대한한의학회지 제28권 제1호(2007년 3월)
J Korean Oriental Med 2007;28(1):198-210

원 저

Protective Effect of *Red Ginseng* and *Paeonia radix* against Nitric Oxide-Induced Apoptosis in Human Neuroblastoma SK-N-MC cells

Young-hoi Park, Yunk-yung Song, Hyung-ho Lim

Dept. of Rehabilitation Medicine, College of Oriental Medicine, Kyung-Won University

Objectives : Nitric oxide(NO) is a reactive free radical and a messenger molecule in many physiological functions. However, excessive release NO of induces neurotoxicity. We investigated whether a mixture of red ginseng and *paeonia radix* possesses a protective effect against sodium nitroprusside(SNP)-induced apoptosis in the human neuroblastoma cell line SK-N-MC.

Methods : We performed 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 activity assay in SK-N-MC cells.

Results : MTT assay showed that SNP treatment significantly reduced the viabilities of cells and that pre-treatment with the red ginseng and *paeonia radix* mixture alleviated SNP-induced cytotoxicity. The cells treated with SNP exhibited several apoptotic features, while those pre-treated for 1 h with the mixture of red ginseng and *paeonia radix* for 1 h prior to SNP expose showed reduced apoptotic features. In addition, the cells pre-treated with the red ginseng and *paeonia radix* mixture for 1 h prior to SNP expose increased bcl-2 expressions, decreased Bax expressions, and decreased caspase-3 enzyme activity.

Conclusions : These results show that the red ginseng and *paeonia radix* mixture exerts a protective effect against SNP-induced apoptosis in SK-N-MC cells.

Key Words: Nitric oxide, apoptosis, SK-N-MC cells, red ginseng, *paeonia radix*

Introduction

Nitric oxide(NO) is a reactive free radical gas and a messenger molecule with many physiological functions[1-2]. NO is generated from L-arginine by nitric oxide synthase(NOS), and is synthesized in neurons, astrocytes, microglial cells, endothelial cells and many other types of cells[3].

Apoptosis, also known as programmed cell death, is an important process in a wide variety of different biological systems including normal cell turnover, the immune system, embryonic development, metamorphosis and hormone-dependent atrophy, and is also involved in chemical-induced cell death[4-6]. Excessive apoptosis is implicated in many human diseases including neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, ischemic damage, autoimmune disorders, and several forms of cancer[7-8].

Caspases, considered to be central players in the apoptotic process, are a class of cysteine

· 접수 : 2007년 2월 27일 · 논문심사 : 2007년 2월 27일
· 채택 : 2007년 3월 9일
· 교신저자 : Hyung-ho Lim, Seoul Oriental Hospital, Kyung-won university, 20-8 Songpa-Dong, Songpa-Gu, Seoul, Korea
(Tel : 02-425-3456, Fax : 02-425-3560
E-mail : omdlimhh@chol.com)

proteases. They trigger a cascade of proteolytic cleavages of many proteins in mammals. In particular, the most widely studied member of the caspase family, caspase-3, is a key initiator of apoptosis, and is partially or totally responsible for many proteins' proteolytic cleavage[9].

Ginseng radix, the root of *Panax ginseng C. A. METER(Araliaceae)*, is one of the best-known medicinal herbs, and has numerous therapeutic applications, such as immune modulatory, anti-mutagenic, adaptogenic, tonic and anti-aging activities[10-11]. Red ginseng(RG) is made by steaming Panax ginseng and drying it repeatedly. The medicinal effects of *ginseng radix* are attributed to the triterpene glycosides known as ginsenosides (saponins)[12].

Paeonia radix(PR) is the root of *Paeonia japonica MIYABE*, which is a perennial plant classified in the family *paeoniaceae*. Aqueous extract of PR has been used in oriental medicine to treat various illnesses including gastralgia, gynopathy, enterorrhagia and headache. PR possesses various pharmacological properties such as sedative, analgesic, anti-inflammatory, anti-stress, anti-microbial and immune augmentative actions[13-15].

The present study investigated the protective effects of the aqueous extracts of RG, PR and a mixture of RG and PR against NO-induced apoptosis in the neuroblastoma cell line SK-N-MC. Apoptosis in SK-N-MC cells was induced by SNP, an NO donor, and the protective effects of RG , PR and the mixture of RG and PR were investigated using following methods.

Materials and Methods

1. Extraction of the aqueous extract of RG and PR

RG and PR used in this experiment were obtained from Korea Pharmacologies(Seoul, Korea). To obtain the aqueous extract of RG and PR, 200 g of RG or PR was added to 2000cc of distilled water, and extraction was performed by heating at 90°C, concentrating with a rotary evaporator, and lyophilization(EYELA, Tokyo, Japan). The amounts of the resulting powders of RG and PR were 10.34g and 17.91g, respectively. The powders were dissolved in saline solution and filtered through a 0.45 μm syringe before use. The mixture of RG and PR was composed of equal parts RG and PR.

2. Cell culture

The human neuroblastoma SK-N-MC cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in Dulbecco's modified eagle medium(DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum(FBS; Gibco BRL) at 37°C in a 5% CO2, 95% O2 humidified cell incubator, and the medium was changed every 2 days.

3. MTT cytotoxicity assay

Cell viability was determined using the MTT assay kit according to the manufacturer's instructions(Boehringer Mannheim GmbH, Mannheim, Germany). The cells were treated with SNP at concentrations of 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, and 1.0 mM for 24 h. To investigate the protective effect of RG, PR and the mixture of RG and PR against cell death induced by SNP, the cells were pre-treated for 1 h with RG, PR and the mixture of RG and PR at 0.001 mg/ml, 0.01 mg/ml, 0.1 mg/ml and 1.0 mg/ml and then treated with SNP for 24 h. Ten μl of MTT

labeling reagent was added to each well, and plates were incubated for 4 h. Subsequently, 100 μl of solubilization solution was added to each well, and plates were incubated for another 12 h. Absorbance was measured at test wavelength of 595 nm using a reference wavelength of 690 nm 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 follows :

The percent viability
= (O.D. of drug-treated sample/control O.D.) × 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 under a phase-contrast microscope(×100, Olympus, Tokyo, Japan).

5. DAPI staining

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

6. TUNEL assay

To detect apoptotic cells in situ, TUNEL assay was performed using ApoTag® peroxidase in situ apoptosis detection kit(Boehringer Mannheim GmbH). The cells(2 × 104 cells/chamber) were cultured on 4-chamber slides (Nalge Nunc International, Naperville, IL, USA), washed with PBS, and fixed in 4% 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, then with a wash buffer for 10 min at room temperature, and finally 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), a peroxidase substrate.

7. DNA fragmentation

DNA fragmentation assay was performed using ApopLadder EXTM DNA fragmentation assay kit (TaKaRa, Shiga, Japan). The cells were pretreated for 1 h with RG, PR and the mixture of RG and PR and then treated with SNP for 24 h, lysed with 100 ?l 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-BLUE total RNA extraction kit according to the manufacturer's instructions (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 10 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 μl of 10 × 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 then incubated at 42°C for 1 h.

PCR amplification was performed in a reaction volume of 40 μl containing 1 μl of the appropriate cDNA, 1 μl of each set of primers at a concentration of 10 pM, 4 μl of 10 × reaction buffer, 1 μl of 2.5 mM dNTP, and 2 units Taq DNA polymerase(TaKaRa). The primer sequences for Bax were 5- GTGCACCAAGGTGCCGGAAC-3 (a 20-mer sense oligonucleotide starting at position 375) and 5-TCAGCCCATCTTCTTCCAGA-3(a 20-mer anti-sense oligonucleotide starting at position 560). The primer sequences for bcl-2 were 5-CGACGACTTCTCCCGCCGCTACCGC-3(a 25-mer sense oligonucleotide starting at position 334) and 5-CCGCATGCTGGGGCCGTACAGTTCC-3(a 25-mer anti-sense oligonucleotide starting at position 628). Primer sequences for the internal control cyclophilin were 5'- ACCCCACCGTGTTCTTCGAC-3' (a 20-mer sense oligonucleotide starting at position 52) and 5'- CATTTGCCATGGACAAGATG-3' (a 20-mer anti-sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 205 bp for Bax, 318 bp for bcl-2, and 291 bp for cyclophilin.

For Bax and bcl-2 the PCR procedure 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 consisting of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec, with an additional extension step at the end of the procedure at 72°C for 5 min. The PCR procedure was carried out under identical conditions except that 25 amplification cycles for cyclophilin was performed. The final amount of RT-PCR product was calculated densitometrically using Molecular Analyst version 1.4.1 (Bio-Rad, Hercules, CA, USA).

9. Western blot analysis

The cells were lysed in the lysis buffer containing 50 mM Tris-HCl(pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid, 1% nonidet-P40 (NP40), 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride(PMSF), and 100 g/ml leupeptin. Protein concentration was measured using a Bio-Rad colorimetric protein assay kit(Bio-Rad). Protein of 50 g was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane(Schleicher & Schuell GmbH, Dassel, Germany). Mouse Bax antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) and rabbit bcl-2 antibody(Santa Cruz Biotech) were used as primary antibody. Horseradish peroxidase-conjugated anti-mouse antibody for Bax and anti-rabbit antibody for bcl-2(Santa Cruz Biotech) were used as secondary antibodies. The detection of the band was performed using the enhanced chemiluminescence(ECL) detection system(Amersham Pharmacia Biotech GmbH, Freiburg, Germany).

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. In brief, the cells were lysed with 50 μl

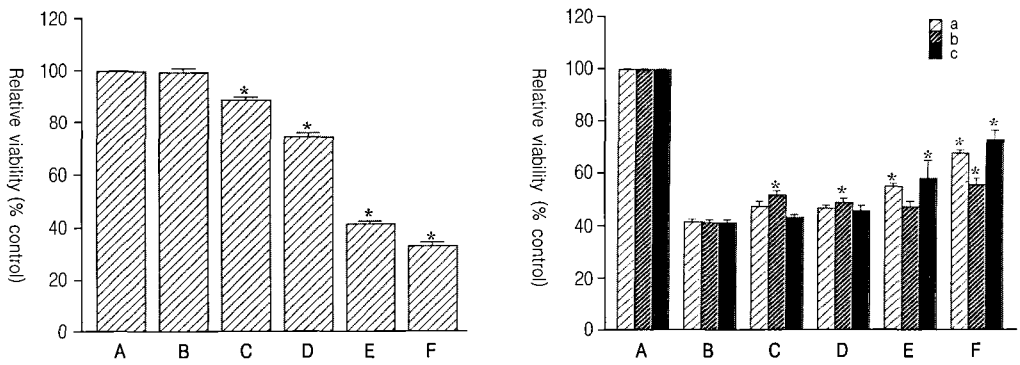

Fig. 1. Protective effect of RG and PR. * represents P<0.05 compared to the control group. Above: Cytotoxic effect of sodium nitroprusside (SNP). (A) Control, (B) 0.01 mM SNP-treated group, (C) 0.05 mM SNP-treated group, (D) 0.1 mM SNP-treated group, (E) 0.5 mM SNP-treated group, (F) 1.0 mM SNP-treated group. Below: a: Protective effect of RG on cell viability. b: Protective effect of PR on cell viability. c: Protective effect of the RG/PR mixture on cell viability. (A) Control, (B) 0.5 mM SNP-treated group, (C) 0.001 mg/ml respective herb pre-treated followed by 0.5 mM SNP-treated group, (D) 0.01 mg/ml respective herb pre-treated followed by 0.5 mM SNP-treated group, (E) 0.1mg/ml respective herb pre-treated followed by 0.5 mM SNP-treated group, (F) 1.0 mg/ml respective herb pre-treated followed by 0.5 mM SNP-treated group.

of chilled cell lysis buffer. Fifty μl aliquot of 2 × 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 using a microtiter plate reader at a test wave length of 405 nm.

11. Statistical analyses

The results were expressed as the mean ± standard error of the mean(SEM). The data were analyzed by one-way ANOVA followed by Duncan's post-hoc test using SPSS for Windows (version 11.0). The difference was considered statistically significant for P<0.05.

Results

1. Cytotoxicity

Viability of cells incubated with SNP for 24 h at concentrations of 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, and 1.0 mM was 99.43 ± 1.66%, 89.18 ± 0.80%, 74.95 ± 1.25%, 41.94 ± 0.93%, and 33.58 ± 1.62%, respectively(Fig. 1. above). 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 41.94 ± 0.93%.

Viability of the cells pre-treated for 1 h with the RG at concentrations of 0.001 mg/ml, 0.01 mg/ml, 0.1 mg/ml, and 1.0 mg/ml, and then exposed to 0.5 mM SNP for 24 h was 48.44 ± 1.50%, 47.91 ± 1.01%, 56.32 ± 0.60%, and 69.08 ± 0.81% respectively(Fig. 1. below. a).

Viability of the cells pre-treated for 1 h with the PR at concentrations 0.001 mg/ml, 0.01 mg/ml, 0.1 mg/ml, and 1.0 mg/ml, and then exposed to 0.5 mM SNP for 24 h was 52.55 ± 1.29%, 50.06 ± 1.31%, 48.88 ± 1.61%, and 57.13 ± 2.08 respectively(Fig. 1. below. b.).

The viability of the cells pre-treated for 1 h with the mixture of RG and PR at concentrations

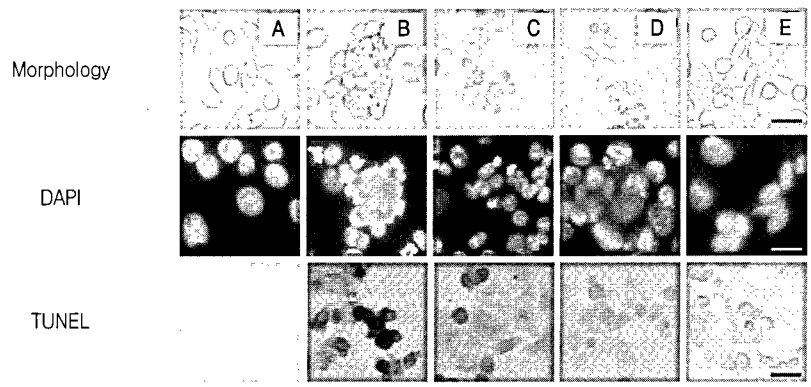

Fig. 2. Morphological analysis. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 1.0 mg/ml RG pre-treated followed by 0.5 mM SNP-treated group. (D) 1.0 mg/ml PR pre-treated followed by 0.5 mM SNP-treated group. (E) 1.0 mg/ml the RG/PR mixture pre-treated followed by 0.5 mM SNP-treated group. The scale bar represents 100 μm. Above: Phase-contrast microscopy. Middle: SK-N-MC cells stained with DAPI. Below: SK-N-MC cells stained via TUNEL assay.

of 0.001 mg/ml, 0.01 mg/ml, 0.1 mg/ml, and 1.0 mg/ml, and then exposed to 0.5 mM SNP for 24 h was increased to 44.46 ± 0.89%, 47.06 ± 1.48%, 59.46 ± 6.47%, and 74.13 ± 3.39% respectively (Fig. 1. below. c).

The data that cell viability was reduced by SNP showed protective effect against SNP-induced cytotoxicity as dose-dependently, RG, PR and the RG/PR mixture. The most potent protective effect against the SNP-induced cytotoxicity was observed for the mixture of RG and PR.(Fig. 1).

2. Morphological changes

To characterize SNP-induced changes in cell morphology, cells were observed by phase-contrast microscopy. The cells treated with 0.5 mM SNP for 24 h detached from the culture dish, and became rounded and irregular in shape with cytoplasmic blebbings. The cells pre-treated for 1 h with the 1.0 mg/ml RG, 1.0 mg/ml PR, 1.0 mg/ml the RG/PR mixture and then exposed to 0.5 mM SNP for 24 h showed meaningful changes from the normal cells(Fig. 2, upper).

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 for 1 h with the 1.0 mg/ml RG, 1.0 mg/ml PR, 1.0 mg/ml the RG/PR mixture, and then exposed to 0.5 mM SNP for 24 h were comparable to the normal cells(Fig. 2, middle).

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 found to be stained dark brown under the light microscope and nuclear condensation was observed, whereas the cells pre-treated for 1 h with the 1.0 mg/ml RG, 1.0 mg/ml PR, 1.0 mg/ml the RG/PR mixture, and then exposed to 0.5 mM SNP for 24 h showed near normal morphology(Fig. 2, lower).

RG, PR and the RG/PR mixture showed protective effect against the SNP-induced morphologic changes. The most potent protective effect against the SNP-induced morphologic changes was obs-

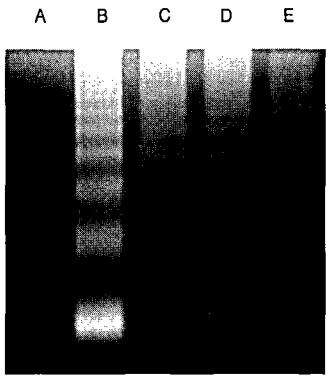

Fig. 3. Electrophoretic examination of the genomic DNA of SK-N-MC cells. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 1.0 mg/ml RG pre-treated followed by 0.5 mM SNP-treated group. (D) 1.0 mg/ml PR pre-treated followed by 0.5 mM SNP-treated group. (E) 1.0 mg/ml the RG/PR mixture pre-treated followed by 0.5 mM SNP-treated group.

erved for the RG/PR mixture.

3. DNA fragmentation

In order to ascertain the protective effect of the RG/PR mixture against SNP-induced apoptosis, DNA fragmentation and the influence of the endonuclease activity were analyzed. SNP treatment at 0.5 mM for 24 h resulted in the formation of definite fragments which could be seen via electrophoresis as a characteristic ladder pattern. Pre-treatment for 1 h with 1.0 mg/ml RG, 1.0 mg/ml PR, 1.0 mg/ml the RG/PR mixture and then exposed to 0.5 mM SNP for 24 h resulted in a significant reduction in the intensity of SNP-induced DNA laddering. The most protective effect on SNP-induced DNA fragmentation was observed for the RG/PR mixture(Fig. 3).

4. RNA isolation and RT-PCR

The RT-PCR was performed to estimate the relative expressions of the Bax and bcl-2 mRNA. 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 for 24 h increased to 9.40 ± 1.13, but it decreased to 5.38 ± 1.00, 5.87 ± 1.01, and 3.68 ± 0.15 in the cells pre-treated with 1.0 mg/ml RG, 1.0 mg/ml PR, and 1.0 mg/ml the RG/PR mixture for 1 h, and then exposed to 0.5 mM SNP for 24 h, respectively.

The mRNA level of bcl-2 in the control was set at 1.00. The level of bcl-2 mRNA following treatment with 0.5 mM SNP decreased to 0.58 ± 0.02, but it increased to 1.01 ± 0.04, 0.95 ± 0.03, and 1.50 ± 0.06 in the cells pre-treated with 1.0 mg/ml RG, 1.0 mg/ml PR, and 1.0 mg/ml the RG/PR mixture for 1 h, and then exposed to 0.5 mM SNP for 24 h, respectively. Treatment with SNP increased Bax mRNA expression and dec- reased bcl-2 mRNA expression. Pre-treatment with RG, PR and the RG/PR mixture alleviated SNP-induced Bax mRNA expression and enhanced bcl-2 mRNA expression. The most potent effect was observed for the RG/PR mixture(Fig. 4).

5. Western blot analysis

When the cells were treated with 0.5 mM SNP for 24 h, Bax(26 kDa) protein expression was

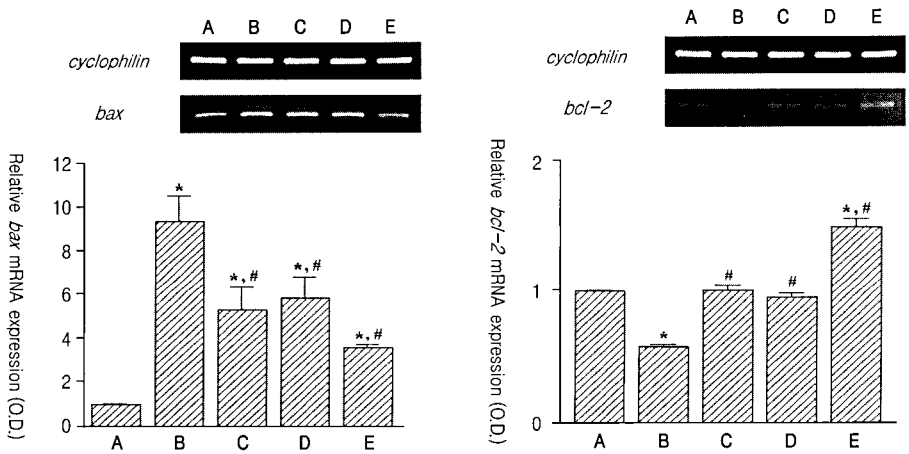

Fig. 4. Results of RT-PCR analysis of the mRNA levels of bcl-2 and Bax. * represents P<0.05 compared to the control group. # represents P<0.05 compared to the SNP-treated group. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 1.0 mg/ml RG pre-treated followed by 0.5 mM SNP-treated group. (D) 1.0 mg/ml PR pre-treated followed by 0.5 mM SNP-treated group. (E) 1.0 mg/ml the RG/PR mixture pre-treated followed by 0.5 mM SNP-treated group. Above: Expression of Bax mRNA levels. Blow: Expression of bcl-2 mRNA levels.

up-regulated and bcl-2 protein(25 kDa) expression was down-regulated. Compared to these cells, those of cells pre-treated with 1.0 mg/ml RG, 1.0 mg/ml PR and 1.0 mg/ml the RG/PR mixture for 1 h, and then exposed to 0.5 mM SNP for 24 h showed lower Bax protein expression and higher bcl-2 protein expression. The most suppressing effect on Bax protein and enhancing effect on bcl-2 protein was observed for the RG/PR mixture(Fig. 5).

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-pNA cleaved significantly increased from 3.88 ± 0.32 nmol(control value) to 12.32 ± 0.20 nmol, but this was decreased to 7.51 ± 0.41, 8.78 ± 1.17, and 6.68 ± 0.44 by pre-treatment with 1.0 mg/ml RG, 1.0 mg/ml PR and 1.0 mg/ml the RG/

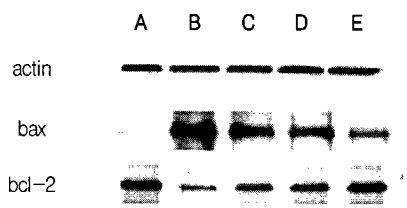

Fig. 5. Results of Western blot analysis of protein levels of Bax and bcl-2. Actin, used as the internal control, was detected at the position corresponding to a molecular weight of 46 kDa. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 1.0 mg/ml RG pre-treated followed by 0.5 mM SNP-treated group. (D) 1.0 mg/ml PR pre-treated followed by 0.5 mM SNP-treated group. (E) 1.0 mg/ml the RG/PR mixture pre-treated followed by 0.5 mM SNP-treated group.

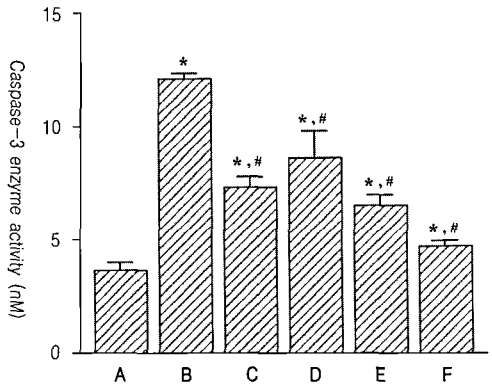

Fig. 6. Results of caspase-3 enzyme assay. The rate of DEVD-pNA cleavage was measured at a wavelength of 405 nm. * represents P<0.05 compared to the control. # represents P<0.05 compared to the SNP-treated group. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 1.0 mg/ml RG pre-treated followed by 0.5 mM SNP-treated group. (D) 1.0 mg/ml PR pre-treated followed by 0.5 mM SNP-treated group. (E) 1.0 mg/ml the RG/PR mixture pre-treated followed by 0.5 mM SNP-treated group, (F) 0.5 mM SNP-treated group with DEVD-fmk added. DEVD-fmk is a caspase-3 inhibitor.

PR mixture, respectively. Caspase-3 enzyme activity was decreased 4.87 ± 0.21 nmol by treatment with 0.5 mM SNP-treated with DEVD-fmk. DEVD-fmk is a caspase-3 inhibitor. The present results demonstrate that SNP increased caspase-3 enzyme activity in SK-N-MC cells and that RG, PR and the RG/PR mixture inhibited caspase-3 enzyme activity. The most potent supp- ressing effect on caspase-3 enzyme activity was observed for the RG/PR mixture(Fig. 6).

Discussion

In the brain, NO at physiological levels generated by neuronal or endothelial NO synthase acts as an intercellular messenger between neurons and other brain cells[16-17]. Under pathological conditions the expression of inducible NO synthase (iNOS) is induced in glia cells (particularly astrocytes and microglia) by inflammatory mediators [18]. The high levels of NO production in glia cells during inflammatory, ischemic and neuronal degenerative diseases result in neuronal dysfunction [19-20]. Furthermore, NO can regulate the apoptotic signaling cascade by regulating several gene expressions, mitochondrial dysfunction and caspases activation[21-22]. The present study investigated the protective effect of RG, PR and a mixture of RG and PR on SNP-induced cell death in human neuroblastoma SK-N-MC cells.

The characteristic morphological changes associated with apoptosis are cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation and the formation of apoptotic bodies[23].

The process of apoptosis is regulated by the expression of several proteins. Two important proteins involved in apoptotic cell death are members of the bcl-2 family[24] and a class of cysteine proteases known as caspases[25]. The bcl-2 family can be classified into two functionally distinct groups: anti-apoptotic and pro-apoptotic. Bcl-2, an anti-apoptotic protein is known to regulate the

apoptosis pathway and to prevent cell death, while Bax, a pro-apoptotic protein in the same family, is known to be expressed abundantly and selectively during apoptosis and to promote cell death[26]. The ratio of bcl-2 to Bax is thought to determine survival or death after apoptotic stimulation[26]. Bax alters the mitochondrial membranes' permeability and triggers caspases' cascade activation[27].

Our MTT assay showed that SK-N-MC cell viability was significantly reduced by SNP treatment, and that RG, PR and the RG/PR mixture exerted a significant protective effect against NO-induced cytotoxicity. The RG/PR mixture showed the most potent protective effect. Under a phase-contrast microscope, the cells treated with SNP showed apoptotic morphologies, i.e., cell shrinkage, cytoplasmic condensation and irregular shapes. Moreover, apoptotic bodies were observed in the SNP-treated cells stained with DAPI. However, the cells pre-treated with RG, PR and the RG/PR mixture prior to SNP treatment showed lower levels of apoptotic morphologic changes. In addition, TUNEL-positive cells, indicative those with apoptotic DNA strand breaks and nicks in DNA molecules, were detected in the SNP-treated cells but the cells pre-treated with RG, PR and the RG/PR mixture showed fewer TUNEL-positive cells. To provide evidence supporting the involvement of apoptosis in SNP-induced cytotoxicity, a DNA fragmentation assay was performed. Apoptotic cell death's distinctive ladder pattern characteristic was detected in cells treated with SNP: on the other hand, pre-treatment with RG, PR and the RG/PR mixture prior to SNP treatment led to lower SNP-induced DNA laddering intensity. The present results show that apoptosis is closely involved in NO-induced cytotoxicity in human neuroblastoma SK-N-MC cells, and that the RG/PR mixture has the most potent protective effect against this cytotoxicity.

Molecular mechanisms underlying NO-mediated apoptosis involve different pathways, which depend on cellular types and the cellular environments [28]. The bcl-2 family of proteins is a key regulator of the mitochondrial response to apoptotic signals and contains both pro- and anti-apoptotic members. Bax is directly involved in the release of cytochrome c from mitochondria[29-31]. When Bax is overexpressed in cells, apoptotic death in response to a death signal is accelerated, result in Bax's designation as a death antagonist. Anti-apoptotic proteins such as bcl-2 are induced by different stimuli[32]. The present results show that NO increased Bax expression and decreased bcl-2 expression in both mRNA and protein levels.

Caspases, a family of cysteine proteases, are integral parts of the apoptotic pathway. Caspase-3, in particular, has many cellular targets and produces the morphological features of apoptosis. The present results revealed that caspase-3 enzyme activity was up-regulated in the cells exposed to NO. We further investigated whether RG, PR and the RG/PR mixture inhibit NO-related cell death pathways involving Bax, bcl-2 and caspase. Our results showed that the RG/PR mixture most potently attenuated NO-induced apoptotic cell death by blocking the pathway for Bax-dependent caspase-3's activation.

Ginsenosides are known to possess triterpene structures in their backbone and to have structural diversity. The ginsenosides Rg3, Rg5, Rg6, Rh2, Rh3 and Rh4 are genuine saponins in RG[33]. Although extracts from ginseng radix have been

extensively tested for their pharmacological effects, their precise biological functions and underlying action mechanisms are still unknown. It has been reported that ginsenoside Rg3 prevents endothelial cell apoptosis via inhibition of a mitochondrial caspase pathway[34]. In a previous study, ginseng radix had a protective effect against 1-Methyl-4-phenlypyridinium (MPP+)-induced apoptosis in PC12 cells[35]. In addition, ginseng radix suppressed an ischemia-induced increase in c-Fos expression and apoptosis in the hippocampal CA1 region in gerbils[36].

PR is a potential anti-aging and anti-carcinogenic agent, as it has been reported to inhibit oxidative DNA cleavage induced by various oxidative DNA damaging chemicals[37]. PR contains a multitude of complex organic compounds. It has been reported that paeoniflorin is one of the compounds extracted from PR and that it has anti-inflammatory and anti-stress effects, and decreases blood pressure[38]. Gallotannin is another group of pharmacologically active components of PR, which has reactive oxygen species-scavenging effects and thus prevents oxidative neuronal cell damage[39]. A previous study found that PR inhibited 5-hydroxytryptamine synthesis and tryptophan hydroxylase expression in the dorsal raphe of exercised rats[40].

The present study tested the viability with 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM and 1.0 mM of SNP in order to find the appropriate density of SNP. The results show that the density of SNP that made the relative viability value closest to 50, is 0.5 mM. Based on this result, 0.5 mM of SNP was chosen for use in the experiment.

The present study showed that RG, PR and the RG/PR mixture reduced apoptotic cell death in a neuroblastoma cell line by inhibiting Bax-dependent caspase-3 activation in human neuroblastoma SK-N-MC cells. The most potent protective effect was observed in the RG/PR mixture. Based on the results presented in this study, the RG/PR mixture could be supposed to have potential therapeutic value in the treatment of a variety of NO-induced brain diseases such as Alzheimer's disease and autoimmune disorders. This research will help the RG/PR mixture to be developed as a new preventive and therapeutic drug that can treat the neuronal dysfunctional and neurodegenerative diseases.

Conclusion

To find the protective effect of the RG/PR mixture against nitric oxide-induced apoptosis in human neuroblastoma SK-N-MC cells, we carried out several experiments. The experimental results were as follows:

1. The extracts of RG, PR and mixture of RG and PR prevented nitric oxide-induced apoptosis.

2. The extracts of mixture of RG and PR most potentively decreased occurrence of apoptotic features than NO-induced apoptosis through DAPI assay and TUNEL assay.

3. The pre-treatment mixture of RG and PR prior to SNP exposure resulted in decreased intensity of SNP-induced DNA laddering.

4. RG, PR and the RG/PR mixture reduced apoptotic cell death in a neuroblastoma cell line by inhibiting Bax-dependent caspase-3 activation in human neuroblastoma SK-N-MC cells.

5. Mixture of RG and PR could be supposed to potential therapeutic value in the treatment of a variety of NO-induced neuronal brain diseases.

References

1. Schmidt HH, Walter U. NO at work. Cell. 1994; 78:919-925.
2. Yun HY, Dawson VL, Dawson TM. Neurobiology of nitric oxide. Crit Rev Neurobiol. 1996;10:291-316.
3. Garthwaite J. Boulton CL. Nitric oxide signaling in the central nervous system. Annu. Rev. Physiol. 1995;57:683-706.
4. Arends MJ, Wyllie AH. Apoptosis : mechanisms and roles in pathology. Int Rev Exp Pathol. 1991;32:223-54.
5. Ellis RE, Yuan JY, Horvitz HR. Mechanisms and functions of cell death. Annu Rev Cell Biol. 1991;7:663-98.
6. Cohen JJ, Duke RC, Fadok VA, Sellins KS. Apoptosis and programmed cell death in immunity. Annu Rev Immunol. 1992;10:267-293.
7. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science. 1995;267: 1456-1462.
8. Nicholson, D.W. ICE/CED3-like proteases as therapeutic targets for the control of inappropriate apoptosis. Nat Biotechnol. 1996; 14:297-301.
9. Cohen GM. Caspases: the executioners of apoptosis. Biochem J. 1997;326;1-16
10. Lee TK, Johnke RM, Allison RR, OBrien KF, Dobbs Jr LJ. Radioprotective potential of ginseng. Mutagenesis. 2005;20:237-243.
11. Kiefer D, Pantuso T. Panax ginseng. Am. Fam. Physician. 2003;68:1539-1542.
12. Attele AS, Wu JA, Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. Biochem Pharmacol. 1999;58:1685-93.
13. Hong ND, Rho YS, Cho YW, Ju SM. Studies on Efficacy of Crude Drug by Processing(Ⅲ) : Effect of Paeoniae Radix alba on CNS and Isolated Ileum. J. Kor. Pharm Sci. 1986;16: 124-131.
14. Hwang JS, Chun HJ, Han YS. Isolation and Identification of Antimicrobial Compound from Jakyak(Paeonia japonica var. pilosa NAKAI). Korean J. Soc. Food Sci. 2000;16,445-452.
15. Park HR, Ham YH, Yee ST, Paik SG., Jo SK. Characteristics of B Cell proliferation by polysaccharide fraction of Paeonia japonica miyabe Immune Network. 2001;1:126-134.
16. Garthwaite J. Boulton CL. Nitric oxide signaling in the central nervous system. Annu. Rev. Physiol. 1995;57:683-706.
17. Prast H. and Philippu A. Nitric oxide as modulator of neuronal function. Prog. Neurobiol. 2001;64:51-68.
18. Murphy M.P. Production of nitric oxide by glial cells: regulation and potential roles in the CNS. Glia. 2000;29: 1-14.
19. Heales SJR, Bolanos JP, Stewart VC, Brookes PS, Land JM, Clark JB. Nitric oxide, mitochondria and neurological disease. Biochim. Biophys. Acta. 1999;1410:215-228.
20. Brown GC, Bal-Price A. Inflammatory neurodegeneration nediated by nitric oxide, glutamate and mitochondria. Mol Neurobiol. 2003; 27:325-355.
21. Brune B, Messmer UK, Sandau K. The role of nitric oxide in cell injury. Toxicol. Lett. 1995; 82/83: 233-237.
22. Kronke KD, Fehsel K, Kolb-Bachofen V. Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. Biol. Chem. Hoppe-Seyler 1995;376:327-343.
23. Wyllie AH, Kerr JF, Currie AR. Cell death: the

significance of apoptosis. Int Rev Cytol. 1980; 68:251-306.
24. Korsmeyer SJ. BCL-2 gene family and the regulation of programmed cell death. Cancer Res. 1999;59:1693-1700.
25. Cohen GM. Caspases: the executioners of apoptosis. Biochem J. 1997;326;1-16
26. Oltavai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerized in vivo with a conserved ho- molog. Bax, that accelerates programmed cell death. Cell. 1993;74:609-619
27. Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol. 1999;15:269-290.
28. Bosca L, Hortelano S. Mechanisms of nitric oxide-dependent apoptosis: Involvement of mitochondrial mediators. Cell Signal. 1999;11:239-244.
29. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004;116:205-219
30. Antonsson B. Mitochondria and the Bcl-2 family proteins in apoptosis signaling pathways. Mol Cell Biochem. 2004 Jan-Feb;256-257:141-55.
31. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death, Science. 2004;305: 626-629.
32. Chao DT, Linette G.P, Boise LH, White LS, Thompson CB, Korsmeyer SJ. Bcl-XL and Bcl-2 repress a common pathway of cell death. J. Exp. Med. 1995;182:821-828.
33. Kitagawa I, Yoshikawa M, Yoshihara M, Hayashi T, Taniyama T. Chemical studies of crude drugs (1). Constituents of Ginseng radix rubra. Yakugaku Zasshi. 1983;103:612-622.
34. Min JK, Kim JH, Cho YL, Maeng YS, Lee SJ, Pyun BJ, Kim YM, Park JH, Kwon YG. 20(S)-Ginsenoside Rg3 prevents endothelial cell apoptosis via inhibition of a mitochondrial caspase pathway. Biochem Biophys Res Commun. 2006;349:987-994.
35. Kim EH, Jang MH, Shin MC, Shin MS, Kim CJ. Protective effect of aqueous extract of ginseng radix against 1-methyl-4-phenylpyridinium-induced apoptosis in PC12 cells. Biol. Pharm. Bull. 2003;26:1668-1673.
36. Park KR, Jang MH, Kim CJ, Lee CY. Ginseng radix suppresses ischemia-induced increase in c-Fos expression and apoptosis in the hippocampal CA1 region in gerbils. Korean J Oriental Physiol & Patholo. 2005;19:224-229.
37. Cheng JT, Wang CJ, Hsu FL. Paeoniflorin reverses guanethidine-induced hypotension via activation of central adenosine A1 receptors in Wistar rats. Clin Exp Pharmacol Physiol. 1999; 26:815-816.
38. Hong ND, Rho YS, Cho YW, Ju SM. Studies on Efficacy of Crude Drug by Processing (III) : Effect of Paeoniae Radix alba on CNS and Isolated Ileum. J. Kor. Pharm Sci. 1986;16: 124-131.
39. Wei T, Sun H, Zhao X, Hou J, Hou A, Zhao Q, Xin W. Scavenging of reactive oxygen species and prevention of oxidative neuronal cell damage by a novel gallotannin, pistafolia A. Life Sci. 2002;70:1889-1899.
40. Hong JA, Chung SH, Lee JS, Kim SS, Shin HD, Kim H, Jang MH, Lee TH, Lim BV, Kim YP, Kim CJ. Effects of PR on 5-hydroxytryptamine synthesis and tryptophan hydroxylase expression in the dorsal raphe of exercised rats. Biol Pharm Bull. 2003;26:166-169.