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Original Article

Neuroprotective Effect of Aqueous Extract of *Polygala tenuifolia Willdenow* on Nitric Oxide-induced Apoptosis in SK-N-MC Cells

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Background: Nitric oxide (NO) is a reactive free radical gas and a messenger molecule. NO has many physiological functions, but excessive NO production induces neurotoxicity.
Objective: The present study investigated whether the aqueous extract of *Polygala tenuifolia Willdenow* possesses a protective effect on NO-induced apoptosis in human neuroblastoma cell line SK-N-MC.
Method: For this study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 4,6-diamidino-2-phenylindole (DAPI) staining, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, DNA fragmentation assay, reverse transcription-polymerase chain reaction (RT-PCR), Western blot, and caspase-3 enzyme assay were performed.
Result: Sodium nitroprusside (SNP) exposure significantly decreased the viability of cells. The cells treated with SNP exhibited several apoptotic features such as increasing of Bax expression, caspase-3 enzyme activity and inhibiting of Bcl-2 expression. On the other hand, the viability of cells pre-treated with the aqueous extract of *Polygala tenuifolia Willdenow* was increased dose-dependently. The cells pre-treated for 1 h with the aqueous extract of *Polygala tenuifolia Willdenow* followed by treatment with SNP showed a decreased occurrence of apoptotic features like decreasing Bax expressions, caspase-3 enzyme activity and increasing Bcl-2 expressions. The aqueous extract of *Polygala tenuifolia Willdenow* reduced apoptotic cell death in neuroblastoma cell line SK-N-MC through the inhibition of Bax-dependent caspase-3 activation and the increasing of Bcl-2 expression.
Conclusion: Based on the present results, it is possible that *Polygala tenuifolia Willdenow* has therapeutic value for the treatment of a variety of NO-induced brain diseases.

Key Words : Nitric oxide, Apoptosis, SK-N-MC cells, *Polygala tenuifolia Willdenow*

Introduction

Polygala tenuifolia Willdenow, which is commonly known as Won-ji, belongs to the polygalaceae and usually used without an inner part of radix. Moreover, *Polygala tenuifolia Willdenow* is a well-known traditional Korean medicine prescribed for amnesia, neurasthenia, palpitation, asthma, rhinitis and insomnia[1,2]. Nitric oxide (NO) is a reactive free radical gas and a messenger molecule with diverse physiological functions[3,4]. NO is generated from L-arginine by nitric oxide synthase (NOS). NO is synthesized in the neurons, astrocytes, microglial cells, endothelial cells, and most other cells[5]. In the mammalian central nervous system, NO modulates many physiological functions including neurotransmission, synaptic plast-

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icity, and memory[6,7]. However, excessive NO formation is emerging as a mediator of neurotoxicity, and NO induces apoptosis in a variety of disorders such as Alzheimer's disease, acquired immune deficiency syndrome (AIDS), dementia, and multiple sclerosis[8,9].

Apoptosis, also known as programmed cell death, is a biological process that plays a crucial role in normal development and in tissue homeostasis[10]. However, this type of cell death also contributes to a variety of human disorders[11]. Characteristic morphological changes of apoptosis are cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and formation of apoptotic bodies[12].

Several genes' expression has been demonstrated in the regulation of apoptosis. A milestone in understanding the function of the anti-apoptotic cytoplasmic protein Bcl-2 was the discovery that Bcl-2 was capable of heterodimerizing with the pro-apoptotic protein Bax at the mitochondrial level, creating a delicate balance of cell death preventing and promoting regulators[13]. P53 is a very short-lived transcriptional activator that induces apoptosis[14]. The activation of p53 regulates the expression of Bax[15]. Bax is a proapoptotic member of the Bcl-2 family of intracellular proteins, which alters the permeability of mitochondrial membrane and triggers caspases cascade activation[16,17]. The caspases, a class of cysteine proteases, are considered as central players of the apoptotic process and trigger a cascade of proteolytic cleavage of many proteins in mammals. In particular, caspase-3 is the most widely studied member of the caspase family and one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many proteins[18].

In the present study, we investigated the protective effect of *Polygala tenuifolia Willdenow* on NO-induced apoptosis in the neuroblastoma cell line SK-N-MC. Apoptosis was induced by sodium nitroprusside (SNP) which is a chemical NO donor, and the protective effect of *Polygala tenuifolia Willdenow* was investigated by using 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 4,6-diamidino-2-phenylindole (DAPI) staining, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, DNA fragmentation assay, reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis, and caspase-3 enzyme assay.

Materials and Methods

1. Preparation of *Polygala tenuifolia Willdenow* extract

Polygala tenuifolia Willdenow was obtained from the Kyung-dong market (Seoul, Korea). To obtain the aqueous extract of *Polygala tenuifolia Willdenow*, 120 g of *Polygala tenuifolia Willdenow* was added to distilled water, heat-extracted at 100°C, concentrated with a rotary evaporator and lyophilization (Eyela, Tokyo, Japan). The resulting powder, weighing 17 g to give a yield rate of 14.16%, was diluted with drinking water and passed through a 0.45 μm syringe filter prior to use.

2. Cell culture

The human neuroblastoma SK-N-MC cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL) at 37°C in 5% CO_2, 95% O_2 in a humidified cell incubator, and the medium was changed every 2 days.

3. MTT cytotoxicity assay

The cell viability was determined using the MTT assay kit according to the manufacturer's protocol (Boehringer Mannheim GmbH, Mannheim, Germany). For the chemical NO donor, sodium nitroprusside (SNP) was used in this study. The cells were induced with SNP at concentrations of 0.001 mM, 0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM

and 1 mM for 24 h. For analysis of the protective effect of *Polygala tenuifolia Willdenow* on cell death induced by SNP, the cells were pre-treated with aqueous extract of *Polygala tenuifolia Willdenow* at concentrations of 1 μg/ml, 5 μg/ml, 10 μg/ml, 50 μg/ml, 100 μg/ml and 500 μg/ml, respectively, for 1 h before treating with 0.5 mM SNP. These cells were stored at 37°C in 5% CO_2, 95% O_2 in a humidified cell incubator for 24 h. After that, 10 μl of MTT labeling reagent was added to each well, and the plates incubated for 4 h. Subsequently, 100 μl of solubilization solution was added to each well, the plates incubated for another 12 h, and finally the absorbance at the test wavelength 595 nm and the reference wavelength 690 nm was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA). The optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. The percent viability was calculated as follow: The percent viability (%) = (O.D. of drug-treated sample/ control O.D.) x 100.

4. Morphological changes

After treatment with SNP, the cells were washed three times in phosphate-buffered saline (PBS) and fixed with 100% methanol at -20°C for 10 min. The cells were then observed with a phase-contrast microscope (Olympus, Tokyo, Japan).

5. TUNEL assay

For in situ detection of apoptotic cells, TUNEL assay was performed using ApoTag® peroxidase in situ apoptosis detection kit (Boehringer Mannheim GmbH). The cells (2×10^4 cells/chamber) were cultured on 4-chamber slides (Nalge Nunc International, Naperville, IL, USA), washed with PBS, and fixed in 4% paraformaldehyde (PFA) for 10 min at 4°C. The fixed cells were incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase-catalyzed reaction for 1 h at 37°C in a humidified atmosphere, incubated with stop/wash buffer for 10 min at room temperature, and then incubated with anti-digoxigenin antibody conjugated with peroxidase for another 30 min. The DNA fragments were stained using 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO, USA) as the substrate for the peroxidase.

6. DAPI staining

To see whether SNP induces apoptosis, DAPI staining was performed[21]. Briefly, the cells were cultured on 4-chamber slides, washed twice with PBS, fixed by incubating with 4% PFA for 30 min, washed with PBS once again, incubated with 1 μg/ml DAPI for 30 min in the dark, and analyzed with a fluorescence microscope (Zeiss, Oberköchen, Germany).

7. DNA fragmentation

DNA fragmentation assay was performed using ApopLadder EXTM DNA fragmentation assay kit (TaKaRa, Shiga, Japan). The cells were pre-treated for 1 h with aqueous extract of *Polygala tenuifolia Willdenow* and treated with SNP, lysed with 100 μl of lysis buffer, incubated with 10 μl of 10% sodium dodecyl sulfate (SDS) solution containing 10 μl of Enzyme A at 56°C for 1 h, and then incubated with 10 μl of Enzyme B at 37°C for another 1 h. This mixture was added with 70 μl of precipitant and 500 μl of ethanol and centrifuged for 15 min. DNA was extracted by washing the pellet in ethanol and resuspending it in Tris-EDTA (TE) buffer. DNA fragmentation was visualized by 2% agarose gel electrophoresis and staining with ethidium bromide.

8. RNA Isolation and RT-PCR

Total RNA was isolated from the SK-N-MC cells using easy-BLUETM total RNA extraction kit according to the manufacturer's instruction (iNtRON,

INC., Seoul, Korea). Two μg of RNA and 2 μl of random hexamers (Promega, Madison, WI, USA) were added together and the mixture was heated at 65°C for 15 min. To the mixture, 1 μl of AMV reverse transcriptase (Promega), 5 μl of 10 mM dNTP (Promega), 1 μl of RNasin (Promega), and 5 μ l of 10 x AMV RT buffer (Promega) were added and the final volume was adjusted to 50 μl with dimethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was incubated at 42°C for 2 h. PCR amplification was performed in the reaction volume 40 μl containing 1 μl of the appropriate cDNA, 1 μl of each set of primers at the concentration 10 pM, 4 μl of 10 x reaction buffer, 1 μl of 2.5 mM dNTP, and 2 units Taq DNA polymerase (TaKaRa). The primer sequences for human Bax were 5'-GTG CAC CAA GGT GCC GGA AC-3' (a 20-mer sense oligonucleotide starting at position 375) and 5'-TCA GCC CAT CTT CTT CCA GA-3' (a 20-mer anti-sense oligonucleotide starting at position 560). The primer sequences for human Bcl-2 were 5'-CGA CGA CTT CTC CCG CCG CTA CCG C-3' (a 25-mer sense oligonucleotide starting at position 334) and 5'-CCG CAT GCT GGG GCC GTA CAG TTC C-3' (a 25-mer anti-sense oligonucleotide starting at position 628). The primer sequences for the internal control cyclophilin were 5'-ACC CCA CCG TGT TCT TCG AC-3' (a 20-mer sense oligonucleotide starting at position 52) and 5'-CAT TTG CCA TGG ACA AGA TG-3' (a 20-mer anti-sense oligonucleotide starting at position 332). The expected sizes of the PCR products are 205 bp for Bax, 318 bp for Bcl-2 and 299 bp for cyclophilin.

For Bax and Bcl-2, the PCR procedures were carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: initial denaturation at 94°C for 5 min, followed by 30 amplification cycles: each amplification cycle consisted of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 45 sec, and there was an additional extension step at the end of the procedure at 72°C for 10 min. For cyclophilin, the PCR procedure was performed under the following conditions: initial denaturation at 94°C for 5 min, followed by 25 amplification cycles: each amplification cycle consisted of denaturation at 94°C for 30 sec, annealing at 55°C, and extension at 72°C for 45 sec, and there was an additional extension step at the end of the procedure at 72°C for 10 min. The final amount of RT-PCR products for each of the mRNA species was calculated densitometrically using Mole- cular AnalystTM version 1.4.1 (Bio-Rad, Hercules, CA, USA).

9. Western blot analysis

Human neuroblastoma SK-N-MC cells were lysed in a ice-cold whole cell lysate buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM magnesium chloride hexahydrate, 1 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 1 mM phenyl-methylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium ortho-vanadate, and 100 mM sodium fluoride, and the mixture was incubated at 4°C for 30 min. The cellular debris was removed by microcentrifugation, and this was followed by quick freezing the supernatant. The protein concentration was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). 40 μg of protein was separated on SDS-polyacrylamide gels and then transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Mouse anti-actin antibody, mouse anti-Bcl-2 antibody, and mouse anti-Bax antibody (1:1000; Santa Cruz Biotech, CA, USA) were used as the primary antibodies. Horseradish peroxidase-conjugated rabbit anti-mouse antibody (1:2000; Amersham Pharmacia Biothech GmbH, Freiburg, Germany) was used as a secondary antibody. Band detection was performed using the enhanced chemiluminescence (ECL) detection system

(Amersham Pharmacia Biothech GmbH).

10. Caspase enzyme activity assay

Caspase enzyme activity was measured using the ApoAlert® caspase-3 assay kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol[23]. In brief, the cells were lysed with 50 µl of chilled Cell Lysis Buffer. A 50 µl aliquot of 2 x reaction buffer (containing DTT) and 5 µl of the appropriate conjugated substrate at a concentration of 1 mM were added to each lysate. The mixture was incubated in a water bath at 37°C for 1 h, and the absorbance was measured with a microtiter plate reader at a test wavelength of 405 nm.

11. Statistical analysis

The results are expressed as the mean ± standard error of the mean (S.E.M.). The data were analyzed by one-way ANOVA followed by Duncan's post-hoc test using SPSS. The difference was considered statistically significant at $P < 0.05$.

Results

1. Effect of *Polygala tenuifolia Willdenow* on SNP-induced cytotoxicity

1) SNP-induced cytotoxicity

The viability of cells incubated with SNP for 24 h at concentrations of 0.001 mM, 0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM and 1mM was 94.40±1.60%, 91.76±1.38%, 89.43±2.51%, 85.75±3.57%, 77.21±2.42%, 58.27±1.81% and 55.35±2.15%, respectively (Fig. 1). As the SNP concentration was increased, the cell viability decreased. The viability of cells exposed to the 0.5 mM SNP for 24 h was 58.27±1.81%.

2) Protective effect of *Polygala tenuifolia Willdenow* on NO-induced Cytotoxicity in SK-N-MC cells.

The viability of the cells pre-treated for 1 h with aqueous extract of *Polygala tenuifolia Willdenow* at the concentrations of 1 µg/ml, 5 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg /ml, and 500 µg/ml, followed by exposing to 0.5 mM SNP for 24 h was 57.87±0.81%, 59.46±1.16%, 60.17±1.52%, 64.53±0.64%, 66.85±1.90% and 74.86±0.58%, respectively (Fig. 2).

The data demonstrated that the viability of the cells was decreased by the SNP treatment, while the viability of the cells pre-treated for 1h with aqueous extracts of *Polygala tenuifolia Willdenow* (at concentrations of 50 µg/ml, 100 µg /ml, and 500 µg/ml) showed significant protective effect on NO-induced

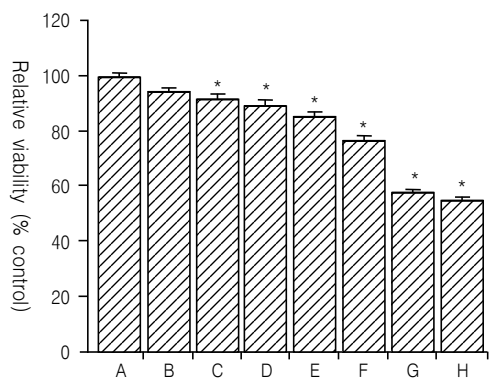

Fig. 1. Sodium nitroprusside (SNP)-induced cytotoxicity.
(A) Control, (B) 0.001 mM SNP-treated group, (C) 0.005 mM SNP-treated group, (D) 0.01 mM SNP-treated group, (E) 0.05 mM SNP-treated group, (F) 0.1 mM SNP-treated group, (G) 0.5 mM SNP-treated group, and (H) 1.0 mM SNP-treated group.

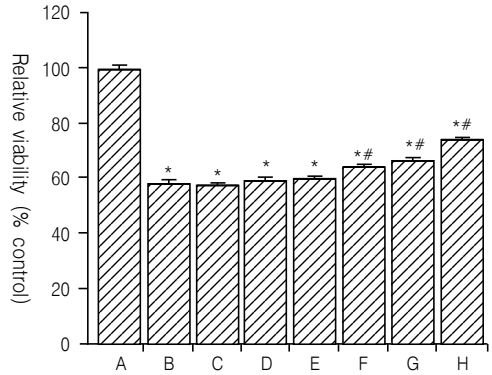

Fig. 2. Protective effect of aqueous extract of *Polygala tenuifolia Willdenow* on cell viability.

(A) Control, (B) 0.5 mM SNP-treated group, (C) 1 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group, (D) 5 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group, (E) 10 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group, (F) 50 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group, (G) 100 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group, and (H) 500 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group. The results are presented as the mean ± standard error of the mean (S.E.M.). * represents $P < 0.05$ compared to the control group. # represents $P<0.05$ compared to the SNP-treated group.

cytotoxicity in SK-N-MC cells

2. Morphological changes

To characterize SNP-induced changes in cell morphology, the cells were examined by phase-contrast microscopy. As shown in Fig. 3, the cells treated with the 0.5 mM SNP for 24 h detached from the culture dish, rounded, and became irregular shape with cytoplasmic blebbings. The cells pre-treated with the 500 μg/ml aqueous extract of *Polygala tenuifolia Willdenow*, followed by SNP exposure were indistinguishable from normal cells. In the DAPI assay, nuclear condensation, DNA fragmentation, and perinuclear apoptotic bodies were detected in the cells treated with the 0.5 mM SNP for 24 h. The cells pre-treated with 500 μg/ml aqueous extract of

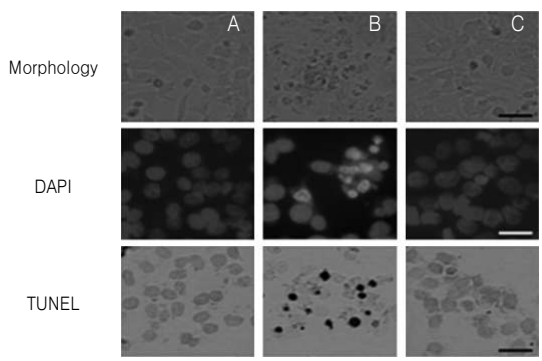

Fig. 3. Morphological analyzes of sodium nitroprusside (SNP)-induced apoptosis and protection of *Polygala tenuifolia Willdenow* on neuroblastoma SK-N-MC cells.

(A) Control group, (B) 0.5 mM SNP-treated group, and (C) 500 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group. The scale bar represents 100 μm.

Polygala tenuifolia Willdenow, followed by SNP exposure were comparable to the normal cells (Fig. 3). In addition, to further confirm the induction of apoptosis by SNP in the SK-N-MC cells, the 0.5 mM SNP-treated cells were analyzed via TUNEL assay. TUNEL-positive cells were shown to be stained dark brown under the light microscope and nuclear condensation was observed, while the cells pre-treated for 1 h with the 500 μg/ml aqueous extract of *Polygala tenuifolia Willdenow*, followed by SNP exposure presented with similar morphology of the normal cells (Fig. 3).

3. Characterization of apoptosis via examination of DNA fragmentation

In order to ascertain the protective effect of aqueous extract of *Polygala tenuifolia Willdenow* on SNP-induced apoptosis, DNA fragmentation, which reflects the endonuclease activity as a feature of apoptosis, was analyzed. As shown in Fig. 4, SNP exposure for 24 h at a concentration of 0.5 mM resulted in the formation of definite fragments which could be seen via electrophoresis as a characteristic ladder pattern. Pre-treatment for 1 h with 500 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* resulted in a significantly decreased intensity of SNP-induced DNA laddering (Fig. 4).

4. Effect of *Polygala tenuifolia Willdenow* on SNP-induced changes in mRNA expressions of Bax and Bcl-2

The RT-PCR analysis of the levels of Bax and Bcl-2 mRNA was performed to estimate the relative level of expressions of these genes.

In the present study, the mRNA level of Bax in the control was set at 1.00. The level of Bax mRNA following treatment with 0.5 mM SNP increased to 4.33±0.22, but decreased to 3.83±0.42 in the cells pre-treated with aqueous extract of *Polygala tenuifolia Willdenow* at concentrations of 100 μg/ml and markedly decreased to 2.80±0.46 in the cells pre-treated with aqueous extract of *Polygala tenuifolia Willdenow* at concentrations of 500 μg/ml.

The mRNA level of Bcl-2 in the control was set at 1.00. The level of Bcl-2 mRNA following treatment with only 0.5 mM SNP significantly decreased to 0.26±0.09, but markedly increased to 0.54±0.06 and 0.81 ±0.03 in the cells pre-treated with aqueous extract of *Polygala tenuifolia Willdenow* at concentrations of 100 μg/ml and 500 μg/ml, respectively (Fig. 5).

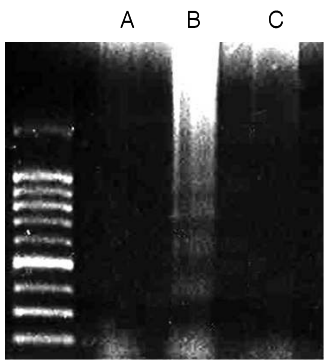

Fig. 4. Electrophoretic examination of the genomic DNA of SK-N-MC cells.
(A) Control group, (B) 0.5 mM SNP-treated group, and (C) 500 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group.

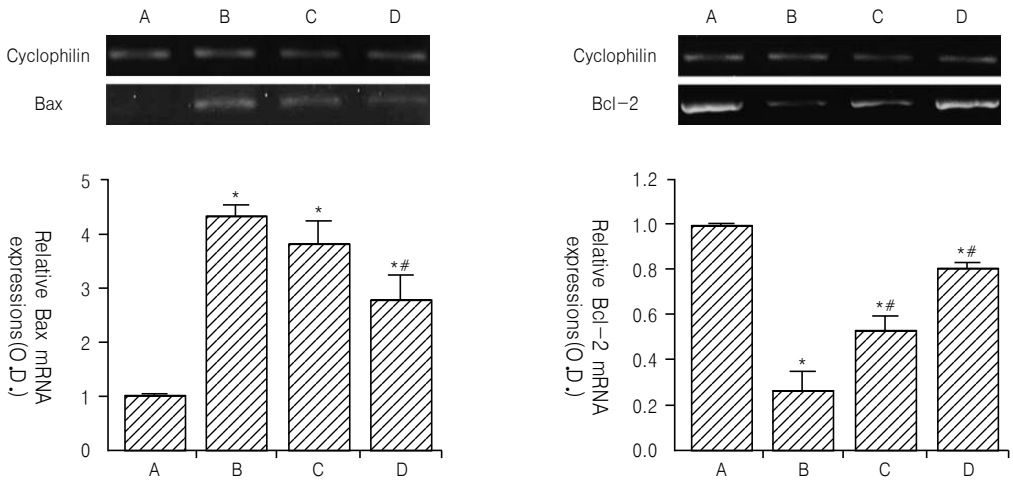

Fig. 5. Results of RT-PCR analysis of the mRNA levels of Bax and Bcl-2.

Cyclophilin mRNA was used as the internal control. The results are presented as the mean ± standard error of the mean (S.E.M.). * represents P < 0.05 compared to the control group. # represents P < 0.05 compared to the SNP-treated group. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 100 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group, and (D) 500 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group.

5. Western blot analysis of Bax and Bcl-2

The effects of aqueous extract of *Polygala tenuifolia Willdenow* on the expression of Bax and Bcl-2 proteins were investigated. The actin expression level indicates that the sample amount was equally loaded. In the cells treated with 0.5 mM SNP, increased expressions of Bax protein (26 kDa) but decreased expression of Bcl-2 protein (25 kDa) was detected. In the cells pre-treated for 1 h with 100 μg/ml and 500 μg/ml aqueous extract of *Polygala tenuifolia Willdenow*, followed by SNP exposure, the expressions of Bax protein (26 kDa) decreased but the expressions of Bcl-2 protein (25kDa) increased (Fig. 6).

6. Caspase-3 enzyme activity assay

Caspase-3 enzyme activity was measured using DEVD-peptide-nitroanilide (pNA). After incubation with 0.5 mM SNP for 24 h, the amount of DEVD-

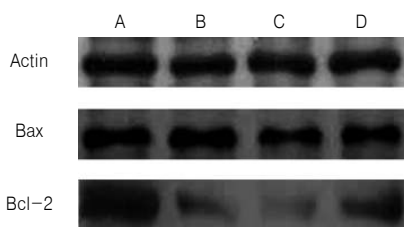

Fig. 6. Results of Western blot analysis.

Actin, used as the internal control, was detected at the position corresponding to a molecular weight of 46 kDa. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 100 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group, and (D) 500 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group.

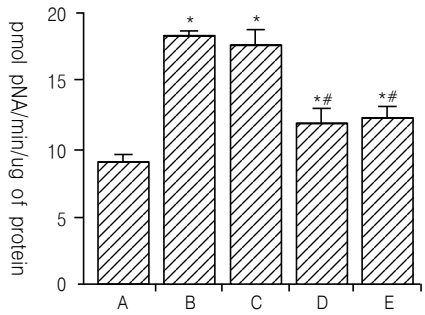

Fig. 7. Inhibitory effect of *Polygala tenuifolia Willdenow* pre-treated on sodium nitroprusside (SNP)-induced caspase-3 enzyme activity.

The rate of DEVD-pNA cleavage was measured at a wavelength of 405 nm. * represents P < 0.05 compared to the control. # represents P < 0.05 compared to the sodium nitroprusside (SNP)-treated group. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 100 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group, (D) 500 μg/ml aqueous extract of *Polygala tenuifolia Willdenow* pre-treated and 0.5 mM SNP-treated group, and (E) 0.5 mM SNP treated group with DEVD-fmk added. DEVD-fmk is a caspase-3 inhibitor.

pNA cleaved was significantly increased from 9.18± 0.36 pmol (control value) to 18.36±0.27 pmol, and this value was decreased to 11.92±1.09 pmol by pre-treatment for 1 h with 500 μg/ml aqueous extract of *Polygala tenuifolia Willdenow*. The present results demonstrated an increase in caspase-3 enzyme activity in the SNP-treated cells and a decline in this increment in the cells pre-treated with aqueous extract of *Polygala tenuifolia Willdenow* (Fig. 7).

Discussion

In the brain, NO is synthesized by neuronal NO synthase, and acts as an intercellular messenger at physiological level. However, a high concentration of NO is induced by certain pathological conditions such as brain ischemia, inflammation, or neurodegenerative disease, and excessive NO production causes apoptotic neuronal cell death, resulting in neuronal dysfunction[19,20]. The present study investigated whether *Polygala tenuifolia Willdenow* has a protective effect on NO-induced cell death in the neuroblastoma cell line SK-N-MC.

The present results of MTT assay showed that cell viability was significantly decreased by SNP exposure, and aqueous extract of *Polygala tenuifolia Willdenow* at concentrations of 50μg/ml, 100 μg/ml and 500μg/ml exerted a significant protective effect on NO-induced cytotoxicity in SK-N-MC cells. Under the phase-contrast microscope, the cells treated with SNP showed apoptotic morphologic change including cell shrinkage, cytoplasmic condensation, and irregularity in shape. Apoptotic body was detected in the SNP-induced cells stained with DAPI. Pre-treatment of aqueous extract of *Polygala tenuifolia Willdenow* showed decrease in the NO-induced apoptotic morphologic changes. In addition, TUNEL-positive cells, indicative of apoptotic DNA strand breaks and nicks in the DNA molecules, were detected in the SNP-treated cells and pre-treatment with aqueous extract of *Polygala tenuifolia Willdenow* showed decrease in the number of TUNEL-positive cells. To provide evidence supporting the involvement of apoptosis in the SNP-induced cytotoxicity, DNA fragmentation assay was performed. The distinctive ladder pattern characteristic of apoptotic cell death was detected in the cells treated with SNP; pre-treatment with aqueous extract of *Polygala tenuifolia Willdenow* showed decrease in the intensity of SNP-induced DNA laddering. The present results

revealed that apoptosis is closely implicated in NO-induced cytotoxicity in human neuroblastoma SK-N-MC cells, and aqueous extract of *Polygala tenuifolia Willdenow* has a protective effect on NO-induced cytotoxicity. The molecular mechanism underlying the NO-mediated apoptosis involves different pathways depending on cell types and different conditions[21].

Apoptosis related genes such as Bcl-2 family are divided into two categories: pro-apoptosis genes and anti-apoptosis genes. Bcl-2 is an important apoptosis repressor, while Bax is one of the most important apoptosis promoters[22,23]. Furthermore, many studies have demonstrated that NO-induced apoptosis occurs through a p53-dependent pathway in various cells, including neuronal cells[24,25]. Lee et al.[24] demonstrated that NO enhances p53 protein expression, and its phosphorylation in myoblast cells. Several pathways have been known to mediate p53-induced apoptosis. Of these, Bax is a well known p53 target gene and a pro-apoptotic member of the Bcl-2 family[26,27]. Bax promotes the release of cytochrome c into the cytosol from mitochondria, which in turn activates caspase-3[28]. In the present study, we observed that SNP increased Bax expressions of mRNA and protein as well as finally increased caspase-3 enzyme activity in SK-N-MC cells.

We further investigated whether an aqueous extract of *Polygala tenuifolia Willdenow* inhibits NO-related cell death pathways such as Bax, Bcl-2 and caspase-3. The results of the present study showed that aqueous extract of *Polygala tenuifolia Willdenow* attenuates NO-induced apoptotic cell death via the Bcl-2 and Bax-dependent caspase-3 pathways.

In this study, we have shown that aqueous extract of *Polygala tenuifolia Willdenow* reduced apoptotic cell death in neuroblastoma cell line SK-N-MC through the inhibition of Bax-dependent caspase-3 activation and the increasing of Bcl-2 expression. Based on the present results, it is possible that *Polygala tenuifolia Willdenow* can have therapeutic value for the treatment of a variety of NO-induced brain diseases such as stroke, ischemia, Alzheimer's disease, and seizures.

Conclusion

Based on the present results, it is possible that *Polygala tenuifolia Willdenow* has therapeutic value for the treatment of a variety of NO-induced brain diseases such as stroke, ischemia, Alzheimer's disease, and seizures.

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