Korean J Physiol Pharmacol
Vol 8: 51-55, February, 2004

Anti-Apoptotic Effect of *Rheum undulatum* Water Extract in Pancreatic β-cell Line, HIT-T15

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Sopungsungi-won has been used as a traditional medicine for diabetes and it has been proved to be a potential remedy for type 2 diabetes mellitus. We previously reported that water extract of Sopungsungi-won exhibits anti-diabetic effects both *in vivo* and *in vitro* experiments. In the present study, we have chosen to examined anti-apoptotic effect of *Rheum undulatum*, which is the main component of Sopungsungi-won, on pancreatic β-cells, HIT-T15, against hydrogen peroxide (H_2O_2). oxidative stress. To investigate the anti-apoptotic effect of *Rheum undulatum* water extract (RUWE) against H_2O_2-induced apoptosis in pancreatic β-cell line of hamster, HIT-T15, MTT assay, DAPI staining, TUNEL assay, RT-PCR and caspase-3 enzyme assay were performed. The morphological analysis demonstrated that cells treated with H_2O_2 exhibited classical apoptotic features, while such changes was reduced in cells pre-treated with RUWE. In addition, RUWE pre-treated cells prior to H_2O_2 treatment induced increase of levels of *bcl*-2 expression and decrease of caspase-3 enzyme activity compared to cells treated with H_2O_2 only. These results provide the possibility of usage of RU in patients with progressively deteriorated diabetes.

Key Words: HIT-T15, Apoptosis, Hydrogen peroxide, *Rheum undulatum* water extract, Oxidative stress

INTRODUCTION

It is well known that oxidative stress impairs various cellular functions and plays important roles in the pathophysiology of many diseases. Diabetic patients are exposed to oxidative stress and complications of diabetes seem to be mediated by oxidative stress. Hyperglycemia is one of the main causes of oxidative stress in type 2 diabetes. Under hyperglycemia, the increased blood level of various reducing sugars promotes protein glycation through the Maillard reaction, which consecutively produces Schiff bases, Amadori products, and advanced glycation end products (AGEs). During this process, reactive oxygen species (ROS) are formed and these eventually trigger tissue damage. Recently, the progressive deterioration of β-cell function in type 2 diabetes has been accounted for in the oxidative stress-induced tissue damage. Pancreatic islet cells are implicated to be vulnerable to oxidative stress, because they express a relatively low amount of antioxidant enzyme genes compared with other tissues and organs (Lenzen et al, 1996). In addition, oxidative stress is reported to enhance apoptosis of β-cells and suppress insulin biosynthesis (Kaneto et al, 1996; Matsuoka et al, 1997). It has also been shown that administration of antioxidant chemicals, such as cysteine or N-acetyl-L-cysteine (NAC), provides some protection to β-cell function both *in vitro* and *in vivo* (Kaneto et al, 1999; Tanaka et al, 1999).

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Apoptosis, also known as programmed cell death, is a form of cell death that occurs in several pathological situations in multicellular organisms. It constitutes a common mechanism of cell replacement, tissue remodeling, and removal of damaged cells (DeLong, 1998). Furthermore, apoptosis is characterized by morphological changes including progressive cell shrinkage with condensation and fragmentation of nuclear chromatin and membrane blebbing (Kerr et al, 1972).

Sopungsungi-won has been used as a traditional Korean medicine for senile constipation and suggested as a potential remedy for type 2 diabetes mellitus. Long-term study with water extract of Sopungsungi-won in Zucker diabetic fatty rats prevented the development and progression of diabetes (Kim et al, 2002). However, the effects of RU, which is the main component of Sopungsungi-won, on H_2O_2-induced apoptosis have not yet been reported. In the present study, the anti-apoptotic effects of *Rheum undulatum* water extract (RUWE) on H_2O_2-induced apoptosis were studied using the pancreatic β-cell line, HIT-T15. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 4,6-diamidino-2-phenylindole (DAPI) staining, terminal deoxy-nucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, reverse transcription-polymerase chain reaction (RT-PCR) and caspase-3 enzyme

ABBREVIATIONS: RUWE, rheum undulatum water extract; ROS, reactive oxygen species; TUNEL, terminal deoxy-nucleotidyl transferase (TdT)-mediated dUTP nick end labeling; RT-PCR, reverse transcription polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tertazolium bromide; DAPI, 4,6-diamidino-2-phenylindole.

assay were performed. Our results showed that RUWE attenuates H_2O_2-induced oxidative stress on pancreatic β-cells and maintains a higher cellular viability.

METHODS

Materials

MTT, DAPI, 3,3'-diaminobenzidine (DAB), and Caspase-3 assay kit were purchased from Sigma (St. Louis, MO, USA), TUNEL assay kit was from Boehringer Mannheim (Mannheim, Germany), and AMV-RT and Oligo dT were from Promega (Madison, WI, USA), and RPMI 1640 medium, fetal bovine serum, and antibiotics were from Gibco (Grand Island, NY, USA).

Preparation of extract

Rheum undulatum (RU) was purchased from Kyungdong herbal market in Seoul, Korea and was extracted with deionized distilled water. The extract was filtered through a 0.45 μm filter, lyophilized, and kept at 4°C. The dried extract was dissolved in deionized distilled water before use.

Cell culture

The HIT-T15 cell line was purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, and cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air, and the medium was changed every other day.

MTT cytotoxicity assay

In a 96-well flat bottom plate, approximately 2,000 cells /100 μl of cell suspension were used to seed each well. The cells were incubated overnight to allow for attachment and recovery. Following treatment for 24 h and additional 3 h incubation with 100 μM H_2O_2, 10 μl of 5 mg/ml of the MTT tetrazolium substrate solution in phosphate buffered saline (PBS) were added and incubated for up to 4 h at 37°C. The resulting violet formazan precipitate was solubilized by the addition of 100 μl of dimethylsulfoxide solution and shaking for 1 h at room temperature. The plate was then analyzed with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at 595 nm with a reference wavelength of 690 nm. The optical density (O.D.) was calculated as the difference between the reference wavelength and the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/O.D. of untreated sample) $\times$ 100.

DAPI staining

In order to determine the effect of RUWE on H_2O_2-induced apoptosis, DAPI staining was performed. In brief, HIT-T15 cells were cultured on four-chamber slides (Nalge Nunc International, Naporvile, IL, USA). After 24 h treatment with 100 μg/ml of RUWE, followed by 3 h incubation with 100 μM H_2O_2, cells were washed twice with PBS and were fixed by incubation in 4% paraformaldehyde for 30 min. Cells were then incubated in 1 μg/ml DAPI solution for 30 min in the dark. The nuclear morphology of cells was examined by fluorescence microscopy (Zeiss, Oberköchen, Germany).

TUNEL assay

For in situ detection of apoptotic cells, TUNEL assay was performed using the ApoTag® peroxidase in situ apoptosis detection kit. HIT-T15 cells were cultured on four-chamber slides at a density of approximately 2×10^4 cells /chamber. After a 24 h exposure to RUWE (100 μg/ml) followed by 3 h incubation with 100 μM H_2O_2, the cells were washed with PBS and fixed in 4% paraformaldehye for 10 min at 4°C. The fixed cells were incubated with digoxigenin-conjugated dUTP in a TdT catalyzed reaction for 60 min at 37°C in a humidified atmosphere, and were then immersed in stop/wash buffer for 10 min at room temperature. The cells were then incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min. The DNA fragments were stained using DAB as the substrate for the peroxidase.

RT-PCR analysis

Total RNA was isolated from HIT-T15 cells with RNAzol™B (TELTEST; Friendswood, TX, USA) according to the manufacturer's protocol. one μg of total RNA was reverse transcribed into cDNA using AMV-reverse transcriptase and Oligo dT as a primer. The forward primer sequence used for analysis of *bcl*-2 was 5'-TAT-AAG CTG TCA CAG AGG G-3' and reverse primer sequence was 5'-CTC TCC ACA CAC ATG ACC-3'. The amplification was initiated at 95°C for 5 min, followed by 40 cycles consisting of denaturation at 94°C for 30 s, annealing at the appropriate primer-pair annealing temperature for 30 s and extension at 72°C for 30 s, and a final extension step of 7 min at 72°C. The GAPDH (forward primer: 5'-GGA AAG ACA ACG GAC AAA TC-3' and reverse primer: 5'-GTC ATC TTC TGG AGC ACC TT-3') was used to verify that equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. The annealing temperature was 52°C for *bcl-2* and 58°C for GAPDH. The amplified fragment sizes were 420 bp and 377 bp for *bcl-2* and GAPDH, respectively. The RT-PCR products were electrophoresed on a 2% agarose gel and visualized by staining with 0.5 μg/ml ethidium bromide.

Caspase-3 activity assay

Caspase-3 activity was measured with Caspase-3 assay kit according to the manufacturer's protocol. HIT-T15 cells were lysed with Lysis Buffer after 24 h treatment with RUWE (100 μg/ml), followed by 3 h incubation with 100 μM H_2O_2. Caspase-3 was used as positive control and Ac-DEVD-CHO was used as caspase-3 inhibitor. Assay buffer followed by Caspase-3 substrate (Ac-DEVD-pNA) was added to each lysate and positive control. The mixture was incubated overnight in humidified circumstance at 37°C. The concentration of the p-NA released from the substrate was measured from the absorbance values at 405 nm, and quantity of p-NA was calculated from a calibration curve of p-NA Standard.

Statistical analysis

Results are expressed as mean$\pm$standard error mean (S.E.M.). The data were analyzed by student's t-test. Mean values were considered significantly different when $P<0.05$.

RESULTS

HIT-T15 cell viability

The viability of cells exposed to H_2O_2 without RUWE was 54.0% of the control value, however, this value was increased to 57.4%, 60.0%, 70.2%, 85.2%, 83.0%, and 80.8% by pretreatment with 5, 10, 50, 100, 200, and 500 μg/ml of concentration of RUWE, respectively, before exposure to 100 μM H_2O_2. It appears that RUWE has protective effects against the cytotoxic action of H_2O_2 (Fig. 1). Based on this result, we selected 100 μg/ml concentration of RUWE in the following experiments.

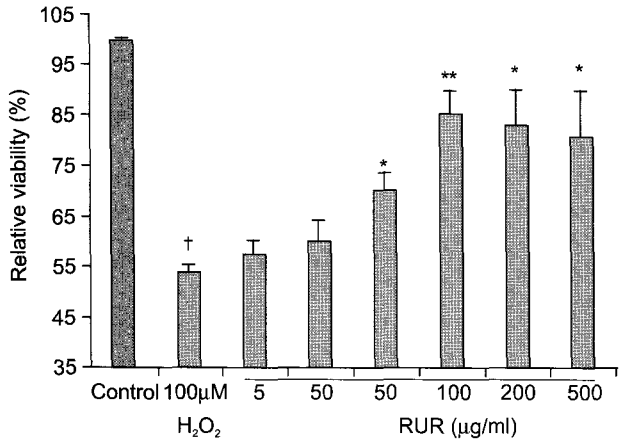

Fig. 1. Effects of RUWE on the viability of cells (Fraction of total cell population surviving) of the HIT-T15. Cells were treated with RUWE at concentrations of 5, 10, 50, 100, 200, and 500 μg/ml for 24 h, followed by exposure to H_2O_2 at a concentration of 100 μM for 3 h. $^{\dagger}P<0.001$ compared to the control group; $^{*}P<0.05$, $^{**}P<0.01$ compared to the 100 μM H_2O_2-treated group.

Morphological analysis

To morphologically observe the protective effects of RUWE against H_2O_2-induced apoptotic changes, cells were examined by phase-contrast microscopy. As shown in Fig. 2, H_2O_2-treated cells without RUWE treatment were shown to be detached from the dish, with cell rounding, cytoplasmic blebbing, and irregularity in shape, while HIT-T15 cells pretreated with RUWE prior to H_2O_2 exposure were morphologically intact and comparable to the control.

TUNEL assay

To confirm the induction of apoptosis by H_2O_2, HIT-T15 cells were biochemically analyzed by TUNEL assay. As shown in Fig. 3, TUNEL-positive cells, which were stained dark brown with nuclear condensation under the light microscope, were observed among the cells treated with H_2O_2 only, whereas the cells pretreated with RUWE prior to H_2O_2 exposure presented morphology similar to that of the control.

DAPI staining

HIT-T15 cells were observed through fluorescence micro-

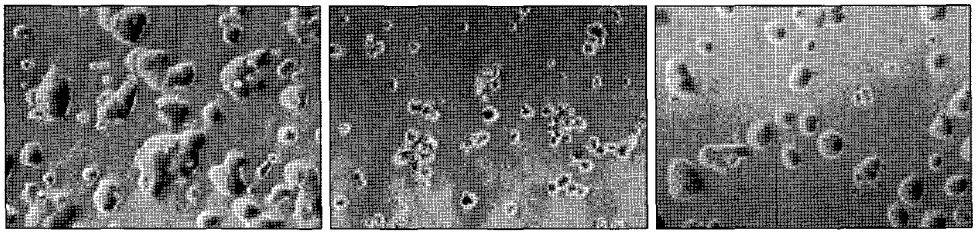

Fig. 2. Morphological changes of HIT-T15 cells pre-treated with RUWE on H_2O_2-induced cytotoxicity. Cells were treated with RUWE at a concentration of 100 μg/ml for 24 h, followed by exposure to 100 μM H_2O_2 for 3 h. Phase-contrast microscopy showed cell shrinkage, irregularity in shape and cellular detachment in the H_2O_2-treated cultures; these morphological characteristics were not observed in the control, and although present, at a far lower degree in the RUWE pre-treated group. Control (left); 100 μM H_2O_2-treated group (middle); 100 μg/ml RUWE pre-treated group (right).

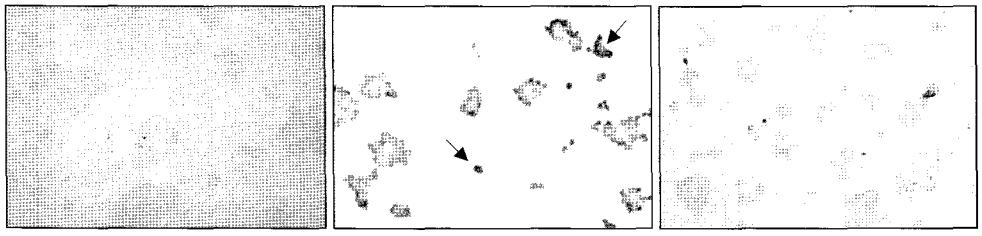

Fig. 3. Protective effects of RUWE against H_2O_2-induced apoptosis. HIT-T15 cells were treated with RUWE at a concentration of 100 μg/ml for 24 h, followed by exposure to 100 μM H_2O_2 for 3 h. Cells were stained according to TUNEL method. Black arrows show labeling of condensed and marginated chromatin. Control (left); 100 μM H_2O_2-treated group (middle); 100 μg/ml RUWE pre-treated group (right).

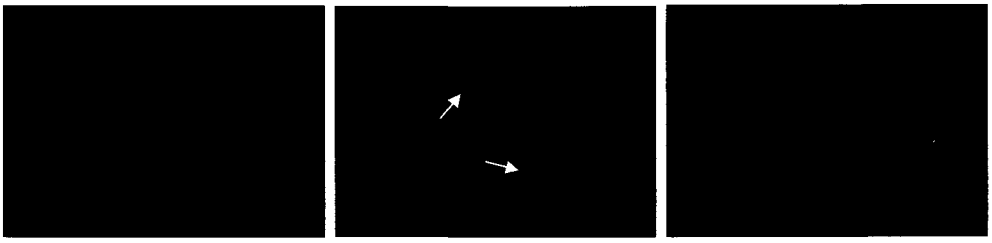

Fig. 4. Effects of RUWE against H_2O_2-induced apoptosis. HIT-T15 cells were treated with RUWE at a concentration of 100 μg/ml for 24 h, followed by exposure to 100 μM H_2O_2 for 3 h. Cells were stained according to DAPI staining protocol. White arrows show labeling of condensed and peri-nuclear apoptotic bodies. Control (left); 100 μM H_2O_2-treated group (middle); 100 μg/ml RUWE pre-treated group (right).

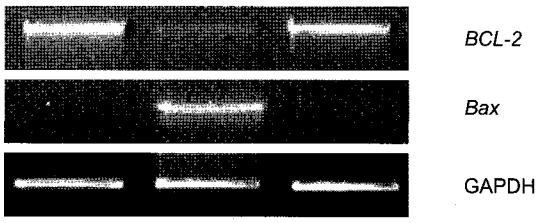

Fig. 5. RT-PCR analysis of *Bcl-2* and *Bax* mRNA level. As the internal control, GAPDH mRNA was also reverse-transcribed and amplified. Control (left); 100 μM H_2O_2-treated group (middle); 100 μg/ml RUWE pre-treated group (right).

scopy, followed by treatment with DAPI solution, which specifically stains the nuclei of cells. The assay revealed the occurrence of nuclear condensation, DNA fragmentation and peri-nuclear apoptotic bodies upon H_2O_2 treatment. RUWE pre-treated cells, however, were morphologically similar to the control.

Bcl-2 and Bax mRNA expression

The effect of RUWE on the expression of anti-apoptotic *bcl-2* and apoptotic *bax* in HIT-T15 cells was examined through RT-PCR. mRNA expression of *bcl-2* was relatively less in the cells treated with 100 μM H_2O_2 than in the cells pre-treated with RUWE. Furthermore, mRNA expression of apoptotic *bax* was relatively high in the cells treated with 100 μM H_2O_2 and that of RUWE pre-treated cells showed relatively low intensity which it was comparable with mRNA expression of the control group (Fig. 5).

Caspase-3 activity

Caspase-3 enzyme activity of HIT-T15 cells stimulated by H_2O_2 was measured, using DEVD peptide-nitroanilide (pNA). As shown in Fig. 6, the quantity of DEVD-pNA cleavage was significantly reduced in the cells pre-treated with RUWE (14.3 ± 0.75) than those treated with H_2O_2 (37.8 ± 1.61) alone. Furthermore, the cells pre-treated with RUWE and H_2O_2 along with caspase-3 inhibitor, Ac-DEVD-CHO, exhibited significantly lower value (11.5 ± 0.07), compared to the cells treated with H_2O_2 and the inhibitor (24.6 ± 1.25).

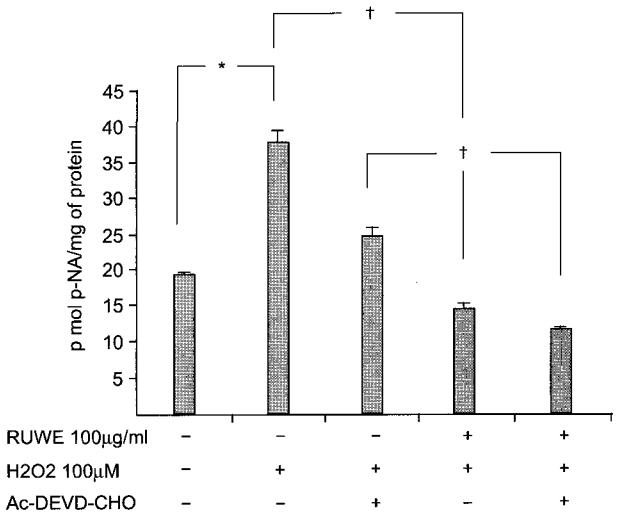

Fig. 6. Caspase-3 enzyme activity assay. The rate of DEVD-pNA cleavage was measured at 405nm. $^{\dagger}P<0.05$ compared to the control group; $^{*}P<0.01$ compared to the 100 μM H_2O_2-treated group.

DISCUSSION

There is increasing evidence to suggest that apoptosis is the main mode of β-cell death leading to type 1 diabetes and that the destruction of β-cells is due to infiltration of lymphocytes into the pancreatic islets (Kay et al, 2000). On the other hand, it is also well known that β-cells undergo apoptosis in type 2 diabetes (Porte, 1991; DeFronzo et al, 1992). In general, the apoptosis cascade is triggered by various kinds of stimuli such as DNA damage, cell cycle perturbation, metabolic imbalance, withdrawal of growth factors, cytokines as well as oxidative stress. Possible sources of oxidative stress in diabetes include an increased production of ROS, especially from enhanced glycation, and decreased enzymatic or non-enzymatic anti-oxidant defense systems. ROS induce toxic effects that lead to necrosis or apoptosis of the β-cells. Chronic hyperglycemia induces protein glycation, and oxidative stress is increased in type 2 diabetes. Ihara et al (1999) examined oxidative stress markers in diabetic GK rats and found increased ROS in pancreatic islets. Progressive impairment of β-cell function in type 2 diabetes has been demonstrated to be due to chronic hyperglycemia (Robertson, 1992; Olson et al, 1993; Poitout et al, 1996).

Because type 2 diabetes is a chronic disease, oxidative stress needs to be prevented not periodically, but as long as the hyperglycemia persists. Hypoglycemic agents and antioxidants have been widely used, however, long-term safety for only a few of them has been established for clinical use. As an alternative, we focused on Korean herbal medicine, because it was shown in several studies that oriental herbal medications possess protective effects against diabetes. Sopungsungi-won has been used as a traditional medicine for diabetes, and it has evidently been proved as a potential remedy for type 2 diabetes mellitus. Both *in vivo* and *in vitro* studies with water extract of Sopungsungi-won have shown anti-diabetic effect (Kim et al, 2002). In this study, we have chosen RU, which is the main component of Sopungsungi-won, to study its protective effect on pancreatic β-cells, HIT-T15, against oxidative stress.

In the present study, we demonstrated that H_2O_2 induced β-cell death was noticeably prevented by the addition of RUWE in HIT-T15 cells. Instead of β-cell-specific toxins such as alloxan or streptozotocin, H_2O_2 was used as a trigger of oxidative stress, since it is known to act physiologically during most oxidative processes. Also, the destruction of β-cells in the present study occurred in a relatively short time period.

The protective effects of RUWE against the cytotoxic action of H_2O_2 were shown by MTT assay. As shown in Fig. 1, the administration of RUWE showed protective effect against the cytotoxic actions of H_2O_2. In addition, DNA strand breaks are known to occur during apoptosis, and nicks in the DNA molecules can be detected via TUNEL asay (Qiao et al, 1998). In this study, TUNEL-positive cells were detected in the H_2O_2-treated group, while the appearance of such cells was decreased in RUWE pre-treated cell cultures.

Furthermore, H_2O_2-treated HIT-T15 cells showed distinctive morphological changes, and RUWE pre-treated cells showed noticeable protective effect against cytotoxic actions of H_2O_2. Under the phase-contrast microscope, the cells of the RUWE pre-treated group appeared morphologically similar to those of the control group, while the cells of the H_2O_2-treated group revealed changes in appearance, including cell shrinkage, cytoplasmic condensation and irregularity in shape. Further confirmation with TUNEL assay and DAPI staining revealed protective effect of RUWE against H_2O_2-induced apoptosis (Figs. 3 and 4). Also, RUWE at a dose of 100 μg/ml significantly inhibited DNA fragmentation (data not shown), and induced the anti-apoptotic *bcl*-2 gene expression (Fig. 5) and caspase-3 activity (Fig. 6). RUWE seems to prevent β-cell damage caused by oxidative stress. Taken together, aqueous extract of *Rheum undulatum* has a potential as a preventive agent for progressively deteriorating type 2 diabetes.

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