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Original article

Effects of Berberine on Hepatic Sirtuin 1-uncoupling Protein 2 Pathway in Non-alcoholic Fatty Liver Disease Rats Induced by High-fat Diet

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ABSTRACT

Objective To investigate the involvement of sirtuin 1 (SIRT1)-uncoupling protein 2 (UCP2) pathway in the development of non-alcoholic fatty liver disease and whether berberine exerts its effects by regulating this pathway. **Methods** Male SD rats were divided into three groups: normal control group, high-fat diet group, and berberine supplement group. The rats in the normal control group were given normal diet while the rats in the other two groups were fed with high-fat diet. Rats in the berberine supplement group were concurrently given berberine (100 mg/kg body weight) once daily. After 16 weeks, the levels of serum, liver lipids, and serum aminotransferase were measured using an automatic biochemical analyzer. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in the liver were measured using commercial kits. Histopathological changes of liver tissues were observed by hematoxylin and eosin (HE) staining and Oil Red O staining. The hepatic mRNA and protein levels of SIRT1 and UCP2 were assayed by reverse transcription polymerase chain reaction (RT-PCR) or Western blotting. **Results** Berberine supplement could significantly decrease the serum and liver lipid contents in rats fed with high-fat diet. Meanwhile, SOD level was significantly elevated, but MDA level was reduced in the liver. The results of HE and Oil Red O staining showed that the hepatic steatosis was alleviated in berberine supplement group. Furthermore, berberine induced an increase in SIRT1 expression but a decrease in UCP2 expression. **Conclusion** The regulation of hepatic SIRT1-UCP2 pathway may be an important mechanism by which berberine exerts the beneficial effects in NAFLD rats.

Key words

berberine; non-alcoholic fatty liver disease; oxidative stress; sirtuin 1; uncoupling protein 2

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is recognized as a hepatic manifestation of metabolic syndrome characterized by predominant macrovesicular steatosis of the liver without alcohol consumption (Loomba and Sanyal, 2013). It comprises a wide spectrum of disease that includes simple steatosis, non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis (Cohen et al, 2011; de Alwis and Day, 2008). NAFLD has become the most common cause of chronic liver disease in Western countries, affecting up to 30% of the general population (Loomba and Sanyal, 2013). In Asia, the prevalence of NAFLD ranges from 15% to 45%, with the epidemic of obesity and type 2 diabetes (Farrell et al, 2013). Although it has been proposed that NAFLD is closely associated with obesity and insulin resistance, the pathogenesis of NAFLD remains ill-defined (Cohen et al, 2011). As a result, the treatment of NAFLD remains controversial and novel therapeutic strategies are needed for the prevention and treatment of NAFLD.

Berberine, also known as umbellatine, is a kind of isoquinoline alkaloid isolated from Chinese medicinal herb *Coptidis Rhizoma*, which has been used in traditional Chinese medicine (TCM) for centuries. It is well known that berberine has many pharmacological properties concerning metabolic diseases, such as obesity and type 2 diabetes (Vuddanda et al, 2010; Wan et al, 2015). Recently, berberine has been reported to have beneficial roles in preventing or treating NAFLD *in vivo* and *in vitro*, suggesting that berberine may be a potential drug for NAFLD (Liu et al, 2013). Sirtuins are the mammalian homologues of silent information regulator-2 (Sir2), a group of class III histone deacetylases, which are NAD^+-dependent protein deacetylases. Sirtuin 1 (SIRT1), first identified among sirtuins, has been demonstrated to regulate the cellular protection against oxidative stress in many diseases including metabolic disorders (Chong et al, 2012; Colak et al, 2011). Since oxidative stress is strongly implicated in the pathogenesis of NAFLD (Videla et al, 2006, Rolo et al, 2012), SIRT1 has been proposed to be a potential therapeutic target in the treatment of NAFLD (Colak et al, 2011). Recently, berberine has been demonstrated to activate SIRT1 *in vivo* and *in vitro* (Chi et al, 2014; Gomes et al, 2012). Uncoupling protein 2 (UCP2) is a member of the super family of anion carrier proteins located in the inner membrane of mitochondria (Fisler and Warden, 2006). Our previous study demonstrated that berberine could improve NAFLD in rats along with a decrease in UCP2 expression, but the underlying mechanisms have not been elucidated (Yang et al, 2011). A previous study has demonstrated that SIRT1 represses mitochondrial UCP2 transcription by binding directly to the UCP2 promoter (Bordone et al, 2006).

Based on these data, it is tempting to speculate that berberine could lead to SIRT1 activation which in turn repressed UCP2 in liver tissues, thereby protect against NAFLD. In the present study, we used an NAFLD rat model to further investigate whether SIRT1-UCP2 pathway and oxidative stress were involved in the effects of berberine in protecting against NAFLD.

2. Materials and methods

2.1 Animals

Thirty specific pathogen-free male Sprague-Dawley (SD) rats aged 6−7 weeks (200 ± 20) g were purchased from the Laboratory Animal Research Center of Guangzhou University of Traditional Chinese Medicine, China (Approval No. SYXK (Yue) 2013−0034). The rats were kept in separate cages under conditions of controlled temperature (24 ± 2) ^oC on a regular 12-h light/dark cycle (lights on from 8:00 am to 8:00 pm), with free access to diet and water.

2.2 Grouping and modeling

After one week adaptive breeding, the rats were randomly distributed into three groups, 10 rats in each group: normal control (NC) group, high-fat diet (HFD) group, and high-fat diet supplemented with berberine (HFB) group. Rat models were duplicated according to the method as described previously with minor modifications (Yang et al, 2011). Rats in NC group got free access to normal chow diet supplied by Experimental Animal Centre of Jinan University, while rats in HFD and HFB groups were fed with a high-fat diet (composed of 88% regular chow, 10% axungiaporci, 1.5% cholesterol, and 0.5% bile salt) which were purchased from Experimental Animal Centre of Guangdong Province. Rats in HFB group were ig administered with berberine (Mysun Pharma, China) by 100 mg/kg, once daily (Gomes et al, 2012). Rats in NC and HFD groups were given distilled water daily. The intervention lasted for 16 weeks. After the last administration, all rats were made to fast for 12 h and then anesthetized by ip injecting 3% pentobarbital (1 mL/kg). Blood samples were collected from abdominal aorta and then centrifuged at 1500 × *g* for 10 min at 4 °C. The clear supernatants were collected for assays. The livers were immediately removed. All rats were treated in compliance with the *Guiding Principles for Animal Experiments* and the protocols were approved by the Animal Experimental Ethnics Committee of Jinan University, Guangzhou, China.

2.3 Biochemical analysis

The concentration of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in serum were measured using automatically biochemical analyzer (Hitachi Company, Japan). Liver tissues were homogenized using a Tissue Lyser-II Homogenizer (Qiagen, Germany) and centrifuged at 3000 × *g* for 10 min at 4 °C. Then the clear supernatants were collected to determine liver levels of TC and TG using an automatic biochemical analyzer.

2.4 Histopathology evaluation

The formalin-fixed and paraffin-embedded sections (about 1.0 cm × 1.0 cm × 1.0 cm) were sliced at a thickness of 4−6 μm and examined by hematoxylin and eosin (HE) staining. The OTC-embedded frozen sections were sliced at a thickness of 10−15 μm and examined by Oil Red O staining (Nanjing Jiancheng Technology Co., Ltd., China). HE staining was used to illustrate the pathological changes of liver tissue while Oil Red O staining was used to detect intracellular lipid droplets. These two kinds of staining were operated following the instructions of corresponding kits. Pathological sections were observed for the histopathological changes under optical microscope.

2.5 Measurement of indicators of oxidative stress

The superoxide dismutase (SOD) activity and malondialdehyde (MDA) content in liver tissues were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions.

2.6 Quantitative RT-PCR analysis

Expression levels of SIRT1 and UCP2 mRNA were assessed using Quantitative RT-PCR. Extraction of total RNA from liver tissues was performed using Trizol reagent (Takara, Japan), and cDNA was synthesized with RT reagent kit (Takara, Japan) according to manufacturer's instructions. Real-time PCR was then performed using the SYBR Green method (Takara, Japan) following the manufacturer's instruction. The primers were all synthesized by Shanghai Generay Biotech Co., Ltd., China. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences were as follows: (1) SIRT1: 5' AACCACC AAAGCGGAAAAAAAGAA 3' (forward) and 5' CCACAG CAAGGCGAGCATAAATA 3' (reverse), 168 bp; (2) UCP2: 5' CAAGACCATTGCACGAGAGGA3'(forward) and 5' GA GGTTGGCTTTCAGGAGAGTAT 3' (reverse), 139 bp; (3) GAPDH: 5' CAACGGGAAACCCATCACCA 3' (forward) and 5' ACGCCAGTAGACTCCACGACAT 3' (reverse), 133 bp.

2.7 Western blotting analysis

Western blotting was used to determine the protein expression levels of SIRT1, UCP2, and GAPDH. GAPDH was used as an internal control. The total protein was extracted from the homogenates with a nuclear extract kit (Thermo Scientific, USA) according to manufacturer's instructions. The protein concentration was measured using a BCA protein assay kit (Key GENBio Tech, China). Equal amounts of protein were loaded onto 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. After being blocked with TBS-T buffer (50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, and 0.1% Tween 20) containing 5% skim milk for 1 h at room temperature, membranes were incubated with a 1:1000 dilution of SIRT1 antibody (Cell Signaling Technology, USA), a 1:500 dilution of UCP2 antibody (Abcam, UK), or GAPDH antibody (KangCheng Bio-tech, China). After being washed, membranes were subsequently incubated with horseradish peroxidase (HRP) conjugated secondary antibody (Southern Biotech, USA). Protein bands were visualized with enhanced chemi-luminescence (Millipore, USA).

2.8 Data analysis

Data were expressed as $\bar{x} \pm s$ and analyzed with SPSS 18.0 for Windows (SPSS Software, USA). Differences among groups were analyzed using one-way ANOVA, followed by Bonferroni for homogeneity of variance or Tamhane's T2 test for heterogeneity of variance. Differences were considered significant when $P < 0.05$.

3. Results

3.1 Histopathology analysis

Liver specimens with HE staining and Oil Red O staining were shown in Figure 1. Light microscope observation showed the typical fatty degeneration in livers of NAFLD rats, was evidenced by excessive lipid droplets inside cytoplasm and hepatocyte ballooning. The histopathological changes showed that NAFLD rat models had been duplicated successfully by feeding with high-fat diet for 16 weeks. In HFD contrast, the severity of hepatic steatosis was slightly ameliorated by berberine supplement.

3.2 Effect of berberine on lipid metabolic parameters

To analyze the possible role of berberine in lipid metabolism, the levels of TC, TG, HDL-C, and LDL-C were measured. As shown in Figure 2, the serum TC, TG, and LDL-C levels were significantly increased ($P < 0.01$), whereas serum HDL-C level was decreased in HFD group in comparison with NC group ($P < 0.01$). These results showed that there was lipid metabolism disorder in NAFLD rats in response to high-fat diet. When supplemented with berberine, the increased serum levels of TC, TG, and LDL-C were significantly suppressed ($P < 0.01$, 0.05), while the decreased serum HDL-C levels were significantly elevated in HFB group compared with HFD group ($P < 0.05$). Moreover, the TC and TG levels in the liver tissues were significantly higher in HFD group compared with NC group ($P < 0.01$). Berberine supplement significantly suppressed the increased levels of TC and TG ($P < 0.05$, 0.01). These results showed that berberine supplement reduced the accumulation of serum and liver lipids in rats fed an HFD.

3.3 Effect of berberine on serum aminotransferase

As important biomarkers of liver damage, the levels of serum aminotransferase were measured. As shown in Figure 3, serum AST levels in HFD group were increased significantly

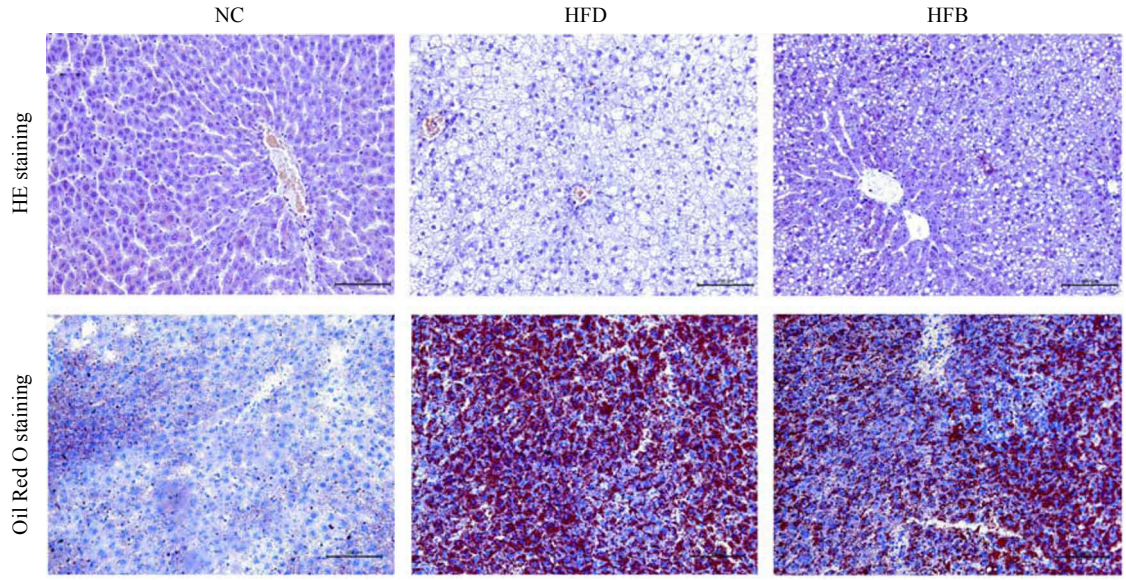

Figure 1 Histological changes of liver sections in different groups

NC group: rats fed with normal diet HFD group: rats fed with HFD HFB group: rats fed with HFD plus berberine

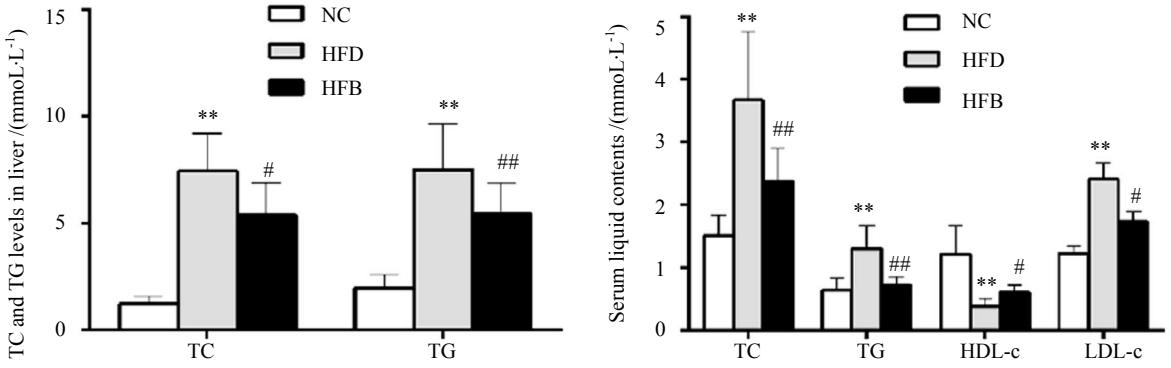

Figure 2 Comparison on lipid contents in different groups ($\bar{x} \pm s$, $n = 10$)

$^{*}P < 0.05$ $^{**}P < 0.01$ *vs* NC group; $^{\#}P < 0.05$ $^{\#\#}P < 0.01$ *vs* HFD group; same as below

($P < 0.01$), whereas ALT levels in HFD group were increased without significance compared with NC group. In HFB group, serum AST levels were significantly decreased compared with HFD group ($P < 0.05$).

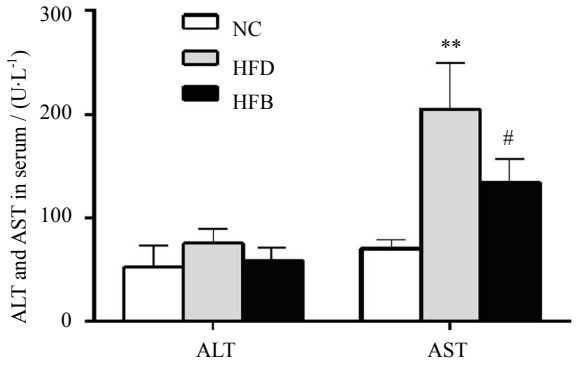

Figure 3 Changes of ALT and AST in serum ($\bar{x} \pm s$, $n = 10$)

3.4 Effect of berberine on SOD and MDA levels

To investigate the effect of berberine on oxidative stress in NAFLD rats, SOD activity and MDA content were measured in the present study. As shown in Figure 4, after 16-week induction of NAFLD, rats had the lower SOD activities and higher MDA levels in HFD group compared with NC group ($P < 0.05$, 0.01). Berberine supplement markedly reduced the MDA levels in rats fed with HFD ($P < 0.05$). In contrast, SOD activities in HFB group were higher than those in HFD group ($P < 0.01$).

3.5 Effect of berberine on expression levels of SIRT1 and UCP2

Because SIRT1 activity has been shown to repress UCP2 expression, we measured the mRNA and protein expression levels of SIRT1 and UCP2 to evaluate the role of SIRT1-UCP2 pathway in the development of NAFLD. As shown in

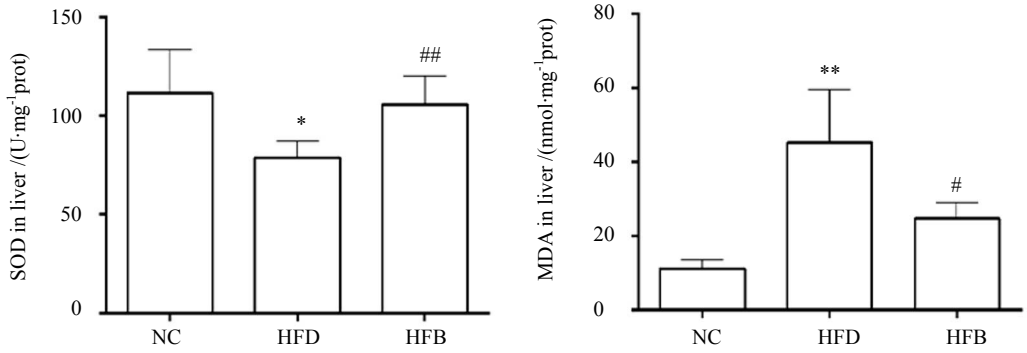

Figure 4 Changes of SOD and MDA levels in liver tissues ($\bar{x} \pm s$, $n = 10$)

Figure 5, the expression of SIRT1 mRNA was significantly decreased in HFD group when compared with NC group ($P < 0.05$). Moreover, in HFB group, berberine supplement increased the expression of SIRT1 mRNA compared with HFD group ($P < 0.05$). In addition, the expression of UCP2 mRNA was significantly increased in HFD group as compared to NC group. In HFB group, berberine supplement evidently reduced the expression of UCP2 mRNA in liver tissues of rats in comparison with HFD group ($P < 0.05$). As shown in Figure 6, Western blotting analysis showed the similar protein expression levels of SIRT1 and UCP2, but the expression of UCP2 protein between HFD and HFB groups had no significant difference ($P > 0.05$). Altogether, these results showed that berberine might regulate to SIRT1-UCP2 in NAFLD rats and the up-regulation of SIRT1 may reduce UCP2 expression by repressing UCP2 mRNA levels.

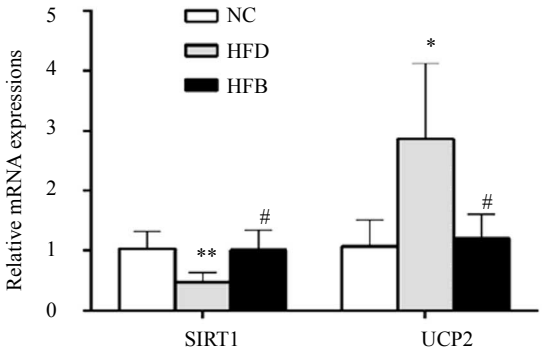

Figure 5 Hepatic mRNA levels of SIRT1 and UCP2 ($\bar{x} \pm s$)

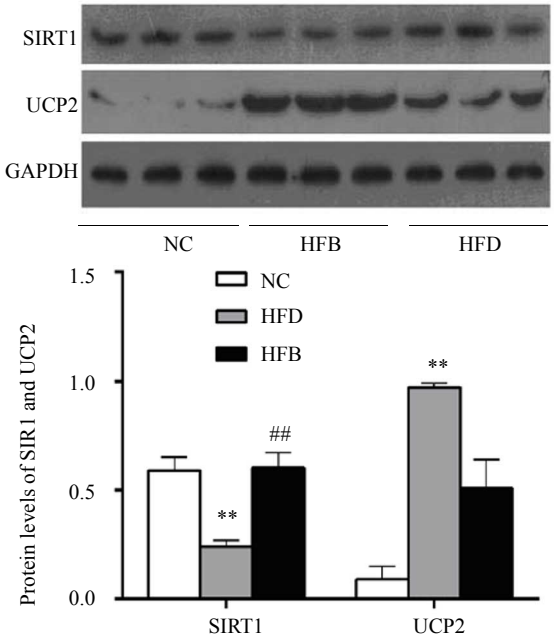

Figure 6 Western blotting analysis of SIR1 and UCP2 proteins ($\bar{x} \pm s$)

4. Discussion

By feeding an HFD for 16 weeks, we established an NAFLD rat model mimicking human NAFLD features. In the HFD group, the serum levels of TC, TG, and LDL-C, as well as the TC and TG levels in liver were markedly increased in rats fed with HFD. Moreover, liver sections from HFD group exhibited the typical lipid accumulation in hepatocytes. Altogether, the results indicated that rats fed with HFD for 16 weeks developed hepatic steatosis, suggesting the successful replication of the NAFLD model.

Although the pathogenesis of NAFLD is far from fully elucidated, the "two-hit" theory (Day and James, 1998) has become a generally accepted framework, guiding the direction of research in this field. According to this theory, the "first hit" includes the fat accumulation in hepatocytes due to insulin resistance, while the "second hit" involves the oxidative stress in the pathogenesis of NAFLD (Malaguarnera et al, 2009). Therefore, various pharmacologic approaches for prevention and treatment of NAFLD focus on the reduction of fat accumulation and reversal of insulin resistance. Berberine is a natural compound derived from the dry roots of *Coptis chinensis* Franch., which has been found to have the beneficial effects in treating metabolic disorders, including obesity and diabetes mellitus (Tang et al, 2006; Xie et al, 2011). A growing body of evidence has demonstrated the therapeutic potential of berberine in protecting against NAFLD (Xing et al, 2011; Zhang et al, 2012; Yuan et al, 2015). In the present study, our results suggest that berberine administration (100 mg/kg) rescues some key features of NAFLD, including hepatic steatosis and hyperlipoidemia. In the HFD group, rats had increased serum and hepatic lipid

content, and liver histology showed marked hepatic steatosis. Meanwhile, we observed an increase in MDA content along with a decrease in SOD activity in the HFD group, suggesting the development of oxidative stress in rats fed an HFD. The abnormality of serum aminotransferase also indicated the impairment of liver function in the HFD group. Berberine supplement markedly ameliorated the deleterious effects. These data are in agreement with several other studies demonstrating that berberine has the potential to reduce the fat accumulation and ameliorate oxidative stress in NAFLD (Yuan et al, 2015; Chang et al, 2010; Kong et al, 2004).

Recently, several studies have suggested that SIRT1 activation represses UCP2 expression, which is associated with ATP producing capacity and insulin sensitivity (Bordone et al, 2006; Xu et al, 2013; Della-Morte et al, 2009). Moderate SIRT activation has shown to offer the significant protection against the oxidative stress in many organs (Hasegawa et al, 2008; Chong et al, 2012; Alcendor et al, 2007). In contrast, lack of SIRT activity has been reported to increase the risk of fatty liver in response to HFD (Xu et al, 2010). Furthermore, SIRT1 has been recognized as a potential target for treatment of NAFLD due to its important roles in multiple functions, including the control of oxidative stress and the regulation of lipid homeostasis (Colak et al, 2011). It has been reported that calorie restriction can improve NAFLD in rats by increasing hepatic SIRT1 expression (Deng et al, 2007). Therefore, we hypothesize that berberine may also lead to the hepatic activation of SIRT1 and thereby have protection against NAFLD.

In the present study, we have found a decrease in SIRT1 levels as well as an increase in UCP2 levels in the HFD group. These findings are partially in agreement with a recent research in which SIRT1 expression is significantly lowered and UCP2 increased in the liver of rats with type 2 diabetes mellitus and NAFLD (Xu et al, 2012). The low hepatic expression of SIRT1, which is implicated in the pathogenesis of NAFLD, may hasten the formation of fatty liver. Moreover, the increased hepatic expression of UCP2 represents a physiological adaptation to increase the reactive oxygen species (ROS) production caused by HFD (Yang et al, 2000). The over expression of UCP2 induced by HFD can reduce not only ROS production, but also ATP production (Fisler and Warden, 2006). The diminished ATP production has been demonstrated to increase the vulnerability to liver injury (Chavin et al, 1999), which may facilitate the development of NAFLD. In this study, the increased amounts of UCP2 are likely not sufficient to control ROS production, evidenced by the changes of SOD activity and MDA content in the HFD group. On the other hand, our data showed that berberine supplement induced an increase in SIRT1 expression, along with a decrease in UCP2 expression in rats fed an HFD. As expected, SIRT1 activation by berberine promotes biochemical and histological improvements in the development of hepatic steatosis. The relief of oxidative stress by berberine supplement was confirmed by changes of MDA and SOD levels in the HFB group, which is consistent with previous studies (Sarna et al, 2010; Zhou and Zhou, 2011). Taken together, our results suggested that the changes of SIRT1-UCP2 pathway existed in the development of NAFLD, and SIRT1-UCP2 pathway might be one of the action targets of berberine in liver.

In conclusion, the results of our study suggest that berberine supplement may regulate the hepatic SIRT1-UCP2 pathway and reduce the deleterious effects of high-fat feeding in NAFLD rats. SIRT1 activation may be an important mechanism by which berberine prevents NAFLD induced by HFD. But the precise mechanism still needs more studies to clarify.

Confict of interest statement

All authors have no conflict of interest to declare.

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