lipids. Furthermore, both H&E and Oil Red O staining indicated that HFD feeding successfully induced hepatic steatosis in rats. Expectedly, the longer HFD feeding duration resulted in more severe body and biochemical parameter abnormalities in NAFLD rats. These data indicated that we had established a rat NAFLD model with obesity and hyperlipidemia by HFD feeding.

In recent years, a growing body of evidence has

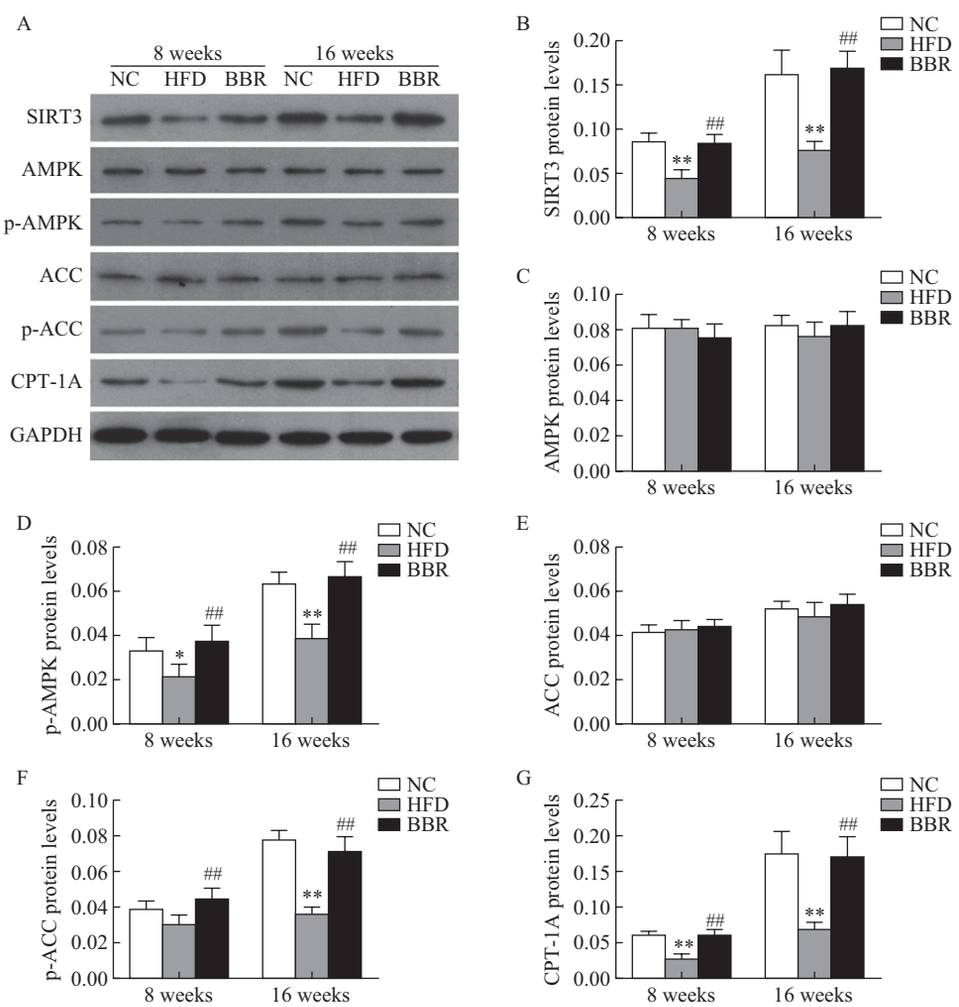

Fig. 2 Western blot analysis of the hepatic SIRT3/AMPK/ACC pathway in rats

A: Western blot of the SIRT3/AMPK/ACC pathway proteins in rat liver; B–G: analysis of protein levels of SIRT3 (B), AMPK (C), p-AMPK (D), ACC (E), p-ACC (F) and CPT-1A (G). GAPDH was used as control. Data are expressed as means±standard deviations. $^{*}P$<0.05, $^{**}P$<0.01 *vs.* the corresponding NC group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs.* the corresponding HFD group

demonstrated that obesity is commonly associated with the development of NAFLD[23, 24], while weight loss has been proven beneficial for patients with NAFLD[25]. Recently, several studies have demonstrated that BBR exerts an anti-obesity effect in animals[21, 26]. Consistent with these studies, our results also showed that BBR effectively reduced the body parameters increased by the HFD, indicating the ability of BBR to improve HFD-induced obesity. Additionally, hyperlipidemia is often regarded as an important feature in patients with NAFLD[27]. A recent study by Yan *et al* demonstrated that BBR can improve serum lipid profiles and reduce hepatic fat content in patients with NAFLD[16]. Consistent with these results, our study showed that BBR markedly improved the serum and liver lipid profiles of rats fed on a HFD. As predicted, BBR also mitigated the increased serum transaminase levels caused by the HFD. In summary, the return of these serum and liver metabolic parameters to control levels supports the protective effect of BBR on attenuating the adverse effects of HFD.

An increasing amount of evidence has demonstrated that SIRT3 plays a critical role in hepatic steatosis formation[8, 28]. A study by Hirschey *et al* demonstrated that in mice, hepatic SIRT3 expression is suppressed by long-term (13-week) HFD feeding but not by an acute (1-week) HFD[28]. In addition, a clinical study provides direct evidence that the down-regulation of sirtuins, including SIRT3, suppresses lipid synthesis in the liver of NAFLD patients, which may exacerbate NAFLD[29]. Thus, it has been proposed that hepatic lipid accumulation may be prevented by the up-regulation of SIRT3[10]. Consistent with these studies, our findings revealed that long-term (at least 8 weeks) HFD feeding resulted in the down-regulation of SIRT3 in the rat liver. Furthermore, several studies have demonstrated that the mechanism by which SIRT3 regulates lipid metabolism may be related to the activation of the AMPK pathway[10, 11]. AMPK plays a critical role in regulating lipid metabolism by both suppressing lipogenesis and stimulating fatty acid oxidation[30]. The activation of AMPK further promotes the phosphorylation and inactivation of ACC, thereby inhibiting fatty acid synthesis. The phosphorylation of ACC decreases the synthesis of malonyl CoA, which results in the disinhibition of CPT-1A and ultimately stimulates fatty acid oxidation[31]. Therefore, it is possible that the SIRT3/AMPK/ACC pathway may play a crucial role in lipid metabolism.

Recently, emerging studies have reported that BBR can activate SIRT3 expression *in vivo* and *vitro*[32, 33]. Therefore, we speculated that the regulation of lipid metabolism by BBR might be mediated through the activation of the SIRT3/AMPK/ACC pathway in the liver. We examined several of the proteins in this pathway in the rat livers to test the hypothesis. Our results showed that BBR markedly increased the hepatic protein expression of SIRT3, which was suppressed by long-term HFD feeding. As predicted, our results showed that BBR administration restored the hepatic phosphorylation of AMPK and ACC, ultimately increasing CPT-1A expression. These results confirmed that BBR activates the AMPK pathway in NAFLD rats, which may be related to SIRT3 activation. However, there are some *in vitro* studies suggesting that BBR may exert beneficial effects independently of AMPK activation[34], indicating that the mechanisms of BBR *in vitro* may be quite different than those *in vivo*. BBR may exert its effects through a wide variety of mechanisms, which need to be further clarified. Taken together, our findings demonstrated that BBR increases the hepatic expression of proteins involved in the SIRT3/AMPK/ACC pathway. Thus, the mechanism by which BBR ameliorates hepatic steatosis may be partially related to the activation of the SIRT3/AMPK/ACC pathway in the liver.

To sum up, in this study, our results demonstrated that a HFD can induce an NAFLD rat model with obesity and hyperlipidemia, and SIRT3 expression can be down-regulated in the rat liver after 8 or 16 weeks of HFD feeding. The concurrent administration of BBR effectively attenuated the adverse effects of the HFD in NAFLD rats. The mechanism by which BBR ameliorates HFD-induced hepatic steatosis may be associated with the activation of the SIRT3/AMPK/ACC pathway in the liver.

Conflict of Interest Statement

The authors declare that they have no competing interests with any financial organization or individuals that could inappropriately influence this work.

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