



Emulsified isoflurane protects beta cells against high glucose-induced apoptosis via inhibiting endoplasmic reticulum stress

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Background: Pancreatic beta cell damage induced by glucose toxicity is an important factor in type 2 diabetes mellitus (T2DM). It has become evident that endoplasmic reticulum stress (ERS)-induced apoptosis was contributed to beta cell dysfunction and insulin resistance. Our previous work showed that emulsified isoflurane (EIso) could alleviate ERS in lung reperfusion injury. This study aimed to elucidate whether EIso could alleviate apoptosis induced by glucose in rat islet RIN-m5F beta cells via inhibiting ERS.

Methods: RIN-m5F cells were divided into five groups: the control group; the 0.1G group, cultured in 0.1M glucose for 24 h; the 0.3G group, cultured in 0.3M glucose for 24 h; the 0.3G + 57E group, cultured in 0.3M glucose with 57 μ M EIso for 24 h, and the 0.3G + 76E group, cultured in 0.3M glucose with 76 μ M EIso for 24 h. First, cell proliferation was measured by MTT assay, and the level of insulin secretion was measured with enzyme-linked immunosorbent assay (ELISA) kit. Second, the expression of B cell leukemia/lymphoma 2 (Bcl-2) associated X (Bax) and Bcl-2 were detected by Western blotting. The level of caspase-3 activity was assessed by colorimetric method. Finally, the ERS marker CHOP and GRP78 expression were detected by Western blotting. The levels of activating transcription factor-6 (ATF6), X-box-binding protein 1 (Xbp1), and eukaryotic translation initiation factor-2 α (eIF2 α) mRNA were assessed by quantitative real-time polymerase chain reaction (qRT-PCR) after being treated with EIso for 24 h.

Results: We found that exposure to high glucose reduced RIN-m5F cell viability, stimulated the secretion of insulin, activated caspase-3, improved the expression of Bax, and down-regulated Bcl-2. EIso improved the survival and protected the function of RIN-m5F. Compared to the 0.3G group, treatment with EIso inhibited the activity of caspase-3, and decreased the expression of Bax. The expression of CHOP and GRP78 were significantly suppressed by EIso at 24 h in a dose-dependent manner. The level of ATF6, Xbp1, and eIF2 α mRNA of RIN-m5F were enhanced by high glucose, but only eIF2 α mRNA was significantly decreased by EIso treatment.

Conclusions: The present study suggests that high glucose induces rat islet beta cell RIN-m5F apoptosis and aggravates the function of beta cells. EIso protects beta cells against high glucose through the ERS-dependent apoptotic pathway and might serve as a potential therapy for diabetes.

Keywords: Emulsified isoflurane (EIso); endoplasmic reticulum stress (ERS); islet beta cell; glucotoxicity; apoptosis

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Introduction

Diabetes mellitus (DM) is a series of diseases characterized mainly by hyperglycemia; the most common forms of DM are type 1 DM (T1DM) and type 2 DM (T2DM). In 2013, the estimated overall prevalence of DM among adults in China was 10.9% and that of prediabetes was 35.7% (1). As a result of obesity, unhealthy diet, sedentary lifestyle, and genetic factors, the prevalence of T2DM in adolescents and young adults has been dramatically increasing (2).

In T2DM patients, during insulin resistance and impending diabetes, the pancreatic beta cell is capable of synthesizing and secreting sufficient insulin for glucohomeostasis (3). Unfortunately, the excessive synthesis of insulin triggers multiple pathological reactions (4-6), which cause progressive loss of beta cell function, insulin deficiency, and overt T2DM if there are no effective interventions. Endoplasmic reticulum stress (ERS) is an adaptive response during stress, and in T2DM patients, it can be triggered by an imbalance between the folding capacity of the endoplasmic reticulum (ER) and irreparably unfolded and misfolded proteins aggregating in the lumen from excessive proinsulin synthesis (7). Recent studies have confirmed that ERS is involved in the development of insulin resistance and progression to T2DM, and that the inhibition of ERS would protect the function of beta cells (7,8).

Emulsified isoflurane (EIso), which is an emulsified preparation of isoflurane, has marked anesthetic potency and a comparable safety index and safety features as propofol for patients and animals (9,10). Furthermore, research has demonstrated that EIso shows extensive protective effects in organs, including the liver, lung, and kidney (11,12). However, whether EIso can protect the pancreas remains poorly understood. Our previous study showed that EIso administered before ischemia could protect the lung against reperfusion injury through inhibiting ERS pathway (published in Chinese). We therefore hypothesized that EIso could contribute to increasing the beta cell mass by inhibiting ERS.

Methods

Cell culture and treatment

Rat islet beta cell line (RIN-m5F) was purchased from the China Center for Type Culture Collection (CCTCC). The

cells were cultured in RPMI-1640 (Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) at 37 °C in a 5% CO₂ atmosphere. RIN-m5F cells were then randomly divided into five doses of glucose and EIso for different cultures. EIso (8%) was obtained from the Laboratory of Anesthesiology and Critical Care Medicine, West China Hospital of Sichuan University (Chengdu, China). On attaining 70–80% confluency, cells were seeded into 6-well plates at a density of 2×10^6 and grown overnight. We chose glucose with final concentrations of 0.1M (0.1G group) and 0.3M (0.3G group) to investigate the influence of high glucose to RIN-m5F cells. Based on preliminary experiments, we used 57 μM (0.3G + 57E group) and 76 μM (0.3G + 76E group) EIso to study the EIso protection.

Cell viability assay

Indicated cells were cultured in 96-well plates, followed by drug exposure for 24 h. The rate of cell apoptosis was examined by MTT assay. Briefly, after exposure to glucose and EIso, 200 μL of MTT solution (0.5 mg/mL, Sangon Biotech, Shanghai, China) was subsequently added. After 4 h, 100 μL of dimethyl sulfoxide (DMSO) was added and mixed gently for 10 minutes. Then, the absorbance was measured at 570 nm using a microplate reader.

Insulin level assay

Cells were seeded overnight in 6-well plates and treated as indicated. Insulin levels in the culture medium were quantified using an insulin rat enzyme-linked immunosorbent assay (ELISA) kit (Beyotime Biotechnology, Nantong, China) according to the manufacturer's instructions.

Caspase-3 activity assay

Caspase-3 activity was measured using the caspase-3 Activity Assay kit (Beyotime Biotechnology, Nantong, China) following the manufacturer's instructions. In brief, the protein samples from cells were obtained and 100 μg protein was added to a reaction buffer containing Ac-DEVD-pNA (2 mM), and incubated at 37 °C for 2 h. The absorbance of yellow pNA (the cleavage product) was measured with microplate reader at 405 nm. The caspase-3 activity was recorded as the ratio to that of the control group.

Western blotting analysis

After treatment, cellular protein was extracted using RIPA lysis buffer (Beyotime Biotechnology, Nantong, China) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min on ice. The supernatant containing soluble total protein was collected after centrifuge at 12,000 rpm for 10 min at 4 °C. The bicinchoninic acid (BCA) protein assay kit (Sangon Biotech, Shanghai, China) was used to evaluate the protein concentrations. Approximately 50 µg of protein was separated by electrophoresis in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and was subsequently transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk powder in 0.01M phosphate buffered saline (PBS) (pH 7.4) and 0.05% Tween-20 at room temperature for 1 h. Subsequently, they were incubated with primary antibodies against B cell leukemia/lymphoma 2 (Bcl-2) associated X (Bax), Bcl-2, GPR78, CHOP (Absin Bioscience, Shanghai, China, 1:1,000), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz, USA, 1:1,000) overnight at 4 °C. Membranes were washed 5 times for 5 min with PBS Tween-20 (PBST) and then incubated with the appropriate HRP-conjugated secondary antibody at room temperature for 2 h. The protein expression was detected by enhanced chemiluminescence (ECL) detection kit (Absin Bioscience, Shanghai, China) using G:BOX F3 gel documentation system (Syngene, UK). Quantification of the band intensities was performed with ImageJ.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The expression of mRNA was detected by qRT-PCR. Total RNA was extracted from cells after treatment with Trizol reagents (Invitrogen, USA) according to the manufacturer's protocol. The concentration of total RNA was measured by spectrophotometer (Thermo Scientific, USA). cDNA was synthesized using a PrimeScript RT reagent kit (Takara, Japan). Then, cDNA was used as a template for qPCR with Premix Ex TaqII (Takara, Japan) on Applied Biosystems 7500 RT-PCR System (Applied Biosystems, CA, USA). The mRNA levels were normalized to GAPDH. Relative quantification was achieved by the comparative $2^{-\Delta\Delta Ct}$ method. The nucleotide sequences of the PCR primers were as follows (5' to 3'): GRPDH forward-AGTGCCAGCCTCGTCTCATA, GRPDH reverse-TACGGCCAAATCCGTTTACA;

activating transcription factor-6 (ATF6) forward-GAGTCGCCTTTTAGTCCGGT, ATF6 reverse-ACTCCCAGTCTTCACCTGGTC; X-box-binding protein 1 (Xbp1) forward-GTCCGCAGCACTCAGACTAC; Xbp1 reverse-ATCTGAAGAGGCAACAGCGT; eukaryotic translation initiation factor-2 α (eIF2 α) forward-GCAATGGAGAAAATTTGCCTTGA, eIF2 α reverse-TCATCTGACCAGGAAGGACAC (Sangon Biotech, Shanghai, China).

Statistical analysis

Data were analyzed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean \pm standard deviation. Different experimental groups were compared by Student's *t*-test. Two-tailed values of $P < 0.05$ were considered statistically significant.

Results

Elso promoted cell viability and restored the function of high glucose-induced RIN-m5F

We evaluated the effect of high glucose on the morphology of RIN-m5F cells. It was revealed that cell shrinkage appeared after exposure to 0.1M and 0.3M glucose for 24 h, and the quantity and rate of cellular attachment were reduced. Cell shrinkage and round off were significantly decreased, while the quantity and rate of cellular attachment were significantly increased when 57 or 76 µM Elso was added (*Figure 1A*).

In addition, an MTT assay was performed to investigate cell viability, and demonstrated that high glucose in the culture media induced significant loss of cell viability in a concentration-dependent manner. Treatment with the 0.1M glucose significantly decreased the viability of RIN-m5F cells compared with the control (0.9603 ± 0.052 , $P < 0.05$). Cell viability of 0.3G group was also markedly decreased compared to the control group (0.5061 ± 0.0140 , $P < 0.05$). Compared to the 0.3G group, 57 µM Elso (1.2717 ± 0.0392 , $P < 0.05$) improved the survival of RIN-m5F, and 76 µM Elso (1.4935 ± 0.0262 , $P < 0.05$) also improved the survival (*Figure 1B*), indicating that Elso improved the survival of RIN-m5F cells.

Furthermore, we assessed the impact of Elso on the insulin secretion of RIN-m5F cells, by measuring the insulin concentration in the culture medium after 24 h

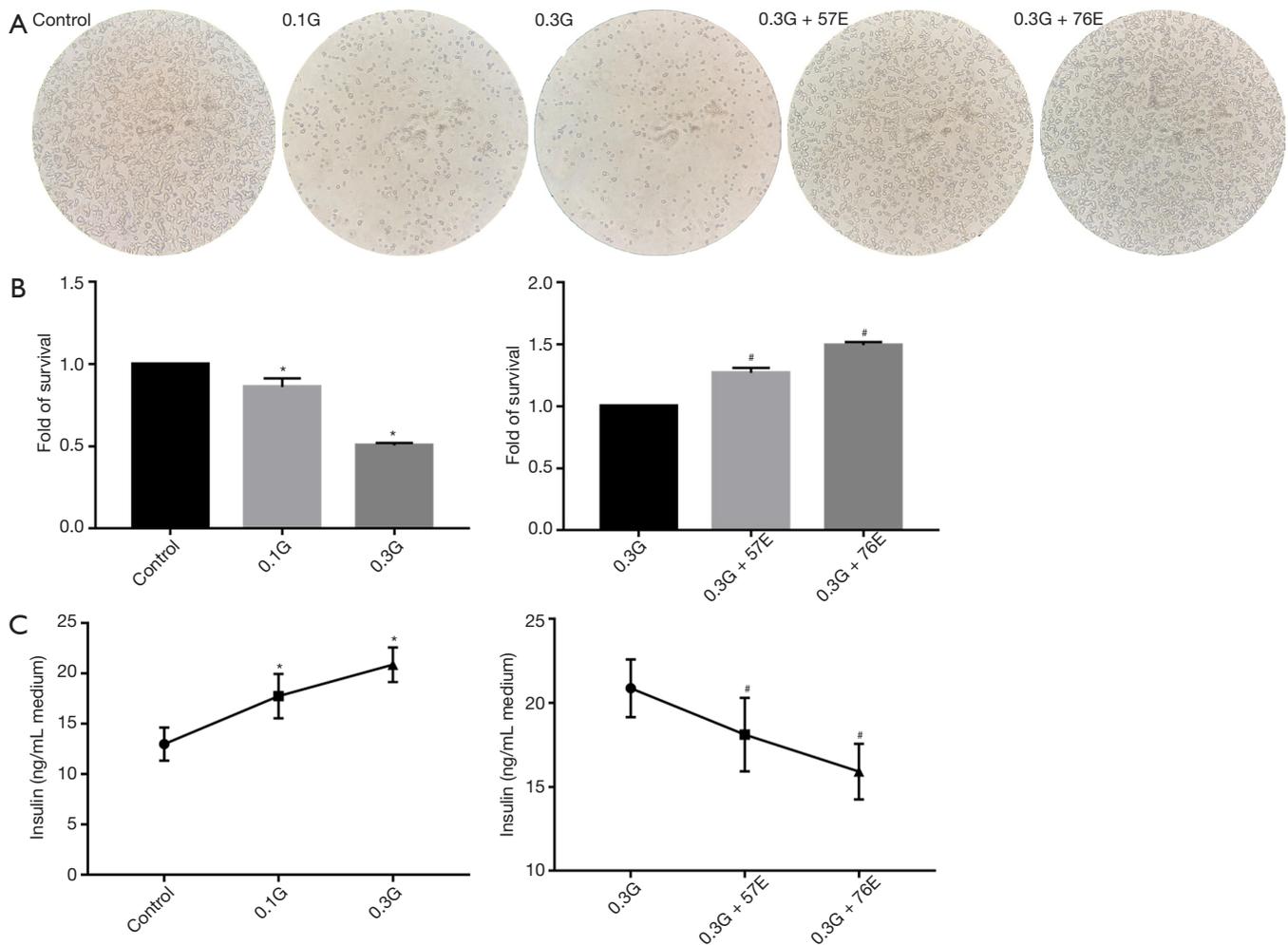


Figure 1 Viability and function of RIN-m5F after cultured with different concentrations of glucose or glucose + EIso for 24 h. (A) Microscopic images of cells taken after 24 h of treatment (10 \times); (B) viability analysis with MTT assay of RIN-m5F after treatment; (C) insulin secretion of RIN-m5F after treatment. *, $P < 0.05$ vs. the control group; #, $P < 0.05$ vs. the 0.3G group. EIso, emulsified isoflurane.

treatment. The results suggested that the insulin secretion was significantly enhanced after 24 h cultivation with 0.1M (17.76 ± 2.20 , $P < 0.05$) and 0.3M (20.88 ± 1.72 , $P < 0.05$) glucose compared with the control group (12.98 ± 1.65). In contrast, cells with 57 μ M (18.12 ± 2.19 , $P < 0.05$) and 76 μ M (15.92 ± 1.66 , $P < 0.05$) EIso maintained the insulin secretion capacity compared with the 0.3G group (Figure 1C).

Taken together, our result indicated that EIso showed protective capacity in a dose-dependent manner in high glucose-induced cell injury in RIN-m5F.

EIso protected RIN-m5F against glucose-induced apoptosis

The above results showed high glucose diminished the

viability of RIN-m5F, and the reduction was reversed by treatment with EIso. We then investigated the mechanism of high glucose damage. First, Ac-DEVD-pNA was used as the catalytic substrate for caspase-3 activity detection. Compared to the control group, caspase-3 activity was increased in the 0.1G group (1.72 ± 0.10 , $P < 0.05$), and reached a maximum value (1.97 ± 0.16 , $P < 0.05$) in the 0.3G group. After EIso treatment, compared to the 0.3G group, caspase-3 activity decreased in the 0.3G + 57E group (0.91 ± 0.02 , $P < 0.05$), and was markedly decreased in the 0.3G + 76E group (0.70 ± 0.15 , $P < 0.05$) (Figure 2A).

In addition to this, we detected the expression of biomarkers of apoptosis: Bcl-2 and Bax. Results showed that, in the 0.1G group (1.54 ± 0.11 , $P < 0.05$), the high

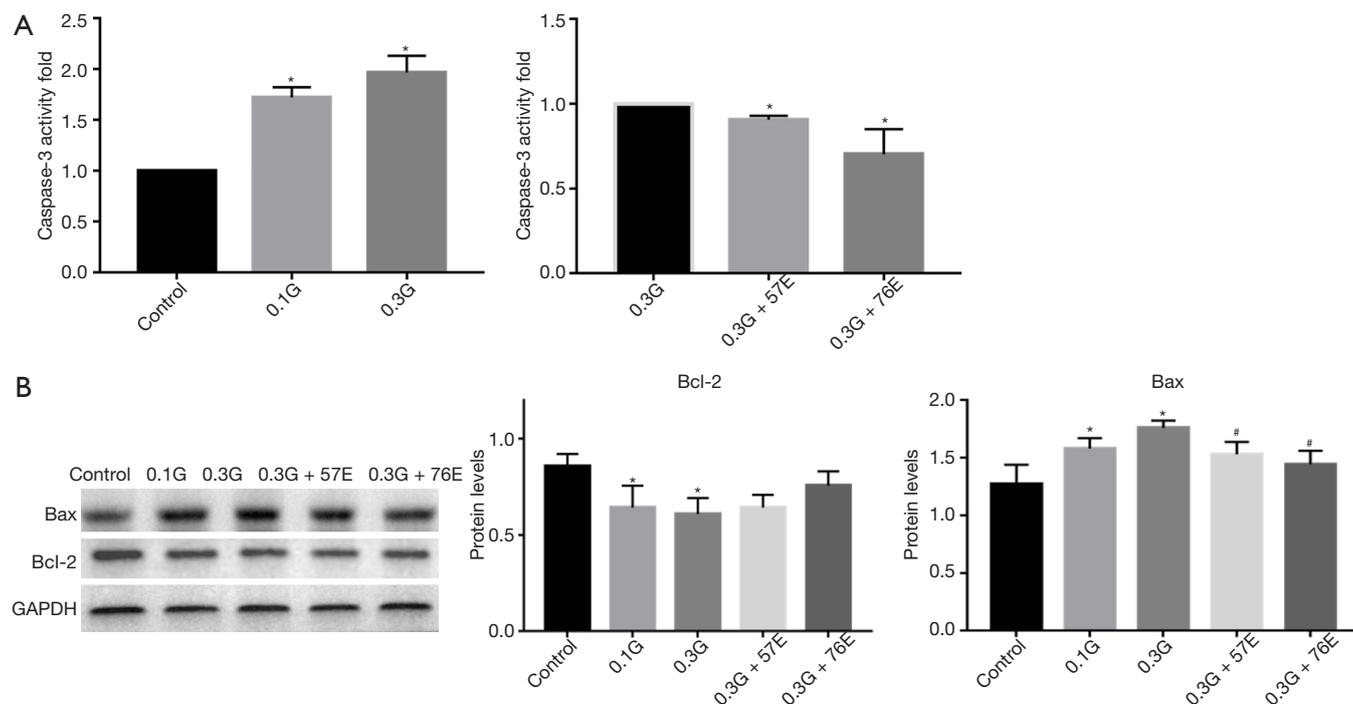


Figure 2 Apoptosis analysis of RIN-m5F with different concentrations of glucose and glucose + Elso for 24 h. (A) Caspase-3 activity analysis of RIN-m5F after glucose and glucose + Elso treatment for 24 h; (B) Bax and Bcl-2 expression of RIN-m5F after glucose and glucose + Elso treatment for 24 h. *, $P < 0.05$ vs. the control group; #, $P < 0.05$ vs. the 0.3G group. Elso, emulsified isoflurane; Bax, Bcl-2 associated X; Bcl-2, B cell leukemia/lymphoma 2.

glucose environment induced higher Bax expression than in the control group (1.27 ± 0.17), and was significantly increased in the 0.3G group (1.76 ± 0.06 , $P < 0.05$) relative to the control group. In contrast, Bax expression was inhibited by treatment with 57 μM (1.53 ± 0.11 , $P < 0.05$) and 76 μM (1.44 ± 0.12 , $P < 0.05$) Elso for 24 h compared to the 0.3G group. Compared to control group, high glucose environment induced Bcl-2 expression decrease in a concentration-dependent manner (0.86 ± 0.06 vs. 0.64 ± 0.11 , 0.86 ± 0.06 vs. 0.61 ± 0.08 , $P < 0.05$). However, no difference was detected between the 0.3G and Elso treatment groups (Figure 2B).

Elso alleviates apoptosis of RIN-m5F by inhibiting ERS

To verify the hypothesis that ERS is involved in the protection of Elso, we detected the expression of GRP78 and CHOP. Compared to the control group, the culture media enhanced the expression of CHOP in the 0.1M glucose (0.37 ± 0.06 vs. 0.61 ± 0.07 , $P < 0.05$) and the 0.3G group (0.37 ± 0.06 vs. 0.87 ± 0.07 , $P < 0.05$). The expression of

GRP78 showed no difference between the control group and the 0.1G group (0.60 ± 0.06 vs. 0.70 ± 0.08 , $P > 0.05$); however, GRP78 expression was significantly increased in the 0.3G group compared with the control group (0.60 ± 0.06 vs. 0.83 ± 0.05 , $P < 0.05$). To our excitement, all the expressions of GRP78 and CHOP were inhibited with Elso treatment for 24 h. Respectively, compared to the 0.3G group, GRP78 expression was significantly decreased in 57 μM (0.83 ± 0.05 vs. 0.66 ± 0.06 , $P < 0.05$) and 76 μM (0.83 ± 0.05 vs. 0.45 ± 0.06 , $P < 0.05$) Elso; the CHOP expression also decreased in 57 μM (0.87 ± 0.07 vs. 0.46 ± 0.10 , $P < 0.05$) and 76 μM (0.87 ± 0.07 vs. 0.31 ± 0.10 , $P < 0.06$) Elso (Figure 3A).

Finally, we detected the expression of the element of the ERS canonical signaling pathway: ATF6, Xbp1, and eIF2 α . Results showed that high glucose increased the mRNA expression of ATF6, Xbp1, and eIF2 α . Compared with the control group, mRNA expression of ATF6 increased in the 0.1G (1.53 ± 0.15 , $P < 0.05$) and in the 0.3G (1.64 ± 0.16 , $P < 0.05$) groups, while mRNA expression of Xbp1 increased in the 0.1G (1.62 ± 0.24 , $P < 0.05$) and in the 0.3G (1.77 ± 0.13 , $P < 0.05$) groups; the mRNA expression of

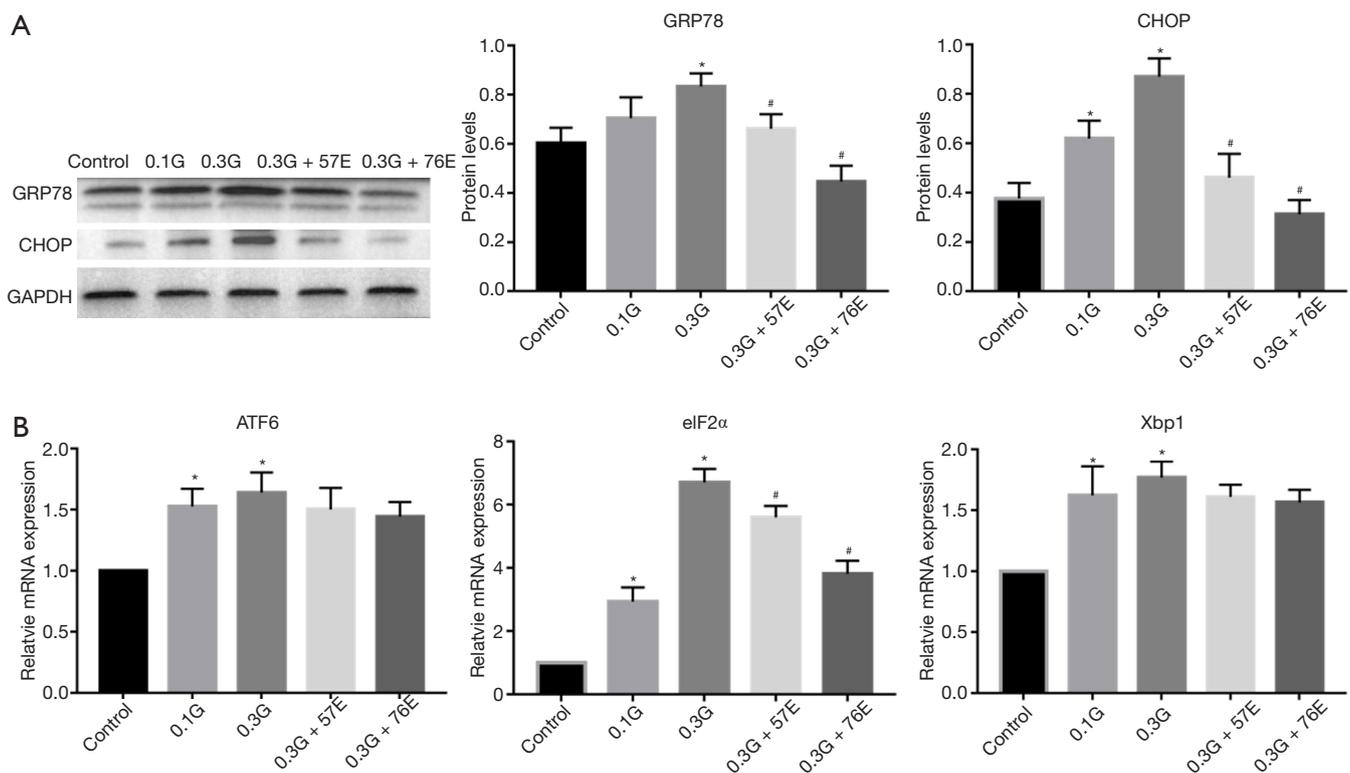


Figure 3 Analysis of ERS canonical signaling pathway of RIN-m5F with different concentrations of glucose and glucose + EIso treatment for 24 h. (A) Detection of GRP78 and CHOP expression of RIN-m5F after treatment for 24 h; (B) mRNA level analysis of ATF6, eIF2 α , and Xbp1 of RIN-m5F after glucose and glucose + EIso treatment for 24 h. *, $P < 0.05$ vs. the control group; #, $P < 0.05$ vs. the 0.3G group. ERS, endoplasmic reticulum stress; EIso, emulsified isoflurane; ATF6, activating transcription factor-6; eIF2 α , eukaryotic translation initiation factor-2 α ; Xbp1, X-box-binding protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

eIF2 α increased in the 0.1G (2.94 ± 0.44 , $P < 0.05$) and 0.3G (6.71 ± 0.43 , $P < 0.05$) groups. After being treated with EIso for 24 h, the expression of eIF2 α mRNA was inhibited in a concentration-dependent manner. Compared to the 0.3G group, the expression of eIF2 α mRNA was significantly decreased by 57 μ M EIso (6.71 ± 0.43 vs. 5.61 ± 0.36 , $P < 0.05$) and 76 μ M (6.71 ± 0.43 vs. 3.81 ± 0.41 , $P < 0.05$) EIso. However, there were no differences in the concentration of 0.3G and EIso treatment when ATF6 and Xbp1 mRNA were detected (Figure 3B).

Our data suggested that the effect of glucose-induced apoptosis in RIN-m5F cells could be restored by EIso at a certain concentration.

Discussion

Glucotoxicity, which refers to the irreversible damage of islet beta cells, plays an important role in the development

of DM (13). There is copious research concerning glucose-induced apoptosis in rodent primary islet cells, INS-1 cells, and MIN6 cells (14,15). However, few articles have given an account of rat islet beta cell RIN-m5F. Thus, in this study, we evaluated the damage of glucose on RIN-m5F. Our results showed that 0.1M and 0.3M glucose stimulated the secretion of insulin, altered the morphology, and decreased the viability within 24 h treatment. Kornelius also revealed that the exposure of RIN-m5F cells to high glucose and free fatty acid (FFA) increases apoptosis (16). While the effect of glucotoxicity on RIN-m5F is still fairly well understood, we sought clarify the protective effects of EIso against glucotoxicity.

Isoflurane is an isomeride of enflurane, which is commonly used in inhalation anesthesia, but it can also induce neurogenetic damage and neurocognitive disorder, and even accelerate the process of Alzheimer's disease (17-19). With a view to identifying the adverse effects of

inhalational anesthetic compared to intravenous anesthetic, EIso was manufactured by the West China Hospital of Sichuan University, China, and liquid isoflurane was mixed with intralipid at a final concentration of 8% (v/v). Except for marked anesthetic potency and safety, Hu demonstrated that EIso pretreatment protects the myocardial ischemia and reperfusion injury in rats, and might be mediated by the inhibition of apoptosis and cell damage (20). In this study, we found that EIso protects the morphology and enhances the viability of RIN-m5F while restoring the synthesis of insulin in 0.1M and 0.3M glucose treatment. Additionally, our results showed that EIso inhibits the activity of caspase-3. Caspase-3 is a predominant player in the execution of apoptosis, which can target key structural and regulatory proteins to bring about apoptotic cell death (21). Cao indicated that caspase-3 is critical for the induction of MIN6 cells apoptosis and its activation is further confirmed to be related to NF- κ B-mediated Bcl-2 down-regulation (22). Bcl-2 family proteins are the key to the regulation and execution of apoptosis, among which Bcl-2 is a pro-survival protein, and Bax is a pro-apoptotic protein (23). In this study, we found that high glucose-induced Bcl-2 expression decreased and Bax expression increased, but EIso down-regulated the expression of Bax and up-regulated the expression of Bcl-2. It thus seems that EIso protecting RIN-m5F against glucotoxicity may be relevant to apoptosis.

The mechanisms of apoptosis are highly complex, as they involve a cascade of molecular events. Increased protein synthesis, imbalance of ER calcium levels, glucose and energy deprivation that trigger ERS can induce beta cell failure by unfolded protein responses (UPRs) (24). GRP78 and CHOP are the best studied inducers of ERS. GRP78 is an important sensor of misfolded proteins in the ER lumen. When misfolded proteins accumulate, GRP78 is competitively bound by misfolded proteins with EIF2AK3, which results in protein kinase RNA (PKR)-like ER kinase (PERK) activation and phosphorylation of eIF2 α (25). An increased availability of GRP78 may reestablish the homeostasis of ER by attenuating UPR (26). UPR protects cells from stress and contributes to the re-establishment of ER; however, prolonged UPR promotes CHOP expression. CHOP induces the expression of growth arrest and DNA damage 34 (GADD34), activates death receptor 5 (DR5), and decreases Bcl-2 anti-apoptotic protein (27). In this study, we demonstrated that EIso increased the expression of GRP78 both in the concentration of 57 and 76 μ M with 24 h treatment. In addition, EIso decreased CHOP

expression in 57 and 76 μ M. ERS triggers downstream response by three canonical signaling sensors: PERK, inositol-requiring enzyme 1 α (IRE1 α), and ATF6 (28). Next, we detected the mRNA expression of ATF6, Xbp1, and eIF2 α . Xbp1 is a molecule that is mediated by the activation of IRE1 α . Our results showed that high glucose increased the protein expression of ATF6, Xbp1 and eIF2 α , and eIF2 α mRNA expression decreased after being treated with 57 and 76 μ M EIso. These results provide evidence indicating that EIso may protect RIN-m5F by partly inhibiting ERS.

Conclusions

The current study demonstrated the ERS-mediated apoptosis was induced by high glucose in rat islet beta cell RIN-m5F. EIso promoted RIN-m5F function and anti-apoptosis by inhibiting ERS. The application of EIso during operations on DM patients may protect the function of the pancreas and impede the stress hyperglycemia.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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