Mulberry leaf flavonoids protect against glucotoxicity-induced INS-1 cell apoptosis

Wang Ying, Li Ming, Yu Xuesong, He Sheng, Wu Xinrong, Wang Yan

OBJECTIVE: To investigate the effect of mulberry leaf flavonoids (MLF) on apoptosis of pancreatic cells induced by high glucose.

METHODS: Long exposure to high glucose induces apoptosis of pancreatic β cells, which can lead to diabetes. In this study, we used the rat insulinoma cell line, INS-1. High glucose (33.3 mM) was used to establish a glucotoxicity model. The MTT assay was used to evaluate the MLF effect on cell viability. INS-1 cells were treated with various concentrations of MLF (125, 250 and 500 mg/L) for 24 h, and then stimulated with 5.5 or 33.3 mM glucose for 48 h. Then, the cell supernatants were collected for enzyme-linked immunosorbent assay to determine the level of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor a (TNF-α) and interleukin 6 (IL-6). Western blotting was used to determine the expression of Bcl-2, Bax, caspase-3 and Caspase-9. Cell apoptosis was measured by Annexin V-FITC/propidium iodide double staining and flow cytometry.

RESULTS: MLF (125-500 mg/L) improved cell viability. Furthermore, MLF (250 and 500 mg/L) inhibited apoptosis induced by high glucose. The anti-apoptosis effect of MLF was associated with increased SOD, CAT and GSH-Px expression, as well as reduced MDA levels in high-glucose-treated INS-1 cells. Moreover, MLF upregulated Bcl-2 expression, downregulated Bax expression, and reduced the expression of caspase-3 and Caspase-9. Finally, MLF decreased the secretion of inflammatory cytokines and insulin in high-glucose-induced INS-1 cells.

CONCLUSION: MLF is a potential therapeutic agent for preventing diabetes and related disorders.

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INTRODUCTION

Type 2 diabetes is a metabolic disorder that features...
high levels of blood glucose due to pancreatic β cells dysfunction. Pancreatic β cells regulate the level of blood glucose by secreting insulin. Pancreatic β cells dysfunction leads to insulin resistance and insulin deficiency, and is critical in the pathogenesis of type 2 diabetes. Although the mechanisms are still unclear, oxidative stress and the inflammatory response have been reported as important causes of apoptosis and dysfunction of these cells.

Via alternative glucose metabolic pathways the mitochondria generates excessive amounts of reactive oxygen species (ROS) when the blood glucose levels are elevated. Additionally, the activity of antioxidant enzymes is diminished, resulting in oxidative stress, cellular damage and apoptosis. Moreover, the expression of inflammatory cytokines such as C-reactive protein, interleukin 6 (IL-6) and monocyte chemoattractant protein (MCP-1) is increased, generating a critical risk for changing the normal structure of β cells and insulin resistance. Thus, it is vital to reduce the risk of β cells dysfunction to attenuate the inflammatory response and oxidative stress induced by high blood glucose levels.

Mulberry leaf flavonoids (MLF) are effective ingredients extracted from the Chinese medicinal plant, Morus alba L., which has multiple therapeutic effects, such as lowering blood glucose levels and improving insulin sensitivity. Previously, mainly the hypoglycemic effects of α-glycosidase inhibitors extracted from mulberry leaves were studied. Furthermore, it has been reported that polysaccharides from mulberry leaves prevent pancreatic islet injury. Nevertheless, the effects of MLF on pancreatic β cells functions as well as survival have not been reported in detail, and the underlying mechanism of MLF’s anti-hyperglycemic effect remains unclear. In this study, we investigated how MLF prevents high-glucose-induced apoptosis and inflammatory response in a rat pancreatic β cell insulinoma cell line (INS-1).

MATERIALS AND METHODS

Chemicals and reagents
MLF were isolated from mulberry leaves (Morus alba L.) (YPA6E0001), which were purchased from Guangzhou medicine company (Guangzhou, China) and identified by Dr. Zeng Lingjie. The isolated compound was analyzed by ultraviolet spectrophotometer, and its purity was measured as 81.42%. An ultra-sensitive rat insulin enzyme-linked immunosorosent assay (ELISA) kit (RAB0904) was obtained from Sigma (St. Louis, MA, USA). MCP-1 (BMS631NST), IL-6 (BMS625) and TNF-α (BMS620) ELISA kits were obtained from eBioscience (San Jose, CA, USA). Superoxide dismutase (SOD, A001-1), glutathione peroxidase (GSH-Px, A005), catalase (CAT, A007-1) and malondialdehyde (MDA, A003-1) detection kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Polyclonal antibodies against Bcl-2 (42235), Bax (50235), caspase-3 (96655), Caspase-9 (#1332) and β-actin (2118S) were obtained from Cell Signaling Technology (CST, Danvers, MA, USA). Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (KGA105) was obtained from Nanjing Keygen Biotech Co. (Nanjing, China).

MLF isolation and purification
We prepared MLF according to Yang et al. Briefly, dry mulberry leaf powder (100 g) was incubated three times with 5-fold (v/v) 70% ethanol under reflux for 1 h. Next, the alcohol was removed by decompression at 60 °C, followed by adsorption with AB-8 resin and polyamide resin. MLF were then successively eluted with 5-fold (v/v) distilled water, 40% ethanol and 80% ethanol. Finally, the 80% ethanol elution was concentrated in a vacuum drier at 60 °C. The extract was stored in a refrigerator until further use.

Cell culture
INS-1 cells obtained from CCTCC (China Center for Type Culture Collection, Wuhan, China) were cultured in RPMI 1640 (Gibco) with 10% (v/v) fetal bovine serum (Gibco), antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Gibco), 11.1 mM glucose, 50 μM β-mercaptoethanol and 1 mM sodium pyruvate at 37 °C in an atmosphere of 5% CO₂.

Cell viability assay
We measured cell viability using the MTT [(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide) assay. We placed INS-1 cells at 2 × 10^3 cells/mL (100 μL per well) in a 96-well plate, and cultured them at 37 °C overnight. We pretreated the cells with different concentrations of MLF for 24 h prior to exposure to glucose (5.5 or 33.3 mM) for another 48 h. Then, 10 μL of MTT solution (5 mg/mL in PBS) were added to each well and cultured for 2 h. After DMSO solubilized the formazan crystals, the absorbance (A) was measured at 570 nm by a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The relative cell viability was computed and examined relative to the performance of the untreated control group.

Measurement of MCP-1, IL-6 and TNF-α expression levels
We treated INS-1 cells with various concentrations of MLF (125, 250 and 500 mg/L) for 24 h, followed by 5.5 or 33.3 mM glucose for 48 h. After the incubation, we collected the medium and stored it at −20 °C. We analyzed the levels of MCP-1, TNF-α and IL-6 in these media with ELISA.

Measurement of SOD, GSH-Px, CAT and MDA expression levels
We treated INS-1 cells with various concentrations of MLF (125, 250 and 500 mg/L) for 24 h, followed by
5.5 or 33.3 mM glucose for 48 h. After the incubation, we collected the medium and stored it at −20 °C. We analyzed the expression levels of SOD, GSH-Px, CAT and MDA in these media with a colorimetric method.

**Glucose-stimulated insulin secretion**
We treated INS-1 cells with different concentrations of MLF (125, 250 and 500 mg/L) for 24 h, followed by 5.5 or 33.3 mM glucose for 48 h. After the incubation, the medium was carefully removed, the cells were washed with PBS, and fresh medium with 3 mM glucose and 2% FBS was added. Five hours later, we activated the cells with Krebs-Ringer buffer (119 mM NaCl, 4.75 mM KCl, 2.54 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 5 mM NaHCO₃ and 20 mM HEPES, pH 7.4) containing 5 or 25 mM glucose for 60 min at 37 °C. Next, we collected the medium for analysis of insulin secretion as previously described.35

**Measurement of Bcl-2, Bax, caspase-3 and Caspase-9 expression levels**
We placed INS-1 cells in a 6-well plate at 4 × 10³ cells/well. After overnight cultivation, we processed the cells with different concentrations of MLF (125, 250 and 500 mg/L) for 24 h, followed by 5.5 or 33.3 mM glucose for 48 h. Protein was extracted from whole-cell lysates to determine the expression level of Bcl-2, Bax, caspase-3 and Caspase-9. For immunoblotting, 50 µg of protein per lane were resolved by SDS-PAGE and blotted onto PVDF membranes (Bio-RAD, Hercules, CA, USA), which were then blocked with 5% BSA solution for 2 h at room temperature. Next, the membranes were incubated overnight at 4 °C with anti-Bcl-2 (1:800), anti-Bax (1:800), anti-caspase-3 (1:1000) and anti-Caspase-9 (1:1000) as the primary antibodies. After washing, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h (dilution of 1:15000) at room temperature. We visualized the proteins by exposing the membranes to Kodak X-Omat films.

**Flow cytometry assessment of apoptosis**
We placed INS-1 cells in a 6-well plate at 4 × 10³ cells/well. After overnight incubation, we added different concentrations of MLF (125, 250 and 500 mg/L) for 24 h, and then stimulated the cells with 5.5 or 33.3 mM glucose for 48 h. Next, we collected the cells and incubated cell suspensions (100 µL) with 5 µL of Annexin-V and 5 µL of PI. We placed the cells in the dark for 15 min in an ice bath. Then, we added 400 µL of binding buffer to the cell suspension. The solution was immediately examined using a flow cytometer (BD, FACS Canto II, San Joes, CA, USA). Apoptotic cells were presented in terms of their percentage of the entire cell count.

**Statistical analysis**
Results are presented as the mean ± standard error of the mean (SEM). We used one-way analysis of variance (ANOVA). Furthermore, we analyzed the results with Tukey’s multiple comparison tests. P < 0.05 was considered statistically significant. We conducted the analyses using SPSS 13.0 for Windows (SPSS company, Chicago, IL, USA).

**RESULTS**

**Effect of MLF on cell viability of high-glucose-treated INS-1 cells**
To examine whether MLF improve the cell viability of INS-1 cells exposed to high-glucose, the MTT assay was conducted (Figure 1). High-glucose considerably decreased the cell viability to 42.4%. However, MLF showed a dose-dependent protective effect against the cellular harm induced by 33.3 mM glucose. MLF at 500 mg/L significantly increased the cell viability to 89.64%.

![Figure 1](image-url)  
**Figure 1** Effect of MLF on cell viability of high-glucose-treated INS-1 cells  
1: treated with 5.5 mM glucose; 2: treated with 33.3 mM glucose; 3: treated with 33.3 mM glucose and 10 mg/L MLF; 4: treated with 33.3 mM glucose and 50 mg/L MLF; 5: treated with 33.3 mM glucose and 100 mg/L MLF; 6: treated with 33.3 mM glucose and 250 mg/L MLF; 7: treated with 33.3 mM glucose and 500 mg/L MLF. Each value is the mean ± standard error of the mean of three independent experiments. a,b,c,d,e,f,g,h,i,j,k,l,m,n,o,p,q,r,s,t,u,v,w,x,y,z P < 0.05 vs group 2, P < 0.05 vs group 5.

**Effect of MLF on inflammatory cytokines expression in high-glucose-treated INS-1 cells**
When we incubated INS-1 cells with high glucose, the secretion of MCP-1, TNF-α and IL-6 underwent considerable enhancement. However, MLF at 250 and 500 mg/L significantly inhibited the level of MCP-1, TNF-α and IL-6 (P < 0.05 or P < 0.01) in a dose-dependent manner (Figure 2).

**Effect of MLF on SOD, GSH-Px, CAT and MDA levels in high-glucose-treated INS-1 cells**
When we incubated INS-1 cells with high glucose, the level of the antioxidant enzymes, SOD, GSH-Px and CAT, was markedly reduced, but the lipid peroxidation marker, MDA, was increased. However, MLF at 250 and 500 mg/L significantly increased the level of these...
antioxidant enzymes and decreased the level of MDA ($P < 0.05$ or $P < 0.01$) in a dose-dependent manner (Table 1).

**Effect of MLF on insulin secretion in high-glucose-treated INS-1 cells**

When we cultured INS-1 cells with high glucose, insulin secretion was significantly decreased. Namely, insulin secretion was checked after 5 and 25 mM glucose exposure, revealing lower insulin secretion in the 25 mM glucose treatment compared with the 5 mM glucose treatment. However, pretreatment with MLF at 250 and 500 mg/L increased the insulin secretion in a dose-dependent manner (Figure 3).

**Effect of MLF on Bcl-2, Bax, caspase-3 and Caspase-9 expression in high-glucose-treated INS-1 cells**

When we cultured INS-1 cells with high glucose, the expression of Bax, caspase-3 and caspase-9 significantly increased, and the expression of Bcl-2 decreased. However, cells pretreated with MLF at 250 and 500 mg/L showed decreased expression of Bax, caspase-3 and Caspase-9, and increased expression of the anti-apoptotic Bcl-2 protein (Figure 4).

**Effect of MLF on apoptosis of high-glucose-treated INS-1 cells**

MLF decreased high-glucose-induced apoptosis of INS-1 cells. To elucidate the rate of apoptosis, we used Annexin V-FITC/PI double staining analysis and flow cytometry. When we cultured the INS-1 cells with high glucose, the count of apoptotic cells markedly increased. However, pretreatment with MLF at 250 and 500 mg/L decreased this high-glucose-induced apoptosis, especially at the 500 mg/L dose. These results demonstrated that MLF protected INS-1 cells against high-glucose-induced apoptosis (Figure 5).

**DISCUSSION**

Pancreatic β cells are crucial for keeping glucose homeostasis in the body by generating insulin. Oxidative stress and the inflammatory response have been regarded as the biggest risk of apoptosis in these cells. After long exposure to a high glucose concentration, pancreatic β cells show increased ROS generation, followed by reduced activity of antioxidant enzymes, including SOD, CAT and GSH-Px, as the ROS generation greatly exceeds the capability of the antioxidant system.
thus inducing oxidative stress and triggering β cells dysfunction and apoptosis.\textsuperscript{20,21} Mulberry leaves have been used to treat diabetes for a long time in China. The Traditional Chinese Medicine dictionary mentions that mulberry leaves have anti-diabetic effect. Flavonoids, which widely exist in nature, have antioxidant and scavenging free radicals effects that have been brought to the attention of the medical world. Our previous studies have shown that MLF have hypoglycemic effects in STZ-induced diabetic rats,\textsuperscript{22,23} but the detailed mechanism is still unclear. This study revealed how MLF protects INS-1 cells against high-glucose-induced apoptosis and oxidative stress.

First, we found that pretreatment with MLF at 100, 250 and 500 mg/L had an obvious protective effect on high-glucose-treated INS-1 cells, suggesting that MLF can protect pancreatic β cells against glucotoxicity. The human antioxidant system includes enzymes such as SOD, CAT and GSH-Px. SOD is an important defense enzyme and is a biological scavenger of oxygen free radicals.\textsuperscript{24} CAT is a peroxidase, ac-

Figure 3 Effect of MLF on insulin secretion in high-glucose-treated INS-1 cells
1: 5.5 mM glucose is the control group; 2: 33.3 mM glucose is the model group; 3: 33.3 mM glucose and 125 mg/L MLF is the low-dose group; 4: 33.3 mM glucose and 250 mg/L MLF is the middle-dose group; 5: 33.3 mM glucose and 500 mg/L MLF is the high-dose group. Each value indicates the mean ± standard error of the mean of three independent experiments. \( \ast P < 0.01 \), \( \ast \ast P < 0.05 \) vs model group.

Figure 4 Effect of MLF on Bcl-2, Bax, caspase-3 and caspase-9 expression in high-glucose-treated INS-1 cells
A: immunobloting with antibody for Bax, Bcl-2, caspase-3, caspase-9. Actin was used as a protein loading control. B: densitometric analysis of Bax, Bcl-2, caspase-3, caspase-9 expression. 1: Bcl-2/Bax ratio; 2: relative expression of caspase-3; 3: relative expression of caspase-9. Control: treated with 5.5 mM glucose; model group: treated with 33.3 mM glucose; low dose MLF intervention: treated with 33.3 mM glucose and 125 mg/L MLF; medium dose MLF intervention: treated with 33.3 mM glucose and 250 mg/L MLF; high dose MLF intervention: treated with 33.3 mM glucose and 500 mg/L MLF. MLF: mulberry leaf flavonoids. Each value indicates the mean ± standard error of the mean of three independent experiments. \( \ast P < 0.01 \), \( \ast \ast P < 0.05 \), vs model group.
INS-1 cells, whereas MLF considerably increased insulin secretion in these cells. Therefore, we inferred that MLF protect INS-1 cells against apoptosis induced by high-glucose-induced cytotoxicity. Furthermore, we found that MLF protected INS-1 cells from high-glucose-induced apoptosis through restoring the ratio of Bcl-2/Bax and decreasing caspase-3 and caspase-9 expression. The flow cytometry assay showed that the ratio of apoptotic INS-1 cells was increased in high-glucose-treated cells compared with the control. However, MLF reduced the rate of apoptosis. Taken together, these results suggest that MLF protect INS-1 cells against apoptosis induced by high glucose.

Pancreatic β cells are crucial for the maintenance of glucose homeostasis by secreting insulin when there is an increase in the blood glucose level. However, glucotoxicity causes apoptosis of β cells, thus obstructing the generation of insulin. In our study, exposure to high glucose greatly reduced insulin secretion from INS-1 cells, whereas MLF considerably increased insulin secretion in these cells. Therefore, we inferred that MLF protect pancreatic β cells against the harm induced by glucotoxicity, thereby benefiting the recovery of insulin secretion. Inflammatory cytokines are important mediators of β cells demise in type 1 and type 2 diabetes. Accumulation of pro-inflammatory cytokines can result in β cells dysfunction, contributing to the insulin resistance implicated in the development of diabetes. In this study, MLF decreased the secretion of MCP-1, TNF-α and IL-6 in a concentration-dependent manner. These results suggest that MLF protects pancreatic β cells against the harm induced by glucotoxicity through its anti-inflammatory effect.

Overall, our results suggest that MLF enhanced β cell viability and insulin secretion through decreasing oxidative stress and the inflammation response. MLF inhibited high-glucose-induced apoptosis of INS-1 cells. Taken together, the results showed that MLF may be a potential therapeutic agent for preventing diabetes and related disorders.

REFERENCES

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