

Original Article

## The Protective Effect of *Paeoniae Radix* Extract against 1-methyl-4-Phenylpyridium-induced Apoptosis on SK-N-MC Neuroblastoma Cells

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**Background :** There are increasing neuro-degenerative disorders with aging. *Paeoniae Radix*(PR) possesses various pharmacological effects such as sedative, analgesic, anti-inflammatory, anti-stress and neuro-protective actions. Also anti-aging and anti-cancer effects of PR were reported. Our purpose was to investigate whether PR is useful on the treatment of Parkinson's disease, one of the neuro-degenerative disorders.

**Objective :** We investigated whether *Paeonia Radix* possesses a protective effect against 1-methyl-4-phenylpyridine(MPP+)-induced cytotoxicity in neuronal cells.

**Methods :** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay, flow cytometry, DNA fragmentation assay, reverse transcription-polymerase chain reaction(RT-PCR), and Western blotting were performed on SK-N-MC neuroblastoma cells.

**Results :** Cells treated with MPP<sup>+</sup> exhibited several apoptotic features, while cells pre-treated with *Paeonia Radix* prior to MPP<sup>+</sup> exposure showed a decrease in the occurrence of apoptotic features.

**Conclusions :** These results suggest that *Paeonia Radix* may exert a protective effect against MPP<sup>+</sup>-induced apoptosis in SK-N-MC neuroblastoma cells.

**Key Words:** *Paeonia radix*, Parkinson's disease, 1-Methyl-4-phenylpyridine, Apoptosis

### Introduction

The neurodegenerative condition of Parkinson's disease is characterized by rigidity and akinesia. A major pathological hallmark of Parkinson's disease is the degeneration of nigrostriatal dopaminergic neurons<sup>1)</sup>. It was reported that MPTP is a neurotoxin that can induce symptoms similar to those observed in Parkinson's disease in mice<sup>2,3)</sup>. The toxicity of MPTP is

known to be mediated by the toxic metabolite MPP<sup>4)</sup>. Numerous studies have shown that MPP<sup>+</sup> induces apoptosis in several cell types<sup>5-8)</sup>.

Apoptosis is thought to play an important role in the neuronal loss in many neurological disorders including Parkinson's disease. It has well been documented that the process of apoptosis is regulated by the expressions of several proteins. Some of them are the Bcl-2 family(Bcl-2, protect against cell death, while Bax, expressed during apoptosis and promotes cell death<sup>9)</sup> and the caspases, one of the key executioners of apoptosis<sup>10)</sup>.

*Paeoniae Radix*(PR), the root of *Paeonia lactiflora* PALLAS, is a crude medicinal herb. It has been used for blood nourishing, circulation activating,

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menstruation regulating, stopping sweats, decreasing blood pressure, alleviating pain and numbness, relieving spasm and cramp, and treating for liver disease in a lot of traditional prescriptions in Korea, China and Japan<sup>11)</sup>. Several polysaccharides isolated from PR was found to have immunological activities<sup>12-13)</sup>.

However, no report has been made on the effect of PR extract against MPP<sup>+</sup>-induced apoptosis in SK-N-MC human neuroblastoma cells. We investigated it and report the results of the study.

## Materials and methods

### 1. Drugs

*Paeoniae Radix*(PR) was purchased from Kyung-Dong marketplace(Seoul, Korea). To obtain the aqueous extract of PR, 200g of PR was added to distilled water, and extraction was performed by heating at 80°C, concentrated with a rotary evaporator, and lyophilized. The resulting powder, weighing 30g, was dissolved in saline.

### 2. Reagents

H<sub>2</sub>O<sub>2</sub> was purchased from Junsei Chemical Co., Ltd.(Tokyo, Japan). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid(MTT) assay kit was purchased from Boehringer Mannheim GmbH(Mannheim, Germany). The DNA fragmentation assay kit was obtained from TaKaRa(Shiga, Japan).

### 3. Cell culture

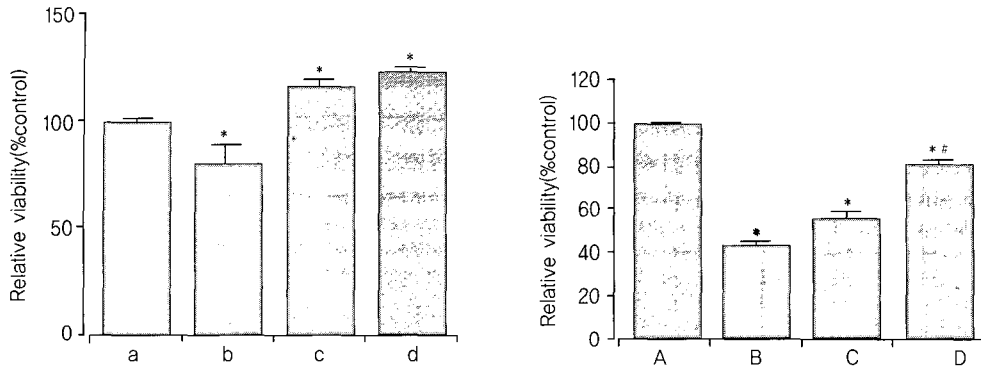
Cells of the human neuroblastoma SK-N-MC were purchased from the Korean Cell Line Bank(KCLB; Seoul, Korea). Cells were cultured in Dulbecco's Modified Eagle's Medium(DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum(FBS; Gibco BRL) at 37°C in 5% CO<sub>2</sub>, 95% O<sub>2</sub> in a humidified cell incubator.

### 4. MTT cytotoxicity assay

Cell viability was determined using the MTT assay kit according to the manufacturer's protocol. To determine the cytotoxicity of *Paeniae Radix*, cells were treated with *Paeniae Radix* at concentrations of 0.1mg/ml, 1mg/ml, and 10mg/ml for 24h. For analysis of the protective effect of PR against cell death induced by MPP<sup>+</sup>, cells were pre-treated with PR at concentrations of 1mg/ml and 10mg/ml for 1h before MPP<sup>+</sup> 100 µg/ml was applied for another 24h. Cultures of the control group were left untreated. The MTT labeling reagent 10 µl was added to each well, and the plates were incubated for 4h. Solubilization solution 100 µl was added to each well, and the cells were incubated for another 12h. The absorbance was then measured with a microtiter plate reader(Bio-Tek, Winooski, VT, USA) at a test wavelength of 595nm and a reference wavelength of 690nm. Optical density(O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Relative viability was calculated as(O.D. of drug-treated sample/control O.D.).

### 5. TUNEL assay

For *in situ* detection of apoptotic cells, TUNEL assay was performed using ApoTag<sup>®</sup> peroxidase *in situ* apoptosis detection kit. SK-N-MC cells were cultured on 4-chamber slides(Nalge Nunc International, Naperville, IL, USA) at a density of 2 x 10<sup>4</sup>cells/chamber. After treatment with PR and MPP<sup>+</sup>, the cells were washed with phosphate buffered saline(PBS) and fixed by incubating in 4% paraformaldehyde(PFA) for 10min at 4°C. The fixed cells were then incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase (TdT)-catalyzed reaction for 60min at 37°C in a humidified atmosphere and were immersed in stop/wash buffer for 10min at room temperature. The cells were then incubated with anti-



**Fig. 1.** MPP<sup>+</sup>-induced cytotoxicity and protective effect of Paeoniae Radix(PR). Above: Effect of Paeoniae Radix on cell viability. (a) Control group; (b) 0.1mg/ml PR-treated group; (c) 1mg/ml PR-treated group; (d) 10mg/ml PR-treated group. Below: Protective effect of Paeoniae Radix on cell viability. (A) Control group; (B) 100µg/ml MPP<sup>+</sup>-treated group; (C) 1mg/ml PR-pre-treated and 100µg/ml MPP<sup>+</sup>-treated group; (D) 10mg/ml PR-pre-treated and 100µg/ml MPP<sup>+</sup>-treated group. Results are presented as mean ± standard error mean(S.E.M.). \* : represents  $p < 0.05$  compared to the control group. # : represents  $p < 0.05$  compared to the MPP<sup>+</sup>-treated group.

digoxigenin antibody conjugated with peroxidase for 30min. DNA fragments were stained using 3,3'-diaminobenzidine(DAB; Sigma Chemical Co., St. Louis, MO, USA) as the substrate for the peroxidase.

## 6. DAPI staining

For DAPI staining, cells were cultured on 4-chamber slides. After treatment with PR and MPP<sup>+</sup>, cells were fixed by incubation in 4% paraformaldehyde(PFA) for 30min. Following washing in PBS, the cells were incubated in 1µg/ml DAPI solution(Sigma Chemical Co.) for 30min in the dark. The cells were then observed under a fluorescence microscope(Zeiss, Oberkochen, Germany).

## 7. Flow cytometric analysis

For flow cytometric analysis, after treatment with PR and MPP<sup>+</sup>, cells were collected and fixed by incubation with 75% ethanol in PBS at -20°C for 1h. Afterwards, the cells were incubated with 100µg/ml RNase and 20µg/ml propidium iodide in PBS for 30min at 37°C and were analyzed using FACSscan(Becton Dickinson, San Jose, CA, USA).

## 8. DNA fragmentation

For detection of apoptotic DNA cleavage, a DNA fragmentation assay was performed using an ApopLadder EXT<sup>TM</sup> DNA fragmentation assay kit. Cells were treated with PR and MPP<sup>+</sup> and then lysed with 100µl of lysis buffer. The lysate was incubated with 10µl of 10% sodium dodecyl sulfate(SDS) solution containing 10µl of Enzyme A at 56°C for 1h followed by treatment with 10µl of Enzyme B at 37°C for 1h. After adding 70µl of precipitant and resuspending the resultant pellet in Tris-EDTA(TE) buffer, genomic DNA was visualized by electrophoresis in a 2% agarose gel containing ethidium bromide.

## 9. RNA isolation and RT-PCR

Total RNA was isolated from SK-N-MC cells using RNAzol<sup>TM</sup>B(TEL-TEST, Friendswood, TX, USA) as per the manufacturer's instruction. RNA 2µg and random hexamers(Promega) 2µl were added together, and the mixture was heated at 65°C for 10min. 1µl AMV reverse transcriptase(Promega), 5µl of 10mM dNTP(Promega), 1µl of RNasin(Promega) and 5µl of 10 x AMV RT buffer(Promega) were then added to the

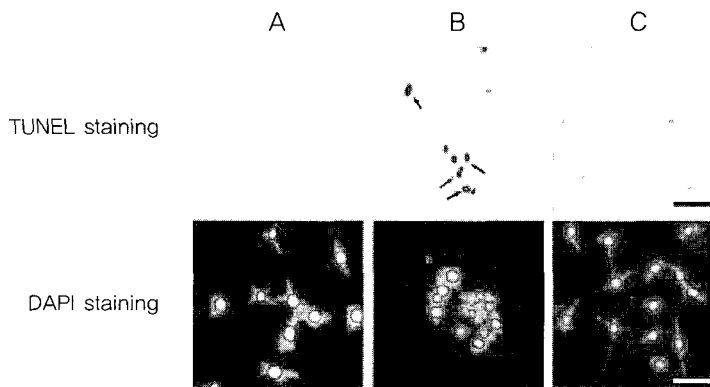
mixture, and the final volume was brought up to 50  $\mu$ l with dimethyl pyrocarbonate(DEPC)-treated water. The reaction mixture was then incubated at 42  $^{\circ}$ C for 1h.

PCR amplification was performed in a reaction volume of 40  $\mu$ l containing 1  $\mu$ l of the appropriate cDNA, 1  $\mu$ l of each set of primers at a concentration of 10pM, 4  $\mu$ l of 10 x RT buffer, 1  $\mu$ l of 2.5mM dNTP, and 2units of *Taq* DNA polymerase(TaKaRa). For human Bax, the primer sequences were 5' - GTGCACCAA-GGTGCCGGAAC-3' (a 20-mer sense oligonucleotide starting at position 375) and 5' - TCAGCCCATC-TTCTTCCAGA-3' (a 20-mer anti-sense oligonucleotide starting at position 560). For human Bcl-2, the primer sequences were 5' - CGACGACTTCTC-CCGCCGCTACCGC-3' (a 25-mer sense oligonucleotide starting at position 334) and 5' -CCGCATGCT-GGGGCCGTACAGTTCC-3' (a 25-mer anti-sense oligonucleotide starting at position 628). For cyclophilin, the internal control used in the study, the primer sequences were 5' -ACCCC-ACCGTGTCTTCGAC-3' (a 20-mer sense oligonucleotide starting at position 52) and 5' -CATTTC-

CATGGACAAGATG-3' (a 20-mer anti-sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 205bp(for Bax), 318bp(for Bcl-2), and 299bp(for *cyclophilin*).

## 10. Western blot analysis

Cells were treated with PR and MPP<sup>+</sup>, and collected. Cells were lysed in a lysis buffer containing 50mM Tris-HCl(pH 7.5), 150mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P40, 0.1% SDS, 1mM PMSF, 100g/ml leupeptin. Protein content was measured using a Bio-Rad colorimetric protein assay kit(Bio-Rad, Hercules, CA, USA). Protein 50g was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane(Schleicher & Schuell GmbH, Dassel, Germany). Mouse caspase-3 antibody(Santa Cruz Biotech, Santa Cruz, CA, USA) was used as a primary antibody. Horseradish peroxidase-conjugated anti-mouse antibody for caspase-3(Amersham Pharmacia Biothech GmbH, Freiburg, Germany) was used as a secondary antibody. Band detection was performed using the enhanced chemiluminescence



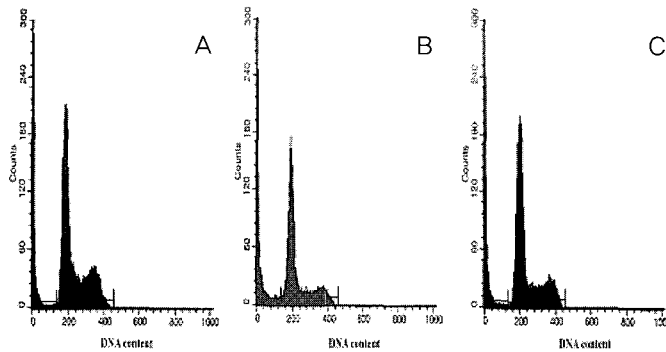
**Fig. 2.** Morphological observations of cells treated with MPP<sup>+</sup> and *Paeoniae Radix*(PR).

Black arrows indicate where condensed and marginated chromatin have been labeled.

(A) Control group;

(B) 100  $\mu$ g/ml MPP<sup>+</sup>-treated group;

(C) 10mg/ml PR-pre-treated and 100  $\mu$ g/ml MPP<sup>+</sup>-treated group. All scale bars represent 100  $\mu$ m.



**Fig. 3.** Flow cytometric analysis. The fraction of cells in the sub-G1 phase was increased in the MPP<sup>+</sup>-treated cultures but reduced in the PR-pre-treated cultures, to a level comparable to that seen in the control group.

- (A) Control group;
- (B) 100 μg/ml MPP<sup>+</sup>-treated group;
- (C) 10 mg/ml PR-pre-treated and 100 μg/ml MPP<sup>+</sup>-treated group.

science(ECL) detection system(Amersham Pharmacia Biothech GmbH).

### 11. Statistical analysis

Results are expressed as mean ± standard error mean(S.E.M.). Data was analyzed by one-way analysis of variance(ANOVA) followed by Duncan's post-hoc test. Differences were considered statistically significant at  $p < 0.05$ .

## EXPERIMENTAL RESULTS

### 1. Effects of *Paeoniae Radix* against MPP<sup>+</sup>-induced cytotoxicity

The viabilities of cells incubated with *Paeoniae Radix*(PR) at concentrations of 0.1 mg/ml, 1 mg/ml, and 10 mg/ml for 24h were  $79.82 \pm 11.33\%$ ,  $108.09 \pm 2.61\%$ , and  $123.57 \pm 2.26\%$  of the control value, respectively. A trend of increasing viability with increasing PR concentration was observed. The viability of the cells exposed with 100 μg/ml MPP<sup>+</sup> for 24h was  $43.74 \pm 0.95\%$  of the control value, while viability of the cells pre-treated with PR at 1 mg/ml and 10 mg/ml

for 1h before exposure to MPP<sup>+</sup> was increased in statistically significant fashion to  $57.05 \pm 2.82\%$  and  $82.91 \pm 0.87\%$ , respectively. Results of the MTT assay showed a significant decrease in the viability of MPP<sup>+</sup>-treated cells, while PR was shown to exert a protective effect against MPP<sup>+</sup>-induced cytotoxicity( Fig. 1).

To further confirm the induction of apoptosis by MPP<sup>+</sup> and the preventive effect of PR in SK-N-MC cells, cells treated with MPP<sup>+</sup> and PR were analyzed using TUNEL assay. TUNEL-positive cells were stained dark brown under the light microscope, and nuclear condensations were observed in cultures treated with 100 μg/ml MPP<sup>+</sup>, while the appearance of cells pre-treated with 10 mg/ml PR prior to MPP<sup>+</sup> exposure was similar to that of the control cells(Fig. 2, upper).

In addition, cells were observed under fluorescence microscope following treatment with DAPI, which specifically stains the nuclei. The assay revealed the occurrence of nuclear condensation, DNA fragmentation and perinuclear apoptotic bodies upon 100 μg/ml of MPP<sup>+</sup> treatment, while the appearance of cells pre-treated with 10 mg/ml PR prior to MPP<sup>+</sup> exposure was similar to that of the control cells(Fig. 2, lower).

### 3. Effects of *Paeoniae Radix* on Cell cycle distribution changes

Flow cytometric analysis was also used to analyze the protective effect of PR against MPP<sup>+</sup>-induced cell death. The fraction of cells in the sub-G1 phase increased from 35.30%(value from the control group) to 61.13% following treatment with 10mg/ml of MPP<sup>+</sup> for 24h, while the figure was decreased to 42.51% with PR-pre-treatment at a concentration of 10mg/ml for 1 h. It indicated that the PR-pre-treatment induced a lesser accumulation of cells in the sub-G1 phase, which has been considered to be the marker of cell death (Fig. 3).

### 4. Effects of *Paeoniae Radix* on DNA fragmentation

In order to ascertain the protective effect of PR against MPP<sup>+</sup>-induced cell death, DNA fragmentation, reflecting the endonuclease activity characteristic of apoptosis, was analyzed. Treatment with MPP<sup>+</sup> at a concentration of 100 $\mu$ g/ml for 24h resulted in the formation of definite fragments which could be seen via electrophoresis as a characteristic ladder pattern; pre-

treatment with 10mg/ml PR for 1h resulted in a decreased intensity of MPP<sup>+</sup>-induced DNA ladder (Fig. 4).

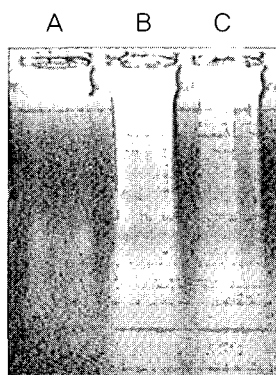
RT-PCR analysis of the mRNA levels of Bax and Bcl-2 were performed in order to provide an estimate of the relative levels of expressions of these genes. In the present study, the mRNA levels of Bax and Bcl-2 in the control cells set at 1.00. The level of Bax mRNA following treatment with 100 $\mu$ g/ml MPP<sup>+</sup> markedly increased to  $2.05 \pm 0.34$ , but decreased to  $1.34 \pm 0.30$  in cells pre-treated with PR at a concentration of 10mg/ml. The level of Bcl-2 mRNA following treatment with 100 $\mu$ g/ml MPP<sup>+</sup> markedly decreased to  $0.39 \pm 0.06$ , but increased to  $0.83 \pm 0.12$  in cells pre-treated with PR at a concentration of 10mg/ml (Fig. 5).

In cells of 100 $\mu$ g/ml MPP<sup>+</sup>-treated groups, increased level of caspase-3 expression(32 kDa) was detected. In the pre-treatment with PR, the level of expression of caspase-3 was decreased (Fig. 6).

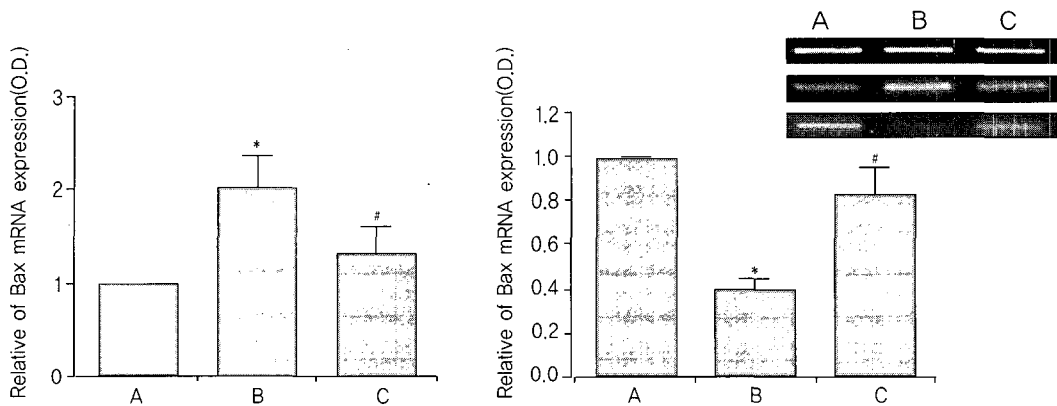
## Discussion

Parkinson's disease(PD) is one of the common neurodegenerative disorders. Its neurodegenerative condition is characterized by rigidity and akinesia. A major pathological hallmark of PD is the degeneration of nigrostriatal dopaminergic neurons<sup>1)</sup>. It was reported that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP) is a neurotoxin that can induce symptoms similar to those observed in PD in mice<sup>2,3)</sup>, and MPTP induces selective loss of dopaminergic neurons in the substantia nigra of mice<sup>4)</sup>. The toxicity of MPTP is known to be mediated by the toxic metabolite 1-methyl-4-phenylpyridine(MPP<sup>+</sup>)<sup>5)</sup>.

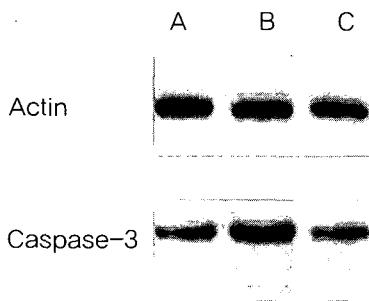
Increasing evidence from epidemiological studies documented that environmental factors are implicated in the selective dopaminergic cell loss in substantia



**Fig. 4.** Electrophoretic examination of the genomic DNA of SK-N-MC human neuroblastoma.  
(A) Control group;  
(B) 100 $\mu$ g/ml MPP<sup>+</sup>-treated group;  
(C) 10mg/ml PR-pre-treated and 100 $\mu$ g/ml MPP<sup>+</sup>-treated group.



**Fig. 5.** RT-PCR analysis of the mRNA levels of Bax and Bcl-2. As the internal control, cyclophilin mRNA was also reverse-transcribed and amplified. Results are presented as mean  $\pm$  standard error mean (S.E.M.). (A) Control group; (B) 100  $\mu$ g/ml MPP<sup>+</sup>-treated group; (C) 10mg/ml PR-pre-treated and 100  $\mu$ g/ml MPP<sup>+</sup>-treated group. \* : represents  $p < 0.05$  compared to the control group. # : represents  $p < 0.05$  compared to the MPP<sup>+</sup>-treated group.



**Fig. 6.** Western blot analysis of the protein levels of caspase-3. (A) Control group; (B) 100  $\mu$ g/ml MPP<sup>+</sup>-treated group; (C) 10mg/ml PR-pre-treated and 100  $\mu$ g/ml MPP<sup>+</sup>-treated group. Actin, used as the internal control, was detected at the position corresponding to a molecular weight of 46 kDa.

nigra. Epidemiological risk factor analyses of PD identified several neurotoxicants. Of these, MPP<sup>+</sup> is converted from MPTP by the monoamine oxidase B in the inner mitochondrial membrane. At the level of the central nervous system, this process takes place mainly in the glial cells. MPP<sup>+</sup> is thus selectively taken up by dopaminergic neurons via the dopamine transporter of the membrane. Various studies reported that MPP<sup>+</sup> induces apoptosis in several cell types such as SK-N-

MC, PC12 cells, cerebellar granule cells, and SHSY-5Y neuroblastoma cells<sup>15-19</sup>.

*Paeoniae Radix*(PR), the root of *Paeonia lactiflora* PALLAS, a traditional medical herb of Korea, China and Japan, has been frequently used for blood nourishing, circulation activating, pain alleviating, menstruation regulating, and treating for liver disease and cancer<sup>11</sup>. There are some experimental reports. Subcutaneous injection of PR has demonstrated marked sedative and suppressant effects on the central nervous system in mice<sup>11</sup>. PR is a potential anti-aging and anti-carcinogenesis agent as it was reported to inhibit oxidative DNA cleavage induced by various oxidative DNA damage chemicals<sup>20-21</sup>.

The extract from PR(PRE) is a complex mixture and its active components are unknown whether the inhibitory effects of PRE are attributable to a single component or more than one component in the drug is uncertain. PRE improves blood flow through its endothelium-dependent vasodilatory action on aorta<sup>22</sup> and its inhibitory effect on thrombosis and platelet aggregation<sup>23</sup>. It was presented that Gallic acid is one of compound isolated from PRE and Gallic<sup>24</sup> and induces

apoptosis in several cancer cell lines<sup>25)</sup> and the induction of apoptosis in several lung cancer cell involved caspase activation<sup>26,27)</sup>. It was informed that benzoic acid was isolated from PR. Different benzoic acid derivatives were reported to induce apoptosis in cancer cells<sup>28,29)</sup>.

Animal models and clinical studies have showed that some herbal remedies including PR are effective in inhibiting the recurrence of malignancies and in cancer therapy<sup>30,31)</sup>. It was revealed that PR can inhibit liver fibrosis and damage induced by CC14 and D-galactosamine in rat; thus PR has a protective role on hepacyte<sup>32,33)</sup>.

Recently, it has been reported that PR has a neuroprotective effect as well as antimicrobial, anticoagulant, cognition-enhancing, immunoregulating, hypoglycaemic, and neuromuscular blocking effects<sup>34,35)</sup>. It was reported that PR is useful for the relieve of stress, pain, and a tonic state<sup>36,37)</sup>.

Lee et al.<sup>38)</sup> reported that PR had inhibitory effect on the growth of human hepatoma cell lines. In addition, Park et al.<sup>37)</sup> showed that *Paeonia japonica* MIYABE improves the immune response of the helper T cells, B cells, and macrophages. Several studies have revealed that PR has a neuroprotective effect against neuronal cell damage<sup>35,39-41)</sup> and has also been used in the treatment of stroke therapy<sup>42,43)</sup>.

In the present study, the protective effect of aqueous extract of PR on apoptosis induced by MPP<sup>+</sup> in SK-N-MC human neuroblastoma cells was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay, 4,6-diamidino-2-phenylindole(DAPI) staining, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling(TUNEL) assay, DNA fragmentation assay, reverse transcription-polymerase chain reaction(RT-PCR), and Western blot analysis.

In this study, MTT assay confirmed that MPP<sup>+</sup> exerts cytotoxicity as a dose-dependent manner, and that

administration of PR has a protective effect against the cytotoxic action induced by MPP<sup>+</sup>. From flow cytometric analysis of DNA contents, an increase in the fraction of cells in the sub-G1 phase was observed in the MPP<sup>+</sup>-treated group. This increased sub-G1-phase fraction was reduced by pre-treatment with PR to a level comparable to that seen in the control group.

It is known that apoptosis involves the activation of endonucleases and that this activation results in the cleavage of genomic DNA into well-defined fragments, which appear as a characteristic ladder pattern upon agarose gel electrophoresis<sup>44)</sup>. To provide evidence supporting the involvement of apoptosis in MPP<sup>+</sup>-induced cytotoxicity and the protective effect of PR against MPP<sup>+</sup>-induced apoptosis, DNA fragmentation assay was performed. The MPP<sup>+</sup>-treated group showed the distinctive ladder pattern characteristic of apoptosis, similar to the results observed by Gonzalez-Polo et al.<sup>18)</sup> in cultured rat cerebellar granule cells and those presented by Chun et al.<sup>45)</sup> in their study involving substantia nigra-derived dopaminergic cell line. On the other hand, the cells pre-treated with PR showed noticeable decrease in the intensity of MPP<sup>+</sup>-induced DNA laddering.

Cells undergoing apoptotic death exhibit several morphological characteristics such as chromatin condensation, nuclear fragmentation, and apoptotic cell body formation<sup>46)</sup>. Apoptotic bodies, one of the stringent morphological criteria of apoptosis, were characteristically present in MPP<sup>+</sup>-treated cells stained with DAPI, while pre-treatment with PR decreased such appearance significantly, in this study. DNA strand breaks are known to occur during apoptosis, and it is known that nicks in the DNA molecules can be detected via TUNEL assay<sup>47)</sup>. In this study, TUNEL-positive cells were detected in the MPP<sup>+</sup>-treated group, while the occurrence of such cells was decreased in the cultures pre-treated with PR.



MPP<sup>+</sup> has been reported to induce neuronal apoptosis<sup>17-19)</sup>, and understanding the molecular events triggering apoptosis is an important step toward the development of effective treatment strategies for such neurological disorders. Bax is a member of the Bcl-2 family that acts as a promotor of cell death<sup>48)</sup>. Administration of MTPT into the mice induced increasing of the expression of Bax in the substantia nigra<sup>49)</sup>. It was reported that MPP<sup>+</sup> induces Bax expression in dopaminergic neuronal cell line MN9D cells<sup>50)</sup>.

It has well been documented that the process of apoptosis is regulated by the expressions of several proteins. The Bcl-2 family can be classified into two functionally distinct groups: anti-apoptotic proteins and pro-apoptotic proteins. Bcl-2, an anti-apoptotic protein, is known to regulate the apoptotic pathways and to protect against cell death, while Bax, a pro-apoptotic protein of the family, is expressed abundantly and selectively during apoptosis and promotes cell death<sup>9)</sup>. The caspases, a class of cysteine proteases, are considered to play a central role in the apoptotic process and to trigger a cascade of proteolytic cleavage events in mammalian cells. Of particular interest is caspase-3, the most widely studied member of the caspase family and one of the key executioners of apoptosis, being responsible either partially or wholly for the proteolytic cleavage of various proteins<sup>10)</sup>.

The present results demonstrated that Bax expression was increased by MPP<sup>+</sup> -treatment in the SK-N-MC cells, and cells pre-treated with PR showed much dampening in this increment. When Bcl-2 is overexpressed, it heterodimerizes with Bax, and cell death is repressed. Presumably, the ratio of Bax to Bcl-2 serves to determine the susceptibility of cells to apoptosis<sup>51)</sup>. In the present study, PR pre-treatment resulted in a decrease in Bax expression.

Caspases, a family of cysteine proteases, are an

integral part of the apoptotic pathway<sup>10)</sup>. Recent reports indicate that caspases play a role in neuronal cell death during development as well as after neuronal injury<sup>52)</sup>. Bilsland et al.<sup>4)</sup> also reported that MPP<sup>+</sup>-induced apoptosis involves the caspase-3 pathway. In the present study, the number of the activated caspase-3 expression was increased by MPP<sup>+</sup>-treatment in the SK-N-MC cells and cells pre-treated with PR showed much dampening in this increment.

The present results showed that *Paeoniae Radix*(PR) exerts a significant neuroprotective effect against MPP<sup>+</sup>-induced apoptosis in the SK-N-MC neuronal cells.

## Conclusion

To find out the protective effect of *Paeoniae Radix*(PR) extract against MPP<sup>+</sup>-induced apoptosis in SK-N-MC human neuroblastoma cells, we carried out several experiments. The experimental results were as follows:

1. The appearance of cells pre-treated with PR 10mg/ml prior to MPP<sup>+</sup> exposure was similar to those of the control cells by TUNEL staining and DAPI staining.
2. The fraction of cells in the sub-G1 phase increased from 35.30%(value from the control group) to 61.13% following MPP<sup>+</sup> exposure, while the figure was decreased to 42.51% in cells pre-treated with PR 10mg/ml prior to MPP<sup>+</sup> exposure.
3. The pre-treatment with PR 10mg/ml prior to MPP<sup>+</sup> exposure resulted in a decreased intensity of MPP<sup>+</sup>-induced DNA laddering.
4. The pre-treatment with PR 10mg/ml prior to MPP<sup>+</sup> exposure resulted in the decrease of the increased level of Bax mRNA expression and in the increase of the decreased level of Bcl-2 mRNA expression.
5. The pre-treatment with PR 10mg/ml prior to MPP<sup>+</sup> exposure decreased the level of expression of caspase-3 .

Based on the present study, we arrive at the conclusion that it is possible for *Paeoniae Radix*(PR) to be a useful drug in the treatment of Parkinson's disease.

## References

1. Lang AE, Lozano AM. Parkinson's disease. First of two parts. *New Engl J Med* 1998;339: 1044-1053.
2. Nicotra A, Parvez SH. Apoptotic molecules and MPTP-induced cell death. *Neurotoxicol Teratol* 2002;24:599-605.
3. Olanow CW, Tatton WG. Etiology and pathogenesis of Parkinson's disease. *Annu Rev Neurosci* 1999;22:123-144.
4. Bilsland J, Roy S, Xanthoudakis S, Nicholson DW, Han Y, Grimm E, Hefti F, Harper SJ. Caspase inhibitors attenuate 1-methyl-4-phenylpyridinium toxicity in primary cultures of mesencephalic dopaminergic neurons. *J Neurosci* 2002;22:2637-2649.
5. Hartley A, Stone JM, Heron C, Cooper JM, Schapira AH. Complex I inhibitors induce dose-dependent apoptosis in PC12 cells: relevance to Parkinson's disease. *J Neurochem* 1994;63:1987-1990.
6. Itano Y, Nomura Y. 1-methyl-4-phenyl-pyridinium ion(MPP<sup>+</sup>) causes DNA fragmentation and increases the Bcl-2 expression in human neuroblastoma, SH-SY5Y cells, through different mechanisms. *Brain Res* 1995;704:240-245.
7. Chalmers-Redman RM, Fraser AD, Carlile W, Pong A, Tatton WG. Glucose protection from MPP<sup>+</sup>-induced apoptosis depends on mitochondrial membrane potential and ATP synthase. *Biochem Biophys Res Commun* 1999;257:440-447.
8. Yoshinaga N, Murayama T, Nomura Y. Apoptosis induction by a dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium ion(MPP<sup>+</sup>), and inhibition by epidermal growth factor in GH3 cells. *Biochem Pharmacol* 2000;60:111-120.
9. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 1993;74:609-619.
10. Cohen G.M. Caspases: the executioners of apoptosis. *Biochem J* 1997;326:1-16.
11. Chen JK, Chen TT. *Chinese Medical Herbology and Pharmacology*. Art of Medicine Press 2004;930-934.
12. Tomoda M, matsumoto K, Shimizu N, Gonda R, Ohara N. Characterization of a neutral and an acidic polysaccharide having immunological activities from the root of *Paeonia lactiflora*. *Biological and Pharmaceutical Bulletin* 1993;16(12):1207-1210.
13. Tomoda M, matsumoto K, Shimizu N, Gonda R, Ohara N, Hirabayashi K. An acidic polysaccharide with immunological activities from the root of *Paeonia lactiflora*. *Biological and Pharmaceutical Bulletin* 1994;17(9):1161-1164.
14. Tipton KF, Singer TP. Advances in our understanding of the mechanisms of the neurotoxicity of MPTP and related compounds. *J Neurochem* 1993;61:1191-1206.
15. Jang MH, Shin MC, Kang IS, Baik HH, Cho YH, Chu JO, Kim EH, Kim CJ. Caffeine induced apoptosis in human neuroblastoma cell line SK-N-MC. *J Korean Med Sci* 2002;17:674-678.
16. Lee M, Hyun D, Halliwell B, Jenner P. Effect of the overexpression of wild-type or mutant alpha-synuclein on cell susceptibility to insult. *J Neurochem* 2001;76:998-1009.
17. Sheng GQ, Zhang JR, Pu XP, Ma J, Li CL. Protective effect of verbascoside on 1-methyl-4-

- phenylpyridinium ion-induced neurotoxicity in PC12 cells. *Eur J Pharmacol* 2002;451:119-124.
18. Gonzalez-Polo RA, Soler G, Alvarez A, Fabregat I, Fuentes JM. Vitamin E blocks early events induced by 1-methyl-4-phenylpyridinium(MPP<sup>+</sup>) in cerebellar granule cells. *J Neurochem* 2003;84:305-315.
  19. Halvorsen EM, Dennis J, Keeney PM, Sturgill TW, Tuttle JB, Bennett Jr JP. Methylpyridinium(MPP<sup>+</sup>)-and nerve growth factor-induced changes in pro- and anti-apoptotic signaling pathways in SH-SY5Y neuroblastoma cells. *Brain Res* 2002;952:98-110.
  20. Ohsugi M, Fan W, Hase K, Xiong Q, Tezuka Y, Komatsu K, et al. Active-oxygen scavenging activity of traditional nourishing- tonic herbal medicines and active constituents of *Rhodiola sacra*. *Journal of Ethnopharmacology* 1999;67(1):111-119.
  21. Okubo T, Nagai F, Seto T, Satoh K, Ushiyama K, Kano I. The inhibition of phenylhydroquinone-induced oxidative DNA cleavage by constituents of Moutan Cortex and *Paeoniae Radix*. *Biological and Pharmaceutical Bulletin* 2000;23(2):199-203.
  22. Goto H, Shimada, Y, Akechi Y, Kohta K, Hattori M, Terasawa K. Endothelium-dependent vasodilator effect of extract prepared from the roots of *Paeonia lactiflora* on isolated rat aorta. *Planta Med* 1996;62:436-439.
  23. Wang Y, Ma R. Effect of an extract of *Paeonia lactiflora* on the blood coagulative and fibrinolytic enzymes. *Zhong Xi Yi Jie He Za Zhi* 1990;10:101-102.
  24. Wu HK, Shen SJ. Capillary electrophoretic determination of the constituents of *Paeoniae Radix*. *Journal of Chromatography A* 1996;753(1):139-146.
  25. Tan H, Liu Y, Fong W, Liu M. Chemical components of decoction of radix *Paeoniae* and radix *Glycyrrhizae*. *Zhongguo Zhong Yao Za Zhi* 1995;20(9):550-551,576.
  26. Ohno Y, Fukuda K, Takemura G, Toyota M, Watanabe M, Yasuda N, et al. Induction of apoptosis by gallic acid in lung cancer cells. *Anticancer Drugs* 1999;10(9):845-851.
  27. Yoshioka K, Kataoka T, Hayashi T, Hasegawa M, Ishi Y, Hibasami H. Induction of apoptosis by gallic acid in human stomach cancer KATO III and colon adenocarcinoma COLO 205 cell lines. *Oncology Reports* 2000;7(6):1221-1223.
  28. Fujimoto K, Hosotani R, Doi R, Wada M, Lee JU, Koshiba T, et al. Induction of cell-cycle arrest and apoptosis by a novel retinobenzoic-acid derivative, TAC-101, in human pancreatic-cancer cells. *International Journal of Cancer* 1999;81(4):637-44.
  29. Shintai Y, Yotsuyanagi H, Moriya K, Fujie H, Tsutsumi T, Kanegae Y, et al. Induction of apoptosis after switch-on of the hepatitis B virus X gene mediated by the Cre/loxP recombination system. *Journal of General Virology* 1999;80(Pt 12):3257-3265.
  30. Sakamoto S, Yoshino H, Shirahata Y, Shimodairo K, Okamoto R. Pharmacotherapeutic effects of kwei-chin-fu-ling-wan(keishi-bukuryogan) on human uterine myomas. *The American Journal of Chinese Medicine* 1992;20(3-4):313-317.
  31. Zee-Cheng RK. Shi-quan-da-bu-tang(ten significant tonic decoction), SQT. A potent Chinese biological response modifier in cancer immunotherapy, potentiation and detoxification of anticancer drugs. *Methods and Findings in Experimental and Clinical Pharmacology* 1992;14(9):725-736.

32. Fu QL. Experimental study on yiqi-huoxue therapy of liver fibrosis. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 1992;12(4):228-229.
33. Qi XG. Protective mechanism of *Salvia miltiorrhiza* and *Paeonia lactiflora* for experimental liver damage. *Zhongguo Xi Yi Jie He Za Zhi* 1991;11(2):102-104,169.
34. Chen LC, Chou MH, Lin MF, Yang LL. Lack of pharmacokinetic interaction between valproic acid and a traditional Chinese medicine, *Paeoniae Radix*, in healthy volunteers. *J Clin Pharm Ther* 2000;25:453-459.
35. Yang J, Wang J, Zhang JX, Jiang W, Ma CG, Xu SY. Improvement effects of total paeony glycoside on ability of learning and memory in model mice induced by drugs. *Chin Pharmacol Bull* 2000;16:46-49.
36. 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* 2002;16:124?131.
37. 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.
38. Lee SM, Li ML, Tse YC, Leung SC, Lee MM, Tsui SK, Fung KP, Lee CY, Waye MM. *Paeoniae Radix*, a chinese herbal extract, inhibit hepatoma cells growth by inducing apoptosis in a p53 independent pathway. *Life Sci* 2002 sep 7;71(19):2267-2277.
39. Ohta H, Matsumoto K, Shimizu M, Watanabe H. Paeoniflorin attenuates learning impairment of aged rats in operant brightness discrimination task. *Pharmacol Biochem Behav* 1994;49: 213-217.
40. Sugaya A, Suzuki T, Sugaya E, Yuyama N, Yasuda K, Tsuda T. Inhibitory effect of peony root extract on pentylenetetrazol-induced intracellular calcium increase. *Bioimages* 1993;1:115-123.
41. Tsuda T, Sugaya A, Ohguchi H, Kishida N, Sugaya E. Protective effects of peony root extract and its components on neuron damage in the hippocampus induced by the cobalt focus epilepsy model. *Exp Neurol* 1997;146:518-525.
42. Zhang WG, Zhang ZS. Anti-ischemia reperfusion damage and anti-lipid peroxidation effects of paeonol in rat heart. *Yao Hsueh Hsueh Pao* 1994;29:145-148.
43. Gong X, Sucher NJ. Stroke therapy in traditional Chinese medicine(TCM): prospects for drug discovery and development. *Trends Pharmacol Sci* 1999;20:191-196.
44. Eastman A, Barry MA. The origins of DNA breaks: a consequence of DNA damage, DNA repair, or apoptosis? *Cancer Invest* 1992;10:229-240.
45. Chun HS, Gibson GE, DeGiorgio LA, Zhang H, Kidd VJ, Son JH. Dopaminergic cell death induced by MPP<sup>+</sup>, oxidant and specific neurotoxicants shares the common molecular mechanism. *J Neurochem* 2001;76:1010-1021.
46. Wyllie AH, Kerr, JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980;68:251-306.
47. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1999;119:493-501
48. Reed JC. Bcl-2 family proteins. *Oncogene* 1998;17:3225-3236.
49. Hassouna L, Wickert H, Zimmermann M, Gillardon F. Increase in Bax expression in substantia nigra following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP) treatment of

mice. *Neurosci Lett* 1996;204:85-88.

50. Choi WS, Canzoniero LMT, Sensi SL, O'Malley KL, Gwag BJ, Sohn S, Kim JE, Oh TH, Lee EB, Oh YJ. Characterization of MPP<sup>+</sup>-induced cell death in a dopaminergic neuronal cell line: role of macromolecule synthesis, cytosolic calcium, caspase, and Bcl-2-related proteins. *Exp Neurol* 1999;159:274-282.
51. Korsmeyer SJ. Bcl-2 gene family and the regu-

lation of programmed cell death. *Cancer Res* 1999;59(7 Suppl):1693-1700.

52. Woo M, Hakem R, Soengas MS, Duncan G.S, Shahinian A, Kocgi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes Dev* 1998;12:806-819.