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Original Research

Ferulic acid confers protection on islet β cells and placental tissues of rats with gestational diabetes mellitus

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Abstract: Gestational diabetes mellitus (GDM) refers to glucose intolerance of variable degree with onset or first recognition during pregnancy. It causes polyhydramnios, ketoacidosis, fetal macrosomia, and neonatal respiratory distress syndrome. The incidence of GDM has greatly increased, hence the search for new interventions that can effectively treat it. The present study investigated the protective effect of ferulic acid on islet β cells and placental tissues of rats with GDM. Female Sprague Dawley rats (n = 30) were used in this study. The rats were randomly assigned to three groups of 10 rats each: control group, GDM group and ferulic acid group. The rats were fed high-fat diet consecutively for 12 weeks, except those in control group. In addition, ferulic acid group rats received ferulic acid at a dose of 20 mg/kg body weight (bwt) intragastrically daily for 12 weeks. The expressions of insulin signal transduction proteins and inflammatory factors were determined in rat placental tissue. Apoptosis and levels of expression of apoptosis-related proteins were assessed in isolated islet β cells. The results showed that the expressions of p-IRS1, p-IRS2, p-PI3K, GLUT1, GLUT3, and GLUT4 were significantly reduced by GDM, but were significantly upregulated after treatment with ferulic acid ($p < 0.05$). The levels of expression of NF-kB, ICAM-1, TNF-α and IL-β in placental tissues were significantly higher in *GDM* group than in control group, but were significantly reduced by ferulic acid treatment ($p < 0.05$). However, the protein expression of visfatin was significantly reduced by GDM, but was significantly increased by ferulic acid treatment ($p < 0.05$). Apoptosis was significantly promoted by GDM in islet β cells, but was significantly and concentration-dependently reduced after treatment with ferulic acid ($p < 0.05$). Gestational diabetes mellitus (GDM) also significantly down-regulated the expression of bcl-2, but markedly upregulated the expressions of bax and caspase-3 ($p < 0.05$). However, treatment with ferulic acid significantly and concentration-dependently upregulated the expression of bcl-2, but down-regulated bax and caspase-3 protein expressions ($p < 0.05$). These results indicate that ferulic acid protects β cells from GDM-induced apoptosis and improves insulin signaling in placenta of female rats with GDM.

Key words: Gestational diabetes mellitus; Ferulic acid; Islet β cells; Gene expression; Apoptosis.

Introduction

Gestational diabetes mellitus (GDM) refers to glucose intolerance of variable degree with onset or first diagnosis during pregnancy (1). It is characterized by insulin resistance accompanied by the inability of islet β cells to secrete appropriate amount of insulin to meet cellular demand (2). The incidence of GDM has greatly increased, and adversely affects the quality of life of sufferers. It predisposes pregnant women to type 2 diabetes mellitus (T2DM) (3). The pathogenesis of GDM has not been fully elucidated, although it has been reported that β cell damage plays a key role in the process (4). Oral hypoglycemic therapy remains the main treatment option for GDM. Oral hypoglycemics are antidiabetic drugs designed to help diabetic patients manage their condition (5).

Ferulic acid, a hydroxycinnamic acid, is a phenolic phytochemical found in plant cell walls, covalently bonded as side chains to biomolecules such as arabinoxylans. As a component of lignin, ferulic acid is a precursor in the biosynthesis of other aromatic compounds. Plant-derived compounds such as black cohosh and ferulic acid are widely used in Traditional Chinese Medicine (TCM) to treat several ailments. They increase coronary blood flow, protect against myocardial ischemia, and prevent platelet aggregation (6, 7). Ferulic acid has been reported to confer protection on kidneys of rats with T2DM (8). However, its effect on GDM has not been reported. This study investigated the protective effect of ferulic acid on islet β cells and placental tissues of rats with GDM.

Materials and Methods

Materials

Ferulic acid (≥ 98 %), collagenase V cell separa-

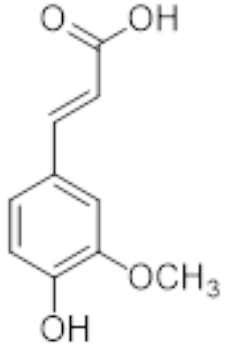

Figure 1. Chemical structure of ferulic acid.

tion solution (histopaque 1077) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (USA). High-fat feed (D12492) was obtained from Research Diet (USA). Insulin enzyme-linked immunosorbent assay (ELISA) kit was produced by Millipore (USA); RPMI-1640 medium was purchased from Gibco (USA), while phosphate-buffered saline (PBS) was product of Solarbio (China). The used antibodies for Bcl-2, Bax, Caspase 3 and β-actin were all purchased from Abcam (Cambridge, UK).

Establishment of rat model of GDM and grouping

Female Sprague Dawley rats used in this study were obtained from the Experimental Animal Department of Jilin University (No. 20190095). The rats were kept in metal cages in a well-ventilated room. Prior to the initiation of the study, the rats were acclimatized to the laboratory environment for one week under controlled conditions at a temperature of 23 ± 2 °C, humidity of 55 – 65 %, and 12 h light/12 h dark cycle. They were allowed access to standard rat feed and clean drinking water. Rat model of GDM was established according to standard methods (9). The rats were randomly assigned to three groups of 10 rats each: control group, GDM group and ferulic acid group. Rats were fed high-fat diet consecutively for 12 weeks, except those in control group. In addition, ferulic acid group rats received 20 mg/kg bwt ferulic acid intragastrically daily for 12 weeks. At the end of the 12th week, the rats were mated with male rats, and the pessary was inserted. This was recorded as day 0 of pregnancy. The treatment then continued throughout the gestation period.

Isolation of islet β cells

After modeling, the primary islet β cells of control and GDM groups were extracted and purified according to standard methods (10). The rats were fasted overnight, and anaesthetized with intraperitoneal injection of pentobarbital sodium. Rat abdominal wall was cut open under aseptic conditions. The liver was turned upside down, the duodenum was pulled down, the entrance to the common bile duct and duodenum was separated with a surgical line. The upper end of the common bile duct near hepatic hilum was ligated, and the middle of the common bile duct was inserted to the distal side using 4.5-gauge needle. After accurate insertion, a yellow-green bile reflux was observed, and the rats were sacrificed using cardiac puncture. Pre-cooled Hank solution was injected retrogradely through the common bile duct, and reperfused with 6 mL of 1 mg/mL collagenase V solution. The inflated pancreas was rapidly cut after perfusion, and it was incubated in a water bath preheated at 37 °C and digested statically for 12 min. The resultant product of pancreatic digestion was vigorously shaken, and the incompletely digested tissue was removed via filtration using 600 μm strainer. The digest was centrifuged at 1000 rpm for 2 min at 4 °C. The islet digestion product was then mixed with 10 mL of histopaque 1077 along the tube wall. This was followed by the addition of 10 mL cold Hank solution, and the purified islet cells were collected via gradient centrifugation.

Cell culture

Islet cells (2 × 10^5/mL) were seeded into 6-well plates, and cultured in RPMI 1640 medium supplemented with 10 % FBS and 1 % penicillin/streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. Cells in logarithmic growth phase were selected and used.

Determination of levels of expression of insulin signal transduction proteins in placental tissue

On the 20th day of pregnancy, the rats were euthanized and their placentas were excised and dissected. The dissected tissue was trypsinized, and the resultant cell suspension was lysed using ice-cold radio-immunoprecipitation assay (RIPA) buffer. Protein expressions of p-IRS1, p-IRS2, p-PI3K, GLUT1, GLUT3, GLUT4, NF-$_k$B, ICAM- 1, tumor necrosis factor α (TNF-α), interleukin β (IL-β) and visfatin were measured using their respective ELISA kits.

Assessment of apoptosis in islet β cell

The islet β cells were seeded in 6-well plates at a density of 3 x 10^6 cells/well, and cultured for 24 h. Then, a range of concentrations of ferulic acid (up to 20 μM) was added to the medium and incubated for another 48 h at 37 °C. The cells were thereafter washed with PBS, and thoroughly mixed with 400 μL binding buffer. The cells were subsequently stained with 5 μL of Annexin V-fluorescein isothiocyanate and 10 μL of propidium iodide within 30 min at room temperature in the dark. Cell apoptosis was assessed using a flow cytometer fitted with argon laser operated at 485 nm, and the apoptotic cell population was counted.

Western blotting

The expressions of apoptosis-related proteins in islet β cells were assessed using Western blotting. The islet β cells (2 x 10^5 cells/well) were incubated with graded concentrations of ferulic acid (0 – 20 μM) for 72 h. The cells were then washed twice with PBS, and trypsinized and lysed with 250 μL of ice-cold RIPA buffer containing protease and phosphatase inhibitors for 1 h. The resultant lysate was centrifuged at 12,000 rpm for 30 min at 4 °C, and the protein concentration of the supernatant was determined using Bradford assay. A portion of total cell protein (30 μg) from each sample was separated on 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 °C with primary antibodies of rabbit polyclonal anti-bcl-2, bax, caspase 3 and β-actin, each at a dilution of 1 to 1000. Then, the membrane was washed thrice with PBS and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using ECL. The respective protein expression levels were normalized to that of β-actin which was used as a standard.

Statistical analysis

Data are expressed as mean ± SD, and statistical analysis was performed using Graph pad Prism (7.0). Groups were compared with Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Expressions of insulin signal transduction proteins in placental tissue

The expressions of p-IRS1, p-IRS2, p-PI3K, GLUT1, GLUT3, and GLUT4 were significantly reduced by GDM, but were significantly upregulated after treatment with ferulic acid ($p < 0.05$). These results are shown in Figure 2.

Expressions of inflammatory factors in placental tissue

The levels of expression of NF-kB, ICAM-1, TNF-α and IL-β in placental tissues were significantly higher in GDM group than in control group, but were significantly reduced by ferulic acid treatment ($p < 0.05$). However, the protein expression of visfatin was significantly reduced by GDM, but was significantly increased by ferulic acid treatment ($p < 0.05$; Figure 3).

Effect of ferulic acid on apoptosis of islet β cells

As shown in Figure 4, apoptosis was significantly promoted by GDM in islet β cells, but was significantly and concentration-dependently reduced after treatment with ferulic acid ($p < 0.05$).

Effect of ferulic acid on the expressions of apoptosis-related proteins in islet β cells

Gestational diabetes mellitus significantly down-regulated the expression of bcl-2, but significantly upregulated the expressions of bax and caspase-3 ($p < 0.05$). However, treatment with ferulic acid significantly and concentration-dependently upregulated the expression of bcl-2, but down-regulated the expressions of bax and caspase-3 concentration-dependently ($p < 0.05$). These results are shown in Figure 5.

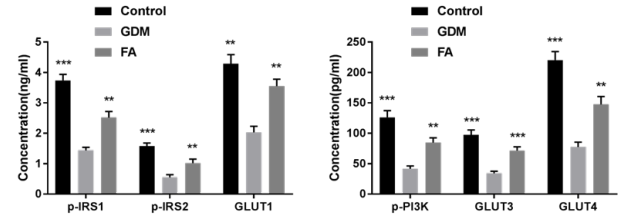

Figure 2. Effect of ferulic acid on the expressions of insulin signal transduction proteins in placenta tissue. $^{**}p < 0.05$; $^{***}p < 0.01$, when compared with *GDM* group.

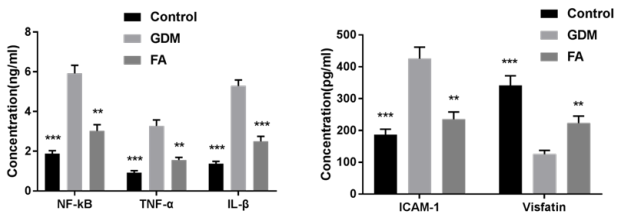

Figure 3. Effect of ferulic acid on the levels of expression of inflammatory factors in rat placenta. $^{**}p < 0.05$; $^{***}p < 0.01$, when compared with *GDM* group.

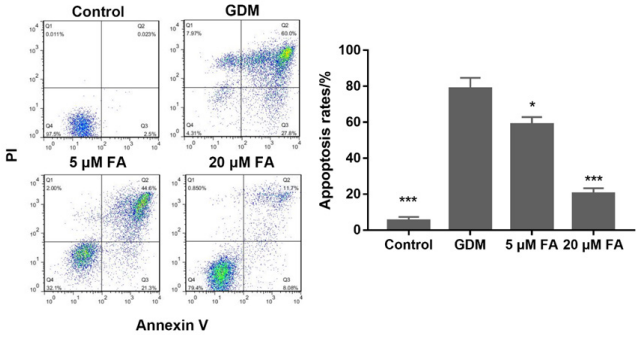

Figure 4. Measurement of apoptosis using flow cytometric analysis. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with *GDM* group.

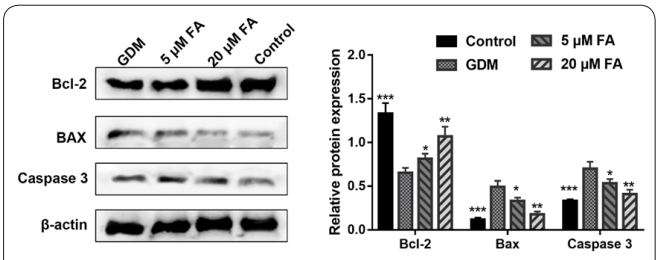

Figure 5. Effect of ferulic acid on the expressions of bax, bcl-2 and caspase-3 in islet β cells. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with *GDM* group.

Discussion

Gestational diabetes mellitus (GDM) refers to glucose intolerance of variable degree with onset or first diagnosis during pregnancy (1). Its pathological features are similar to those of T2DM (11). In China, the incidence of GDM has greatly increased, and it seriously threatens maternal and child health (12). Administration of insulin via subcutaneous route cannot effectively treat T2DM. Ferulic acid is a hydroxycinnamic acid isolated from wild celery and *Ligusticum striatum* (13). It possesses sedative, analgesic, antibacterial, anti-inflammatory, antioxidant, thrombolytic, hypolipidemic, antiatherosclerotic, and antitumor properties (14). It also improves microcirculation.

The placenta is an important accessory organ during pregnancy. It provides oxygen and nutrients for the fetus and removes waste products from the fetal blood. This organ is involved in insulin resistance that leads to GDM. Insulin receptor substrates 1 and 2 (IRS1 and IRS2) are important molecules in insulin signal transduction in the placenta. On phosphorylation, they promote the phosphorylation of downstream signal molecule phosphoinositide 3-kinase (PI3K), which in turn activates GLUTs 1, 3, and 4 (15, 16). Glucose transporter 1 (GLUT1) catalyzes the transport of glucose between the mother and fetus; GLUT3 aids glucose transport between the systemic circulation of the placenta and umbilical cord blood, while GLUT4 catalyzes the transport and metabolism of glucose in placental villi (17).

This study investigated the protective effect of ferulic acid on islet β cells and placental tissues of rats with GDM. The results showed that the expressions of p-IRS1, p-IRS2, p-PI3K, GLUT1, GLUT3, and GLUT4 were significantly reduced by GDM, but were signifi-

cantly upregulated after treatment with ferulic acid. These results suggest that abnormal insulin signal transduction mediated by IRS1 and IRS2 in the placenta may be responsible for the occurrence of GDM, and that ferulic acid may improve insulin signal transduction in placentas of rats with GDM.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is a key transcription factor in the regulation of inflammatory response. Under physiological conditions, the inactive form of the protein binds the corresponding inhibitory protein. However, under pathophysiological conditions, NF-kB is activated and dissociates from the inhibitory protein, and is translocated into the nucleus where its initiates the transcription of genes of inflammatory cytokines (18). Intercellular adhesion molecule 1 (ICAM-1), TNF-α, and IL-β are inflammatory cytokines regulated by NF-kB. The ICAM-1 mediates cell-to-cell adhesion and promotes the infiltration of inflammatory cells at the site of inflammation, thereby amplifying the inflammatory response (19). Tumor necrosis factor α (TNF- α) and IL-β directly mediate inflammatory response and inhibit the activation of IRS1 and IRS2, thereby repressing the transcription of genes of insulin signaling proteins (20, 21). Visfatin, a cytokine with insulin sensitization domain, regulates the biosynthesis of NAD^+ and increases the sensitivity of peripheral tissues to insulin (22). In this study, the levels of expression of NF-kB, ICAM-1, TNF-α and IL-β in placental tissues were significantly higher in GDM group than in control group, but were significantly reduced by ferulic acid treatment. However, the protein expression of visfatin was significantly reduced by GDM, but was significantly increased by ferulic acid treatment. It is likely that ferulic acid inhibits the inflammatory response of the placenta in rats with GDM, thereby improving insulin resistance.

In GDM, persistent insulin resistance accompanied by compensatory enhancement of secretory function of β cells leads to β cell damage (23). The mitochondria-dependent pathway of apoptosis is a key regulatory mechanism of β cell damage. Bax and bcl-2 are important regulators of mitochondria-dependent apoptosis pathway. Both molecules are localized in mitochondrial membrane. While bax forms homodimer pores and promotes the release of cytochrome c into the cytosol where it activates caspase-3 via cascade reactions, Bcl-2 forms a heterodimeric complex with bax, and suppress its action, thereby preventing the release of cytochrome c, thereby inhibiting apoptosis (24). In this study, the results of Western blotting showed that GDM significantly down-regulated the expression of bcl-2, but significantly upregulated the expressions of bax and caspase-3. However, treatment with ferulic acid significantly and concentration-dependently upregulated the expression of bcl-2, but concentration-dependently down-regulated the expressions of bax and caspase-3. These results indicate that ferulic acid may inhibit GDM-induced apoptosis in islet cells via the mitochondrial pathway.

Ferulic acid protects β cells from GDM-induced apoptosis and improves insulin signaling in placentas of female rats with GDM.

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Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Jian Zhao; Jian Zhao, Jianrong Gao, Huan Li collected and analysed the data; Jian Zhao wrote the text and all authors have read and approved the text prior to publication.

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