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Oxya chinensis sinuosa Mishchenko (Grasshopper) Extract Protects INS-1 Pancreatic β cells against Glucotoxicity-induced Apoptosis and Oxidative Stress

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Type 2 diabetes is a serious chronic metabolic disease, and the goal of diabetes treatment is to keep blood glucose at a normal level and prevent complications from diabetes. Hyperglycemia is a key pathologic feature of type 2 diabetes that mainly results from insulin resistance and pancreatic β-cell dysfunction. Chronic exposure of β-cells to elevated glucose concentrations induces glucotoxicity. In this study, we examined whether an 80% ethanol extract of *Oxya chinensis sinuosa* Mishchenko (OEE) protected INS-1 pancreatic β-cells against glucotoxicity-induced apoptosis and oxidative stress. Pre-treatment with a high concentration of glucose (high glucose = 30 mM) induced glucotoxicity and apoptosis of INS-1 pancreatic β cells. Treatment with OEE significantly increased cell viability. Treatment with 0.01-0.20 mg/ml OEE dose dependently decreased intracellular reactive oxygen species, lipid peroxidation, and nitric oxide levels and increased insulin secretion in high glucose-pretreated INS-1 β cells. OEE also significantly increased the activities of antioxidant enzymes in response to high-glucose-induced oxidative stress. Moreover, OEE treatment significantly reduced the expressions of pro-apoptotic proteins, including Bax, cytochrome C, caspase-3, and caspase-9, and increased anti-apoptotic Bcl-2 expression. Apoptotic cells were identified using Annexin-V/propidium iodide staining, which revealed that treatment with OEE significantly reduced high-glucose-induced apoptosis. These findings implicate OEE as a valuable functional food in protecting pancreatic β-cells against glucotoxicity-induced apoptosis and oxidative stress.

Key words : Apoptosis, INS-1 pancreatic β cells, oxidative stress, *Oxya chinensis sinuosa* Mishchenko, type 2 diabetes

Introduction

Dysfunction of pancreatic beta (β) cells is an important part of the pathogenesis of type 2 diabetes. The exact mechanism of β cell destruction remains unknown. However, oxidative stress induced by hyperglycemia is likely a major contributor to the destruction of pancreatic β cells [30]. When the pancreatic β cells are continuously exposed to high concentrations of blood glucose, the expression of the insulin gene decreases, as do insulin synthesis and secretion. This results in a malfunction of the pancreatic β cells and irreversible toxicity, which is termed glucotoxicity [28]. Therefore, protecting pancreatic β-cells from hyperglycemia is important to prevent the progression of diabetes.

Glucotoxicity causes the excess production of reactive oxygen species (ROS) through alternative metabolic pathways that induce oxidative stress in mitochondria [12]. Excess ROS causes lipid peroxidation of cell membranes and decreases insulin gene expression by altering the expression of specific genes in pancreatic β cells [26, 32]. In addition, pancreatic β cells are vulnerable to oxidative stress because of the relatively low expression of antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px) [3, 34]. Therefore, increased oxidative stress and exposure to high blood glucose ultimately increases apoptosis and adversely affects pancreatic β cells, which is evident as defective insulin secretion [8, 10, 14]. A fundamental medical treatment for diabetes does not yet exist. The best treatment method for diabetes currently is to protect pancreatic β cells and prevent diabetes complications by controlling blood glucose so it is maintained at a normal level [1, 22].

Oxya chinensis sinuosa Mishchenko (*O.* Mishchenko) is a grasshopper species belonging to the phylum Arthropoda (Order, 54 Orthoptera; Family, Acrididae; Subfamily, Oxyinae). This species is well known as a "famine relief insect" and has recently been registered as a food product in the

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Korean Food Standards Codex of the Ministry of Food & Drug Safety [16]. *O.* Mishchenko contains abundant quantities of protein, unsaturated fatty acids like linoleic acid (C18:2) and oleic acid (C18:1), micronutrients, and polyphenols. The value of *O.* Mishchenko in the treatment of various diseases is unclear. However, anti-inflammatory, cellular protective, and antioxidant effects have been described [27, 37]. However, the ability of *O.* Mishchenko in ameliorating the effects on high glucose-induced apoptosis and oxidative stress in pancreatic β cells remains unclear.

In this study, the potential protective effect of the 80% ethanol extract of *O.* Mishchenko (OEE) on glucotoxicity-induced apoptosis and oxidative stress was investigated in INS-1 pancreatic β cells. The underlying mechanisms were also investigated.

Materials and Methods

Materials

O. Mishchenko was collected from Mugapsan Mountain grasshopper farm (Gwangju, Gyeonggi-do, Korea) at late October, 2019, and pulverized using a grinder (Shinhan Science & Technology Co., Kyunggi, Korea). The sample was extracted three times with 10 volumes of 80% ethanol for 12 hr at room temperature. The filtered sample was vaporized by vacuum (BUCHI Co., Flawil, Switzerland) to obtain OEE, which was stored in a deep freezer. RPMI 1640 medium was purchased from Welgene (Gyeongsan, Gyeongsangbuk-do, Korea). Other chemicals, including fetal bovine serum (FBS), streptomycin, and penicillin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The rat/mouse insulin enzyme-linked immunosorbent assay (ELISA) kit was purchased from LINCO Research Inc. (St. Charles, MO, USA). EZ-SOD, EZ-Catalase, and EZ-Glutathione assay kits were purchased from DoGenBio (Seoul, Korea). Antibodies against Bcl-2, Bax, cytochrome c, Caspase-9, and Caspase-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

INS-1 pancreatic β cells were cultured in RPMI 1640 medium (Welgene, Gyeongsan, Gyeongsangbuk-do, Korea) supplemented with 10% FBS, streptomycin (100 μg/ml), penicillin (100 units/ml), and 50 μM β-mercaptoethanol at 37℃ in a humidified atmosphere containing 5% CO_2.

Cell viability assay

Cell viability was assessed using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The assay measures the conversion of yellow MTT to purple formazan by mitochondrial enzymes in viable cells. Cells (2×10^4 per well) were pre-incubated with glucose (5.5 or 30 mM) in the wells of a 96-well plate for 48 hr and then incubated with 0, 0.01, 0.05, 0.1, 0.15, and 0.2 mg/ml OEE for 48 hr. MTT solution (1 mg/ml, 100 μl) was added to each well prior to incubation for 4 hr at 37℃. One hundred microliters of dimethyl sulfoxide (Sigma-Aldrich) was added to dissolve the formazan crystals in the viable cells. The absorbance of each well was measured at 540 nm using a microplate reader.

Assay of intracellular ROS

Intracellular ROS levels were measured using a dichlorofluorescein assay. In the assays 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), a cell-permeable and nonfluorescent probe, is deacetylated and reacts quantitatively with intracellular radicals (mainly hydrogen peroxide, H_2O_2). DCF-DA is converted to a fluorescent product, DCF, which is retained within the cells. The assay was used to evaluate the generation of ROS under oxidative stress. Cells (2×10^4 per well) were pre-incubated with 5.5 or 30 mM glucose in the wells of a 96-well plate for 48 hr before incubation with 0, 0.01, 0.05, 0.1, 0.15, and 0.2 mg/ml OEE for 48 hr. The cells were washed with phosphate-buffered saline (PBS) and incubated with 5 μM DCF-DA for 30 min at room temperature. Finally, fluorescence was measured using a model LS-3B fluorescence plate reader (Perkin-Elmer, Waltham, MA, USA).

Assay of lipid peroxidation

Lipid peroxidation was assessed by measuring the production of thiobarbituric acid reactive substances (TBARS). Cells (2×10^4 per well) were pre-incubated with 5.5 or 30 mM glucose in the wells of a 96-well plate for 48 hr, before incubation with 0, 0.01, 0.05, 0.1, 0.15, and 0.2 mg/ml OEE for 48 hr. Two hundred microliters of each sample was mixed with 400 μl of TBARS solution then heated at 95℃ for 20 min. The absorbance was measured at 532 nm. The TBARS concentration was determined using a standard curve constructed with serial dilutions of 1,1,3,3-tetraethoxypropane serial dilution standard curve. The TBARS values are expressed as equivalent nanomoles of malondialdehyde

(MDA).

Assay of nitric oxide (NO)

Cells (2×10^4 per well) were pre-incubated with 5.5 or 30 mM glucose in the wells of a 96-well plate for 48 hr, before incubation with 0, 0.01, 0.05, 0.1, 0.15, and 0.2 mg/ml OEE for 48 hr. NO levels in the supernatant were measured using the Griess reaction. Aliquots of cell culture media (50 µl) were mixed with an equal volume of Griess reagent (0.1% N-(1-naphthyl)-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) and incubated at room temperature for 10 min. Absorbance was measured at 550 nm using a microplate absorbance reader. A series of known concentrations of sodium nitrite were used as standards.

Antioxidant enzyme assay

Cells were pre-incubated with 5.5 or 30 mM glucose in 10-mm dishes for 48 hr and then incubated with 0, 0.01, 0.05, 0.1, 0.15, and 0.2 mg/ml OEE for 48 hr. Cells were washed with cold PBS, centrifuged, and the supernatant was discarded. To assess SOD and catalase activities, the cell pellet was sonicated on ice in 1-2 ml cold PBS and 1 mM EDTA, and centrifuged at 10,000× g for 15 min at 4℃. The cell supernatant was collected to assess SOD and catalase activities using EZ-SOD and EZ-catalase assay kits, respectively (DoGenBio). The reaction medium was composed of 171 mM potassium phosphate buffer, 4.28 mM sodium azide, 2.14 mM EDTA, 6 mM reduced glutathione, 0.9 mM NADPH, and 2 U/ml glutathione reductase. The reaction at 22℃ (±1) was initiated with the addition of 0.72 mM H_2O_2. The absorbance of the samples was measured at 340 nm using a spectrophotometer. To assess glutathione content, each cell pellet was resuspended in 200-500 µl ice-cold 5% MPA and homogenized. Each suspension was transferred to a microfuge tube and centrifuged at 12,000 rpm for 5 min at 4℃. The cell supernatant was collected and the glutathione content was determined using the EZ-Glutathione assay kit (DoGenBio).

Glucose-Stimulated Insulin Secretion (GSIS)

Cells (2×10^4 per well) were pre-incubated with 5.5 or 30 mM glucose in the wells of a 96-well plate for 48 hr before incubation with 0, 0.01, 0.05, 0.1, 0.15, and 0.2 mg/ml OEE for 48 hr. The cell culture medium was carefully removed before washing the cells with PBS. Fresh medium containing 3 mM glucose and 2% FBS was added. After 5 hr, cells were stimulated with Krebs - Ringer buffer (119 mM NaCl, 4.75 mM KCl, 2.54 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, and 20 mM HEPES, pH 7.4) containing 5 mM glucose for 60 min at 37℃. The insulin secreted into the collected fluid was determined using the rat/mouse insulin ELISA kit (LINCO Research Inc.).

Western blot analysis

Cells (2×10^4 per well) were pre-incubated with 5.5 or 30 mM glucose in wells of a 96-well plate for 48 hr before incubation with 0, 0.01, 0.05, 0.1, 0.15, and 0.2 mg/ml OEE for 48 hr. Cell lysates were prepared using ice-cold lysis buffer containing 250 mM NaCl, 25 mM Tris - HCl (pH 7.5), 1% (v/v) NP-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (10 µg/ml aprotinin, 1 µg/ml leupeptin). Cell lysates were washed by centrifugation and the protein concentrations were determined using a BCA protein assay kit (Bio-Rad, Hercules, CA, USA). The lysate containing 20 µg of protein was electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel. The separated proteins were transferred to nitrocellulose membranes. The membranes were incubated separately with antibodies against Bax, Bcl-2, cytochrome c, caspase-9, caspase-3, or β- actin in Tris buffered saline Tween (TBST; 25 mM Tris - HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.4) containing 5% skim milk for 2 hr. The membranes were then washed with TTBS and incubated with secondary antibodies. Signals from the secondary antibodies were detected using the enhanced chemiluminescence (ECL) Western blotting detection kit (Bio-Rad) and images were captured using X-ray films. Relative protein expression was quantified by densitometric means using Multi Gauge v3.1 (FujiFilm, Tokyo, Japan) and calculated according to the reference β-actin bands.

Flow cytometry assessment of apoptosis

Death by apoptosis was quantified in 5.5 mM and 30 mM glucose-treated cells in the absence or presence of OEE using Annexin-V/propidium iodide (PI) staining. One hundred microliters of a cell suspension was incubated with 5 µl Annexin-V and 5 µl PI. This mixture was kept in the dark for 15 min in an ice bath. Four hundred microliters of binding buffer was added to the cell suspension and flow cytometry analysis was immediately performed using a FACSCalibur device and Cell Quest software (Becton Dickinson, San Jose, CA, USA). Apoptotic cells were ex-

pressed as a percentage of the total number of cells.

Statistical analyses

Each experiment was performed in triplicate. Results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA). Treatment groups were compared by one-way analysis of variance (ANOVA) followed by a post hoc Duncan's multiple-range test. A p-value < 0.05 was considered statistically significant.

Results

Effect of OEE on cell viability

The effect of OEE on the viability of INS-1 pancreatic β cells was investigated using the MTT assay (Fig. 1). When pancreatic β cells were exposed to 30 mM glucose, cell viability was significantly reduced to 34.73% compared to cells treated with 5.5 mM glucose. However, treatment with 0.01, 0.05, 0.10, 0.15, and 0.20 mg/ml OEE significantly increased the viability rates to 39.68, 41.49, 45.12, 50.11 and 67.57%, respectively. These results indicated that OEE protects pancreatic β cells against cell damage induced by high glucose.

Effect of OEE on intracellular ROS

In pancreatic β cells treated with 30 mM high glucose, the level of intracellular ROS was significantly increased by 197.81% compared with cells treated with 5.5 mM normal glucose (Fig. 2A). However, following treatment with OEE, ROS levels decreased in a dose-dependent manner. Treatment with 0.01, 0.05, 0.10, 0.15, and 0.20 mg/ml OEE significantly reduced high glucose-induced ROS production (194.85, 175.99, 163.34, 148.01, and 141.06%, respectively). Thus, OEE significantly reduced intracellular ROS excessively produced in response to high glucose levels in pancreatic β cells.

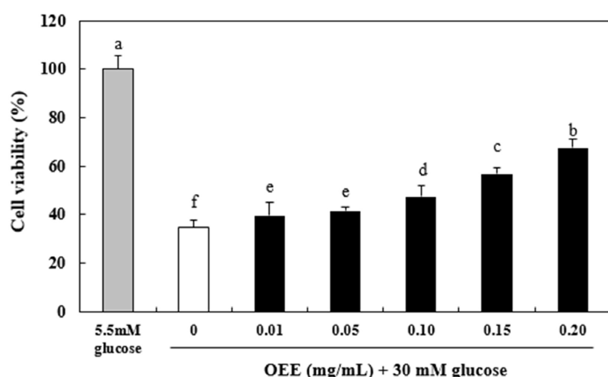

Fig. 1. Effect of OEE on cell viability in INS-1 β cells treated with a high concentration of glucose. INS-1 β cells (2×10^4 per well) were pre-incubated in 96-well plates with 5.5 or 30 mM glucose for 48 hr. The cells were then incubated with 0.01, 0.05, 0.1, 0.15, and 0.2 mg/ml OEE for 48 hr. The 5.5 mM and 30 mM concentrations of glucose represents normal and high glucose levels, respectively . Each value is expressed as the mean ± SD (n= 3). $^{a-f}$Values are significantly different at $p<0.05$, as analyzed by Duncan's multiple-range test.

Effect of OEE on generation of TBARS

The effect of OEE on lipid peroxidation was investigated by measuring the lipid peroxidation product TBARS (Fig. 2B). The level of TBARS in cells treated with 5.5 mM glucose was measured as 0.13 nmol MDA. The TBARS level in cells treated with 30 mM glucose was significantly increased to 0.27 nmol MDA. However, treatment with 0.01, 0.05, 0.10, 0.15, and 0.20 mg/ml OEE significantly reduced high glucose-induced TBARS generation (0.22, 0.21, 0.19, 0.17, and 0.16 nmol MDA, respectively). The findings indicated that OEE significantly inhibited TBARS formation, indicating the inhibition of lipid peroxidation.

Effect of OEE on NO level

Treatment of INS-1 pancreatic β cells with 30 mM glucose significantly increased NO levels to 183.11% compared with control cells treated with 5.5 mM glucose (Fig. 2C). However, cells pretreated with OEE before exposure to 30 mM glucose displayed decreased levels of NO in an OEE dose-dependent manner. The NO production induced by the high glucose concentration in the 0.01, 0.05, 0.10, 0.15, and 0.20 mg/ml OEE pretreated cells was 174.67, 161.40, 130.04, 111.96, and 108.32%, respectively. Thus, OEE progressively inhibited NO production caused by high glucose.

Effect of OEE on SOD, catalase, and GSH-Px activities

Oxidative stress is caused by glucotoxicity and can be alleviated by antioxidant enzymes [34]. In this study, the effect of OEE on SOD, catalase, and GSH-Px activities was examined. As shown in Table 1, the activities of antioxidant enzymes, including SOD, catalase, and GSH-Px, were significantly reduced in INS-1 pancreatic β-cells exposed to 30 mM glucose compared with 5.5 mM glucose. However, treatment with OEE in INS-1 β-cells exposed to 30 mM glucose significantly increased the activities of SOD, catalase, and GSH-

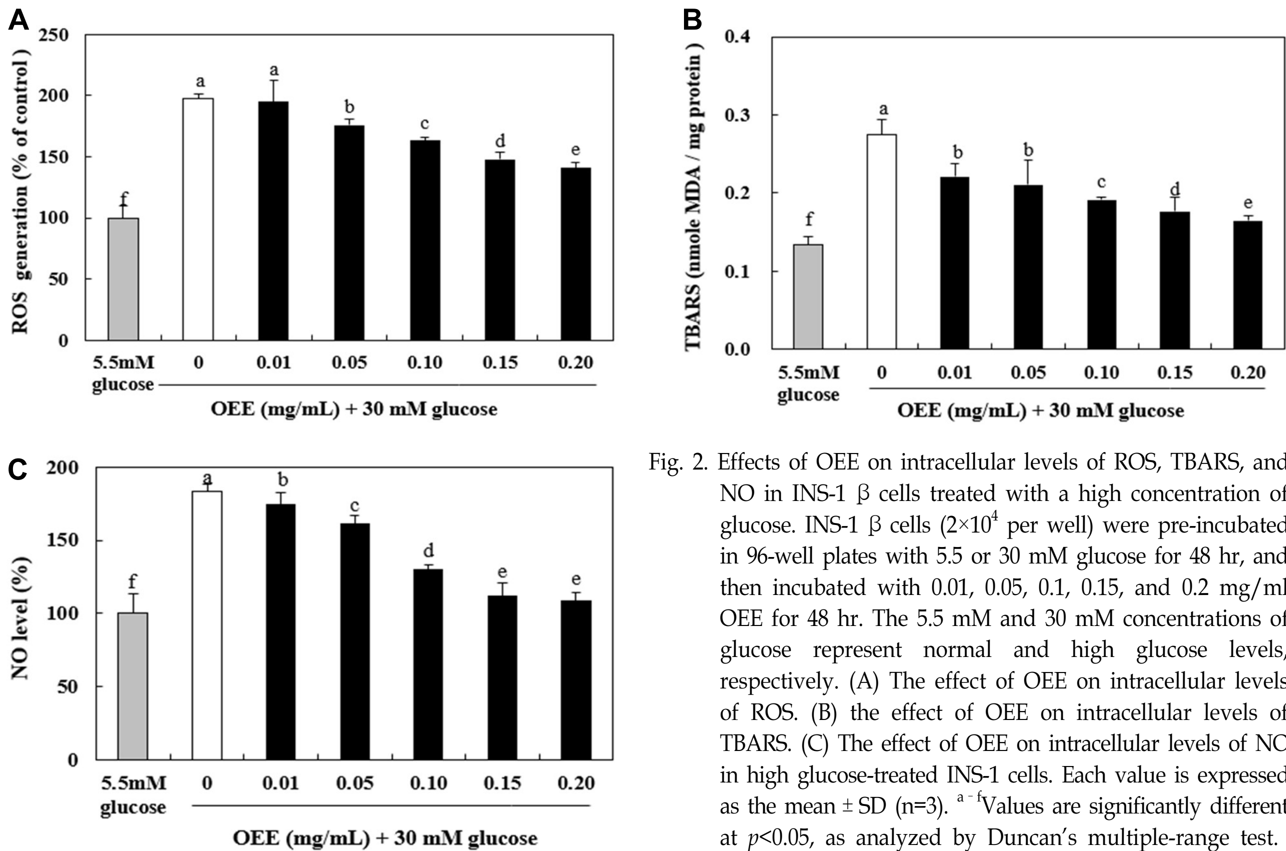

Fig. 2. Effects of OEE on intracellular levels of ROS, TBARS, and NO in INS-1 β cells treated with a high concentration of glucose. INS-1 β cells (2×10^4 per well) were pre-incubated in 96-well plates with 5.5 or 30 mM glucose for 48 hr, and then incubated with 0.01, 0.05, 0.1, 0.15, and 0.2 mg/ml OEE for 48 hr. The 5.5 mM and 30 mM concentrations of glucose represent normal and high glucose levels, respectively. (A) The effect of OEE on intracellular levels of ROS. (B) the effect of OEE on intracellular levels of TBARS. (C) The effect of OEE on intracellular levels of NO in high glucose-treated INS-1 cells. Each value is expressed as the mean ± SD (n=3). [a-f]Values are significantly different at p<0.05, as analyzed by Duncan's multiple-range test.

Table 1. Effects of OEE antioxidant enzymes in high glucose-treated INS-1 cells

	5.5 mM glucose	30 mM glucose + OEE (mg/ml)		
		0	0.1	0.2
SOD (U/ml)	28.15±1.12^a	13.19±0.68^d	15.4±0.47^c	26.58±1.06^b
Catalase (U/ml)	3.89±0.06^a	1.24±0.03^d	1.83±0.05^c	3.41±0.07^b
GSH-Px (U/ml)	2.62±0.03^a	1.12±0.03^d	1.74±0.10^c	2.30±0.09^b
GSH contents (μM)	6.29±0.04^a	1.98±0.11^e	2.87±0.12^d	4.68±0.06^c

INS-1 cells (2×10^4 per well) were pre-incubated in 96-well plates with 5.5 or 30 mM glucose for 48 hr, and then incubated with 0.1 and 0.2 mg/ml OEE for 48 hr. The 5.5 mM and 30 mM concentrations of glucose represent normal and high glucose levels, respectively. Each value is expressed as the mean ± SD (n=3). [a-d]Values are significantly different at p<0.05, as analyzed by Duncan's multiple-range test.

Px in a dose-dependent manner. After treatment with 0.20 mg/ml OEE, SOD and catalase and GSH-Px activities were significantly increased to 26.58 U/ml, 3.41 U/ml, and 2.30 U/ml, respectively. Furthermore, total GSH content was significantly reduced to 1.98 mg/ml following treatment with 30 mM glucose. In contrast, 0.20 mg/ml OEE treatment significantly increased GSH content to 4.68 mg/ml.

Effect of OEE on apoptosis-related protein expression

To investigate whether OEE could alter the expression of genes associated with high glucose-induced apoptosis. Protein levels of Bax, Bcl-2, cytochrome *C*, caspase-9, and caspase-3 were measured by Western blotting. As shown in Fig. 3, the phosphorylation levels of Bax, cytochrome c, caspase-9, and caspase-3 were significantly increased in pancreatic β-cells treated with high glucose. In contrast, expression of the anti-apoptotic protein Bcl-2 was significantly decreased. Treatment with 0.1 and 0.2 mg/ml OEE significantly decreased the levels of Bax, cytochrome c, caspase-9 and caspase-3, and significantly increased levels of Bcl-2. These results indicated that treatment with OEE decreases pro-apoptotic protein expression and increases anti-apop-

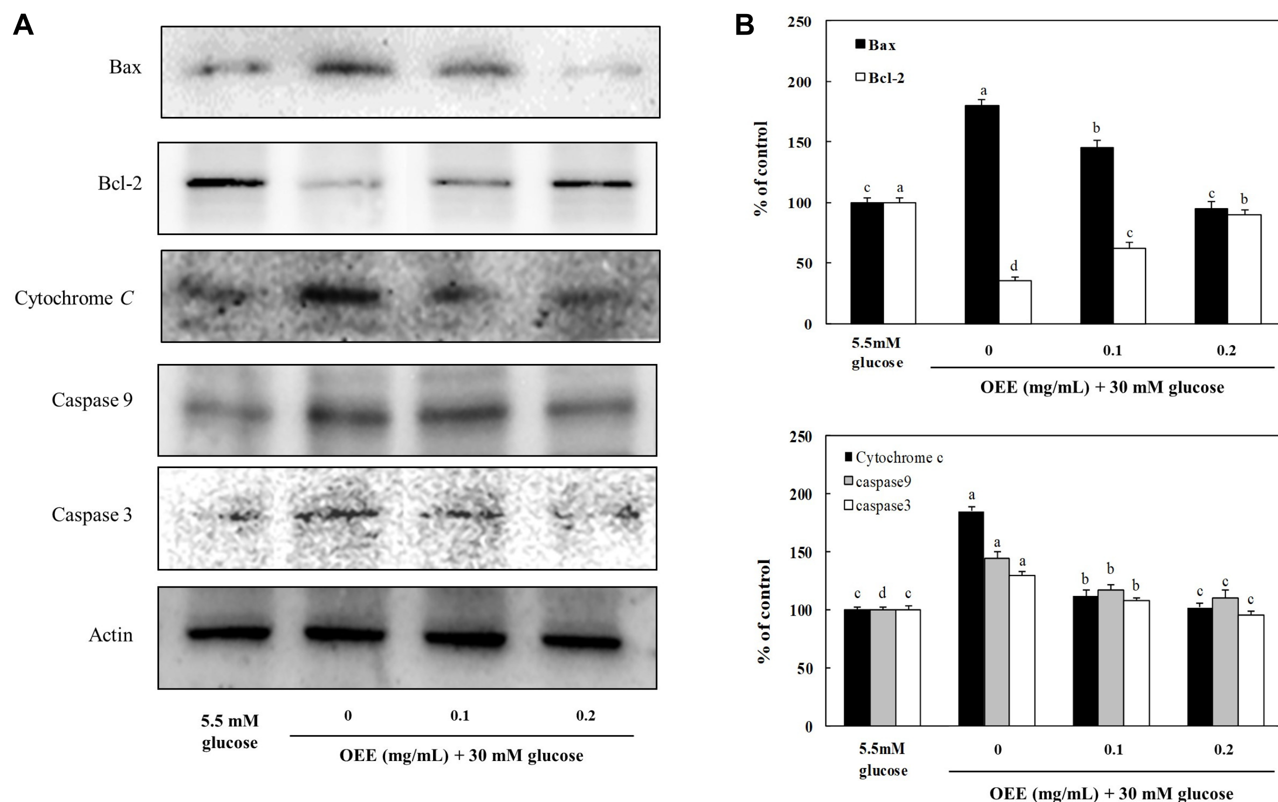

Fig. 3. Effects of OEE on Bax, Bcl-2, cytochrome C, caspase-9, and caspase-3 protein expression in high glucose-treated INS-1 β cells. INS-1 β cells were pre-incubated in 96-well plates with 5.5 or 30 mM glucose for 48 hr, followed by 0.1 and 0.2 mg/ml OEE for 48 hr. The 5.5 mM and 30 mM concentrations of glucose represents normal and high glucose levels, respectively. Equal amounts of cell lysates were electrophoresed and analyzed for Bax, Bcl-2, cytochrome C, caspase 9, and caspase 3 protein using Western blots. β-actin was used as an internal control. (A) Bax, Bcl-2, cytochrome C, caspase-9, and caspase-3 protein expression. (B) expression levels of Bax, Bcl-2, cytochrome C, caspase-9, and caspase-3. Each value is expressed as the mean ± SD (n=3). [a-d]Values are significantly different at $p<0.05$, as analyzed by Duncan's multiple-range test.

totic protein expression in cells damaged by exposure to high glucose.

Cell apoptosis

After treatment with OEE, the apoptosis rate in INS-1 pancreatic β-cells was assessed by flow cytometry using annexin-V/PI staining. The early and late stages of apoptosis are shown in Fig. 4 at the bottom and top of the right quadrant, respectively. Under high glucose conditions (30 mM), the level of apoptosis was significantly increased compared with that in cells treated with normal glucose (5.5 mM). However, treatment with 0.1 and 0.2 mg/ml OEE reduced the rates of apoptosis in cells pretreated with 30 mM glucose. When INS-1 pancreatic β-cells were treated with 30 mM glucose alone, the rate of apoptosis was 57.48%. However, the rate of apoptosis decreased to 31.05% following treatment with 0.2 mg/ml OEE. These findings demonstrated that OEE protected INS-1 pancreatic β cells from apoptosis induced by exposure to high glucose.

Effect of OEE on insulin secretion

Insulin secretion was significantly decreased as a result of treatment with 30 mM glucose versus 5.5 mM glucose in INS-1 pancreatic β cells (Fig. 5). Treatment of INS-1 pancreatic β cells with 0.01, 0.05, 0.10, 0.15, and 0.20 mg/ml OEE increased the level of insulin secretion to 7.20, 9.85, 11.86, 14.36, and 16.00 ng/hr, respectively. Thus, OEE increased insulin secretion in high glucose-pretreated INS-1 pancreatic β cells.

Discussion

In type 2 diabetes, chronic hyperglycemia causes pancreatic β cell failure, leading to impaired insulin secretion [21].

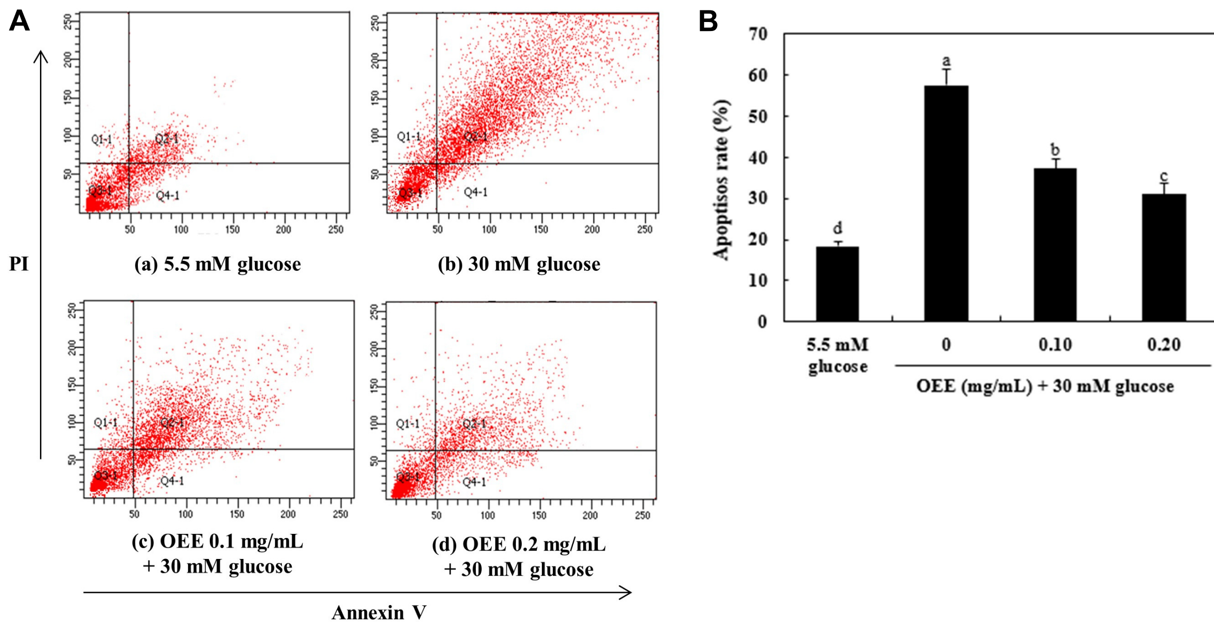

Fig. 4. Identification of the apoptotic stage of INS-1 β cells with different treatments using Annexin-V/PI staining. (A) Apoptotic cell death was determined by flow cytometry enumeration of INS-1 β cells stained with Annexin-V-fluorescein isothiocyanate/PI. Cells were pre-incubated with glucose and then incubated in the presence or absence of 0.1 and 0.2 mg/ml OEE. The right lower and upper quadrants show the numbers of early and late apoptotic cells, respectively. (B) Mean rates of apoptosis. $^{a-d}$Values are significantly different at p<0.05, as analyzed Duncan's multiple-range test.

This is associated with hyperglycemia-induced ROS production [31]. Hyperglycemia can induce excessive formation

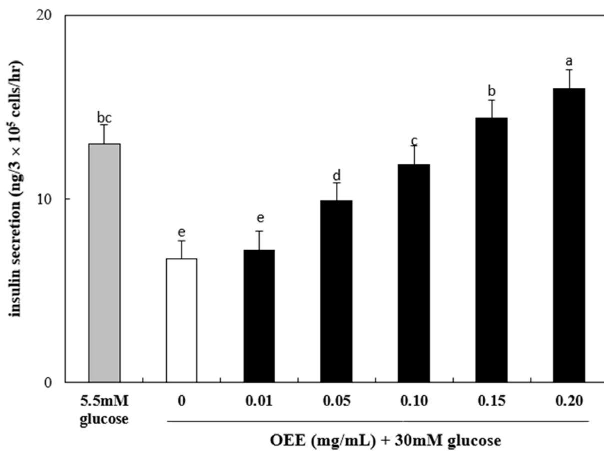

Fig. 5. Effect of OEE on insulin secretion in high glucose-treated INS-1 β cells. INS-1 β cells (2×10^4 per well) were pre-incubated in 96-well plates with 5.5 or 30 mM glucose for 48 hr, and then incubated with 0.01, 0.05, 0.1, 0.15, and 0.2 mg/ml OEE for 48 hr. Thereafter, the cells were stimulated with Krebs–Ringer buffer containing 5 mM glucose for 60 min. The 5.5 mM and 30 mM concentrations of glucose represent normal and high glucose levels, respectively. Each value is expressed as the mean ± SD (n=3). $^{a-e}$Values are significantly different at p< 0.05, as analyzed by Duncan's multiple-range test.

of ROS that eventually leads to oxidative stress. This damages β cells that subsequently produce relatively low levels of antioxidant enzymes like catalase and GSH-Px. The oxidative stress caused by high levels of glucose should be alleviated to lessen or prevent pathological damage of β cells [11]. *O.* Mishchenko is an edible grasshopper, which contains abundant amounts of protein, unsaturated fatty acids, trace nutrients, and pigments that include chlorophyll, carotenoids, and polyphenols [17]. *O.* Mishchenko has been used in traditional anti-diabetic medicines [37]. Therefore, we examined the effect of *O.* Mishchenko in protecting pancreatic β-cells against oxidative stress and apoptosis induced by elevated concentrations of glucose.

Recently, there has been some focus on the toxic effects of fluctuating high glucose on pancreatic β-cell functions and survival [4]. Presently, OEE on cell viability in INS-1 pancreatic β cells was first examined using the MTT assay. Exposure of β-cells to high glucose levels significantly decreased cell viability, but OEE treatment recovered their viability rate. This result demonstrated that OEE protected INS-1 pancreatic β cells against high glucose-induced cytotoxicity .

β cells exposed to high concentrations of glucose concentrations increase their production of ROS due to the over-

production of electron donors by the tricarboxylic acid cycle. This leads to increased oxidative stress [31]. Excessive cellular levels of ROS can interact with nearby cellular components that include proteins, DNA, lipids, and cellular organs like mitochondria. These interactions cause wide-ranging damage [34]. In this study, treatment of INS-1 β cells with a high concentration of glucose (30 mM) resulted in the significant increase of intracellular ROS levels. Pretreatment of cells with OEE inhibited the production of ROS. The damage to cellular components by excess ROS particularly includes RNA, potentially leading to gene expression changes [19]. Therefore, our results revealed that OEE could prevent high glucose-induced pancreatic β cell damage through the reduction of ROS generation.

Freeze-dried *O.* Mishchenko was shown to contain 14.06 - 17.61 mg or polyphenols per 100 g of sample [17, 15]. OEE contains polyphenolic compounds, such as pyroglutamic acid, oxalic acid, and cinnamic acid [2]. These polyphenolic compounds exhibit strong antioxidant activity and act as hydrogen or electron donating agents. They are considered one of the most important classes of natural antioxidants [36]. Thus, the ROS scavenging effect of OEE in β cells seems to be due to the polyphenolic constituents of OEE.

ROS also cause lipid peroxidation and formation of reactive lipid aldehyde. These contribute to the progressive deterioration of β-cell function in type 2 diabetes. Lipid peroxidation can lead to cell apoptosis and has been implicated in a number of pathophysiological conditions [13]. In this study, treatment with a high concentration of glucose-induced lipid peroxidation in INS-1 β cells. Treatment with OEE effectively inhibited TBARS formation likely due to radical scavenging and anti-peroxidative activity of the extract. Polyphenol compounds in *O.* Mishchenko reportedly suppress lipid peroxidation in a concentration-dependent manner [9]. Cinnamic acid and its derivatives are among the most abundant groups of compounds found in grasshoppers. These compounds may at least partly contribute to the inhibition of TBARS formation [2]. The ethanol extract of *Brachytrupes orientalis* (Gryllidae), another common edible insect species consumed by different tribes of North East India, reduces lipid peroxide in the liver [7]. Since ethanol is a polar protic solvent, it extracts polar compounds better than other solvents, such as water extract. The polyphenol compounds in the ethanol extract were reported to potently inhibit lipid peroxide. Therefore, the inhibitory effect of OEE on lipid peroxide seems likely to be due to the polyphenol compounds (pyroglutamic acid, oxalic acid, cinnamic acid, etc.) contained in the extract.

Hyperglycemia rapidly increases NO production, which can affect the function of β-cells [6, 23]. In addition, hyperglycemia induces the production of superoxide (O^-_2) in the presence of NO and forms peroxynitrite ($ONOO^-$), a powerful oxidizing agent [25]. This response leads to β-cell death during the development of type 2 diabetes and impaired glucose tolerance [20]. In this study, exposure to a high concentration of glucose significantly elevated NO levels in INS-1 pancreatic β-cells. However, OEE treatment significantly reduced NO production, suggesting that OEE may protect β-cells under hyperglycemic conditions.

Antioxidant enzymes, such as SOD, catalase, and GSH-Px, are important in removing toxic intermediates generated by oxidative stress caused by glycotoxicity *in vivo*. If hyperglycemia persists, it inhibits the activities of antioxidant enzymes due to impaired regulation of the antioxidant enzyme system [29, 33]. Presently, treatment of INS-1 pancreatic β cells with a high concentration of glucose reduced SOD, catalase, and GSH-Px activities. Treatment with OEE significantly restored these antioxidant enzyme activities. SOD protects cells from oxidative damage by converting O^-_2 radicals to H_2O_2 and oxygen. H_2O_2 induced by SOD is detoxified by catalase and eliminated by GSH-Px [20].

The use of extracts from natural edible insects to increase the activities of antioxidant enzymes has become a research interest. In one study, administering silkworm to rats resulted in increased SOD and GSH-Px activities in liver tissue [24]. In another study, SOD and GSH-Px activities were also increased in rats given *O.* Mishchenko extract [18]. *O.* Mishchenko extract is rich in unsaturated fatty acids such as linoleic acid (C18:2) and oleic acid (C18:1). These unsaturated fatty acids prevent oxidative stress by increasing the activities of biological defense antioxidant enzymes, such as SOD and GSH-Px [18]. Therefore, the effects of OEE on increasing antioxidant enzyme activity noted above might be due to these components.

ROS cause cellular oxidative stress and inflict serious damage to gene proteins, nucleic acids, and membrane lipids. They also control the processes involved in the initiation of apoptotic signaling and have critical pro-apoptotic roles. The mitochondrial apoptosis pathway is regulated by the Bcl 2 family of proteins, including the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2. Pro-apoptotic proteins promote the release of cytochrome c, which

triggers caspase-9 activation. Anti-apoptotic proteins prevent the release of cytochrome c [5]. In the present study, the treatment of INS-1 pancreatic β cells with a high concentration of glucose triggered the activation of the mitochondrial apoptotic pathway. However, treatment with OEE significantly reduced the level of Bax and increased the level of Bcl-2 expression. OEE also markedly reduced the activation of cytochrome c, caspase-9, and caspase-3. These findings indicate that OEE protected INS-1 pancreatic β- cells from apoptosis induced by exposure to the high level of glucose. This effect might be explained by the increased expression of the anti-apoptotic Bcl-2 and decreased expression of the pro-apoptotic Bax, cytochrome c, and caspase-9.

Flow cytometry was performed to investigate the rate of apoptosis, using Annexin-V as a cell death marker [35]. After treatment with the high concentration of glucose, INS-1 pancreatic β-cells displayed an increased rate of apoptosis. The prevalence of apoptotic cells was markedly decreased in OEE treated samples. These results indicate that OEE can protect β-cells from apoptosis induced by hyperglycemia. Hyperglycemia-related damage to pancreatic β-cells can lead to reduced insulin secretion [11]. In this study, insulin secretion was also significantly decreased in cells treated with 30 mM glucose. However, treatment with OEE in high glucose-pretreated β cells significantly increased insulin secretion. These results suggest that OEE can protect pancreatic β cells and restore their capacity for insulin secretion.

In conclusion, exposure of INS-1 pancreatic β cells to a high level of glucose increased the levels of ROS, lipid peroxides, and NO and decreased the activities of SOD, catalase, and GSH-Px. β cell apoptosis increased and the level of insulin secretion decreased. However, OEE treatment protected pancreatic β-cells exposed to the high concentration of glucose by reducing oxidative stress and apoptosis, which significantly restored insulin secretion. OEE treatment significantly decreased the levels of intracellular ROS, lipid peroxide, and NO, and increased cell viability and the activities of antioxidant enzymes. OEE also significantly enhanced anti-apoptotic gene expression and significantly decreased the expression of pro-apoptotic genes. These results indicate the potential value of OEE as a nutraceutical material for the protection of pancreatic β-cells against high glucose-induced oxidative stress and apoptosis.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

References

1. Brownlee, M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature* **414**, 813-820.
2. Buszewska-Forajta, M., Struck-Lewicka, W., Bujak, R., Siluk, D. and Kaliszan, R. 2014. Determination of water-soluble components of abdominal secretion of grasshopper (*Chorthippus* spp.) by GC/MS/MS in search for potential wound healing agents. *Chromatographia* **77**, 1091-1102.
3. Ceriello, A. 2002. Oxidative stress and glycemic regulation. *Metabolism* **49**, 27-29.
4. Del, S., Grupillo, M., Masini, M., Lupi, R., Bugliani, M., Torri, S., Boggi, U., Del, M., Vistoli, F., Mosca, F., Del, S. and Marchetti, P. 2007. Gliclazide protects human islet beta-cells from apoptosis induced by intermittent high glucose. *Diabetes Metab. Res. Rev.* **23**, 234-238.
5. Dhanasekaran, D. N. and Reddy, E. P. 2008. JNK signaling in apoptosis. *Oncogene* **27**, 6245-6251.
6. Du, X., Stockklauser, K. and Rösen, P. 1999. Generation of reactive oxygen intermediates, activation of NF-κB, and induction of apoptosis in human endothelial cells by glucose: role of nitric oxide synthase? *Free Radic. Biol. Med.* **27**, 752-763.
7. Dutta, P., Dey, T., Dihingia, A., Manna, P. and Kalita, J. 2017. Antioxidant and glucose metabolizing potential of edible insect, Brachytrupes orientalis via modulating Nrf2/AMPK/GLUT4 signaling pathway. *Biomed. Pharmacother.* **95**, 556-563.
8. Hink, U., Li, H., Mollnau, H., Oelze, M., Matheis, E., Hartmann, M., Skatchkov, M., Thaiss, F., Stahl, R. A. K., Warnholtz, A., Meinertz, T., Griendling, K., Harrison, D. G., Forstermann, U. and Munzel, T. 2001. Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ. Res.* **88**, 14-22.
9. Im, A. R., Park, I., Ji, K. Y., Lee, J. Y., Kim, K. M., Na, M. and Chae, S. 2019. Protective effects of Oxya chinensis sinuosa Mishchenko against ultraviolet B-induced photodamage in hairless mice. *BMC Complement. Altern. Med.* **19**, 286.
10. Jacqueminet, S., Briaud, I., Rouault, C., Reach, G. and Poitout, V. 2002. Inhibition of insulin gene expression by long-term exposure of pancreatic beta cells to palmitate is dependent on the presence of a stimulatory glucose concentration. *Metabolism* **49**, 532-536.
11. Kaneto, H., Nakatani, Y., Kawamori, D., Miyatsuka, T., Matsuoka, T. A., Matsuhisa, M. and Yamasaki, Y. 2005. Role of oxidative stress, endoplasmic reticulum stress, and c-Jun

N-terminal kinase in pancreatic beta-cell dysfunction and insulin resistance. *Int. J. Biochem. Cell Biol.* **37**, 1595-1608.
12. Kaplan, M., Aviram, M. and Hayek, T. 2012. Oxidative stress and macrophage foam cell formation during diabetes mellitus-induced atherogenesis: role of insulin therapy. *Pharmacol. Ther.* **136**, 175-185.
13. Kappus, H. 1987. Oxidative stress in chemical toxicity. *Arch. Toxicol.* **60**, 144-149.
14. Kawahito, S., Kitahata, H. and Oshita, S. 2009. Problems associated with glucose toxicity: Role of hyperglycemia-induced oxidative stress. *World J. Gastroenterol.* **15**, 4137.
15. Kim, H. J., Kang, S. J., Kim, S. G., Kim, J. E., Koo, H. Y., Park, J. H. and Choi, H. C. 2015. Antioxidant activity and antimicrobial activity of the grasshopper, Oxya chinensissinuosa. *J. Seric. Entomol. Sci.* **53**, 130-134.
16. Kim, I. W., Markkandan, K., Lee, J. H., Subramaniyam, S., Yoo, S., Park, J. and Hwang, J. S. 2016. Transcriptome profiling and in silico analysis of the antimicrobial peptides of the grasshopper *Oxya chinensis sinuosa*. *J. Microbiol. Biotechnol.* **26**, 1863-1870.
17. Kim, T. S., Lee, J. H., Choi, B. D. and Ryu, H. S. 1987. Nutritional value of dried paddy grasshopper, Oxya chinensis formosana. *J. Kor. Soc. Food Nutr.* **16**, 98-104.
18. Kim, Y. S. and Kwon, T. D. 2018. Effects of grasshopper diet and treadmill exercise performance of blood lipid profile and antioxidant enzyme in rats. *Kor. J. Sports Sci.* **27**, 1605-1614.
19. Kong, Q. and Lin, C. L. 2010, Oxidative damage to RNA: mechanisms, consequences, and diseases. *Cell Mol. Life Sci.* **67**, 1817-1829.
20. Lenzen, S. 2008. Oxidative stress: the vulnerable β-cell. *Biochem. Soc. Ttans.* **36**, 343-347.
21. Maedler, K., Sergeev, P., Ris, F., Oberholzer, J., Joller-Jemelka, H. I., Spinas, G. A., Kaiser, N., Halban, P. A. and Donath, M. Y. 2002. Glucose-induced β cell production of IL-1β contributes to glucotoxicity in human pancreatic islets. *J. Clin. Invest.* **110**, 851-860.
22. Marshak, S., Leibowitz, G., Bertuzzi, F., Socci, C., Kaiser, N., Gross, D. J., Cerasi, E. and Melloul, D. 1999. Impaired beta-cell functions induced by chronic exposure of cultured human pancreatic islets to high glucose. *Diabetes* **48**, 1230-1236.
23. Mcdaniel, M. L., Kwon, G., Hill, J. R., Marshall, C. A. and Corbett, J. A. 1996. Cytokines and nitric oxide in islet inflammation and diabetes. *Exp. Biol. Med.* **211**, 24-32.
24. Oh, H. G., Lee, H. Y., Kim, J. H., Kang, Y. R., Moon, D. I. and Seo, M. Y. 2012. Effects of male silkworm pupa powder on the erectile dysfunction by chronic ethanol consumption in rats. *Lab. Anim. Res.* **28**, 83-90.
25. Pacher, P., Beckman, J. S. and Liaudet, L. 2007. Nitric oxide and peroxynitrite in health and disease. *Physiol. Rev.* **87**, 315-424.
26. Paolisso, G. and Giugliano, D. 1996. Oxidative stress and insulin action. Is there a relationship? *Diabetologia* **39**, 357-363.
27. Park, J. Y., Heo, J. C., Woo, S. U., Yun, C. Y. and Kang, S. W. 2006. Anti-inflammatory and cellular protective effects on hydrogen peroxideinduced cytotoxicity of grasshopper extracts. *Kor. J. Food Preserv.* **13**, 796-802.
28. Poitout, V. and Robertson, R. P. 2002. Minireview: secondary beta-cell failure in type 2 diabetes－convergence of glucotoxicity and lipotoxicity. *Endocrinology* **143**, 339-342.
29. Rajarajeswari, N. and Pari, L. 2011. Antioxidant role of coumarin on streptozotocin-nicotinamide-induced type 2 diabetic rats. *J. Biochem. Molecul. Toxicol.* **25**, 355-361.
30. Rashid, M. A., Lee, S., Tak, E., Lee, J., Choi, T. G., Lee, J. W., Kim, J. B., Youn, J. H., Kang, I., Ha, J. and Kim, S. S. 2010. Carbonyl reductase 1 protects pancreatic beta-cells against oxidative stress-induced apoptosis in glucotoxicity and glucolipotoxicity. *Free Radic. Biol. Med.* **49**, 1522-1533.
31. Robertson, R. P. 2004. Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet β cells in diabetes. *J. Biol. Chem*. **279**, 42351-42354.
32. Sakai, K., Matsumoto, K., Nishikawa, T., Suefuji, M., Nakamaru, K., Hirashima, Y., Kawashima, J., Shirotani, T., Ichinose, K., Brownlee, M. and Araki, E. 2003. Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic beta-cells. *Biochem. Biophys. Res. Commun*. **300**, 216-222.
33. Suryanarayana, P., Satyanarayana, A., Balakrishna, N., Kumar, P. U. and Reddy, G. B. 2007. Effect of turmeric and curcumin on oxidative stress and antioxidant enzymes in streptozotocin-induced diabetic rat. *Med. Sci. Monit*. **13**, BR286-BR292.
34. Tiedge, M., Lortz, S., Drinkgern, J. and Lenzen, S. 1997. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin producing cells. *Diabetes* **46**, 1733-1742.
35. Vermes, I., Haanen, C. and Reutelingsperger, C. 2000. Flow cytometry of apoptotic cell death. *J. Immunol. Methods* **243**, 167-190.
36. Wang, D. Q., Yang, J. and Xu, J. 2020. Polyphenol and flavonoids content and antioxidant activity of different solvent extracts from Artemisia argyi. *Asian J. Immunol.* **3**, 30-35.
37. Yoon, Y. I., Chung, M. Y., Hwang, J. S., Goo, T. W., Ahn, M. Y., Lee, Y. B., Han, M. S. and Yun, E. Y. 2014. Anti-inflammatory effect of Oxya chinensis sinuosa ethanol extract in LPS-induced RAW 264.7 cells. *J. Life Sci.* **24**, 370-376.

초록 : INS-1 췌장 베타 세포에서 벼메뚜기(*Oxya chinensis sinuosa* Mistshenk) 추출물의 당독성 개선 효과

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당뇨병은 만성대사성 질환으로 우리나라 국민의 사망요인 중 5위를 차지하고 있다. 제 2형 당뇨병에서 나타나는 인슐린 분비 감소는 베타세포의 자가사멸에 의한 베타세포질량의 급격한 감소로 인한 것으로 보고되고 있으며, 베타세포의 자가사멸을 촉진하는 요인으로 고혈당에 의한 당독성 및 활성산소종들의 증강에 의한 산화스트레스 등이다. 벼메뚜기 추출물은 고농도 포도당 처리된 INS-1 췌장 베타 세포에서 세포 생존율을 증가시키고, 지질과산화물, 세포 내 ROS 및 NO 수준을 감소시켰다. 세포사멸 관련 인자의 유전자 발현 결과 pro-세포자가사멸 인자인 Bax, Cytochrome C, caspase-3 및 caspase-9의 단백질 발현을 유의적으로 감소시켰고, anti-세포자가사멸 인자인 Bcl-2 발현을 증가시켰다. Annexin V/I propidium iodide 염색법을 통하여 벼메뚜기 추출물이 고농도 포도당으로 유도된 세포 사멸을 감소시키고, INS-1 췌장 베타세포에서의 인슐린 분비능을 증가시키는 것으로 사료된다. 그러므로 벼메뚜기 추출물이 고농도 포도당으로 손상된 INS-1 췌장 베타세포의 보호효과를 나타낸다. 본 연구의 결과는 벼메뚜기 추출물이 당뇨병 치료에 도움을 주는 항당뇨 기능성 식품 소재 및 개발에 기여할 수 있음을 시사한다.