

Effects of Flavonoids in *Morus indica* on Blood Lipids and Glucose in Hyperlipidemia-diabetic Rats

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Abstract: **Objective** To investigate the effect and possible mechanism of flavonoids extracted from *Morus indica* (FMI) on blood lipids and glucose. **Methods** The experimental hyperlipidemia-diabetic rats were induced by high-fat diet (HFD) and low dose of Streptozotocin (STZ). Flavonoids-treated rats were pretreated with FMI (50, 100, and 200 mg/kg). The plasma, skeletal muscle, and livers were isolated for biochemical assays, HE staining, immunohistochemistry, and Western blotting analysis. **Results** The results showed that the body weight in flavonoids-treated (100 and 200 mg/kg) rats was reduced ($P < 0.05, 0.01$) compared to HFD-fed rats. FMI obviously reduced total cholesterol ($P < 0.01$), triglycerides ($P < 0.05$), and low-density lipoprotein cholesterol ($P < 0.05$), increased high-density lipoprotein ($P < 0.05$), and significantly decreased the atherosclerosis index ($P < 0.01$); FMI (100 and 200 mg/kg) also down-regulated the elevation of blood glucose induced by STZ ($P < 0.05, 0.01$); At the meantime, FMI increased hepatic superoxide dismutase (SOD) activity and reduced hepatic malondialdehyde (MDA) content obviously. In addition, the results showed that the expression of hepatic CYP2E1 was markedly decreased while the expression of GLUT-4 in skeletal muscles was increased by FMI. **Conclusion** The above results demonstrate that the effect of FMI is related to its up-regulation of hepatic SOD activity, reduction of hepatic MDA content, down-regulation of hepatic CYP2E1 expression, and increase of GLUT-4 expression in skeletal muscle, which suggests that FMI may prevent or improve hyperlipidemia and hyperglycemia caused by an excessive HFD.

Key words: blood glucose; blood lipid; diabetes; flavonoid; hyperlipidemia; *Morus indica*

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Introduction

Diabetes mellitus (DM) is a group of disorders with different etiologies, which is characterized by derangements in carbohydrate, protein, and fat metabolism. It is caused by complete or relative insufficiency of insulin secretion and/or insulin action (Qiao, Nakagami, and Tuomilehto, 2000). Hypercholesterolemia is a common phenomenon in diabetes, contributing to the high prevalence of accelerated atherosclerosis and coronary heart disease (Andallu *et al.*, 2001).

Flavonoids exist naturally in fruit, vegetables, nuts, seeds, flowers, and so on. They play an important role in the prevention of cardiovascular disease, chronic diseases, with the function of oxidation resistance, antiviral, and anti-inflammation (Duarte *et al.*, 1993). *Morus indica* L. is a traditional edible and medicinal

plant in China, and is well known for its economic importance in the sericulture industry as its foliage is used to feed silkworms (Andallu, Radhika, and Suryakantham, 2003). Flavonoids are the main active components in *M. indica*, which possess hypoglycemic and hypolipidemic activities. It has been reported that flavonoids extracted from *M. indica* (FMI) has an antidiabetic activity in experimental animals (Hansawasdi and Kawabata, 2006). Therefore, it is of special interest to understand whether FMI has a positive influence on blood lipids in diabetic state.

In this study, a repeatable experimental model of hyperlipidemia-diabetic rat induced by Streptozotocin (STZ) and high-fat diet (HFD) was established to investigate the effects of FMI on blood lipids and glucose as well as its possible mechanism.

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Materials and methods

Preparation of FMI

Mulberry leaves were crushed into powder by versatile plant pulverizer after being dried at 80 °C for 3 h. The powder was degreased by petroleum ether. Then, the powder of the samples was extracted with methanol at 80 °C for 4 h, and the solid-liquid ratio was 1:40 (g/mL). After extraction, the extract was evaporated by using a rotary evaporator. SP825 macroporous resin was used to separate flavonoids. The coloration method was used to determine the content of flavonoids in the sample by UV-Vis detector; AlCl_3 was used as the color developing reagent, 420 nm as determination wavelength, rutin was used as the reference substance for a calibration curve. The flavonoid content was calculated using the following linear equation based on the calibration curve: $A = 37.547 C - 0.0012$, $r = 0.9997$, where A is the absorbance, C is the content in mg/mL. Flavonoids (62.2%) were further diluted with distilled water to obtain different concentration and then kept at room temperature.

Animal models and treatment protocols

Sixty male Wistar rats of body weight (BW) 180–200 g were obtained from Experimental Animal Center of Hubei Province. They were maintained in a temperature-controlled room [(25 ± 1) °C] on a 12 h-12 h light-dark cycle (lighting from 6:00 AM to 18:00 PM). After 7 d of acclimation, the rats were randomly divided into six groups: control, model, low-, mid-, and high-dose (50, 100, and 200 mg/kg) FMI, and Zhibituo (positive control, 1.5 g/kg, once daily) groups. All rats were weighed weekly, respectively. After administration for four weeks, blood samples were withdrawn from caudal vein. Samples were centrifuged at $2000 \times g$ for 5 min at 4 °C for the separation of plasma. The plasma levels of triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were detected using diagnostic kits (Jiancheng, Nanjing, China), respectively. All samples were analyzed in triplicate.

After administration for four weeks, the rats in model, FMI or Zhibituo groups were treated with STZ (ip injection, 30 mg/kg, Sigma, No. S0130). And the blood glucose of the rats was measured on day 7 after STZ treatment. Blood glucose was detected using diagnostic kits (Jiancheng, Nanjing, China). All

animals were sacrificed on the day 7 after STZ treatment, and then the livers and skeletal muscle were isolated for biochemical assays, HE staining, immunohistochemistry, and Western blotting analysis.

Measurement of SOD and MDA in liver

Livers were homogenized (20%) with phosphate buffer (50 mmol/L K_2HPO_4 containing 0.1 mmol/L EDTA, pH 7.4). The homogenate was centrifuged at $2000 \times g$ for 10 min at 4 °C, and then the supernatant was recentrifuged at $9000 \times g$ for 20 min. Mitochondrial pellet was collected, resuspended in 0.25 mol/L sucrose solution, and stored at -80 °C until detection of superoxide dismutase (SOD) and malondialdehyde (MDA) using the corresponding kits (Jiancheng, Nanjing, China), respectively.

Hepatic pathological evaluation and immunohistochemical examination

Harvested livers were placed into 10% formalin immediately after excision, and immersed for 24 h. Liver specimens were then embedded in paraffin and sections were cut into 4–5 μm pieces. Half of the sections were used for HE staining and were examined histologically to evaluate the liver necrosis. And the other sections were used to perform a standard peroxidase-antiperoxidase technique as described previously (Hsu, Raino, and Fanger, 1981), using CYP2E1 rabbit anti-mouse polyclonal antibody (Abcam Inc, UK) at a 1:150 dilution and a biotinylated goat anti-rabbit or goat anti-mouse antibody (Santa Cruz, CA, USA) as the secondary antibody. Brown colour in cytoplasm of the hepatocytes was evaluated as positive staining.

Western blotting analysis

The liver tissue and skeletal muscle were removed from rats in each group and then washed twice with cold PBS before lysed in Proteo JET™ Mammalian Cell Lysis Reagent (MBI fermentas). The lysate was centrifugated for 45 min at 4 °C, 16 000 r/min. Proteins of liver homogenate or skeletal muscle were separated by SDS-PAGE and proteins were transferred onto a polyvinylidene difluoride membrane by electroblotting. Membrane was blocked overnight and then incubated for 2 h with a 1:1000 dilution of anti-cytochrome P4502E1 antibody (ab8146) (Abcam Inc, UK). After incubation with the secondary antibody, proteins were detected with an electrochemiluminescence detection

kit (Amersham Biosciences, UK), and scanned with a Typhoon 9200 Scanner (Amersham Biosciences Europe GmbH, Germany). The amount of protein expression was corrected by the amount of β -actin in the same sample.

Statistical analysis

All the data were statistically evaluated with SPSS11.0 software. Hypothesis testing methods included One-way analysis of variance (ANOVA) followed by least significant difference (LSD) test; $P < 0.05$ was considered to indicate statistical significance. All the results were expressed as $\bar{x} \pm s$ for 10 animals in each group.

Results and discussion

Effect of total FMI on BW, blood lipids, blood glucose, and liver tissue injury in experimental hyperlipidemia-diabetic rats

The BW, plasma TC, TG, LDL-C, and HDL-C in the experimental groups at the end of four weeks were shown in Fig. 1. The HFD caused a significant increase in BW, TC, TG, and LDL-C and a decrease in HDL-C compared with the control group. The mid- and high-dose (100 and 200 mg/kg) of FMI significantly reduced plasma total TC level compared with the model rats ($P < 0.05, 0.01$). The FMI (50, 100, and 200 mg/kg) decreased plasma TG level in the model rats ($P < 0.05, 0.01$). The high-dose (200 mg/kg) FMI decreased BW ($P < 0.01$), LDL-C levels ($P < 0.01$) and increased HDL-C ($P < 0.01$). Similar results were seen in rats

treated with Zhibituo [1.5 g/(kg·d)].

A pretreatment of HFD and sequential STZ injection resulted in experimental DM, which is probably due to the destruction of β -cells of islets of Langerhans (Maiti *et al*, 2004). In this study, a significant increase on blood glucose was observed in the rats treated with HFD and successive STZ injection (Fig. 1, $P < 0.01$). All experimental doses of FMI decreased the blood glucose in the model rats on day 7.

To analyze the extent of liver injury, liver sections were stained with HE (Fig. 2). It was found that the hepatocytes were clear and integrity in liver sections from control group (Fig. 2a). However, the liver tissue in the model rats showed the significant hepatic lipid accumulation, scattered inflammatory cells, and mild hepatocytes necrosis compared to the control group (Figs. 2a and 2b). The results also indicated that the rats treated with 100 or 200 mg/kg FMI showed considerably lower hepatic lipid accumulation and more liver injury recovery than those of the model rats (Figs. 2b, 2c, and 2d). Furthermore, the effect of FMI on liver tissue injury shows in a dose-dependent manner.

Effect of total FMI on levels of SOD and MDA in liver tissues of experimental hyperlipidemia-diabetic rats

SOD is an anti-oxidative enzyme that converts superoxide anion radicals into hydrogen peroxides (Saitoh *et al*, 2001). Its sole function is to remove the superoxide anion formed via the univalent reduction of

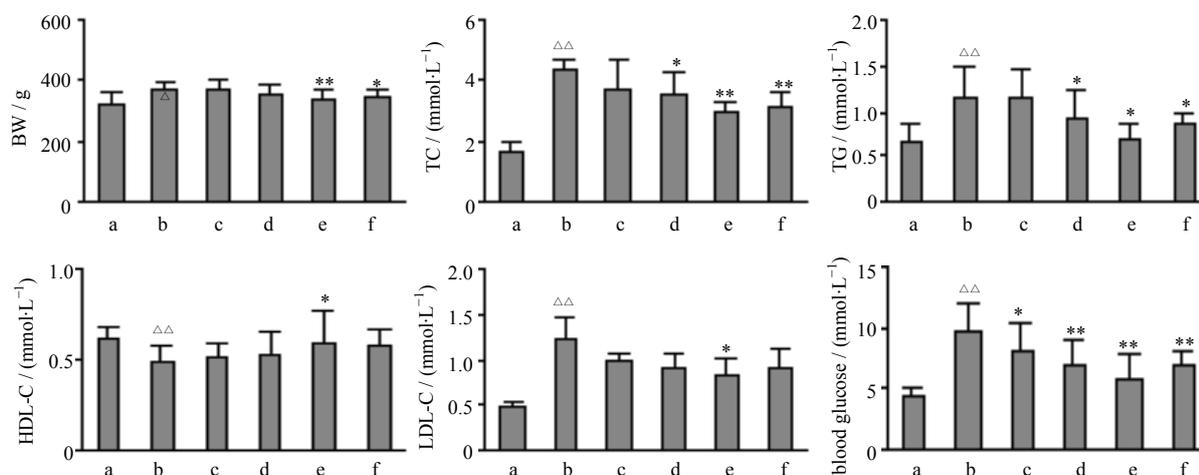


Fig. 1 Effect of FMI on BW, TC, TG, HDL-C, and LDL-C after four weeks and blood glucose on day 7 of STZ-treatment in HFD-diabetic rats ($\bar{x} \pm s, n = 10$)

a: control group b: model group c—e: FMI groups (50, 100, and 200 mg·kg⁻¹) f: Zhibituo group (1.5 g·kg⁻¹), same as below

$\Delta P < 0.05$ $\Delta\Delta P < 0.01$ vs control group; * $P < 0.05$ ** $P < 0.01$ vs model group; same as below

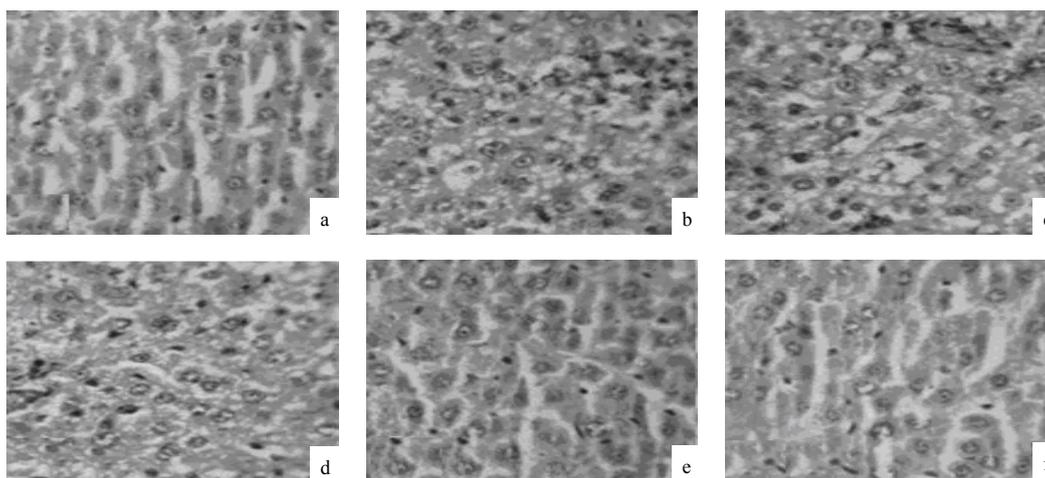


Fig. 2 Histological photographs of livers in rats treated with FMI and Zhibituo

oxygen, and thus to protect the cells against toxicity (Angelove *et al*, 1996). The reduced activities of SOD are associated with the tissue damage in the diabetic rats. Hepatic SOD levels were illustrated in Fig. 3. It showed a significant reduction on hepatic SOD activity in HFD-fed and following STZ-treatment rats compared to control group ($P < 0.01$); At the meantime, after treatment with FMI (200 mg/kg) or Zhibituo [1.5 g/(kg·d)], the hepatic SOD level was increased ($P < 0.05$).

MDA is the principal and most studied product of polyunsaturated fatty acid peroxidation. Several methods quantify the level of oxidative stress *in vivo* and *in vitro* have been developed to assess this molecule in order to (Rio *et al*, 2005). The result also showed that HFD combined with STZ-treatment raised the hepatic MDA level in model group compared to control group ($P < 0.05$), treatment with 200 mg/kg of FMI inhibited the

increase in MDA level compared with HFD - fed combined with STZ-treatment rats ($P < 0.05$) (Fig. 3).

Effect of total FMI on expression of glucose transporter subtype 4 in skeletal muscle and CYP2E1 in hepatic tissues in experimental hyperlipidemia-diabetic rats

Biotransformation of chemical toxicants and carcinogens is mediated by cellular enzymes including the cytochromes P450 (CYP) system (Kang *et al*, 2008). Among these enzymes, CYP2E1 is specifically implicated in the metabolism of several compounds including toxicants and low molecular weight procarcinogens (Koop, 1992). In addition, CYP2E1 plays a major role in the generation of oxidative stress and has been found to be elevated in diabetic liver (Cederbaum, 2006). In this study, in order to demonstrate whether the effect of total FMI was involved in CYP2E1 pathway, hepatic CYP2E1 expression was determined by immunohistochemistry and Western blotting (Fig. 4). There were few positive immunostained cells in the control group, which was also evidenced by Western blotting. However, treatment with HFD and STZ obviously increased CYP2E1 expression compared with the control group; However, FMI obviously down-regulated CYP2E1 expression compared with that of the model group.

Glucose transporter subtype 4 (GLUT-4) is a 12-transmembrane domain protein that mediates the transport of glucose in direction of glucose gradient (Barret, Walmsley, and Gould, 1999). Insulin promotes GLUT-4 incorporation into plasma membrane, and this translocation from intracellular compartments appears

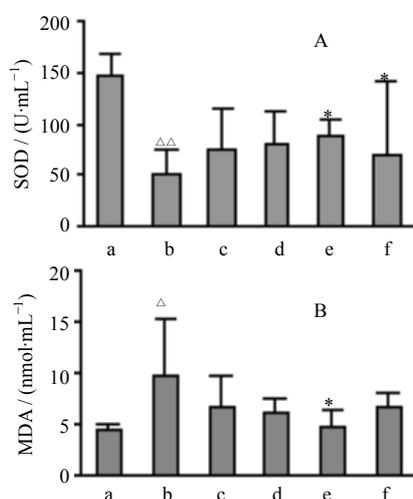


Fig. 3 Effect of FMI on hepatic SOD (A) and MDA (B) in hyperlipidemia-diabetic rats ($\bar{x} \pm s$, $n = 10$)

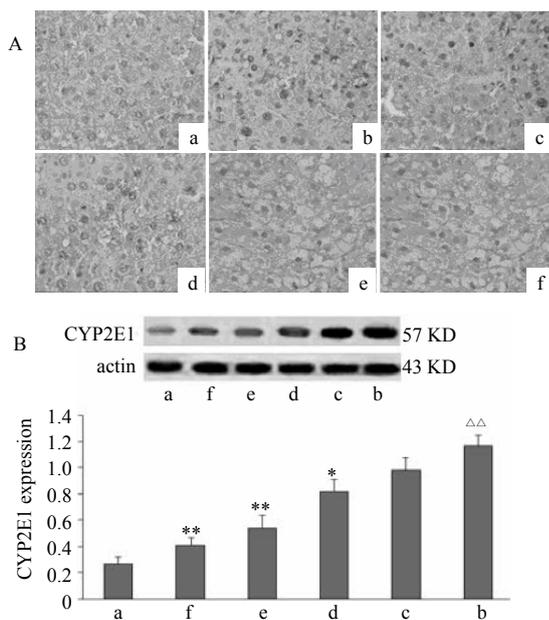


Fig. 4 Immunostaining photomicrographs (A) and Western blotting analysis (B) for hepatic CYP2E1 from rats treated with FMI and Zhibituo

to fail in the insulin resistance present in some form of diabetes (Chiappe De Cingolani and Caldiz, 2004). This study was to detect the expression level of GLUT-4 in skeletal muscles. Western blotting analysis showed band of GLUT-4 protein in skeletal muscles was located at 50 000 (Fig. 5). Treatment with HFD and STZ decreased GLUT-4 expression compared with that of the control group. At the meantime, GLUT-4 expression was increased after treatment with FMI in the doses of 100 and 200 mg/kg.

Conclusion

Above all, the present study showed that FMI could inhibit BW gain and body fat accumulation in diabetes rats caused by HFD and reverse the elevation of blood glucose in HFD-fed rats treated with STZ. The effects of FMI on blood lipids, blood glucose, and liver protection were related to its up-regulation of hepatic SOD activity, reduction of hepatic MDA content, and down-regulation of hepatic CYP2E1 expression and increase of GLUT-4 expression in skeletal muscle. The results also suggested that FMI-treatment may prevent or improve hyperlipidemia and hyperglycemia caused by excessive HFD.

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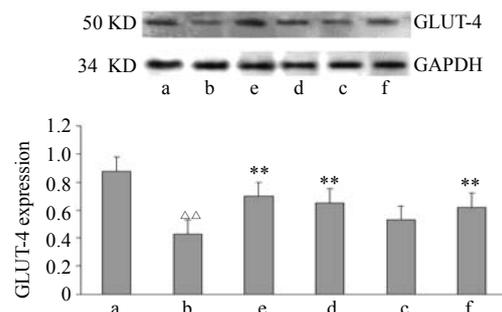


Fig. 5 Western blotting analysis for skeletal muscle GLUT-4 of expression of GLUT-4 from rats treated with FMI and Zhibituo

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