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Protective Effect of *Coriolus versicolor* Cultivated in Citrus Extract Against Nitric Oxide-Induced Apoptosis in Human Neuroblastoma SK-N-MC Cells

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Nitric oxide (NO) is a reactive free radical and a messenger molecule in many physiological functions. However, excessive NO is believed to be a mediator of neurotoxicity. The medicinal plant *Coriolus versicolor* is known to possess anti-tumor and immune-potentiating activities. In this study, we investigated whether *Coriolus versicolor* possesses a protective effect against NO donor sodium nitroprusside (SNP)-induced apoptosis in the human neuroblastoma cell line SK-N-MC. We utilized 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry, 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. MTT assay showed that SNP treatment significantly reduces the viability of cells, and the viabilities of cells pre-treated with the aqueous extract of *Coriolus versicolor* cultivated in citrus extract (CVE$_{citrus}$) was increased. However, aqueous extract of *Coriolus versicolor* cultivated in synthetic medium (CVE$_{synthetic}$) showed no protective effect and aqueous citrus extract (CE) had a little protective effect. The cell treated with SNP exhibited several apoptotic features, while those pre-treated for 1 h with CVE$_{citrus}$ prior to SNP expose showed reduced apoptotic features. The cells pre-treated for 1 h with CVE$_{citrus}$ prior to SNP expose inhibited p53 and Bax expressions and caspase-3 enzyme activity up-regulated by SNP. We showed that CVE$_{citrus}$ exerts a protective effect against SNP-induced apoptosis in SK-N-MC cells. Our study suggests that CVE$_{citrus}$ has therapeutic value in the treatment of a variety of NO-induced brain diseases.

Key words: *Coriolus versicolor*, nitric oxide, apoptosis, human neuroblastoma

INTRODUCTION

Coriolus versicolor, known as Yun-zhi in Korean, is a mushroom and belongs to the class Basidiomycetes. Medicinal mushrooms have been used to treat various diseases. Of theses, *Coriolus versicolor* has been demonstrated to possess a wide range of biological activities, which include anti-tumor, anti-bacterial, anti-viral, and

immune-potentiating activities (Ng, 1998; Borchers et al., 1999).

Nitric oxide (NO) is a reactive free radical gas and a messenger molecule with many physiological functions (Schmidt and Walter, 1994; Yun et al., 1996). NO is generated from L-arginine by nitric oxide synthase (NOS), and it is synthesized in neurons, astrocytes, microglial cells, endothelial cells, and many other cell types (Garthwaite and Boulton, 1995). Moreover, in the mammalian central nervous system, NO modulates many physiological functions including neurotransmission, synaptic plasticity, and memory (Hawkins, 1996; Hölscher, 1997). However, excessive NO formation is now believed to be a mediator of neurotoxicity, and NO is known to induce apoptosis in a variety of disorders, such as Alzheimer disease, acquired immune deficiency syndrome (AIDS) dementia, and multiple sclerosis (Gross and Wolin, 1995; Dawson and Dawson, 1996).

Apoptosis, also known as programmed cell death, is a biological process that plays a crucial role in normal development and tissue homeostasis (Woodle and Kulkarni, 1998). However, this type of cell death also contributes to a variety of human disorders (Thompson, 1995). The characteristic morphological changes associated with apoptosis are cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and the formation of apoptotic bodies (Wyllie et al., 1980; Chandra et al., 2000; Jang et al., 2002). Several gene expressions have been demonstrated to be involved in the regulation of apoptosis. P53 is a short-lived transcriptional activator that induces apoptosis (Lowe and Ruley, 1993) and the activation of p53 regulates the expression of Bax (Xiang et al., 1998) which is a proapoptotic member of the Bcl-2 family of intracellular proteins. Bcl-2 family proteins also play important roles in regulation of apoptosis. The Bcl-2 family proteins are classified into anti-apoptotic proteins, including Bcl-2 and Bcl-2XL, and pro-apoptotic proteins, such as Bax and Bid. The balance between pro-apoptotic and anti-apoptotic Bcl-2 family members determines the mitochondrial response to apoptotic stimuli (Kim et al., 2010; Upadhyay et al., 2003). Bax alters the permeability of mitochondrial membranes and triggers caspases cascade activation (Budihardjo et al., 1999; Korsmeyer, 1999; Upadhyay et al., 2003). The caspases are a class of cysteine proteases, and are considered to be central players of the apoptotic process and to trigger a cascade of proteolytic cleavages of many proteins in mammals (Aggarwal, 2000). In particular, the most widely studied member of the caspase family, caspase-3, is a key executioner of apoptosis, and is partially or totally responsible for the proteolytic cleavage of many proteins (Cohen, 1997; Ko et al., 2009).

Citrus fruits contain sugar, organic acids, and a number of physiologically active components, such as citric acid, ascorbic acid, minerals, coumarins, and flavonoids (Tanizawa et al., 1992; Kawaii et al., 1999). Moreover, the aqueous extract of *Coriolus versicolor* cultivated in citrus extract has been reported to have anti-cancer and anti-oxidant effects (Lee et al., 2003). In this study, we tried to find out the cultivation method to improve the efficacy of *Coriolus versicolor* on neuronal apoptosis, that is the basic underlying mechanism of many neurodegenerative disorders.

In the present study, we investigated the protective effects of the aqueous extracts of *Coriolus versicolor* cultivated in synthetic medium (CVE$_{synthetic}$), aqueous extract of citrus (CE), and the aqueous extract of *Coriolus versicolor* cultivated in citrus extract (CVE$_{citrus}$) against NO-induced apoptosis in the neuroblastoma cell line SK-N-MC. For this study, apoptosis in SK-N-MC cells was induced using sodium nitroprusside (SNP), a NO donor, and the protective effect of *Coriolus versicolor* was investigated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry, 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

Preparation of Coriolus versicolor extract

Coriolus versicolor was cultivated in synthetic medium for 6 days at 30°C with constant shaking at 150 rpm. After filtering through a #4 Whatman filter paper, the medium was concentrated using a rotary evaporator, and then lyophilized to yield 5 g/L of a powder was obtained (CVE$_{synthetic}$). *Coriolus versicolor* was also cultivated in citrus extract media using identical conditions, to yield 7 g/L powder (CVE$_{citrus}$). Citrus extract was obtained from the Jeju Provincial Development Corporation (Jeju, Korea) and was concentrated using a rotary evaporator, and lyophilized, to yield 88 g/L powder (CE).

Cell culture

The human neuroblastoma SK-N-MC 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% CO_2, 95% O_2 humidified cell incubator, and the medium was changed every 2 days.

MTT cytotoxicity assay

The cell viability was determined using the MTT assay kit according to the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany)(Lee et al., 2005). 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 *Coriolus versicolor* against cell death induced by SNP, the cells were pre-treated for 1 h with CVE$_{synthetic}$, CE, and CVE$_{citrus}$ each at 0.01 mg/ml, 0.5 mg/ml, 0.1 mg/ml, 0.5 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 was calculated by (O.D. of drug-treated sample/control O.D.)×100.

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 (Olympus, Tokyo, Japan) as a previously described method (Jang et al., 2002).

TUNEL assay

To detect apoptotic cells *in situ*, TUNEL assay was performed using ApoTag® peroxidase *in situ* apoptosis detection kit (Boehringer Mannheim GmbH) (Lee et al., 2005). 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, then with a stop/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.

DAPI staining

To determine whether SNP induces apoptosis, DAPI staining was performed as a previously described method (Lee et al., 2005). 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, incubated with 1 μg/ml DAPI for 30 min in the dark, and analyzed under a fluorescence microscope (Zeiss, Oberköchen, Germany).

DNA fragmentation

DNA fragmentation assay was performed using ApopLadder EX™ DNA fragmentation assay kit (TaKaRa, Shiga, Japan) (Lee et al., 2005). The cells were pre-treated for 1 h with CVE$_{citrus}$ 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.

Flow cytometric analysis

Flow cytometric analysis was performed as a previously described method (Jang et al., 2002). Briefly, after pre-treatment for 1 h with CVE$_{citrus}$ and the treating them with SNP, the cells were collected, fixed by incubating with 75% ethanol in PBS at −20°C for 1 h, then incubated with 100 μg/ml RNase and 20 μg/ml propidium iodide in PBS for 30 min at 37°C, and analyzed using FACScan (Becton Dickinson, San Jose, CA, USA).

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) (Lee et al., 2005). 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 *p53* were 5'-CAG CCA AGT CTG TGA CTT GCA CGT AC-3' (a 26-mer sense oligonucleotide) and 5'-CTA TGT CGA AAA GTG TTT CTG TCA TC-3' (a 26-mer anti-sense oligonucleotide). The primer sequences for *Bax* were 5'-GTG CAC CAA GGT GCC GGA AC-3' (a 20-mer sense oligonucleotide) and 5'-TCA GCC CAT CTT CTT CCA GA-3' (a 20-mer anti-sense oligonucleotide). 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 were 293 bp for *p53*, 205 bp for *Bax*, and 291 bp for *cyclophilin*.

For *Bax*, the PCR procedure was 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 35 amplification cycles for *p53* and 25 amplification cycles for *cyclophilin* were performed. The final amount of RT-PCR product was calculated densitometrically using Molecular Analyst™ version 1.4.1 (Bio-Rad, Hercules, CA, USA).

Western blot analysis

Western blot was performed as a previously described method (Kim et al., 2010). 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 μg/ml leupeptin. Protein concentration was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad). Protein of 50 μg was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). Mouse anti-p53 antibody (1 : 500; Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit anti-phospho p53 (Thr 18) antibody (1 : 200; Santa Cruz Biotech), and mouse anti-Bax antibody (1 : 1,000; Santa Cruz Biotech) were used as primary antibody. Horseradish peroxidase-conjugated anti-mouse antibody for p53 and Bax, and anti-rabbit antibody for phospho p53 (1 : 1,000; Santa Cruz Biotech) were used as secondary antibody. The detection of the band was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech GmbH, Freiburg, Germany).

Caspase-3 enzyme activity assay

Caspase-3 enzyme activity was measured using the ApoAlert® caspase-3 assay kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocols (Kim et al., 2003). In brief, the cells were lysed with 50 μl of chilled Cell Lysis Buffer. A 50 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 wavelength of 405 nm.

Statistical analyses

The results are 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. The difference was considered statistically significant at $p < 0.05$.

RESULTS

Effect of Coriolus versicolor against SNP-induced cytotoxicity

The viability of cells incubated with SNP for 24 h at concentrations of 0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, or 1.0 mM was 95.50±0.64%, 88.09± 0.60%, 78.20±0.77%, 52.72±1.13%, or 34.14±0.69%, respectively (Fig. 1A). As the SNP concentration was increased, the cell viability was decreased. The viability of cells exposed to the 0.5 mM SNP for 24 h was 52.72±1.13%.

The viability of the cells pre-treated for 1 h with the CVE$_{synthetic}$ at concentrations of 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml, or 1.0 mg/ml, and then exposed to 0.5 mM SNP for 24 h was 52.69±0.81%, 53.47±0.54%, 55.67±1.02%, or 53.42±1.08%, respectively (Fig. 1B).

The viability of the cells pre-treated for 1 h with the CE at concentrations 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml, or 1.0 mg/ml, and then exposed to 0.5 mM SNP for 24 h was 58.39±0.64%, 60.07±1.26%, 66.48±0.77%, or 66.14±2.01, respectively (Fig. 1C).

The viability of the cells pre-treated for 1 h with the CVE$_{citrus}$ at concentrations of 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml, or 1.0 mg/ml, and then exposed to 0.5 mM SNP for 24 h was increased to 56.22±1.39%, 64.03±3.01%, 77.24±0.70%, or 83.37±1.36%, respectively (Fig. 1D).

The above data demonstrated that the cell viability was reduced by SNP and that CVE$_{synthetic}$ showed no significant protective effect against NO-induced cytotoxicity in SK-N-MC cells and that CE had a little protective effect against NO-induced cytotoxicity. The most potent protective effect against the SNP-induced cytotoxicity was observed for CVE$_{citrus}$. Therefore, we selected CVE$_{citrus}$ for further study (Fig. 1).

Morphological changes

To characterize SNP-induced changes in cell morphology, the cells were examined 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 CVE$_{citrus}$ and exposed to 0.5 mM SNP for 24 h were indistinguishable 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
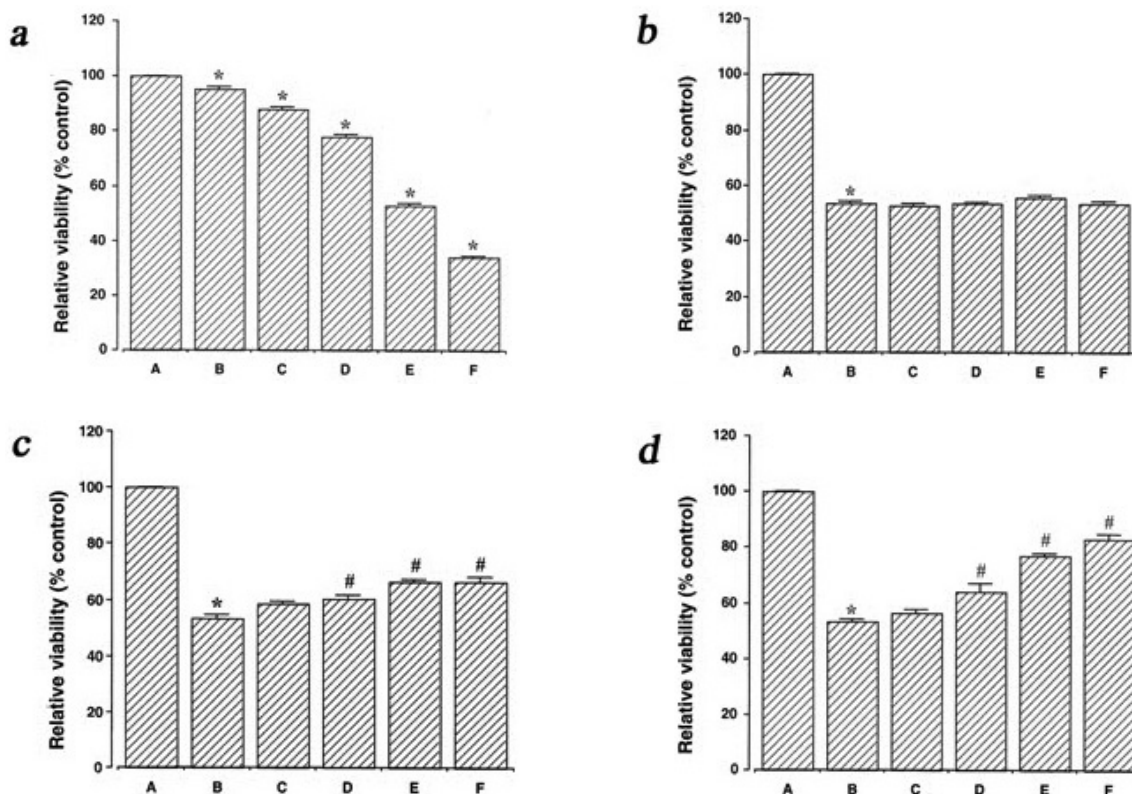
Fig. 1. Protective effect of *Coriolus versicolor*. *Represents p<0.05 compared to the control group. #Represents p<0.05 compared to the SNP-treated group. a: Cytotoxic effect of SNP. (A) Control, (B) 0.01 mM SNP-treated group, (C) 0.05 mM SNP-treated group, (D) 0.1 mM SNP-treated group, (E) 0.5 mM SNP-treated group, (F) 1.0 mM SNP-treated group. b: Protective effect of CVE$_{synthetic}$ on cell viability. (A) Control, (B) 0.5 mM SNP-treated group, (C) 0.05 mg/ml CVE$_{synthetic}$ followed by 0.5 mM SNP group, (D) 0.1 mg/ml CVE$_{synthetic}$ followed by 0.5 mM SNP group, (E) 0.5 mg/ml CVE$_{synthetic}$ followed by 0.5 mM SNP group, (F) 1.0 mg/ml CVE$_{synthetic}$ followed by 0.5 mM SNP group. c: Protective effect of CE on cell viability. (A) Control, (B) 0.5 mM SNP-treated group, (C) 0.05 mg/ml CE followed by 0.5 mM SNP group, (D) 0.1 mg/ml CE followed by 0.5 mM SNP group, (E) 0.5 mg/ml CE followed by 0.5 mM SNP group, (F) 1.0 mg/ml CE followed by 0.5 mM SNP group. d: Protective effect of CVE$_{citrus}$ on cell viability. (A) Control, (B) 0.5 mM SNP-treated group, (C) 0.05 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group, (D) 0.1 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group, (E) 0.5 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group, (F) 1.0 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group.
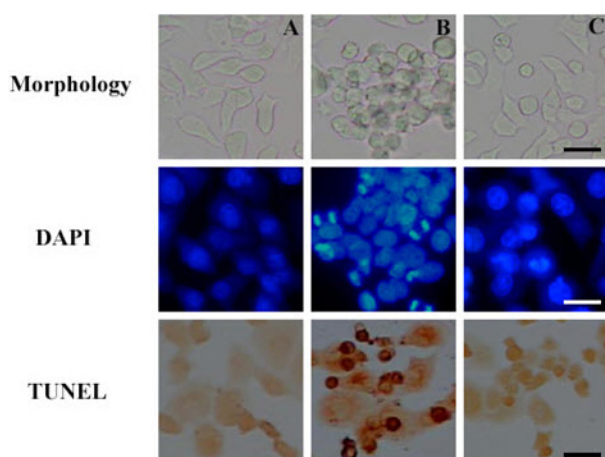

Fig. 2. Morphological analysis. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 1.0 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP. The scale bar represents 100 μm. Above: Phase-contrast microscopy. Middle: SK-N-MC cells stained with DAPI. Below: SK-N-MC cells stained *via* TUNEL assay.

with the 0.5 mM SNP for 24 h. The cells pre-treated for 1 h with the 1.0 mg/ml CVE$_{citrus}$ and 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 CVE$_{citrus}$ and exposed to 0.5 mM SNP for 24 h had near normal morphology (Fig. 2, Lower).

Characterization of apoptosis by DNA fragmentation

In order to ascertain the protective effect of CVE$_{citrus}$ against SNP-induced apoptosis, DNA fragmentation, reflecting the endonuclease activity characteristic of apoptosis, was 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 CVE$_{citrus}$ and exposed to 0.5 mM SNP for 24 h resulted in a significant reduction in the intensity of SNP-induced DNA laddering (Fig. 3).

Cell cycle distribution changes

Through flow cytometric analysis of DNA content using the DNA-specific dye PI, we assessed the protective effect of CVE$_{citrus}$ against SNP-induced cell death. The population of cells in the sub-G1 phase in the 0.5 mM SNP-treated group increased from 12.90% (control level) to 24.77%, whereas this figure was reduced by CVE$_{citrus}$ pre-treatment at 0.5 mg/ml or 1 mg/ml for 1 h and exposed to 0.5 mM SNP for 24 h to 18.50% or 16.38%, respectively (Fig. 4).

Expressions of p53 and Bax mRNA

The RT-PCR was performed to estimate the relative expressions of the p53 and Bax mRNA. In the present study, the mRNA level of p53 in the control was set at 1.00. The level of p53 mRNA following treatment with 0.5 mM SNP for 24 h increased to 13.79±4.21, but it was only 3.30±0.57 or 2.28±0.27 in the cells pre-treated with CVE$_{citrus}$ at 0.5 mg/ml or 1.0 mg/ml for 1 h and exposed to 0.5 mM SNP for 24 h, respectively.

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 6.19±0.54, but it was only 4.49±0.43 or 1.30±0.06 in the cells pre-treated with CVE$_{citrus}$ at 0.5 mg/ml or 1.0 mg/ml for 1 h and exposed to 0.5 mM SNP for 24 h, respectively (Fig. 5).
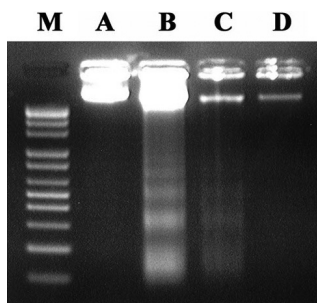

Fig. 5. RT-PCR analysis of the mRNA levels of p53 and Bax. *Represents $p < 0.05$ compared to the control group. #Represents $p < 0.05$ compared to the SNP-treated group. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 0.5 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group, (D) 1.0 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group.

Fig. 3. Electrophoretic examination of the genomic DNA of SK-N-MC cells. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 0.5 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group, (D) 1.0 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group.
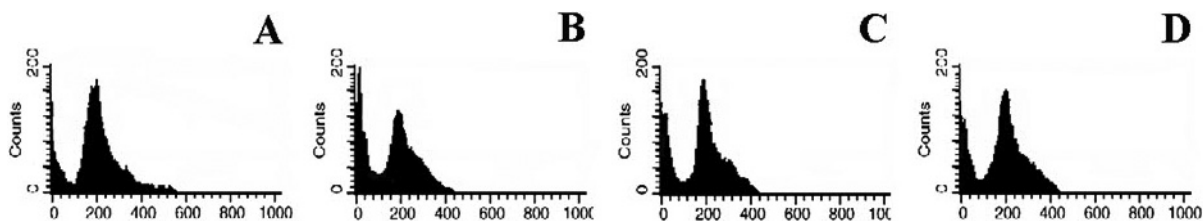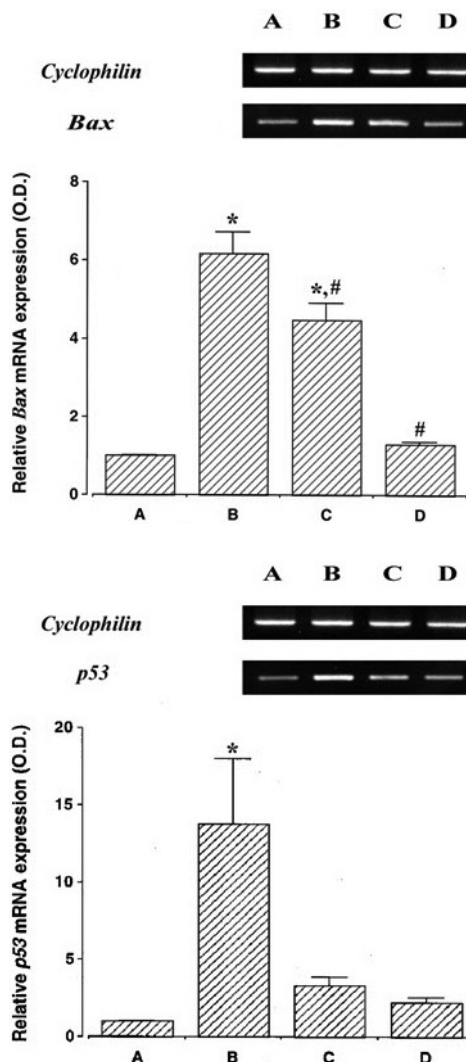

Fig. 4. Flow cytometric analysis. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 0.5 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group, (D) 1.0 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group.

Western blot analysis of p53 and Bax

When the cells were treated with 0.5 mM SNP for 24 h, Bax (21 kDa) and p53 protein (53 kDa) expressions were up-regulated. Compared to these cells, those of cells pre-treated with CVE$_{citrus}$ at 0.5 mg/ml or 1.0 mg/ml for 1 h and exposed to 0.5 mM SNP for 24 h showed lower Bax and p53 protein expressions.

When the expressions of p53, phosphor-p53, and Bax in the control cells were set at 1.00, p53 expression after treatment with 0.5 mM SNP for 24 h increased to 13.20±1.29, but it was only 5.43±1.28 or 1.72±0.25 in the cells pre-treated with CVE$_{citrus}$ at 0.5 mg/ml or 1.0 mg/ml for 1 h and exposed to 0.5 mM SNP for 24 h, respectively. The phpspho-p53 (Thr 18) expression after treatment with 0.5 mM SNP for 24 h increased to 11.60±1.28, but it was only 5.99±0.86 or 3.92±0.83 in the cells pre-treated with CVE$_{citrus}$ at 0.5 mg/ml or 1.0 mg/ml for 1 h and exposed to 0.5 mM SNP for 24 h, respectively. The Bax expression after treatment with 0.5 mM SNP for 24 h increased to 4.23±0.40, but it was only 2.08±0.17 or 1.43±0.25 in the cells pre-treated CVE$_{citrus}$ at 0.5 mg/ml or 1.0 mg/ml for 1 h and exposed to 0.5 mM SNP for 24 h, respectively (Fig. 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 was significantly increased from 4.31±0.14 pmol (control value) to 10.43±0.70 pmol, but this was only 6.46±0.49 pmol by pre-treatment with CVE$_{citrus}$ at 1 mg/ml for 1 h and exposed to 0.5 mM SNP for 24 h. The present results demonstrated that SNP increased caspase-3 enzyme activity in SK-N-MC cells and that CVE$_{citrus}$ attenuated this increase (Fig. 7).

DISCUSSION

In the brain, NO is synthesized by neuronal nitric oxide synthase (NOS) and acts as an intercellular messenger at the physiological level. However, the high concentrations of NO induced by certain pathological conditions, such as brain ischemia, inflammation, neurodegenerative diseases, and may result in neuronal dysfunction (Heales et al., 1999; Murphy, 1999; Lee et al., 2010). NO also causes apoptotic neuronal cell death (Lee et al., 2005). In the present study, we investigated whether *Coriolus versicolor* has a protective effect on NO-induced cell death in SK-N-MC cells, a neuroblastoma cell line.

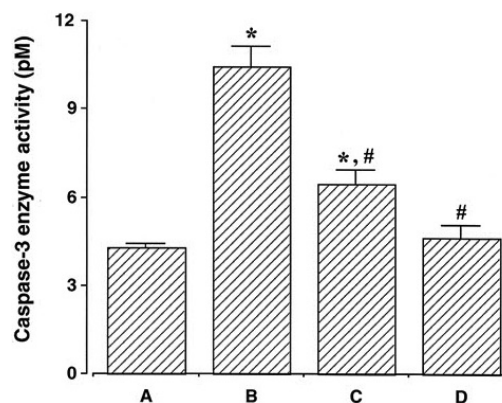Our MTT assay results showed that SK-N-MC cell viability was

Fig. 7. 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) 0.5 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group, (D) 1.0 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group.
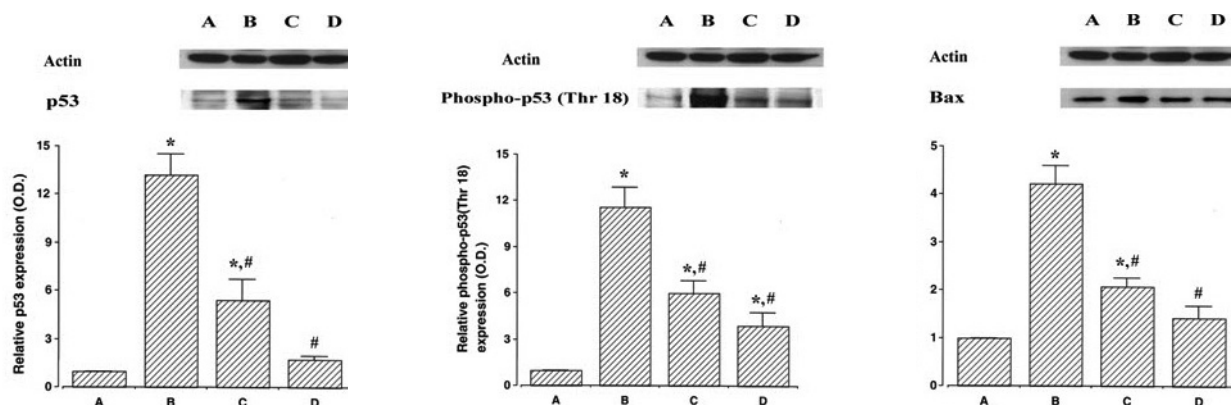
Fig. 6. Western blot analysis of protein levels of p53 and Bax. Actin, used as the internal control, was detected at the position corresponding to a molecular weight of 46 kDa. *Represents $p<0.05$ compared to the control. #Represents $p<0.05$ compared to the SNP-treated group. (A) Control group, (B) 0.5 mM SNP-treated group, (C) 0.5 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group, (D) 1.0 mg/ml CVE$_{citrus}$ followed by 0.5 mM SNP group. Above: Expression level of p53. Middle: Expression level of phosphor-p53 (Thr 18). Below: Expression level of Bax.

significantly reduced by SNP treatment, and that CVE$_{citrus}$ exerted a significant protective effect against NO-induced cytotoxicity. However, CVE$_{synthetic}$ showed no protective effect and CE has a little protective effect. Flow cytometric analysis of DNA contents showed an increase in the population of cells in the sub-G1 phase after SNP treatment, whereas the cells pre-treated with CVE$_{citrus}$ prior to SNP showed a decrease in the sub-G1 phase. Under the phase-contrast microscope, the cells treated with SNP only showed apoptotic morphologies, i.e., cell shrinkage, cytoplasmic condensation, and irregularity in shape. Moreover, apoptotic bodies were observed in the SNP-treated cells stained with DAPI. However, the cells pre-treated with CVE$_{citrus}$ prior to SNP showed lower levels of apoptotic morphologic changes.

In addition, TUNEL-positive cells, indicative of apoptotic DNA strand breaks and nicks in DNA molecules, were detected in the SNP-treated cells, but the cells showed lower levels of TUNEL-positive cells. To provide evidence supporting the involvement of apoptosis in the SNP-induced cytotoxicity, the DNA fragmentation assay was performed. Distinctive ladder pattern characteristic of apoptotic cell death was detected in the cells treated with SNP, on the other hand pre-treatment with CVE$_{citrus}$ prior to SNP showed lower SNP-induced DNA laddering intensity.

The present results showed that apoptosis is closely implicated to NO-induced cytotoxicity in human neuroblastoma SK-N-MC cells and that CVE$_{citrus}$ has a protective effect against this cytotoxicity. Molecular mechanisms underlying the NO-mediated apoptosis involve different pathways which depend on cell type and the cellular environment (Bosca and Hortelano, 1999). Many studies have demonstrated that NO-induced apoptosis occurs through a p53-dependent pathway in various cells including neuronal cells (Yung et al., 2004; Lee et al., 2005). It was demonstrated that NO enhances p53 protein expression and its phosphorylation in myoblast cells (Lee et al., 2005). The present study also showed that NO increased p53 protein expression and its phosphorylation at Thr 18 in SK-N-MC cells.

Apoptosis-regulatory proteins have been repeatedly implicated in the susceptibility of neurons to cell death (Xu et al., 2007; Kim et al., 2010; Baek et al., 2011). Caspase-3 is one of the most widely studied members of the caspase family and it is involved in apoptosis as the principal executor (Cohen, 1997; Kim et al., 2010). Several pathways have been shown to mediate p53-induced apoptosis, and Bax is a well known p53 target gene and a proapoptotic member of the Bcl-2 family (Miyashita and Reed, 1995; McCurrach et al., 1997). Bax promotes the release of cytochrome c into the cytosol from mitochondria, which in turn activates caspase-3 (Reed, 1995; Upadhyay et al., 2003). In the present study, we observed that SNP increased Bax expressions at

the mRNA and protein levels in SK-N-MC cells, and that it finally increased caspase-3 enzyme activity. We further investigated whether CVE$_{citrus}$ inhibits NO-related cell death pathways involving p53, Bax, and caspase. Our results showed that CVE$_{citrus}$ attenuated NO-induced apoptotic cell death by blocking a p53- and Bax-dependent caspase-3 pathway. Suppression of DNA fragmentation and caspase-3 expression is known to be closely related with inhibition of apoptosis of neurons, resulting in facilitation of memory recovery (Quindry et al., 2007; Ko et al., 2009; Baek et al., 2011).

Under normal conditions, NO modulates many physiological functions including neurotransmission, synaptic plasticity, and memory in the mammalian central nervous system (Hawkins, 1996; Hölscher, 1997). However, under excessive NO formation conditions, NO exerts neurotoxicity inducing apoptosis, and NO-induced apoptosis causes many brain disorders (Gross and Wolin, 1995; Dawson and Dawson, 1996). The present study showed that CVE$_{citrus}$ reduced NO-induced apoptotic cell death in a neuroblastoma cell line. The pro-apoptotic proteins, p53- and Bax, are known to activate caspase-3, resulting in apoptosis. The possible mechanisms of neuroprotective effect of CVE$_{citrus}$ can be ascribed to the inhibition of p53- and Bax-dependent caspase-3 activation. Our results suggest that CVE$_{citrus}$ potentially has therapeutic value in the treatment of a variety of NO-induced brain diseases such as stroke.

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