Malus toringoides (Rehd.) Hughes Improves Metabolic Syndrome and Liver Injury in High-Fructose-Induced Mice

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Research

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Abstract

Background: *Malus toringoides* (Rehd.) Hughes, as a traditional medicinal and edible plant used in Tibet, China, is used to treat hypertension, hyperlipemia and liver diseases. This present study was designed to investigate the effects of ethanol extract of *M. toringoides* (EMT) on metabolic syndrome (MS) and liver injury in high-fructose-induced mice.

Methods: The C57BL/6J male mice were divided into five groups (n=8). Con group was drunk with standard water, Fru group and the other three with 30% high-fructose water for 8 weeks. EMT (195 mg/kg, 390 mg/kg, 780 mg/kg) was administered to each of high fructose groups simultaneously. Glucose tolerance tests (GTT) were performed. Blood samples were collected from eyeball. The mice were euthanized. Liver and epididymal fat were weighed. The palmitic acid (PA)-induced HepG2 cells were used to evaluate the protective effect of EMT on liver lipid accumulation.

Results: The administration of EMT is helpful to maintain near normal body weight, blood glucose, insulin, organ index, glucose tolerance, and serum levels of TC, TG, LDL-C, HDL-C, Apo-B, and Apo-A1 ($P < 0.05$ or $P < 0.01$). EMT treatment significantly improved liver injury by the down-regulation of liver lipid accumulation, oxidative stress and inflammatory mediators in high-fructose-induced mice ($P < 0.05$ or $P < 0.01$). *In vitro*, EMT (25 $\mu\text{g/mL}$ -200 $\mu\text{g/mL}$) significantly decreased lipid droplet accumulation and TG content in PA-induced HepG2 ($P < 0.05$ or $P < 0.01$).

Conclusion: EMT can obviously improve high fructose-induced MS in mice. *In vitro*, EMT can inhibit PA-induced lipid accumulation in HepG2 cells, which may emphasize the use of *M. toringoides* supplementation in everyday life of over-weighted persons and opens perspectives for clinical trials.

Introduction

Metabolic syndrome (MS) is defined as a cluster of metabolic disorders, including obesity, hyperglycemia, hypertension, hyperlipemia, insulin resistance, etc[1, 2]. A variety of factors can cause cardiovascular disease, type 2 diabetes, non-alcoholic fatty liver (NAFLD), and chronic kidney disease by inducing MS[3, 4, 5]. Among them, the excessive intake of fructose is closely related to the occurrence and development of MS[6]. Fructose, as a common edible sugar, exists in honey and fruits. Using fructose instead of granulated sugar in the daily diet can reduce calorie intake under the same sweetness, and its glycemic index is also very low. Therefore, fructose has been considered better in the prevention and control of diabetes in the past. But this view has been refuted. There is increasing evidence that a high-fructose diet (HFruD) may cause hyperglycemia, hyperlipemia, and insulin resistance, especially NAFLD in humans and animals[7, 8, 9]. It is proved that most of the fructose ingested was absorbed and metabolized by the liver. Due to the lack of a rate-limiting step catalysed by phosphofructokinase, liver fructose metabolism is rapid than glucose metabolism, resulting in high burden of de novo lipogenesis (DNL)[10, 11]. HFruD can increase visceral lipid accumulation, lipolysis rate, free fatty acids in blood circulating through portal

vein into liver causing hyperlipemia and insulin resistance, which are not only components of MS, but also play a key role in the development of MS[12, 13].

Malus toringoides (Rehd.) Hughes is a traditional medicinal plant used in Tibet, China. According to record in 'Tibetan Medicine the crystal mirror Materia Medica', this plant has the effect of treating dyspepsia, abdominal pain. The leaves of the plant is called "e se" in Tibetan, which is used by local doctors to treat hypertension, hyperlipemia and liver diseases[14]. Phytochemical studies have shown that *M. toringoides* contains the bioactive components of phenolics, flavonoids, polysaccharides, fatty acids and amino acids[15]. The bioactive ingredients of *M. toringoides* flavonoids had hyperglycemic effect in STZ or alloxan-induced diabetes animal models[16]. Our previous studies[17] have shown that *M. toringoides* contains phlorizin and phloretin, and that exert significant hypolipidemic and antioxidant properties in high-fat diet (HFD)-induced hyperlipidemic rats.

However, the improvement effects of this plant in fructose induced MS and liver injury has not been reported. Hence, the present study was designed to investigate the effects of ethanol extract of *M. toringoides* (EMT) on high-fructose-induced obesity, hyperlipemia, fat accumulation and liver injury in mice.

Materials And Methods

Chemicals and Reagents

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The kits used in the present study were as follows: total cholesterol (TC), Triglyceride (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and aspartic proteinase (ALP), superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-px) (Jiancheng Biotechnology Science, Inc. Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for apolipoprotein (Apo)-A1, Apo-B, insulin, tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6 and C reactive protein (CRP) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Roswell Park Memorial Institute (RPMI)-1640, fetal bovine serum (FBS), and other tissue culture reagents were purchased from GIBCO BRL Co. (Grand Island, NY, USA).

Extraction and analysis of the plant material

The leaves of *M. Toringoides* were obtained from Changdu, Tibetan Autonomous Prefecture and identified by Professor Jule Wang (College of Medicine, Tibet University, Lhasa, Tibet, China) in September 2018. This plant was refluxed and extracted with 70% ethanol twice for 1.5 h each time. The obtained extractive solution was filtered and evaporated in vacuum to obtain powers (The yield obtained was 43.2%). The voucher specimen (No. CBTM-E375) was deposited in Component Bank of Tibetan Medicine, Lhasa, Tibet, China. For cell experiments, ethanol extract was dissolved in dimethyl sulfoxide (DMSO, final culture concentration was 0.05%), and then kept at -20 °C for subsequent studies.

Experimental animals and experimental design

The C57BL/6J male mice (25 ± 2 g) were placed and adapted to the environmental conditions (temperature of 22 ± 2 °C, relative humidity of $55 \pm 5\%$ and a 12 h of day and night cycle) for 7 days. The experimental program of the animal care and use was approved by the Ethics Committee of Qingdao University of Science and Technology (approval number: ACQUST-2019-047).

Animals were randomly divided into five groups ($n = 8$): normal control group (Con); high-fructose group (Fru); high-fructose group treated with low-dose EMT (L-EMT, 195 mg/kg, i.g.), high-fructose group treated with medium-dose EMT (M-EMT, 390 mg/kg, i.g.), high-fructose group treated with high-dose EMT (H-EMT, 780 mg/kg, i.g.). Con group and Fru group were orally taken saline to mice once a day. The Con group drank normal water and the high fructose groups drank 30% high fructose water[18]. After 56 days of treatment, fasting blood glucose of mice was detected. Mouse were euthanized. Blood samples were obtained by enucleation of the eyeball. Serum was obtained from blood samples by centrifugation at 3500 rpm/min for 15 min and used for analysis. Liver was weighed and stored at -80 °C for biochemical and pathological analysis.

Glucose tolerance test (GTT)

On the 56th day, mouse fasted overnight were intraperitoneally injected with glucose (2 g/kg). Tail blood was collected at 0, 30, 60 and 120 min and the level of glucose was determined using a glucometer.

Histopathological evaluation

Hepatic tissue fixed with 4% paraformaldehyde for more than 24 h was gradient eluted with water and ethanol, dealcoholized with xylene, and embedded with paraffin. Paraffin blocks were sliced, transparent with xylene, and then eluted with ethanol and water in reverse gradient. Hematoxylin and eosin (H&E) staining were used to evaluate histopathological changes. The livers sections were collected by frozen sectioning (20 μ m) for Oil-red O staining (Leica CM1860, Germany). The staining was observed and photographed under an inverted optical microscope.

Biochemical assays

The contents of TC, TG, LDL-C, HDL-C, ALT, AST and ALP in serum and the levels of MDA, SOD and GSH-Px in liver by the kit. ELISA kits were used to measure the contents of Apo-A1 and Apo-B in serum and the levels of TNF- α , IL-6, IL-1 β and CRP in liver homogenate. The serum concentrations of glucose were detected by portable glucometer. The serum concentrations of insulin were analyzed by ELISA according to the manufacturer's instructions, and HOMA-IR was calculated to judge the insulin tolerance.

$\text{HOMA-IR index} = \text{insulin (mIU/L)} \times \text{blood glucose (mmol/L)} / 22.5$.

Cell culture

HepG2 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) maintained in RPMI containing 10% foetal bovine serum and 100 U/mL penicillin G and incubated in a humidified incubator containing 5% CO₂ at 37 °C, and 80% confluent cells were rendered quiescent by incubation for 12 h in serum-free medium before treatment with EMT or palmitic acid (PA, Sigma).

Cell viability assay

Cell viability was measured by MTT assay. In short, HepG2 cells (8×10^3 cells/well) were inoculated into a 96-wells culture plate, pre-incubated with EMT of concentration gradient for 24 h, treated with or without PA (500 μ M) for 24 h, incubated with 50 μ L MTT (2.5 mg/mL) at 37 °C for 4 h per well, then crystals were dissolved with 150 μ L/well DMSO, and the absorbance was detected at 490 nm.

TG assay and Oil red O staining in cells

HepG2 cells were cultured in 6-well plates, overnight, pre-treated for 24 h with the indicated concentrations of EMT, and then treated for 24 h with PA (500 μ M). The TG of cell were detected by commercial kit. Oil-red staining was observed under optical microscope.

Statistical analysis

All data were expressed as mean $\pm$ SD. Double-tailed t-test was used for determining differences between groups. Statistical analysis was carried out using the GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Significant differences were established when $P < 0.05$.

Results

Effects of EMT on body, liver and fat weight in HFruD-induced mice

Our results showed that the Fru group had a significant weight gain after high-fructose administration compared with that of the Con group. Compared with the Fru group, the weight gain of the M-EMT and H-EMT groups was decreased significantly ($P < 0.01$). As shown in Table 1, the liver weight, hepatic index, fat weight and fat index were significantly increased in Fru group ($P < 0.01$ or $P < 0.05$). After 8 weeks of preventive administration, these parameters of EMT groups had decreased in a dose-dependent manner. Among them, the effect of H-EMT group was significant ($P < 0.01$ or $P < 0.05$). These results suggest that EMT improve obesity, at least in HFruD-induced mice.

Table 1
Effects of EMT on body, liver and fat weight of HFruD-induced mice.

Parameter	Con	Fru	L-EMT	M-EMT	H-EMT
Initial Body weight(g)	31.76 ± 2.90	32.11 ± 2.42	33.49 ± 3.49	31.39 ± 3.52	30.19 ± 3.09
Final Body weight(g)	42.57 ± 5.50	47.13 ± 5.58	46.18 ± 4.33	41.88 ± 2.51*	39.54 ± 2.82**
Weight gain (g/8 weeks)	10.81 ± 2.62	15.02 ± 3.16#	12.69 ± 0.84	9.48 ± 1.01**	9.08 ± 0.27**
Liver weight (g)	1.68 ± 0.18	1.94 ± 0.23##	1.69 ± 0.28*	1.61 ± 0.20**	1.60 ± 0.18**
Hepatic index (%)	3.51 ± 0.11	4.43 ± 0.31##	4.11 ± 0.24*	3.97 ± 0.27**	3.75 ± 0.39**
Fat weight (g)	0.98 ± 0.16	1.39 ± 0.36##	1.16 ± 0.33	1.15 ± 0.36	1.00 ± 0.29*
Fat index (%)	2.10 ± 0.77	3.08 ± 0.69#	2.76 ± 0.86	2.95 ± 0.82	2.25 ± 0.43**

Hepatic index: ratio of liver weight to body weight. Fat weight: the weight of epididymal adipose tissue. Fat index: ratio of fat weight to body weight. The data are expressed as the mean ± SD, n = 8. #P < 0.05 or ##P < 0.01 compared to Con group; *P < 0.05 or **P < 0.01 compared to Fru group.

Effect of EMT on serum glucose, insulin, and GTT in HFruD-induced mice

As shown in Fig. 1a-c, compared with the Con group, the Fru group had significant increase in serum glucose, serum insulin, and HOMA-IR (P < 0.01). After 8 weeks of EMT (195 mg/kg, 390 mg/kg, 780 mg/kg) treatment, serum glucose and insulin in serum can be significantly reduced, and insulin resistance can be improved (P < 0.01 or P < 0.05). In order to determine whether EMT treatment improves glucose homeostasis, GTT was conducted (Fig. 1d, e). EMT administration can obviously restore fasting blood glucose to the level before taking glucose within 120 min (P < 0.01 or P < 0.05).

Effect of EMT on serum lipid profiles and liver function in HFruD-induced mice

High fructose can induce hyperlipemia and hepatic dysfunction. As shown, serum TC, TG, LDL-C and Apo-B of HFruD-induced mice increased significantly (P < 0.01) when compared to control mice (Fig. 2a-d). Conversely, the levels of HDL-C and Apo-A1 were significantly lowered (Fig. 2e, f). These changes were dose dependently reversed by EMT (195 mg/kg, 390 mg/kg, 780 mg/kg) treatment significantly (P < 0.01

or $P < 0.05$). Furthermore, after 8 weeks of EMT preventive treatment, the high serum contents of AST, ALT, and ALP were significantly ($P < 0.01$ or $P < 0.05$) attenuated in HFruD-induced mice by a dose-dependent manner (Fig. 3).

Effect of EMT on hepatic antioxidants in HFruD-induced mice

As shown in Fig. 4 (a, b), compared with Con group, the hepatic levels of SOD and GSH-Px were significantly decreased in Fru group ($P < 0.01$). After 8 weeks of preventive treatment with EMT, the hepatic contents of SOD and GSH-Px in HFruD-induced mice were significantly increased by a dose-dependent manner ($P < 0.01$ or $P < 0.05$). Figure 4c showed that the hepatic level of MDA in Fru group was significantly increased, which can significantly be reversed by EMT treatment ($P < 0.01$).

Effect of EMT on hepatic inflammatory factors in HFruD-induced mice

As shown in Fig. 5, compared with the Con group, the hepatic levels of TNF- α , IL-1 β , IL-6, and CRP were increased significantly in Fru group ($P < 0.01$). However, after 8 weeks of treatment, the levels of these inflammatory factors in HFruD-induced mice were by significantly reduced by EMT in a dose-dependent manner ($P < 0.01$ or $P < 0.05$).

Effect of EMT on histopathological changes in HFruD-induced mice

The improvement effect of EMT on HFruD-induced mice fatty liver was further confirmed by evaluation of liver histopathology. As shown in Fig. 6a, hepatic tissue was histologically examined by H&E staining. Compared with the Con group, Fru group had enlarged the number of lipid droplets and inflammatory cells. EMT treatment can improve the above pathological changes in a dose-dependent manner. Furthermore, the Oil-red O staining confirmed that the treatment of EMT can significantly reduce the accumulation of lipid in the liver induced by high-fructose (Fig. 6b).

Effect of EMT on PA-induced HepG2 cells injury

MTT assay was used to detect cell viability. As shown in Fig. 7a, EMT showed no cytotoxicity at concentration range of 25 $\mu\text{g/mL}$ -200 $\mu\text{g/mL}$. Pretreatment with EMT for 24 h protected HepG2 cells from PA (500 μM) -mediated toxicity (Fig. 7b).

Effects of EMT on lipid accumulation in PA-induced HepG2 cells

In order to explore the effects of EMT on the process of lipid accumulation in vitro, HepG2 cells were treated with PA (500 μM) in the presence or absence of EMT (25 $\mu\text{g/mL}$ -200 $\mu\text{g/mL}$) for 24 h. As shown in Fig. 8a, the result of Oil-red O staining showed that EMT could significantly reduce the number of lipid droplets in PA-induced HepG2 cells, which was corresponded to the change of TG contents (Fig. 8b).

Discussion

MS plays an important role in pathogenesis of chronic diseases, which can reduce of human quality of life and increase mortality[19, 20]. The comprehensive treatment of metabolic syndrome should be to reverse hyperglycemia, hypertension, obesity, insulin resistance, etc. There are some drugs against MS, most of which are lipid-lowering and hypoglycemic drugs, but they are still far from optimal. Natural plants with multiple targets and low toxicity are increasingly favored by people. As a potential medicinal plants, *M. toringoides* have been preliminarily confirmed had hypoglycemic and hepatoprotective effects. In our previous studies, phloridzin and phloretin was the main components of EMT, and the content of phloridzin was as high as 10%. Phloridzin has been reported can improve obesity, insulin resistance and liver lipid degeneration [21]. Phloretin can prevent HFD-induced obesity and improve metabolic homeostasis [22]. Based on the hypoglycemic and hypolipidemic potential of related species in animal models, and diversified *in vitro* and *in vivo* pharmacological properties of EMT, the current experiment was designed to evaluate the anti-MS effect of *M. toringoides* leaves against HFruD-induced mice.

Insulin resistance plays a central role in the development of multiple sclerosis. Insulin insensitivity requires islet β cells to secrete more insulin to maintain glucose homeostasis, resulting in hyperinsulinemia, and chronic hypersecretion of insulin may lead to β cell dysfunction[23]. Evidence showed that in rats fed 66% fructose, insulin receptor mRNA and insulin receptor numbers in skeletal muscle and liver were obviously decreased[24, 25]. In our studies, Fru group mice showed insulin resistance, which include hyperglycemia, hyperinsulinemia and high HOMA-IR. Furthermore, in GTT, the fast blood glucose in Fru group increased significantly and could not return to normal level within 120 min, which was related to the decrease of insulin sensitivity in mice after high fructose stimulation. EMT can significantly reduce hyperglycemia and insulin resistance caused by high-fructose.

Obesity, occurred in MS, is mainly caused by unhealthy lifestyle, such as intake of a large amount of fat and glucose, and lack of exercise[26, 27]. Some researchers[28, 29] had found that the intake of high fructose corn syrup was an important factor in the prevalence of obesity. Consistent with the report, in this experiment, C57BL/6J mice fed 30% fructose water for 8 weeks showed weight gain, liver and fat index increased. EMT prevention and treatment can significantly reduce the above phenomenon, which proved that EMT had certain weight loss effect. Fructose was once called "healthy sugar" because its metabolism can directly be absorbed by the intestine without relying on insulin. It will be partially oxidized in the liver, partially converted into glycogen or lactic acid, and most of it will enter DNL[30]. It is reported that HFruD can up-regulate lipid production pathway, thus increasing triglyceride synthesis[31, 32]. In present study, high fructose stimulated the increase of TC, TG, LDL-C and Apo-B in serum, and the decrease of HDL-C and Apo-A1. Interestingly, EMT preventive treatment completely reversed above change, which showed that EMT had a good hypolipidemic effects.

The liver is the main organ for fructose metabolism. The experimental and clinical evidenced that fructose precipitates fat accumulation in the liver, due to both increased lipogenesis and impaired fat oxidation. Fructose is rapidly decomposed into a large amount of lipid synthesis substrates pyruvate and

acetyl-CoA under the action of liver fructokinase after excessive intake. Furthermore, fructose can also stimulate liver endogenous lipid synthesis by up-regulating SREBP-1c, CHREBP and lipid synthases[33, 34, 35]. It is reported that liver lipid deposition and liver injury are also the main pathological results of MS. EMT can significantly reduce the elevation of liver injury markers AST, ALP and ALT levels in serum caused by high fructose. More and more evidences had shown that HFruD-induced MS had oxidative stress and chronic inflammation in liver or whole body[36, 37, 38]. In this study, we founded that mice fed 30% fructose water for 8 weeks had oxidative stress and inflammatory in liver. In histopathology, the Fru group an also be obviously observed a large number of inflammatory cell infiltration and lipid accumulation in the liver. Surprisingly, EMT therapy reversed this phenomenon. *In vitro*, we found that EMT can inhibit the formation of lipid droplets and high TG level in PA-induced HepG2 cells. The results showed that EMT has the potential to protect liver function by improving liver antioxidant and anti-inflammatory capabilities and reducing liver lipid synthesis and accumulation.

Conclusion

In conclusion, our results showed that EMT had obvious improvement obesity, hyperlipemia, hyperglycemia, insulin resistance and liver injury on MS in HFruD-induced mice. *In vitro*, the studies had shown that EMT can inhibit PA-induced HepG2 cells lipid accumulation. Our research provides the *M. toringoides* may be beneficial in the prevention and management of diet-induced MS and its associated complications. Such results should emphasizes the use of *M. toringoides* supplementation in everyday life of over-weighted persons and opens perspectives for clinical trials.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XHQ and QG performed biochemical assays, conducted data analysis, and wrote the manuscript. XL and YL performed biochemical assays. MC and JL assisted in data analysis. SH and BL assisted in design of the experiments. XHQ and BL designed the experiments, and revised the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Effect of EMT on serum glucose, insulin, and GTT in HFruD-induced mice. (a) serum glucose, (b) insulin, (c) GTT, (d) the AUC of the GTT. #P < 0.05 or ##P < 0.01 compared to Con group; *P < 0.05 or **P < 0.01 compared to Fru group.

Figure 2

Effect of EMT on serum lipid profiles in HFrD-induced mice. (a) TC, (b) TG, (c) LDL-C, (d) Apo-B, (e) HDL-C, (f) Apo-A1. ^{##} $P < 0.01$ compared to Con group; ^{*} $P < 0.05$ or ^{**} $P < 0.01$ compared to Fru group.

Figure 3

Effect of EMT on liver function in HFrD-induced mice. The levels of AST (a), ALT (b), ALP (c) in serum were measured. ^{##}P < 0.01 compared to Con group; *P < 0.05 or **P < 0.01 compared to Fru group.

Figure 4

Effect of EMT on liver oxidative stress of liver in HFrD-induced mice. (a) SOD, (b) GSH-px, (c) MDA. ^{##}P < 0.01 compared to Con group; *P < 0.05 or **P < 0.01 compared to Fru group.

Figure 5

Effect of EMT on inflammatory mediators of liver in HFruD-induced mice. (a) TNF- α , (b) IL-1 β , (c) IL-6 and (d) CRP. ##P < 0.01 compared to Con group; *P < 0.05 or **P < 0.01 compared to Fru group.

Figure 6

Effect of EMT on histopathological changes of liver in HFruD-induced mice. (a) H&E staining, (b) Oil-red O staining(200 ×).

Figure 7

Effect of EMT on cell viability in HepG2 cells. (a) Cells were treated with different concentrations of EMT (25 μg/mL-400 μg/mL) for 24 h. (b) Effect of EMT on cell injury induced by PA (500μM) for 24 h. Values are mean ± SD of 3 independent experiments with triplicate samples. ^{##}P < 0.01 compared to the control group; ^{ΔΔ}P < 0.01 compared to the group treated with PA.

Figure 8

Effect of EMT on lipid accumulation in PA-induced HepG2 cells. (a) The Oil-red O staining, (b) TG analysis. Values are mean $\pm$ SD of 3 independent experiments with triplicate samples. ## $P < 0.01$ compared to the control group; $\Delta P < 0.05$ or $\Delta\Delta P < 0.01$ compared to the group treated with PA.