The metabolic syndrome creates risk factors for coronary heart disease, diabetes, fatty liver, obesity and several cancers. Our group has already reported that the essential oil from leaves of *Pinus koraiensis* SIEB (EOPK) exerted antihyperlipidemic effects by upregulating the low-density lipoprotein receptor and inhibiting acyl-coenzyme A, cholesterol acyltransferases. We evaluated in the current study the anti-diabetic effects of EOPK on mice with streptozotocin (STZ)-induced type 1 diabetes and on HIT-T15 pancreatic β cells. EOPK significantly protected HIT-T15 cells from STZ-induced cytotoxicity and reduced the blood glucose level in STZ-induced diabetic mice when compared with the untreated control. EOPK consistently and significantly suppressed the α-amylase activity in a dose-dependent manner and enhanced the expression of insulin at the mRNA level in STZ-treated HIT-T15 cells, while the expression of insulin was attenuated. EOPK also significantly abrogated the population of reactive oxygen species when compared to the untreated control in STZ-treated HIT-T15 cells. Furthermore, EOPK significantly reduce nitric oxide production, suppressed the phosphorylation of endothelial nitric oxide (NO) synthase and suppressed the production of vascular endothelial growth factor (VEGF) in STZ-treated HIT-T15 cells, implying its potential application to diabetic retinopathy. Overall, our findings suggest that EOPK had hypoglycemic potential by inhibiting reactive oxygen species (ROS), endothelial NO synthase (eNOS) and VEGF in STZ-treated mice and HIT-T15 pancreatic β cells as a potent anti-diabetic agent.

**Key words:** HIT-T15 cell; streptozotocin; *Pinus koraiensis*; vascular endothelial growth factor (VEGF)

Diabetes mellitus is a metabolic disorder involving a high level of blood glucose which can affect many organs, including the heart, blood vessels, nerves, eyes and kidneys. In particular, type 2 diabetes mellitus (T2DM), the most common type of diabetes resulting from insulin resistance, leads to such diabetes complications as heart attacks, strokes, amputation, diabetic retinopathy, and kidney failure, while type 1 diabetes mellitus (T1DM) is typically an autoimmune disease resulting from the specific destruction of β-cells in the pancreatic islets. Although many anti-diabetic drugs have been developed to reduce the blood glucose level, such as biguanides (metformin, phenformin and buformin) and thiazolidinediones (rosiglitazone, pioglitazone and troglitazone), such adverse effects from them as gastrointestinal symptoms, lactic acidosis and hepatotoxicity have been of serious concern for diabetes treatment. Recent hypoglycemic herbal medicines are therefore considered attractive, since they are suitable for long-term diabetes control with little toxicity.

The chloroform fraction of *Andrographis paniculata* has recently exerted an anti-diabetic effect on diabetic albino mice, a cinnamon extract has shown a moderate effect in reducing the fasting plasma glucose concentration in diabetic patients with poor glycaemic control, and a curcumin extract has improved the overall function of β-cells from T2DM patients with very minor adverse effects. The metabolic syndrome includes obesity, hyperlipidemia and diabetes, and we can hypothesize on the anti-diabetic potential of EOPK, given that our previous study has shown the essential oil from leaves of *Pinus koraiensis* (EOPK) to exert its antihyperlipidemic effects by upregulating the low-density lipoprotein receptor and inhibiting cholesterol acyltransferases. We verify this hypothesis by identifying the effect of EOPK on glucose and on ROS that are known to mediate diabetes and such diabetic retinopathy-related biomarkers as eNOS and VEGF in mice with streptozotocin (STZ)-induced T1DM and HIT-T15 pancreatic β cells.

**Materials and Methods**

Preparation of the essential oil from leaves of *Pinus koraiensis* and the reagents used. Essential oil from leaves of *Pinus koraiensis* SIEB (EOPK) was prepared by using the hydrodistillation method. Dried...
and pulverized *P. koraiensis* leaves were immersed in distilled water and subjected to steam distillation, using a rotary evaporator with a condenser (Hanil Labtech, Seoul, Korea). The distillation continued for 3–4 h at 90°C, and then the volatile compounds contained in the water-soluble fraction were allowed to settle down for 20 min. The essential oil layer was finally separated and purified by microfiltration. STZ was obtained from Sigma Chemicals (St. Louis, MO, USA). RPMI 1640 supplemented with fetal bovine serum (FBS), penicillin and streptomycin (PEST), and trypsin EDTA were purchased from Gibco (Carlsbad, CA, USA). The enhanced chemiluminescence (ECL) western blotting detection reagents and hyperfilm ECL were from Amersham-Pharmacia Korea (Seoul, Korea).

**Animals.** Four-week-old male ICR mice weighing 20 to 25 g were purchased from Nara Biotech (Seoul, Republic of Korea) and given food and water ad libitum. The mice were housed in a room maintained at 22–24°C with 55% relative humidity and controlled lighting for 12 h (07:00–19:00). All materials, including bedding and feed, were sterilized for 15 min by UV radiation before treating the mice. The experiments were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of Kyung Hee University [KHUASP(SE)-11-007].

**Diabetic mouse model and EOPK administration.** Type 1 diabetes mellitus was induced in the mice by an i.p. injection of streptozotocin (STZ). The mice were assigned to four groups (n = 10/group): the normal group, STZ control group, STZ + EOPK group, and STZ + acarbose group. The normal group mice were given distilled water. STZ was dissolved in a 0.05 M sodium citrate buffer at pH 4.5 and intraperitoneally injected into the mice at 200 mg/kg of body weight. One day after this STZ treatment, EOPK and acarbose (as a positive control) were diluted in corn oil and orally administered for 4 d.

**Measurement of the blood glucose level.** The blood glucose concentration in blood obtained from the lateral tail vein was measured by using a CareSens® N glucometer (Life Bioscience, Victoria, Australia).

**α-Amylase assay.** EOPK (3.00 or 6.00 μg/mL) was mixed with a 0.02 m sodium phosphate buffer containing 0.5 mg/mL of α-amylase (1:1 (v/v)) and incubated at 25°C for 10 min. The same volume of a 1% starch/0.02 m sodium phosphate buffer was added, and the mixture was maintained at 25°C for 10 min. The optical density (OD) was measured at 540 nm after the colorimetric reaction by using 96 mM dinitrosalicylic acid. The inhibition rate of α-amylase was calculated by the equation: percentage inhibition = [(Aasscontrol−Aaseopk)/Aasscontrol] × 100%.

**Cell culture.** HIT-T15 pancreatic β-cells were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea) and maintained in the RPMI 1640 medium (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS), 2 μg/mL l-glutamine and penicillin/streptomycin.

**Cytotoxicity assay.** A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay was performed to investigate the cytotoxicity of EOPK and/or STZ against the HIT-T15 cells. HIT-15 cells were seeded on 96-well microplates at a density of 1 × 10^4 cells per well and treated with EOPK and/or STZ for 24 h. An MTT working solution was added to the microplates at 37°C for 2 h. The MTT extraction buffer (20% SDS/50% dimethylformamide) was treated overnight at 37°C. The optical density (OD) was measured at 570 nm after incubation by using a Sunrise microplate reader (Tecan, Mannedorf, Switzerland). The cell viability was calculated by applying the equation: cell viability (%) = (OD (EOPK) − OD (blank))/ [OD (control) − OD (blank)] × 100.

**Western blotting.** A whole cell lysate was prepared by using a lysis buffer (20 mM Tris pH 7, 4, 250 mM NaCl, 2 mM EDTA at pH 8.0, 0.1% Triton X-100, 0.01 mg/mL of aprotinin, 0.003 mg/mL of leupeptin, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), and 4 mM NaVO_4_). The lysate was spun at 13,000 × g for 15 min to remove the insoluble material and then resolved on 10% SDS-PAGE gel. The proteins were electro-transferred to a nitrocellulose membrane after electrophoresis, blocked with 5% non-fat milk, and probed overnight with antibodies against phospho-eNOS (Cell Signaling Tech., Danvers, MA, USA) and β-actin (Sigma, St. Louis, MO, USA). The blots were washed, exposed for 2 h to horseradish peroxidase (HRP)-conjugated secondary antibodies, and finally examined by enhanced chemiluminescence (ECL; GE Health Care Bio-Sciences, Piscataway, NJ, USA). Band intensity was quantified by using NIH Image-J software.

**RT-PCR analysis.** Total RNA was extracted by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. cDNA was synthesized from 2 μg of total RNA and subjected to the PCR reaction by using a Superscript One Step reverse transcription-PCR (RT-PCR) kit (Invitrogen, Carlsbad, CA, USA). The PCR conditions were 30 cycles of 96°C for 30 s, 59°C for 30 s and 72°C for 1 min. The primer sequences for insulin were (forward) 5′-AGAAGGCATCAAGCAAGCAGG-3′ and (reverse) 5′-AGAGTGGCTTCCCAAAAGTG-3′, and for β-actin were (forward) 5′-TTCCTCCACGGTAGTGG-3′ and (reverse) 5′-GTACCCAGC-ATTGAAGGGGC-3′. The PCR products were run on 2% agarose gel and then stained with ethidium bromide. The stained bands were visualized under UV light and photographed.

**Enzyme-linked immunosorbent assay (ELISA) for VEGF.** ELISA was performed by using a Human Vascular Endothelial Growth Factor ELISA kit (Biosource International, Camarillo, CA, USA). Briefly, 50 μL of the collected culture supernatant was added to a 96-well plate and then incubated at room temperature for 2 h with 50 μL of a dilution buffer and 50 μL of an incubation buffer. The plate was then washed four times with a washing buffer, and 100 μL of the biotin conjugate was put into each well for 1 h at room temperature. After washing four times with the washing buffer, 100 μL of the stabilized chromogen was added to each well and incubated for 30 min at room temperature in the dark. Finally, 100 μL of a stop solution was added to each well, and the absorbance was measured at 450 nm.

**Measurement of the generation of reactive oxygen species (ROS).** A 2,7-dichlorofluorescein diacetate (DCFDA) fluorescence dye was used to detect the level of ROS. HIT-T15 cells in the absence or presence of EOPK were incubated for 30 min with 1 μM DCFDA. BD FACSCalibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) was used to measure the fluorescence intensity.

**Statistical analysis.** Each experiment was conducted at least three times. All data are expressed as the mean ± standard deviation (SD) of three independent experiments. Statistically significant differences were calculated by Student’s *t*-test.

**Results**

**Effect of EOPK on the viability of STZ-treated HIT-T15 cells**

STZ significantly decreased the viability of HIT-T15 cells in a dose-dependent manner (Fig. 1A), whereas EOPK exerted weak cytotoxicity toward HIT-T15 pancreatic β-cells (Fig. 1B). However, co-treatment with EOPK and STZ significantly suppressed the cytotoxicity of STZ in HIT-T15 pancreatic β-cells (Fig. 1C).

**Effect of EOPK on the body weight of EOPK or STZ-induced diabetic mice**

STZ is used to induce experimental type 1 diabetes. In the present study, male ICR mice were i.p. injected with STZ at a dose of 200 mg/kg to induce diabetes. EOPK was then administered at a dose of 150 mg/kg for 4 d after the STZ injection. Figure 2A shows that the body weights in the normal group were not significantly changed for 4 d, while the STZ treatment reduced the
Anti-Diabetic Potential of EOPK in Vivo and in Vitro

Effects of EOPK on the Body Weight and Blood Glucose Level of STZ-Induced Diabetic Mice

Four d after the STZ administration, the blood glucose concentration had significantly increased by 548 ± 27.35 mg/dL when compared with the normal group (216.50 ± 7.50 mg/dL). In contrast, a significant decrease in blood glucose level was revealed in the EOPK-treated group (200.12 ± 23.72 mg/dL) when compared with the STZ control group, almost similar to the efficacy of acarbose as a positive anti-diabetic agent (Fig. 2B).

Effect of EOPK on the α-amylase activity

The inhibitory effect of EOPK on α-amylase was evaluated by an in vitro α-amylase enzyme assay. Figure 3 shows that EOPK significantly inhibited the activity of α-amylase in a concentration-dependent manner.

Effect of EOPK on the oxidative stress and angiogenic biomarkers in STZ-treated HIT-T15 cells

We found in our study that ROS generation was clearly decreased to 38.84 % when compared with the untreated control (Fig. 4A). In contrast, EOPK effectively reduced ROS production to 26.98 % and 23.95 % at respective concentrations of 3 μg/mL and 6 μg/mL.
Discussion

Diabetes with malfunctioning β-cells, regardless of the type of diabetes mellitus, features the lack or reduction of insulin leading to hyperglycemia, or an abnormally high level of blood sugar. There is accumulating evidence that herbal extracts have been found attractive in controlling diabetes with little toxicity.7–9 Previous evidence has shown d-limonene to have anti-inflammatory and anti-cancer actions,23 and an anti-diabetic effect.24 Camphene has also been shown to have a hypolipidemic action in an in vivo model.25 Nonetheless, there has been no evidence until now on the anti-diabetic effect of EOPK, except for the in vivo antioxidative activity of Pinus koraiensis.26 The EOPK treatment protected against STZ-induced cytotoxicity in HIT-T15 pancreatic β-cells, since it is well known that an STZ treatment has induced cell death in cultured pancreatic β-cells,27 implying the protective effect of EOPK on STZ-induced cell death. In addition, no loss of body weight was apparent in the EOPK-treated group, while some loss of body weight was induced by STZ in the mice.

β-Cells are responsible for creating and releasing the insulin and amylin hormones which serve to regulate the glucose level in the blood.28 Amylin, called islet amyloid polypeptide (IAPP), also works in conjunction with insulin by regulating the glucose level in the blood in a more short-term manner.29 In this present study,
EOPK significantly suppressed the α-amylase activity and blood glucose level in a concentration-dependent manner and enhanced the expression of insulin at the mRNA level in STZ-treated HIT-T15 cells, indicating the hypoglycemic potential of EOPK. The cytotoxicity of STZ toward pancreatic β-cells is known to be mediated by the production of free radicals. It has therefore been well established that ROS derived from multiple sources plays a causal role in multiple types of insulin resistance and contributes to β-cell dysfunction, thus enhancing the development and progression of T2DM. As we expected, EOPK partially abrogated the population of ROS when compared to the untreated control in STZ-treated HIT-T15 cells, implying the inhibitory potency of EOPK toward ROS-mediated insulin resistance. Diabetic retinopathy is one of the important complications associated with hyper-angionesis. NO produced by eNOS is an important mediator of the VEGF function facilitating vascular growth and maturation. VEGF also plays a key role in vasculogenesis and angiogenesis related to diabetic retinopathy. We found in the present study that EOPK reduced the NO production, and suppressed the phosphorylation of eNOS and the production of VEGF in STZ-treated HIT-T15 cells, strongly suggesting the anti-diabetic potential of EOPK via the inhibition of ROS, eNOS and VEGF to act as a potent anti-diabetic agent.

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References