Formononetin exhibits anti-hyperglycemic activity in alloxan-induced type 1 diabetic mice

Guizhen Qiu¹, Wei Tian², Mei Huan², Jinlong Chen³ and Haitao Fu⁴

¹Department of Health, Linyi People's Hospital of Shandong University, Linyi 276003, Shandong Province, China; ²Department of Nursing, Linyi Oncosurgical Hospital, Linyi 276002, Shandong Province, China; ³Department of Ophthalmology, Chinese Medicine Hospital of Linshu County, Linshu 276700, Shandong Province, China; ⁴Department of Ophthalmology, Linyi People's Hospital of Shandong University, Linyi 276003, Shandong Province, China

Corresponding author: Haitao Fu. Email: haitaofu163@163.com

Abstract

The aim of this study was to investigate the anti-hyperglycemic activity and mechanism of formononetin in alloxan-induced type 1 diabetic mice by determining its effect on some diabetes-related indices as described below. Body weight, fasting blood glucose, hepatic glycogen, serum insulin, and serum glucagon were determined by electronic scales, glucometer, and ELISA kits. Fas, Caspase-3, pancreatic and duodenal homeobox-1, insulin receptor substrate 2, glucokinase and glucose transporter 2, mRNA and proteins levels in pancreas tissue, and glucokinase and glucose-6-phosphatase mRNA, and proteins levels in liver tissue were detected by fluorogenic quantitative-polymerase chain reaction and Western blot assays. The results indicated that formononetin (5, 10, and 20 mg/kg; oral administration) reversed the alloxan-induced increase of some indices (fasting blood glucose level and Fas and Caspase-3 mRNA and proteins levels in pancreas tissue) and reduction of some indices (body weight gain, oral glucose tolerance, insulin activity, hepatic glycogen level, pancreatic and duodenal homeobox-1, insulin receptor substrate 2, glucokinase and glucose transporter 2, mRNA and proteins levels in pancreas tissue, and glucokinase mRNA and protein levels in liver tissue). The glucagon level and glucose-6-phosphatase mRNA and protein levels in liver tissue were not affected by the drugs administration. In conclusion, formononetin exhibited anti-hyperglycemic activity in alloxan-induced type 1 diabetic mice by inhibiting islet B cell apoptosis and promoting islet B cell regeneration, insulin secretion, hepatic glycogen synthesis, and hepatic glycolysis.

Keywords: Formononetin, type 1 diabetes, anti-hyperglycemic activity, islet B cell apoptosis and regeneration, insulin secretion, glycolysis

Experimental Biology and Medicine 2017; 242: 223–230. DOI: 10.1177/1535370216657445

Introduction

Diabetes, a chronic metabolic disease, afflicts approximately 3% of the world population and is characterized by hyperglycemia (excessive hepatic glycogenolysis and gluconeogenesis) resulting from the deficiency in the production of insulin or its action.¹ Diabetes is classified into types 1 and 2, and the etiology of type 1 diabetes is the absolute deficiency of insulin secretion, while the etiology of type 2 diabetes is a combination of insulin resistance with inadequate compensatory of insulin.^{1,2} Therefore, the important strategies in treating types 1 and 2 diabetes are to offset absolute deficiency of insulin secretion and control blood glucose level, respectively.^{1,2} Current oral anti-diabetic drugs mainly include α -glucosidase inhibitors used to decrease the intestinal glucose uptake, insulin releasers used to increase the insulin release, and insulin sensitizers used to increase the insulin action.³ Since long-term use of anti-diabetic agents is

the necessary condition for diabetic patients, decreased efficacy over time is an increasing problem for treating diabetes.⁴ Therefore, it is important and urgent to research and develop novel drugs for treating diabetes.

Plants are always considered as a wonderful source for medicines, and it is estimated that 1200 species of plants can be used to treat diabetes.⁵ Formononetin (FMN, Figure 1), an isoflavanone, is widely distributed in leguminous plants such as *Trifolium pratense* L. and *Pueraria lobata* (Willd.) Ohwi.^{6,7} It is reported that FMN can facilitate the fangchinoline-induced insulin release in streptozotocin-diabetic mice, and it indicated that FMN may exhibit anti-hyperglycemic activity.⁸

In this work, the anti-hyperglycemic activity and mechanism of FMN against alloxan-induced type 1 diabetes in mice were investigated by determining its effect on some diabetes-related indices including body weight, fasting

Figure 1 Chemical structure of FMN

blood glucose (FBG), glucose tolerance, hepatic glycogen, serum insulin, serum glucagon, Fas, Caspase-3, pancreatic and duodenal homeobox-1 (PDX-1), insulin receptor substrate 2 (IRS2), glucokinase (GK), glucose transporter 2 (GLUT2) mRNA, and proteins levels in pancreas tissue and GK and glucose-6-phosphatase (G-6-P) mRNA, and proteins levels in liver tissue. The results of this study showed that FMN exhibited anti-hyperglycemic activity in alloxan-induced type 1 diabetic mice by inhibiting islet B cell apoptosis and promoting islet B cell regeneration, insulin secretion, hepatic glycogen synthesis, and hepatic glycolysis.

Materials and methods

Chemicals and reagents

Alloxan monohydrate, an accepted reagent used to induce type 1 diabetic model in animal,^{9,10} was purchased from Abcam (Cambridge, MA, USA). Xiaoke pill, an accepted drug used to treat type 1 diabetes,¹¹ was purchased from Guangzhou Baiyun Mountain Pharmaceutical Co., Ltd (Guangzhou, Guangdong, China). FMN was obtained from Chengdu Mansite Biology (purity $\geq 98\%$, Chengdu, Sichuan, China). Insulin, glucagon, and hepatic glycogen ELISA kits were provided by Huijia Biotechnology (Xiamen, Fujian, China) and Keshun Biotechnology (Shanghai, China). Trizol reagent and AMV first strand cDNA synthesis kit were provided by Invitrogen Life Technologies (Carlsbad, CA, USA) and JK GREEN (Beijing, China), respectively. SybrGreen qPCR master mix was obtained from DBI Bioscience (Ludwigshafen, Germany). Enhanced BCA protein assay kit was purchased from Beyotime (Haimen, Jiangsu, China). Primary antibodies for GAPDH, Fas, Caspase-3, PDX-1, IRS2, GK, GLUT2, and G-6-P were obtained from Abcam (Cambridge, MA, USA) or Cell Signaling Technology (Beverly, MA, USA). HRP-conjugated goat anti-rabbit secondary antibody was provided by OriGene (Beijing, China).

Animal

Male KM mice (20 ± 2 g body weight) were provided by Shandong University Laboratory Animal Center (Jinan, Shandong, China) and housed in a temperature controlled vivarium (25°C) with relative humidity of 65% and 12/12-h light-dark cycle. All mice had free access to water and food. All animal treatments were conducted in strict accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.¹² All animal experiments were

performed with the approval of the Ethics Committee of Linyi People's Hospital.

Establishment of type 1 diabetic model, grouping and treatment

The type 1 diabetic mouse model was established by alloxan based on the previous method.^{9,10} After fasting for 24 h, mouse was treated with 1% alloxan at a dose of 85 mg/kg by tail vein injection. Three days after fasting for 12 h, the FBG of mouse was determined using a Sinocare Inc. glucometer (Changsha, Hunan, China). If the FBG >11.1 mmol/L, it indicated that the type 1 diabetic mouse model was established successfully.

Mice with type 1 diabetes were randomly divided into five groups ($n=10$): model group, Xiaoke pill group, and low, middle and high doses of FMN groups. Normal mice without type 1 diabetes served as normal group ($n=10$). Mice in the normal and model groups were administrated orally with 0.5% sodium carboxyl methyl cellulose (CMC-Na) once a day for 28 days. Mice in the Xiaoke pill group were administrated orally with 200 mg/kg Xiaoke pill once a day for 28 days. Mice in the low, middle, and high doses of FMN groups were administrated orally with 5, 10, and 20 mg/kg FMN once a day for 28 days, respectively. Xiaoke pill powder and FMN were suspended in 0.5% CMC-Na to obtain their different concentrations so that the mice received an intragastric volume of 10 mL/kg.

Determination of body weight and FBG

After fasting for 12 h, mice body weight was determined by electronic scales on 0th, 7th, 14th, 21st, and 28th days during the drugs administration. Meanwhile, blood from the tail vein of each mouse was used to determine FBG level using a Sinocare Inc. glucometer on 0th, 7th, 14th, 21st, and 28th days during the drugs administration.

Oral glucose tolerance test

After fasting for 12 h on 28th day, mice in the different groups were treated with the corresponding drugs. After 0.5 h of drugs treatment, mice were administrated orally with 25% glucose solution at a dose of 2.0 g/kg. Then, the blood glucose of each mouse was determined using a Sinocare Inc. glucometer after 0, 0.5, 1, and 2 h of 25% glucose treatment. The blood glucose-time curve was plotted, and the blood glucose value and time served as Y and X axis, respectively. The area under the blood glucose-time curve (AUC) was calculated as follows: $\text{AUC (mmol/L} \times \text{h)} = (\text{Y}_1 + \text{Y}_4)/2 + \text{Y}_2 + \text{Y}_3$.¹³ Y_1 , Y_2 , Y_3 , and Y_4 were the blood glucose value of mouse after 0, 0.5, 1, and 2 h of 25% glucose treatment, respectively. AUC was used to evaluate the effect of FMN on the oral glucose tolerance of mice with type 1 diabetes. The oral glucose tolerance test was performed independently to prevent the effect of glucose administration on determination of subsequent indices.

Determination of insulin and glucagon

After determination of mice body weight and FBG, the blood of each mouse was collected from eyeball and centrifuged at 3000 r/min for 10 min at 4°C to obtain serum. Serum was used to determine insulin activity and glucagon level by the corresponding ELISA kit according to the manufacturer's instructions for each. After reactions were completed, absorbance was determined for each sample using a 680 Microplate Reader (Bio-Rad, USA). The absorbance for insulin and glucagon was used to calculate their level or activity according to the corresponding standard curves.

Determination of hepatic glycogen

After blood was collected from eyeball, mice were sacrificed directly by decapitation to collect their pancreas and liver tissues. The part liver tissue was homogenized and centrifuged at 3000 r/min for 10 min at 4°C to obtain the supernatant, which was used to determine hepatic glycogen level by ELISA kit according to the manufacturer's instructions. The rest of the liver tissue and pancreas tissue were separately put into cryopreserved tubes and then were put into liquid nitrogen. Finally, they were stored in a refrigerator at -80°C.

Fluorogenic quantitative polymerase chain reaction assay

Liver and pancreas tissues were taken out from refrigerator, and then 100 mg liver tissue or pancreas tissue was ground into powder in a mortar with liquid nitrogen. Then, total RNA was isolated based on the standard protocol of Trizol reagent. The purity and concentration of total RNA samples were analyzed using a TU-1901 UV spectrophotometer (Persee, Beijing, China). Total RNA of pancreas tissue was used to determine Fas, Caspase-3, PDX-1, IRS2, GK and GLUT2 mRNA levels, and total RNA of liver tissue was used to determine GK and G-6-P mRNA levels. Equal amounts of total RNA (3 µg) for each sample were reversely transcribed using the AMV first strand cDNA Synthesis Kit. The primers for all genes were designed by Primer Premier 5.0 software based on the Genebank, and all primers (Table 1) were synthesized by Sangon Biotech (Shanghai, China). GAPDH gene was selected as

internal reference gene. Then, the reverse transcribed product (cDNA) along with primer and SybrGreen qPCR master mix were used for fluorogenic quantitative-polymerase chain reaction FQ-PCR amplification with an ABI 7500 PCR gene amplifier (Scottsdale, AZ, USA) programmed to 40 cycles at 95°C for 3 min initial step, then 95°C for 15 s, and 60°C for 40 s, with a final elongation step of 60°C for 40 s. Once the PCR was completed, gene amplification curves were plotted by the LightCycler480 Software Setup, and the Ct value for each gene was obtained. The relative level of target mRNA was calculated as follows: $2^{-\Delta\Delta Ct} = 2^{-[(Ct1-Ct2)-(Ct3-Ct4)]}$. Ct1, Ct2, Ct3, and Ct4 were the target gene Ct in the test sample, the GAPDH gene Ct in the test sample, the target gene Ct in the calibrator sample, and the GAPDH gene Ct in the calibrator sample, respectively. The calibrator sample consisted of appropriate concentrations of target gene and GAPDH gene and was used to eliminate the error resulting from experimental operation process and instrument performance.

Western blot assay

After pretreatment with liquid nitrogen, tissue protein extraction reagent and centrifugation, total protein of liver tissue or pancreas tissue was extracted, and their concentrations were determined using Enhanced BCA Protein Assay Kit. Total protein of pancreas tissue was used to detect Fas, Caspase-3, PDX-1, IRS2, GK, and GLUT2 proteins levels. Total protein of liver tissue was used to detect GK and G-6-P proteins levels. Equal amounts of total protein (about 40 µg) were separated by sodium dodecyl sulfate/polyacrylamide and transferred to a PVDF membrane. After blocking with 5% non-fat milk, PVDF membranes were incubated with the corresponding primary antibodies overnight at 4°C. After washing with Tris-buffered saline-Tween (TBS-T), PVDF membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody in TBS-T for 2 h at room temperature. PVDF membranes were washed with TBS-T, and proteins were detected by chemiluminescence. GAPDH was selected as internal reference protein to assess the protein loading, and the relative level of target protein was represented as the target protein level/GAPDH level.

Table 1 Primer sequence and product

Gene	Primer sequence		Product (bp)
	Forward	Reverse	
GAPDH	5'-GGTTGTCTCCTGCGACTTCA-3'	5'-TGGTCCAGGGTTTCTTACTCC-3'	183
Fas	5'-GGTTGTTGACCATCCTTGTTTT-3'	5'-CTGTCTCCTTTCCAGCACTTT-3'	86
Caspase-3	5'-TACTCTACAGCACCTGGTTACTATTC-3'	5'-TTCCGTTGCCACCTTCCT-3'	151
PDX-1	5'-AGGCGTCGCACAAGAAGAA-3'	5'-TCAGTTTGGAGCCCAGGTT-3'	197
IRS2	5'-ATTGAGCCAGGAGACACGAAC-3'	5'-TATTGCTTCACTCTTTCACGAC-3'	199
GLUT2	5'-TGGCTCGGGGACAAACTT-3'	5'-AGCAATGATGAGGGCGTGT-3'	120
GK	5'-TAAAGATGTTGCCACCTACG-3'	5'-GGAATACATCTGGTGTTCGTCT-3'	164
G-6-P	5'-ATCAATCTCCTCTGGGTGGC-3'	5'-TGTTGCTGTAGTAGTCGGTGTCC-3'	115

Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD). The differences among different groups were analyzed by one-way ANOVA of SPSS 21.0 (IBM SPSS Statistics, Armonk, New York City, USA). Differences were considered to be statistically significant at $P < 0.05$ or 0.01 .

Results

Effect of FMN on body weight and FBG level of mice with type 1 diabetes

As shown in Table 2, the mice body weight in all groups was gradually increased during the 28-day observation period. The growth rate of body weight in model group was lower than that in the other groups. The statistical analysis of the body weight on 28th day suggested that the body weight in the model group was significantly ($P < 0.01$) lower than that in the normal group. After treatment with Xiaoke pill or FMN (5, 10, or 20 mg/kg), the body weight was increased significantly ($P < 0.01$) relative to that in the model group.

As shown in Table 3, during the 28-day observation period, the FBG level in the normal and model groups was relatively stable, and the FBG level in the Xiaoke pill and FMN (5, 10, and 20 mg/kg) groups was gradually reduced. The statistical analysis of the FBG level on 28th day showed that the FBG level in the model group was significantly ($P < 0.01$) higher than that in the normal group. After treatment with Xiaoke pill or FMN (5, 10 or

20 mg/kg), the FBG level was reduced significantly ($P < 0.01$) relative to that in the model group.

Effect of FMN on oral glucose tolerance of mice with type 1 diabetes

As shown in Figure 2(a), after treatment with glucose, the FBG level in the all groups was increased quickly from 0 to 0.5 h, and then the FBG level was gradually reduced. The AUC of the model group was increased significantly ($P < 0.01$), compared with the normal group (Figure 2(b)). After treatment with Xiaoke pill or FMN (5, 10, or 20 mg/kg), the AUC was reduced significantly ($P < 0.01$) relative to that in the model group.

Effect of FMN on insulin activity, glucagon level, and hepatic glycogen level of mice with type 1 diabetes

As shown in Figure 3(a) and (c), the insulin activity and hepatic glycogen level in the model group were decreased significantly ($P < 0.01$) relative to those in the normal group. After treatment with Xiaoke pill or FMN (5, 10, or 20 mg/kg), the insulin activity and hepatic glycogen level were increased significantly ($P < 0.01$) relative to those in the model group. The hepatic glycogen level was not affected significantly by the drugs administration (Figure 3(b)).

Table 2 FMN promoted body weight gain of mice with type 1 diabetes

Group	Body weight of mice (g)				
	0th day	7th day	14th day	21st day	28th day
Normal	20.59 $\pm$ 1.24	22.93 $\pm$ 1.87	24.71 $\pm$ 2.05	27.92 $\pm$ 2.18	31.59 $\pm$ 3.43
Model	20.13 $\pm$ 1.07	20.71 $\pm$ 1.44	21.02 $\pm$ 1.83	21.79 $\pm$ 2.07	22.13 $\pm$ 1.76 ^{##}
Xiaoke pill	20.56 $\pm$ 1.38	22.37 $\pm$ 1.71	23.98 $\pm$ 2.18	26.56 $\pm$ 2.03	29.56 $\pm$ 3.25 ^{**}
FMN 5 mg/kg	20.43 $\pm$ 1.52	21.56 $\pm$ 1.52	22.37 $\pm$ 1.52	24.69 $\pm$ 1.52	26.01 $\pm$ 2.33 ^{**}
FMN 10 mg/kg	20.22 $\pm$ 1.61	22.18 $\pm$ 1.92	23.20 $\pm$ 2.05	24.89 $\pm$ 2.16	27.45 $\pm$ 2.47 ^{**}
FMN 20 mg/kg	20.31 $\pm$ 1.39	22.36 $\pm$ 2.11	23.63 $\pm$ 2.31	25.47 $\pm$ 2.29	28.31 $\pm$ 2.68 ^{**}

FMN: Formononetin.

^{##} $P < 0.01$ vs that in the normal group, ^{**} $P < 0.01$ vs that in the model group.

Table 3 FMN reduced FBG level of mice with type 1 diabetes

Group	FBG level (mmol/L)				
	0th day	7th day	14th day	21st day	28th day
Normal	5.01 $\pm$ 0.82	4.93 $\pm$ 0.71	5.12 $\pm$ 0.66	5.05 $\pm$ 0.74	4.99 $\pm$ 0.89
Model	23.37 $\pm$ 2.16	22.84 $\pm$ 2.52	22.56 $\pm$ 1.97	24.11 $\pm$ 2.81	23.83 $\pm$ 2.45 ^{##}
Xiaoke pill	22.13 $\pm$ 1.95	18.53 $\pm$ 2.01	15.97 $\pm$ 1.97	12.75 $\pm$ 2.10	9.29 $\pm$ 1.88 ^{**}
FMN 5 mg/kg	23.04 $\pm$ 2.29	21.42 $\pm$ 1.96	20.07 $\pm$ 2.12	18.39 $\pm$ 1.91	16.42 $\pm$ 1.73 ^{**}
FMN 10 mg/kg	22.85 $\pm$ 2.63	20.38 $\pm$ 1.96	18.57 $\pm$ 2.20	16.25 $\pm$ 1.86	14.12 $\pm$ 1.81 ^{**}
FMN 20 mg/kg	22.16 $\pm$ 2.73	19.27 $\pm$ 2.34	17.31 $\pm$ 1.76	13.37 $\pm$ 2.16	10.48 $\pm$ 1.26 ^{**}

FMN: Formononetin.

^{##} $P < 0.01$ vs that in the normal group, ^{**} $P < 0.01$ vs that in the model group.

Figure 2 FMN improved oral glucose tolerance of mice with type 1 diabetes. (a) FBG level after 0, 0.5, 1, and 2 h of 25% glucose treatment. (b) AUC. $^{##}P < 0.01$ vs. that in the normal group, $^{**}P < 0.01$ vs. that in the model group

Effect of FMN on Fas, Caspase-3, PDX-1, IRS2, GK and GLUT2 mRNA and proteins levels in pancreas tissue, and GK and G-6-P mRNA and proteins levels in liver tissue of mice with type 1 diabetes

The Fas and Caspase-3 mRNA and proteins levels of pancreas tissue in the model group were significantly ($P < 0.01$) higher than those in the normal group (Figures 4(a) and 5(a)). The PDX-1, IRS2, GK and GLUT2 mRNA and proteins levels of pancreas tissue in the model group were significantly ($P < 0.01$) lower than those in the normal group (Figures 4(b) and (c) and 5(b) and (c)). The GK mRNA and protein levels of liver tissue in the model group were significantly ($P < 0.01$) lower than those in the normal group (Figures 4(d) and 5(d)). Compared with those in the model group, Xiaoke pill or FMN (5, 10 or 20 mg/kg) down-regulated significantly ($P < 0.01$) the Fas and Caspase-3 mRNA and proteins levels of pancreas tissue (Figures 4(a) and 5(a)), up-regulated the PDX-1, IRS2, GK and GLUT2, mRNA and proteins levels of pancreas tissue (Figures 4(b) and (c) and 5(b) and (c)) and the GK mRNA and protein levels of liver tissue (Figures 4(d) and 5(d)). The G-6-P mRNA and protein levels of liver tissue were not affected significantly by the drugs administration (Figures 4(d) and 5(d)).

Discussion

It has been reported that after treatment with alloxan, the blood glucose level and islet B cell apoptosis of mice were increased, and the body weight gain, glucose tolerance, hepatic glycogen level, and insulin secretion of mice

Figure 3 FMN increased insulin activity and hepatic glycogen level of mice with type 1 diabetes and did not affect glucagon level of mice with type 1 diabetes. (a) Insulin activity. (b) Glucagon level. (c) Hepatic glycogen level. $^{##}P < 0.01$ vs. those in the normal group, $^{**}P < 0.01$ vs. those in the model group

Figure 4 FMN down-regulated Fas and Caspase-3 mRNA levels in pancreas tissue, up-regulated PDX-1, IRS2, GK and GLUT2 mRNA levels in pancreas tissue, and GK mRNA level in liver tissue and did not affect G-6-P mRNA level in liver tissue. (a) Fas and Caspase-3 mRNA levels in pancreas tissue. (b) PDX-1 and IRS2 mRNA levels in pancreas tissue. (c) GK and GLUT2 mRNA levels in pancreas tissue. (d) GK and G-6-P mRNA levels in liver tissue. ## $P < 0.01$ vs. those in the normal group, ** $P < 0.01$ vs. those in the model group

were decreased.^{14–16} Therefore, alloxan is an accepted reagent to induce type 1 diabetes. In the present study, the changes of all these indices had been observed in the alloxan-induced type 1 diabetic mice, and these changes were reversed by Xiaoke pill, an accepted drugs used to treat type 1 diabetes.¹¹ These results indicated that the alloxan-induced type 1 diabetic mouse model and Xiaoke pill were established and administrated successfully, respectively. In this work, we first investigated the anti-hyperglycemic activity of FMN by observing its effect on the alloxan-induced changes of FBG level and body weight gain in mice. Then, the anti-hyperglycemic mechanism of FMN was explored by observing its effect on the alloxan-induced changes of islet B cell apoptosis and regeneration, insulin secretion, hepatic glycogen synthesis, and hepatic glycolysis in mice.

Body weight and FBG level are two general indices to evaluate whether the diabetic mouse model is established successfully or drug exhibits anti-hyperglycemic activity.¹⁷ Our results found that FMN reversed the alloxan-induced reduction of body weight gain and increase of FBG level in mice with alloxan-induced type 1 diabetes (Tables 2 and 3), suggesting that FMN exhibited anti-hyperglycemic activity in alloxan-induced type 1 diabetic mice.

Islet B cell apoptosis and regeneration have an important influence on diabetes treatment.¹⁸ Islet B cell can regulate the blood glucose level to be normal by secreting insulin.¹⁹ If the balance between the islet B cell apoptosis and

regeneration were broken, the blood glucose level is out of control. PDX-1 induces the differentiation of pancreatic duct cells or some other cells into islet B cell and inhibits islet B cell apoptosis.^{20–22} IRS2 is a vital regulatory factor in promoting islet B cell proliferation and differentiation and inhibiting islet B cell apoptosis.²³ Fas and Caspase-3 are two important factors in promoting cell apoptosis.²⁴ Our results indicated that FMN reversed the alloxan-induced increase of islet B cell apoptosis and inhibition of islet B cell regeneration by down-regulating the Fas and Caspase-3 mRNA and proteins levels and up-regulating the PDX-1 and IRS2 mRNA and proteins levels in pancreas tissue of mice with alloxan-induced type 1 diabetes (Figures 4(a) and (b) and 5(a) and (b)).

GK and GLUT2 play important roles in the insulin-secreting function of islet B cell. GK promotes glucose metabolism, which can induce insulin secretion.²⁵ GLUT2 can induce insulin secretion by promoting the glucose transportation from blood into islet B cell and the release of insulin from islet B cell into blood.²⁶ Our results indicated that FMN reversed the inhibitory effect of alloxan on insulin secretion (Figure 3(a)) by up-regulating the GK and GLUT2 mRNA and proteins levels in pancreas tissue of mice with alloxan-induced type 1 diabetes (Figures 4(c) and 5(c)).

Liver is the main organ of glucose metabolism, and glycolysis, gluconeogenesis, and glycogen synthesis are the key methods of liver to regulate blood glucose level.²⁷ In the normal body, hexokinase is the rate-limiting enzyme of

Figure 5 FMN down-regulated Fas and Caspase-3 proteins levels in pancreas tissue, up-regulated PDX-1, IRS2, GK and GLUT2 proteins levels in pancreas tissue, and GK protein level in liver tissue and did not affect G-6-P protein level in liver tissue. (A) Fas, Caspase-3, PDX-1, IRS2, GK and GLUT2 proteins in pancreas tissue. (B) GK and G-6-P proteins in liver tissue. (a) Fas and Caspase-3 proteins levels in pancreas tissue. (b) PDX-1 and IRS2 proteins levels in pancreas tissue. (c) GK and GLUT2 proteins levels in pancreas tissue. (d) GK and G-6-P proteins levels in liver tissue. $^{##}P < 0.01$ vs. those in the normal group, $^{**}P < 0.01$ vs. those in the model group

hepatic glycolysis,²⁸ but in the body with diabetes, the rate-limiting enzyme is GK.²⁹ G-6-P is a key enzyme for hepatic gluconeogenesis.³⁰ Glucagon promotes hepatic glycogen degradation and inhibits hepatic glycogen synthesis.³¹ Oral glucose tolerance is an important index to evaluate the glucose metabolism of body.³² Our results found that the glucagon level (Figure 3(b)) and the G-6-P mRNA and protein levels in liver tissue (Figures 4(d) and 5(d)) were not affected by the drugs administration, suggesting that FMN did not promote hepatic glycogen degradation and gluconeogenesis. FMN reversed significantly the inhibitory effects of alloxan on hepatic glycogen synthesis (Figure 3(c)) and hepatic glycolysis by up-regulating the GK mRNA and protein levels in liver tissue of mice with alloxan-induced type 1 diabetes (Figures 4(d) and 5(d)).

Meanwhile, the results of oral glucose tolerance test also indicated that FMN promoted hepatic glycogen synthesis or glycolysis of mice with alloxan-induced type 1 diabetes (Figure 2).

In conclusion, this work showed that FMN exhibited anti-hyperglycemic activity in alloxan-induced type 1 diabetic mice by inhibiting islet B cell apoptosis and promoting islet B cell regeneration, insulin secretion, hepatic glycogen synthesis, and hepatic glycolysis. Therefore, FMN may be used as a drug candidate to treat type 1 diabetes, and there is a need to further investigate FMN in this regard.

Authors' contributions: GZQ and HTF participated in the design of the experiments, performed the statistical analyses, and wrote the manuscript. GZQ, WT, MH and JLC

carried out the experiments. All authors read and approved the submission.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING STATEMENT

There was no specific funding for this work.

REFERENCES

- Umar A, Ahmed QU, Muhammad BY, Dogarai BB, Soad SZ. Anti-hyperglycemic activity of the leaves of *Tetracera scandens* Linn. Merr. (Dilleniaceae) in alloxan induced diabetic rats. *J Ethnopharmacol* 2010;**131**:140–5
- Hsu YJ, Lee TH, Chang CL, Huang YT, Yang WC. Anti-hyperglycemic effects and mechanism of *Bidens pilosa* water extract. *J Ethnopharmacol* 2009;**122**:379–83
- Li TH, Hou CC, Chang CL, Yang WC. Anti-hyperglycemic properties of crude extract and triterpenes from *Poria cocos*. *Evid Based Complement Alternat Med* 2011;**2011**:128402
- Grover JK, Yadav S, Vats V. Medicinal plants of India with anti-diabetic potential. *J Ethnopharmacol* 2002;**81**:81–100
- Marles RJ, Farnsworth NR. Antidiabetic plants and their active constituents. *Phytomedicine* 1995;**2**:137–89
- Ma Q, Lei HM, Zhou YX, Wang CH. Studies on chemical constituents of *Trifolium pratense*. *Chin Pharm J* 2005;**40**:1057–9
- Li GH, Zhang QW, Wang YT. Chemical constituents from roots of *Pueraria lobata*. *Chin J Chin Mater Med* 2010;**35**:3156–60
- Ma W, Nomura M, Takahashi-Nishioka T, Kobayashi S. Combined effects of fangchinoline from *Stephania Tetrandra* Radix and formononetin and calycosin from *Astragalus membranaceus* Radix on hyperglycemia and hypoinsulinemia in streptozotocin-diabetic mice. *Biol Pharm Bull* 2007;**30**:2079–83
- Yang RJ, Li QW, Zhao R. Comparison between the effects of alloxan and streptozotocin on inducing diabetes in mice. *J Northwest Sci Tech Univ Agri Nat Sci Ed* 2006;**34**:17–20
- Chen HY, Yang XB, Wang JH, Lu LJ, Cao WB, Huang ZM. Diabetic model induced by alloxan in mice and its affecting factors. *Bull Acad Mil Med Sci* 2005;**29**:535–7
- Yang B. Study on anti-hyperglycemic activity and mechanism in experimental type I diabetic mouse of water extract from *Echinops latifolius* Tansch. PhD thesis. Inner Mongolia Agricultural University 2014;90–1
- The National Research Council of the National Academy of Sciences. *Guide for the care and use of laboratory animals*. 8th ed. Washington, D.C.: The National Academies Press, 2010
- Liu CJ, Wang YG. Functional assessment of islet α -cell and β -cell in patients with gestational diabetes mellitus. *Chin J Endocrinol Metab* 2012;**28**:835–8
- Szkudelski T. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiol Res* 2001;**50**:537–46
- Badole S, Patel N, Bodhankar S, Jain B, Bhardwaj S. Antihyperglycemic activity of aqueous extract of leaves of *Cocculus hirsutus* (L.) Diels in alloxan-induced diabetic mice. *Indian J Pharmacol* 2006;**38**:49–53
- Tomita T, Lacy PE, Natschinsky FM, McDaniel ML. Effect of alloxan on insulin secretion in isolated rat islets perfused in vitro. *Diabetes* 1974;**23**:517–524
- Ahmed MF, Kazim SM, Ghori SS, Mehjabeen SS, Ahmed SR, Ali SM, Ibrahim M. Antidiabetic activity of vinca rosea extracts in alloxan-induced diabetic rats. *Int J Endocrinol* 2010;**2010**:841090
- Meier JJ, Bhushan A, Butler AE, Rizza RA, Butler PC. Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? *Diabetologia* 2005;**48**:2221–8
- Zhang CY, Baffy G, Perret P, Krauss S, Peroni O, Grujic D, Hagen T, Vidal-Puig AJ, Boss O, Kim YB, Zheng XX, Wheeler MB, Shulman GI, Chan CB, Lowell BB. Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, β cell dysfunction, and type 2 diabetes. *Cell* 2001;**105**:745–55
- Offield MF, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA, Hogan BL, Wright CV. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 1996;**122**:983–95
- Kritzik MR, Krah T, Good A, Krakowski M, St-Onge L, Gruss P, Wright C, Sarvetnick N. Transcription factor expression during pancreatic islet regeneration. *Mol Cell Endocrinol* 2000;**164**:99–107
- Johnson JD, Ahmed NT, Luciani DS, Han Z, Tran H, Fujita J, Misler S, Edlund H, Polonsky KS. Increased islet apoptosis in Pdx1+/- mice. *J Clin Invest* 2003;**111**:1147–60
- Gui S, Yuan G, Wang L, Zhou L, Xue Y, Yu Y, Zhang J, Zhang M, Yang Y, Wang DW. Wnt3a regulates proliferation, apoptosis and function of pancreatic NIT-1 beta cells via activation of IRS2/PI3K signaling. *J Cell Biochem* 2013;**114**:1488–97
- Al-Assaf AH, Algahtani AM, Alshatwi AA, Syed NA, Shafi G, Hasan TN. Mechanism of cadmium induced apoptosis in human peripheral blood lymphocytes: the role of p53, Fas and Caspase-3. *Environ Toxicol Pharmacol* 2013;**36**:1033–9
- Maiztegui B, Roman CL, Barbosa-Sampaio HC, Boschero AC, Gagliardino JJ. Glucokinase, glucose metabolism, and insulin pathway in the enhancing effect of islet neogenesis-associated protein on glucose-induced insulin secretion. *Pancreas* 2015;**44**:959–66
- Thorens B. GLUT2, glucose sensing and glucose homeostasis. *Diabetologia* 2015;**58**:221–32
- Nordlie RC, Foster JD, Lange AJ. Regulation of glucose production by the liver. *Annu Rev Nutr* 1999;**19**:379–406
- Gu JJ, Singh A, Mavis C, Yanamadala V, Xue K, Grau M, Lenz P, Lenz G, Czuczman M, Hernandez F. Up-regulation of hexokinase II (HK) alters the glucose metabolism and disrupts the mitochondrial potential in aggressive b-cell lymphoma contributing to rituximab-chemotherapy resistance and is a clinically relevant target for future therapeutic development. *Blood* 2014;**124**:1767
- Castro A. Kinase activators as a novel class of antidiabetic agents. *Drug Discov Today* 2012;**17**:528–9
- Nachar A, Vallerand D, Musallam L, Lavoie L, Badawi A, Arnason J, Haddad PS. The action of antidiabetic plants of the Canadian James bay Cree traditional pharmacopeia on key enzymes of hepatic glucose homeostasis. *Evid Based Complement Alternat Med* 2013;**2013**:189819
- Roden M, Perseghin G, Petersen KF, Hwang JH, Cline GW, Gerow K, Rothman DL, Shulman GI. The roles of insulin and glucagon in the regulation of hepatic glycogen synthesis and turnover in humans. *J Clin Invest* 1996;**97**:642–8
- Aekplakorn W, Tantayotai V, Numsangkul S, Sripho W, Tatsato N, Burapasiriwat T, Pipatsart R, Sansom P, Luckanajantachote P, Chawarokorn P, Thanonghan A, Lakhamkaew W, Mungkung A, Boonkean R, Chantapoon C, Kungsri M, Luanseng K, Chaiajit K. Detecting prediabetes and diabetes: agreement between fasting plasma glucose and oral glucose tolerance test in Thai adults. *J Diabetes Res* 2015;**2015**:396505

(Received February 18, 2016, Accepted June 1, 2016)