Mori Cortex extract ameliorates nonalcoholic fatty liver disease (NAFLD) and insulin resistance in high-fat-diet/streptozotocin-induced type 2 diabetes in rats

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[ABSTRACT] Nonalcoholic fatty liver disease (NAFLD) and type 2 Diabetes Mellitus (T2DM) are highly prevalent diseases and are closely associated, with NAFLD being present in the majority of T2DM patients. In Asian traditional medicine, Mori Cortex is widely used for the treatment of diabetes and hyperlipidemia. However, whether it has a therapeutic effect on T2DM associated with NAFLD is still unknown. The present study showed that the oral treatment with Mori Cortex extract (MCE; 10 g·kg⁻¹·d⁻¹) lowered the blood lipid levels and reversed insulin resistance (IR) in high-fat-diet/streptozotocin-induced type 2 diabetes in rats. The expression levels of sterol receptor element-binding protein-1c (SREBP-1c) and carbohydrate-responsive element binding protein (ChREBP), which are involved in steatosis in NAFLD rats, were measured in the liver samples. MCE decreased the protein and mRNA expression levels of SREBP-1c and ChREBP. In conclusion, down-regulation of SREBP-1c and ChREBP might contribute to the protective effect of MCE on hepatic injury and IR in the rats with T2DM associated with NAFLD.

[KEY WORDS] Mori Cortex extract; Type 2 diabetes; Non-alcoholic fatty liver; Sterol receptor element-binding protein-1c; Carbohydrate-responsive element binding protein

[CLC Number] R965

Introduction

Non-alcoholic fatty liver (NAFLD) is the presence of hepatosteatosis without significant alcohol consumption. It is related to insulin resistance (IR) and metabolic syndromes such as type 2 diabetes (T2DM) and dyslipidemia [1]. The prevalence of NAFLD in the general population is about 20%–30%, but is estimated to be as high as 75% in patients with T2DM [2]. Furthermore, recent studies suggest that NAFLD may promote the development of T2DM and its macrovascular complications, such as diabetic nephropathy (DN) [3]. T2DM associated with NAFLD not only aggravates hyperglycemia, but also increases the risk of NAFLD converting to non-alcoholic steatohepatitis (NASH), which may progress to hepatic fibrosis and cirrhosis [4-5]. Thus, NAFLD represents a serious burden of disease for patients with T2DM. It has been reported that NAFLD is a consequence, but not a cause, of insulin resistance. According to the two-hit hypothesis, an excessive fat accumulation in the liver occurs first, and then oxidative stress and lipid peroxidation lead to hepatic inflammation, fibrosis, and a progressive form NASH to cirrhosis [6]. Under insulin-resistant conditions, fatty acid supply is increased, as the adipose tissue becomes resistant to the anti-lipolytic effect of insulin [7]. Insulin resistance is also the main reason for hyperglycemia in obesity and T2DM [8]. Thus, interventions to ameliorate NAFLD are appropriate for the prevention and treatment of insulin resistance and excessiveness of fatty acid, which is equal to inhibiting the onset of the “first hit” of NAFLD.
To control the progress of NAFLD, it is necessary to first understand the precise mechanism of lipid metabolism regulation in the liver. Over recent years, studies have reported that the gene expression and protein content of sterol receptor element-binding protein-1c (SREBP-1c) and carbohydrate-responsive element binding protein (ChREBP) are significantly increased in the liver of obese-hyperglycemic (ob/ob) mice and they play a pivotal role in accelerating hepatic steatosis and insulin resistance [9]. ChREBP is essential for activating both SREBP-1c and glucose metabolism; it also acts in synergy with lipogenic genes, such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), in response to insulin and glucose [10]. Research has found that these genes are up-regulated in NAFLD patients [11]. SREBP-1c is activated in patients with NAFLD and has been speculated to be a dominant regulator of the onset of NAFLD [12]. It could be a potential therapeutic target for these pathological states.

The root bark of mulberry tree (Mori Cortex, MC), called “Sang-Bai-Pi” in China, is one of the traditional Chinese medicines that have been used for at least 4000 years. It is a rich source of biologically active components, and has been used as anti-diabetes, anti-phlogistic, liver protective, kidney protective, and hypotensive drugs [13-14]. Recent studies have indicated that Mori Cortex extract (MCE dose-dependently reduces serum total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), inhibits the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and increases high-density lipoprotein cholesterol (HDL-C) [15]. Moreover, MC can modulate hepatic glucose metabolism and gluconeogenesis [16], and has insulin-sensitizing activity [17]. We hypothesized that MC is a potential natural material to treat T2DM associated with NAFLD. The specific mechanism by which MC lowers blood glucose and lipids remains unknown. In the present study, we investigated if MC could prevent NAFLD and inhibit insulin resistance via the down-regulation of SREBP-1c and ChREBP.

Materials and Methods

Preparation of Mori Cortex extract (MCE)

The raw medicinal herb MC was obtained from Shanghai Yan-He-Tang Traditional Chinese Medicine Company in Shanghai, China. The raw herb was botanically authenticated by Prof. LI Yi-Ming in the School of Pharmacy, Shanghai University of Traditional Chinese (Shanghai, China). The voucher specimen (No. MC001) was deposited at the Herbarium of the Department of TCM Chemistry, School of Pharmacy, Shanghai University of Traditional Chinese Medicine.

MC was extracted using the following process: one kg of MC was reflux extracted with five kg of 70% alcohol twice, 90 min each time. The extract solution was let stand overnight and then filtered. The filtrate was concentrated to a small volume under reduced-pressure evaporation and then dried by lyophilization. The extract yield after freeze drying was 11.6% and was named MCE.

Experimental model and treatments

Thirty male adult Sprague-Dawley (SD) rats aged 8 weeks (275 ± 25 g) were purchased from Zhejiang Laboratory Animal Center (Zhejiang, China, Certificate No. 0012371) and housed in a room of 23 ± 2 °C with a 12h/12 h light/dark cycle and were acclimatized for one week with normal diet. The experimental and animal care procedures were approved by the Animal Ethics and Welfare Committee (AEWC) of Ningbo University (AEWC-2015-32) on January 20, 2015. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH). The rats were then randomly divided into the following five groups, control: normal control rats (n = 6); MC: normal control rats treated with MC (n = 6); DM: type 2 diabetic control rats (n = 6); MC + DM = type 2 diabetic rats receiving preventive and therapeutic treatment of MC (n = 6); and DM + MC: type 2 diabetic rats receiving preventive and therapeutic treatment of MC (n = 6). The rats of the control and MC groups were fed with normal diet (10% of calories from fat), while those in the other groups were fed with a high-fat diet (60% of calories from fat); all foods were provided by Shanghai Lab-oratory Animal Co. Ltd. In order to investigate whether MCE has prevention on NAFLD, the rats of the MC + DM + MC group were orally administered with MCE at a dose of 10 g·kg⁻¹·d⁻¹ at 9 a.m. daily for 4 months as the preventive and therapeutic treatment after 8 weeks. After 12 weeks of high fat diet (HFD) feeding, the rats of the DM, MC + DM + MC, and DM + MC groups were intraperitoneally injected with a freshly prepared solution of streptozotocin (STZ; 40 mg·kg⁻¹, Sigma, Aldrich Chemicals Pvt. Ltd. Saint Louis, MO, USA) in 0.1 mol·L⁻¹ citrate buffer (pH 4.5) to induce type 2 diabetes, while those of the control and MC groups were injected with equal volume of the citrate buffer.

Biochemical sampling and analysis

At the end of the experiment, all the rats were fasted overnight. The fasting blood glucose (FBG) was measured from the tail vein with One Touch Ultra test strips and a glucometer (One Touch; Johnson and Johnson, New Brunswick, NJ, USA). The blood samples were collected from the retro-orbital sinus before cervical dislocation. The serum insulin level was measured with a Rat Insulin ELISA kit (Mipilore, Darmstadt, Germany) using a Scientific Multiskan FC Microplate Photometer (Thermo Scientific, Norwood, MA, USA). The hepatic function parameters, ALT and AST, were analyzed with a MODULAR P800 Automation Biochemist Analyzer (Roche, Basel, Switzerland). TG, TC, and free fatty acids were measured with a MODULAR P800 Automation Biochemist Analyzer (Roche, Basel, Switzerland).
acids (FFA) were measured with the corresponding kits (Nanjing Jian-cheng Bioengineering Institute). Homeostasis model assessment (HOMA) of IR (HOMA-IR) was calculated by the HOMA method using the following equation: IR (HOMA-IR) = fasting glucose (mmol·L⁻¹) × fasting insulin (μIU·mL⁻¹)/22.5.

Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed at the end of the experiment. The overnight-fasted rats were given glucose (2 g·kg⁻¹) through oral gavage and subsequently blood was drawn from a tail vein to measure blood glucose levels at 0, 30, 60, and 120 min after glucose administration, using a glucometer.

Liver histological evaluation

Liver tissues were fixed overnight in 10% buffered formalin and embedded in paraffin. Serial sections (5 μm) were cut using a microtome (Leica RM 2125; Leica, Heidelberg Germany). The degree of steatosis was assessed by hematoxylin-eosin (HE), Sudan III, and Oil Red O staining.

Western blotting analysis

Total protein was extracted from the liver and lysed in RIPA lysis buffer supplemented with a protease inhibitor cocktail, 2 mmol·L⁻¹ PMSF, and 1 mmol·L⁻¹ sodium orthovanadate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Total protein levels were determined by the bicinchoninic acid (BCA) method (Cwbio, Beijing, China). Equal amounts of protein samples were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to PVDF membranes. The membranes were blocked with 10% skim milk in TBS-T (TBS with 0.03% Tween 20) and then incubated at 4°C overnight, with one of the following primary antibodies: β-actin (diluted to 1 : 10000 with TBS-T; Abcam, Cambridge, UK), goat anti-rat ChREBP polyclonal antibody, goat anti-rat SREBP-1c polyclonal antibody, goat anti-rat ACC polyclonal antibody, and goat anti-rat FAS polyclonal antibody (all diluted to 1 : 10000 with TBS-T; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After three washes with TBS-T, the membranes were incubated with 1 : 10000 HRP-conjugated anti-rabbit antibody (MultiSciences Biotech Co., Hangzhou, China) at room temperature for 1 h. The membranes were developed with the ECL system (Tanon, Shanghai, China). The density values of protein bands were quantified using the ImageJ program (NIH, MD, USA).

RT-qPCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, San Diego, CA, USA) from liver according to manufacturer's instructions. The RNA concentrations were determined by measuring the absorbance at 260 nm and 280 nm, and 500 ng RNA was reverse transcribed to cDNA by using HiFiScript 1st Strand cDNA Synthesis Kit (Cwbio, Beijing, China). Real-time PCR was performed on Light-Cycler 480 (Roche, Basel, CH) with the Light Cycler 480 SYBR Green I Master (Roche, Basel, CH). The primer sequences were listed in Table 1.

### Table 1  Primers used in the present study

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<td>5'-GAAGACACCAAGACCAAGATGC-3'</td>
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</tr>
<tr>
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<td>5'-CGCAGAAGACGACCCATTACT-3'</td>
</tr>
<tr>
<td>FAS S</td>
<td>5'-CAGGAAACAAACTCATCGTTCTCT-3'</td>
</tr>
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<td>5'-GGACCGTAGATATGCGGTTCGA-3'</td>
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<tr>
<td>β-actin S</td>
<td>5'-CTGACCCCTAAGGGAACCA-3'</td>
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<td>β-actin AS</td>
<td>5'-GCCAGAGGCCATACAGGGACAA-3'</td>
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Thermal cycling conditions were 95 °C, 5 min, for the initial denaturation step, followed by 45 cycles of 94 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s. The 2⁻∆∆CT method was used to determine relative amounts of product, and data are presented as fold change with β-actin as an endogenous control.

Statistical Analysis

The results were expressed as means ± SEM. The differences between two groups were analyzed with Student's t-test, and one-way ANOVA was used to compare mean differences among three or more groups. A two-tailed P value of < 0.05 was considered statistically significant. SPSS 13.0 for Windows (SPSS Software, Chicago, IL, USA) was used for statistical analysis.

Results

**Effects of Mori Cortex extract on FBG and insulin sensitivity in HFD/STZ-induced type 2 diabetic rats**

At 12 weeks after STZ injection, FBG in the DM group was significantly higher than that in the control group (P < 0.05). MCE decreased FBG (P < 0.05) and increased the insulin level in the DM rats (Figs. 1A and B). As a result, the HOMA-IR value was significantly reduced in the MC + DM + MC and MC + DM groups (Fig. 1C), indicating increased insulin sensitivity in the MCE-treated rats. OGTT analysis revealed that the glucose clearance rate was considerably lower in rats of the DM group than that in the control group (Fig. 1D). The therapeutic effect of MCE was stronger with both preventive and therapeutic treatments than that with the therapeutic treatment alone.

**Effects of Mori Cortex extract on hepatic histology and lipid metabolism in HFD/STZ-induced T2DM rats**

Compared with the control rats, the DM rats had greater hepatic lipid deposit and higher TG, TC, and FFA concentrations (Fig. 2). Fibrosis was not observed in any group. HE, Oil Red O, and Sudan III staining showed that MCE alleviated the liver injury, as there were fewer lipid droplets in the hepatic lipid deposit and higher TG, TC, and FFA concentrations (Fig. 2).

Liver histological evaluation

Liver tissues were fixed overnight in 10% buffered formalin and embedded in paraffin. Serial sections (5 μm) were cut using a microtome (Leica RM 2125; Leica, Heidelberg Germany). The degree of steatosis was assessed by hematoxylin-eosin (HE), Sudan III, and Oil Red O staining.

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parenchymal cells (Fig. 2). As shown in Figs. 2B–D, preventive and therapeutic treatment or therapeutic treatment of MCE in diabetic rats also resulted in reductions in serum TC (by 37.9% and 34.5%, respectively), serum TG (by 45.2% and 41.9%, respectively), and serum FFA (by 37.3% and 25.4%, respectively), compared with the DM group. In addition, the preventive and therapeutic treatment of MCE was more effective than therapeutic treatment alone (Fig. 2).

Effects of MCE on hepatic function in HFD/STZ-induced T2DM rats

The levels of AST and ALT were significantly higher in the DM rats than that in the control rats. MCE significantly decreased AST and ALT levels (Fig. 3). As shown in Fig. 3, the serum AST levels in MC + DM + MC and MC + DM groups were reduced by 42.7% and 27.5%, respectively; and serum ALT level in MC + DM + MC group and MC + DM group were reduced by 24% and 21.1%, respectively, compared with that of the DM group. The MC + DM + MC group showed a greater decrease in AST and ALT levels than the MC + DM group. These results suggested that hepatosteatosis induced liver injury and MCE restored hepatic function.

Effects of MCE on the regulation of the expression of genes involved in lipid metabolism in the liver of T2DM rats

To test whether MCE exerts the observed therapeutic effect through SREBP-1c and lipid metabolism enzymes, we measured the expression of SREBP-1c, and ChREBP. MCE down-regulated SREBP-1c and ChREBP (Figs. 4A–B and D), which are known to be involved in TG and cholesterol metabolism and required for activating and transcribing FAS. As shown in Figs. 4C–D, decreases in mRNA and protein levels of FAS were observed in rats receiving MCE, and the preventive and therapeutic treatment showed greater effect than the therapeutic treatment alone. These results suggested that MCE regulates fatty acid synthesis to improve lipid metabolism. Moreover, an increase in total AMP-activated protein kinase (AMPK) and a decrease in total ACC were observed with the administration of MCE in the diabetic rats, in comparison with the DM group (data not shown). These findings suggest that MCE ameliorates fatty liver via reducing lipid biosynthesis and regulates hepatic lipid metabolism by down-regulating SREBP-1c and ChREBP.
Discussion

Insulin resistance and defects in lipid metabolism are the major reasons for the link between NAFLD and T2DM [18]. Studies have found that NAFLD is present in the majority of T2DM patients, and the presence of NAFLD in T2DM patients accelerates the progression of macro- and microvascular complications, such as diabetic nephropathy [19]. However, there is still low awareness of this association and few treatment options exist [20]. Moreover, the use of insulin and sulfonylureas in T2DM is considered responsible for the presence of NASH and fibrosis [21]. Prompt diagnosis and appropriate glucose-lowering therapy may help prevent both hepatic and systemic diabetic complications.

In the present study, we generated a rat model of T2DM-associated NAFLD by HFD and a STZ injection at a dose of 40 mg·kg⁻¹ body weight [22]. Our data revealed that glucose tolerance and lipid metabolism in untreated diabetic rats were significantly impaired, as assessed by FBG values, OGTT, and serum lipid levels. Liver is the major organ that metabolizes glucose in response to insulin [23]. In the present study, MCE significantly enhanced glucose metabolism, as the FBG was restored to normal level and OGTT showed improved glucose tolerance with MCE treatment in the diabetic rats. Moreover, in the MCE-treated diabetic rats, blood insulin was increased and HOMA-IR was significantly decreased, indicating improved insulin responsiveness. In addition, the efficacy of the preventive and therapeutic treatment was better than the therapeutic treatment, indicating that supplementation of MCE at the prediabetic stage might reduce the burden of metabolic syndrome in patients.

The hepatic protection effect of MC has been investigated for and attributed to lipid modification and antioxidant mechanisms [24]. In the present study, MCE decreased serum TG, TC, and FFA contents and lowered lipid droplet accumulation in the liver. It is known that fatty acid supply increases in various insulin-resistant states [7], and FFA induces hepatic insulin resistance [25]. MCE significantly reduced FFA level significantly in diabetic rats, and the effect of the preventive and therapeutic treatment was nearly 1.5 fold greater than that of the therapeutic treatment. Furthermore, it has been reported that ALT can be used as a marker to predict the development of T2DM in NAFLD patients [26]. In our study, liver enzyme levels in DM rats treated...
with MCE were significantly decreased, revealing that MCE could reverse fatty liver damage and slow the process of NAFLD.

Fig. 3  MCE restores hepatic function in HFD/STZ-induced T2DM rats. (A) AST values; (B) ALT values. control: normal control rats \( (n = 6) \); MC: normal control rats treated with Mori Cortex \( (n = 6) \); DM: type 2 diabetic control rats \( (n = 6) \); MC + DM + MC: type 2 diabetic rats receiving preventive and therapeutic treatment of Mori Cortex \( (n = 6) \); and MC + DM: type 2 diabetic rats receiving therapeutic treatment of Mori Cortex \( (n = 6) \). AST: aspartate aminotransferase; ALT: alanine aminotransferase. Data is presented as mean ± SEM \( (n = 6 \) male Sprague-Dawley rats in each group). * \( P < 0.05 \) vs NC; # \( P < 0.05 \) vs DM; + \( P < 0.05 \) vs DM + MC

Fig. 4  MCE regulates lipid metabolism by down-regulating SREBP-1c and ChREBP. (A–C): relative levels of SREBP-1c (A); and ChREBP (B); FAS (C); (D) representative western blots for each protein of interest. control: normal control rats \( (n = 6) \); MC: normal control rats treated with Mori Cortex \( (n = 6) \); DM: type 2 diabetic control rats \( (n = 6) \); MC + DM + MC: type 2 diabetic rats receiving preventive and therapeutic treatment of Mori Cortex \( (n = 6) \); and MC + DM: type 2 diabetic rats receiving therapeutic treatment of Mori Cortex \( (n = 6) \). Data are presented as means ± SEM \( (n = 6 \) male rats in each group). * \( P < 0.05 \) vs NC; # \( P < 0.05 \) vs DM; + \( P < 0.05 \) vs DM + MC

The possible mechanisms of MC action on lipid metabolism are largely unknown. In the present study, we used high fat-diet/streptozotocin-induced type 2 diabetic rats and showed that MCE down-regulated the expression of SREBP-1c and ChREBP. This result suggested that SREBP-1c and ChREBP are the critical components involved in the MCE-induced...
inhibition of lipid synthesis and improvement of insulin responsiveness. Down-regulation of SREBP-1c and ChREBP is mediated by the activation of AMPK during glucagon secretion and fatty acids mobilization in hepatocytes in a previous report [1]. However, the levels of phosphorylated AMPK (pho-AMPK) and phosphorylated ACC (pho-ACC) showed no difference between the MC + DM + MC and DM groups, but increased levels of total AMPK and decreased levels of total ACC were observed with the administration of MCE in diabetic rats (data not shown). The reason might be that phosphorylation of AMPK and ACC occurred at a time point that we did not investigate. Our results therefore suggested the importance of MCE for systemic glucose and lipid homeostasis via the down-regulation of SREBP-1c and ChREBP.

Taken together, the present study demonstrated the effects of MCE on glucose and lipid metabolism in T2DM rats with NAFLD for the first time. The results indicated that the administration of MCE could reduce blood glucose and improve insulin sensitivity. Both prevented the progress of T2DM and NAFLD. Moreover, MCE lowered lipid droplet accumulation in the liver and the fatty acid levels in the circulation, likely via down-regulation of SREBP-1c and ChREBP. However, whether MCE decreases the expression of SREBP-1c and ChREBP by a mechanism dependent on pho-AMPK is still unclear and requires further investigation.

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