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Neuroprotective Effects of *Carpinus tschonoskii* MAX on 6-Hydroxydopamine-Induced Death of PC12 Cells

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Abstract − The present study investigated the neuroprotective effect of *Carpinus tschonoskii* MAX and its intracellular protective mechanism on 6-hydroxydopamine (6-OHDA)-induced oxidative damage in PC12 cells. We found that pretreatment of PC12 cells with *C. tschonoskii* extract significantly inhibited the cell death induced by 6-OHDA in a dose dependent manner. *C. tschonoskii* extract decreased 6-OHDA-induced apoptotic events such as chromatin condensation, DNA fragmentation, the decrease of Bcl-2/Bax ratio, caspase-3 activation and PARP cleavage. *C. tschonoskii* extract also reduced generation of 6-OHDA-induced reactive oxygen species and nitric oxide. Furthermore, *C. tschonoskii* extract up-regulated the myocyte enhancer factor 2 D (MEF2D), a critical transcription factor for neuronal survival, and Akt activity, whereas it inhibited the activity of ERK1/2 and JNK. The results suggest that *C. tschonoskii* extract decreases 6-OHDA-induced oxidative stress and could prevent PC12 cell apoptosis induced by 6-OHDA via the up-regulation of MEF2D and Akt activity, and thus may have application in developing therapeutic agents for Parkinson's disease.

Keywords: *Carpinus tschonoskii* MAX, 6-OHDA, PC12 cells, Apoptosis, MEF2D, Akt

INTRODUCTION

Parkinson's disease (PD) is currently the most common degenerative disorder of the aging brain, after Alzheimer's disease (Bové *et al.*, 2005). The pathological hallmark of PD involves the loss of the nigrostriatal dopaminergic pathway, resulting in a marked impairment of motor control (Blum *et al.*, 2001). Although the pathogenic processes of PD remain unknown, recent findings indicate that an increase of oxidative stress may be a critical mediator of dopaminergic neuron destruction in PD (Jenner and Olanow, 1996; Przedborski and Ischiropoulos, 2005). The death of dopaminergic neurons caused by oxidative stress might produce altered activities of survival signaling factors, including myocyte enhancer factor 2 (MEF2) (Mao *et al.*, 1999), MAP Kinase pathways (Veeranna *et al.*, 2000) or the PI3K/Akt pathway (Shimoke and Chiba, 2001; Greggio and Singleton, 2007). To date, however, no therapeutic drugs for PD have been identified which do not have certain adverse effects. Many studies have focused on plant components and extracts that can scavenge ROS and protect dopaminergic neurons from oxidative damage.

Recent studies showed that *Carpinus* leaves have flavonoid compounds such as myricetin, quercetin, kaempferol, apigenin and luteolin, which are known to be antioxidants (Chang and Jeon, 2004; Jeon *et al.*, 2007). In addition, cytoprotective activities of *Carpinus tschonoskii* MAX methanol extract have been reported (Zhang *et al.*, 2007). An extract of *C. tschonoskii* exhibited ROS scavenging activity which preserved V79-4 Chinese hamster lung fibroblast viability against H_2O_2 induced oxidative stress (Zhang *et al.*, 2007). However, a possible neuroprotective effect of *C. tschonoskii* extract, and its mechanism in a PD model, have not previously been assessed.

This study was conducted to investigate the neuroprotective effect of *C. tschonoskii* extract and its intracellular protective mechanism, using PC12 cells treated with 6-hydroxydopamine (6-OHDA) as a PD model.

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MATERIALS AND METHODS

Materials

EtOH extract (80%) and several solvent fractions from *Carpinus tschonoskii* MAX were purchased from Jeju hi-Tech Industry Development Institute, which has professional facilities and provides extracts from plants growing on Jeju Island for research purposes in biotechnology and related industrial fields. The HPLC profile of *C. tschonoskii* EtOH extract was also obtained from Jeju hi-Tech Industry Development Institute (Fig. 1). 6-hydroxydopamine (6-OHDA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorofluorescin diacetate (DCFH-DA) and β-actin monoclonal antibody were purchased from Sigma-Aldrich (Yongin, Kyunggi-do, Korea). MEF2D monoclonal antibody was purchased from BD Biosciences (CA, USA). ERK1/2, p-ERK1/2, p38, p-p38, SAPK/JNK, p-SAPK/JNK, Akt and p-Akt polyclonal antibodies were obtained from Cell Signaling Technology (MA, USA). HRP- conjugated goat anti-rabbit and horse anti-mouse IgGs were purchased from Vector Laboratories (MA, USA). Aprotinin, leupeptin and Nonidet P-40 were purchased from Roche Applied Science (IN, USA).

Cell culture

PC12, a rat pheochromocytoma cell line, was supplied by KCLB (Korea Cell Line Bank). PC12 cells were incubated in RPMI 1640 medium (Hyclone, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO Inc, NY, USA) at 37°C under an atmosphere of 95% air and 5% CO_2. All experiments used cells plated at a density of 1.0×10^5 cells/ml and were conducted 24 h after cells were seeded.

MTT assay for cell viability

Cell survival was evaluated by MTT reduction (Scudiero *et al.*, 1988). PC12 cells (1.0×10^5 cells/ml) were seeded in 24-well plates for 24 h. The PC12 cells were pretreated with 25, 50 or 100 μg/ml of 80% EtOH extract or solvent fractions from *C. tschonoskii* for 30 min prior to incubation with 250 μM 6-OHDA for 24 h. MTT was added to the cells at a final concentration of 250 μM and cultures were further incubated at 37°C with 5% CO_2 for 4 h to produce a dark blue formazan product formed by MTT reduction. Media were aspirated and the resulting formazan crystals were dissolved in DMSO (Amresco, OH, USA). The absorbance of each well was measured using a microplate reader (Amersham Pharmacia Biotech, NY, USA) at 540 nm excitatory emission wavelength. PC12 cell viability was determined as a percent of inhibition due to reduced absorbance compared to the untreated controls.

Evaluation of intracellular reactive oxygen species (ROS)

Intracellular ROS levels were determined using the fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Rosenkranz *et al.*, 1992). This molecule is cleaved intra-cellularly by esterases to form non-fluorescent 2',7'-dichlorofluorescin (DCFH), which is transformed to the fluorescence compound 2',7'-dichlorofluorescein (DCF) upon oxidation by ROS. PC12 cells (1.0×10^5 cells/ml) were seeded in 6-well plates for 24 h. The cells were pretreated with 100 μg/ml of *C. tschonoskii* 80% EtOH extract for 30 min prior to 250 μM 6-OHDA treatment for 3 h. The cells were incubated with 50 μM DCFH-DA for 20 min at 37°C under 5% CO_2 in the dark. PC12 cells were washed twice with PBS and fluorescence was monitored by a COULTER® EPICS® XL™ Flow Cytometer (Coulter, Miami, FL, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

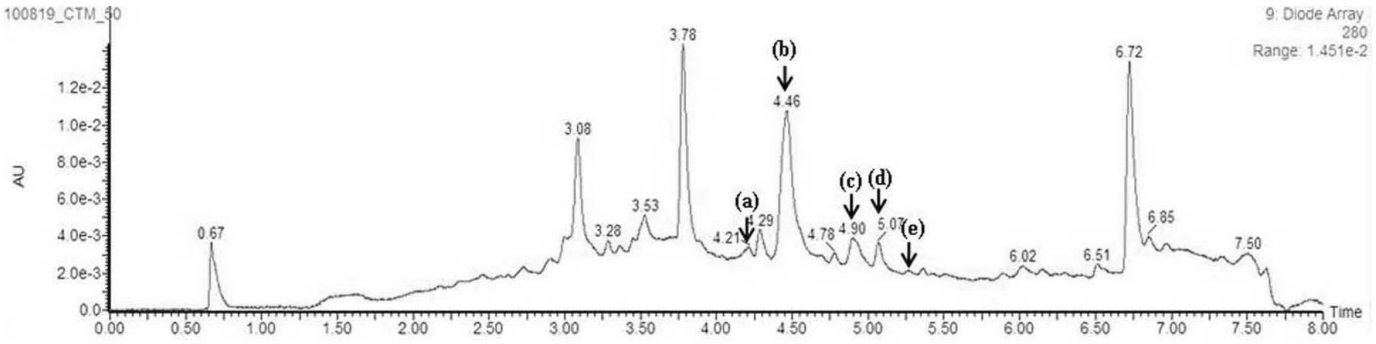

Fig. 1. LC/MS Chromatogram of flavonoids from *Carpinus tschonoskii* MAX EtOH extract by Jeju Hi-Technology Development Institute. Each peak indicates Quercetin-3-O-glucoside (a), quercitrin (b), quercetin (c), apigenin (d) and kaempferol (e).

Measurement of nitric oxide (NO) production

After PC12 cells (1.0×10^5 cells/ml) were pre-plated for 24 h, the cells were treated with 100 μg/ml of *C. tschonoskii* 80% EtOH extract for 30 min prior to incubation with 250 μM 6-OHDA for 3 h. Nitrite in culture supernatants was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100 μl samples of media. All measurements were performed in triplicate. The concentration of NO_2^- was determined from a standard curve prepared using $NaNO_2$.

Morphological analysis of apoptosis by Hoechst 33342 staining

Changes in nuclear morphologies of apoptotic cells were investigated by labeling the cells with Hoechst 33342 nuclear stain, followed by fluorescent microscopy. Briefly, PC12 cells were pre-plated in 24-well plates (1.0×10^5 cells/ml) and treated with 250 μM 6-OHDA for 7 h after pre-treatment with *C. tschonoskii* extract at 1, 10, 50 or 100 μg/ml for 30 min. Cells were then stained with Hoechst 33342 (5 μg/ml), and observed using fluorescence microscopy (IX-71, Olympus, Japan).

Detection of DNA fragmentation due to apoptosis

PC12 (1.0×10^5 cells/ml) cells were pre-plated for 24 h and then treated with 250 μM 6-OHDA for 7 h after pre-treatment with *C. tschonoskii* extract (100 μg/ml) for 30 min. After incubation, the cells were collected and washed twice with cold-PBS. DNA was extracted using a Promega

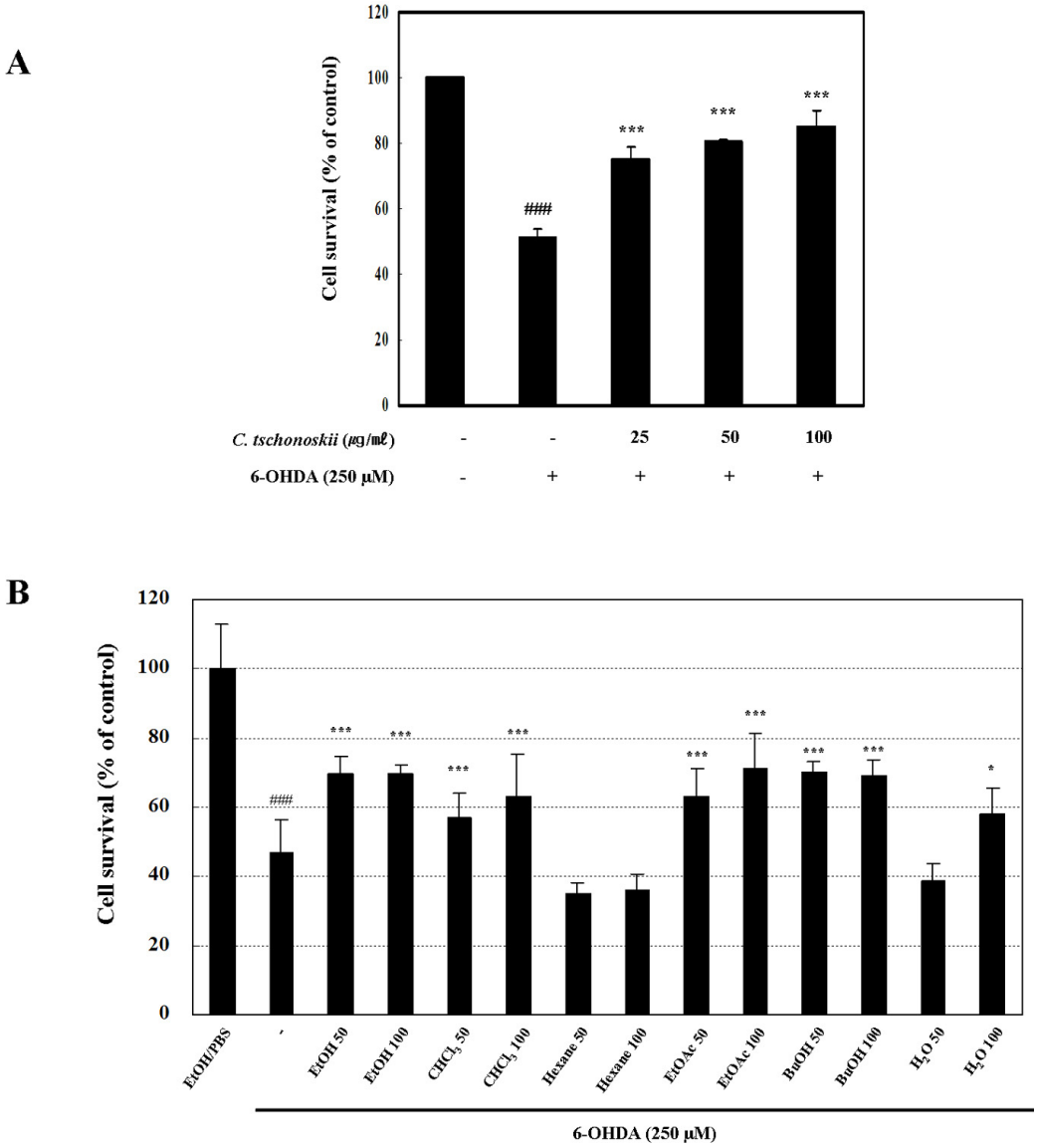

Fig. 2. Neuroprotective effect of EtOH extract or solvent fractions from *Carpinus tschonoskii* MAX on 6-OHDA-induced death of PC12 cells. (A) PC12 cells (1.0×10^5 cells/ml) were pre-treated with 25, 50 or 100 μg/ml of 80% EtOH extract from *C. tschonoskii* for 30 min prior to 250 μM 6-OHDA exposure for 24 h. (B) PC12 cells (1.0×10^5 cells/ml) were treated with 250 μM 6-OHDA after pre-treatment with 50 or 100 μg/ml of 80% EtOH extract or solvent fractions from *C. tschonoskii* for 30 min and measured for viability using the MTT assay method for 24 h. Results are mean ± S.D. (n=3). $^{###}p<0.005$ vs. control, $^{*}p<0.05$ vs. 6-OHDA treatment group, $^{***}p<0.005$ vs. 6-OHDA treatment group using student's t-test.

Wizard® Genomic DNA Purification Kit (Promega, WI, USA). DNA samples (10 μl) were electrophoresed on a 1.2% agarose gel in 450 mM Tris borate-EDTA buffer, pH 8.0. DNA was observed under a UV transiluminator (Spectronics Corporation Westbury, NY, USA).

Immunoblotting

Equal amounts of protein were loaded onto a sodium dodecyl sulfate polyacrylamide electrophoresis gel and then transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, CA, USA). After blocking with 1% bovine serum albumin (BSA) in TBS-Tween (TBS-T) (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20), the membrane was incubated with the primary antibodies in 10 ml of buffer (Tris-buffered saline and 0.1% Tween-20) with gentle shaking at 4°C, and then incubated with the respective conjugated secondary antibodies. Signals were detected using western blot detection reagents (Intron Biotechnology, Gyeonggi-do, Korea).

Statistical analysis

Results are expressed as means ± standard deviation (SD) of at least 3 independent experiments performed in triplicate. Comparison between groups was performed by the t-test. Statistically significant differences were set at $p < 0.05$, $p < 0.01$, $p < 0.005$ and $p < 0.001$.

RESULTS

The MTT assay was used to determine if *C. tschonoskii* extract protected PC12 cells against 6-OHDA-induced neuronal cell death. *C. tschonoskii* 80% EtOH extract prevented PC12 cell death induced by 24 h treatment with 250 μM 6-OHDA in a dose-dependent manner (Fig. 2A). When PC12 cells were pre-treated with 50 or 100 μg/ml of EtOH extract and different solvent fractions of *C. tschonoskii*, PC12 cell death following 6-OHDA exposure markedly decreased (Fig. 2B).

6-OHDA is known to induce PC12 cell death by the production of intracellular ROS and NO (Saito *et al.*, 2007). We therefore examined whether *C. tschonoskii* extract could inhibit the ROS and NO production associated with 6-OHDA-induced cell death. Pre-treatment with *C. tschonoskii* extract significantly reduced 6-OHDA-induced ROS generation, indicating that the *C. tschonoskii* extract attenuated the pro-oxidant effects of 6-OHDA (Fig. 3A). 6-OHDA significantly increased NO levels, but this was suppressed by pre-treatment with *C. tschonoskii* extract (Fig. 3B).

The protective effect of *C. tschonoskii* extract against 6-OHDA-induced apoptosis was examined by Hoechst 33342 staining and microscopic detection of DNA fragmentation. Chromatin condensation and DNA fragmentation were increased by treatment with 250 μM 6-OHDA for

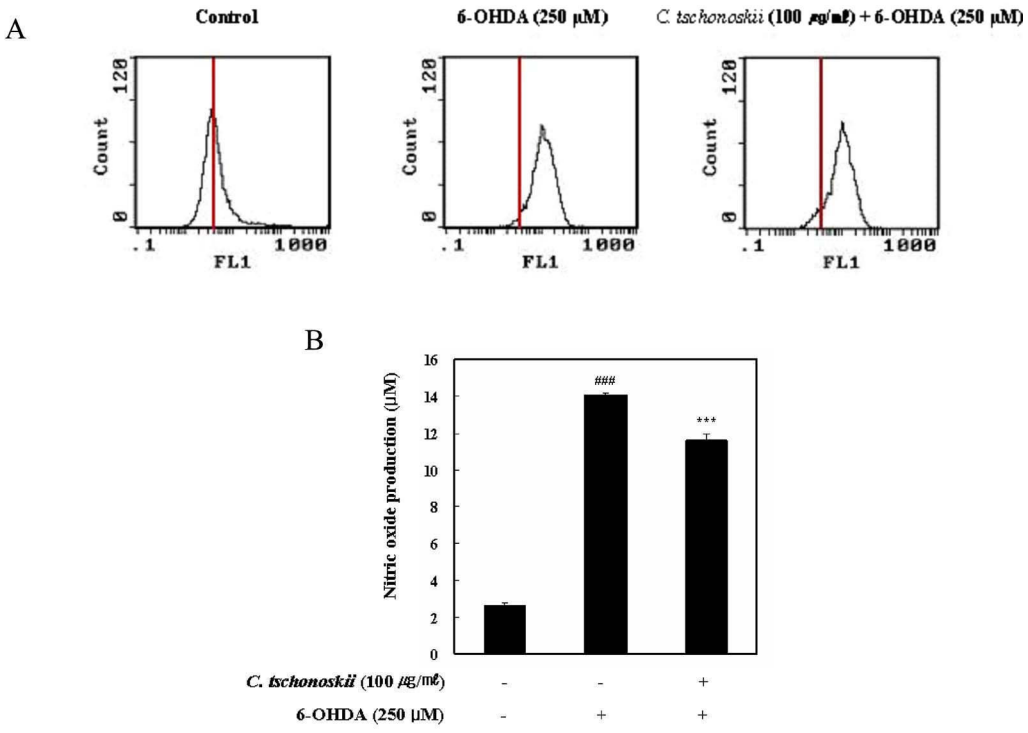

Fig. 3. Neuroprotective effect of *Carpinus tschonoskii* MAX extract on the 6-OHDA-induced increases of ROS and NO in PC12 cells. PC12 cells (1.0×10^5 cells/ml) were pre-treated with *C. tschonoskii* extract (100 μg/ml) for 30 min prior to 6-OHDA treatment for 3 h and intracellular ROS and NO were then measured. (A) *C. tschonoskii* extract suppressed 6-OHDA-induced intracellular ROS. (a) control, (b) 250 μM 6-OHDA for 3 h, (c) 250 μM 6-OHDA treatment for 3 h after pre-treatment with 100 μg/ml of *C. tschonoskii* extract for 30 min. (B) *C. tschonoskii* reduced 6-OHDA-induced production of NO. Results are mean ± SD (n=3). $^{###}p < 0.005$ vs. control, $^{***}p < 0.005$ vs. 6-OHDA treatment group using student's t-test.

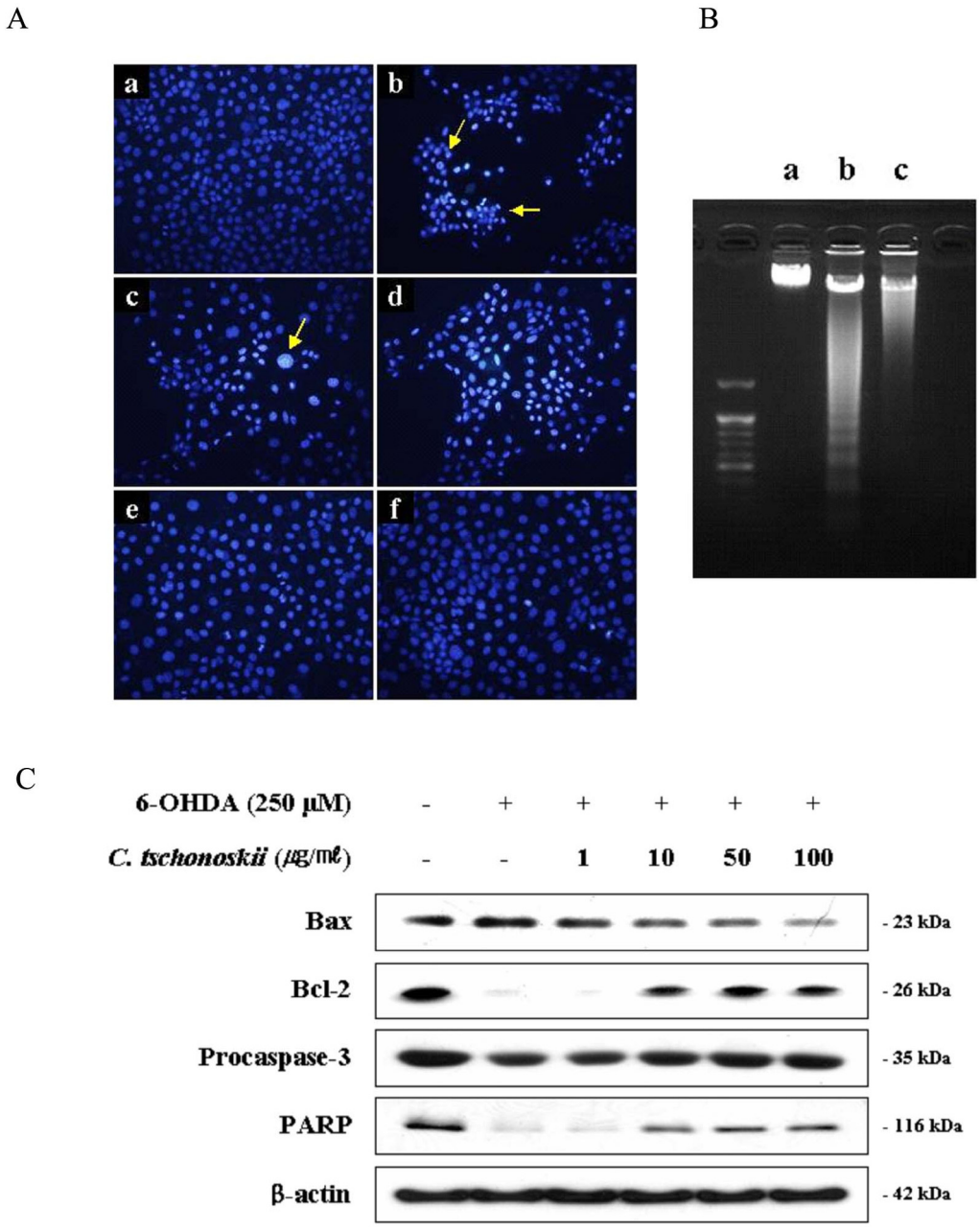

Fig. 4. Neuroprotective effect of *Carpinus tschonoskii* MAX extract on 6-OHDA-induced apoptosis in PC12 cells. (A) *C. tschonoskii* extract reduced 6-OHDA-induced chromatin condensation. PC12 cells (1.0×10^5 cells/ml) were treated as follows. (a) control, (b) with 250 μM 6-OHDA for 7, with 1 (c), 10 (d), 50 (e) and 100 (f) μg/ml of *C. tschonoskii* extract for 30 min prior to 250 μM 6-OHDA treatment for 7 h. (B) *C. tschonoskii* extract inhibited 6-OHDA-induced DNA fragmentation. PC12 cells (1.0×10^5 cells/ml) were treated as follows. (a) control, (b) 250 μM 6-OHDA for 7 h, (c) 250 μM 6-OHDA treatment for 7 h after pre-treatment with 100 μg/ml of *C. tschonoskii* extract for 30 min. (C) PC12 cells (1.0×10^5 cells/ml) were treated with 250 μM 6-OHDA for 7 h after pre-treatment with 1, 10, 50 or 100 μg/ml of *C. tschonoskii* extract for 30 min. Levels of Bax, Bcl-2, proaspase-3, PARP and β-actin were determined by western blotting.

7 h, while 100 μg/ml of *C. tschonoskii* extract significantly reduced 6-OHDA-induced apoptosis (Fig. 4A and B). We examined neuroprotective effects of *C. tschonoskii* extract on the activation of apoptotic signaling by 6-OHDA. Treatment with 250 μM 6-OHDA for 7 h increased the expression of Bax, while the levels of Bcl-2, procaspase-3 and PARP were decreased. These effects were reduced by *C. tschonoskii* pre-treatment in a dose-dependent manner (Fig. 4C).

We also assessed if *C. tschonoskii* extract could ameliorate the decrease of MEF2D levels induced by 6-OHDA. Pre-treatment with *C. tschonoskii* extract could compensate for the decrease of 6-OHDA induced MEF2D expression in a dose-dependent manner (Fig. 5).

To determine if there were any effects of *C. tschonoskii* extract on the 6-OHDA-induced activation of MAPK pathways, PC12 cells were pre-treated with *C. tschonoskii* extract at final concentrations of 1, 10, 50 or 100 μg/ml for 30 min, and then treated with 250 μM 6-OHDA for 2 h. Treatment of PC12 cells with 6-OHDA increased the phosphorylations of ERK 1/2, JNK and p38, while pre-treatment with *C. tschonoskii* EtOH extract decreased the phosphor-

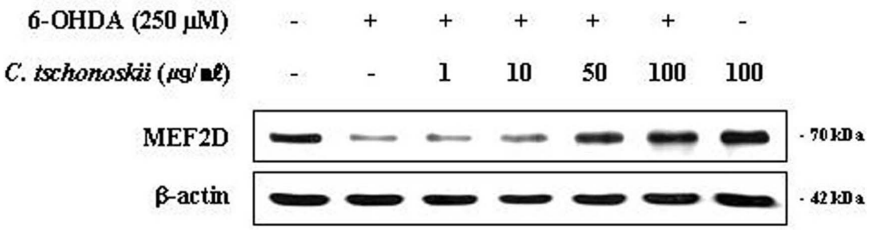

Fig. 5. Effect of *Carpinus tschonoskii* MAX extract on 6-OHDA-induced down-regulation of MEF2D in PC12 cells. PC12 cells (1.0×10^5 cells/ml) were treated with 250 μM 6-OHDA for 7 h after being pre-treated with 1, 10, 50 or 100 μg/ml of *C. tschonoskii* extract for 30 min; level of MEF2D was determined by western blotting. β-actin was used as a loading control after re-probing the same membrane.

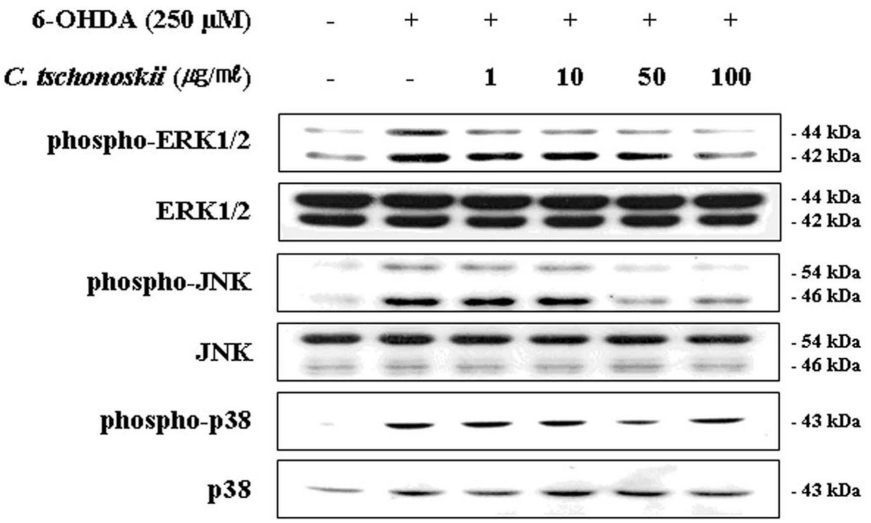

Fig. 6. Effect of *Carpinus tschonoskii* MAX extract on 6-OHDA-induced activation of MAP kinases in PC12 cells. PC12 cells (1.0×10^5 cells/ml) were treated with 250 μM 6-OHDA for 2 h after being pre-treated with 1, 10, 50 or 100 μg/ml of *C. tschonoskii* extract for 30 min; Levels of the phosphorylated form of each MAPK (ERK: Thr202/Tyr204; JNK: Thr183/ Tyr185; p38: Thr180/Tyr182) and the corresponding total protein were determined by western blots.

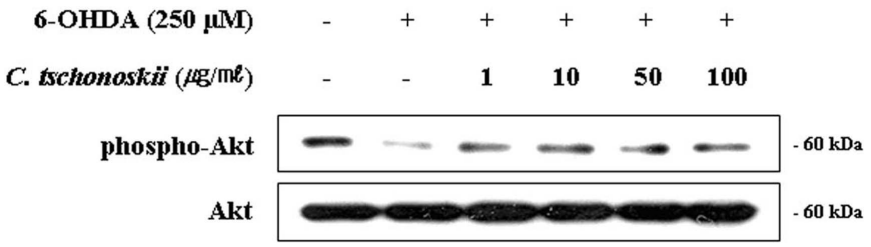

Fig. 7. Effect of *Carpinus tschonoskii* MAX extract on 6-OHDA-induced inactivation of Akt in PC12 cells. PC12 cells (1.0×10^5 cells/ml) were treated with 250 μM 6-OHDA for 2 h after being pre-treated with 1, 10, 50 or 100 μg/ml of *C. tschonoskii* extract for 30 min; levels of the phosphorylated form of Akt (Ser473) and total Akt were determined by western blots.

ylations of ERK 1/2 and JNK in a dose-dependent manner (Fig. 6). However, *C. tschonoskii* EtOH extract did not affect 6-OHDA-induced phosphorylation of p38 (Fig. 6).

We investigated whether *C. tschonoskii* extract could ameliorate inactivation of Akt by 6-OHDA. 6-OHDA decreased the phosphorylation of Akt, while the pre-treatment with *C. tschonoskii* EtOH extract increased the phosphorylation of Akt in a dose-dependent manner (Fig. 7).

DISCUSSION

To our knowledge, this is the first study to demonstrate the neuroprotective effect of *C. tschonoskii* extract on 6-OHDA-induced death of PC12 cells. In the study, we showed that *C. tschonoskii* extract could prevent apoptosis of PC12 cells induced by 6-OHDA via the up-regulation of MEF2D and Akt activity, as well as the down-regulation of ERK1/2 and JNK.

PD is a chronic neurodegenerative disorder and pathologically involves the loss of the nigrostriatal dopaminergic pathway (Blum *et al.*, 2001). 6-OHDA, a well established neurotoxin, can induce nigrostriatal dopaminergic lesions via the generation of reactive oxygen radicals, followed by the induction of apoptosis (Heikkila and Cohen, 1972; Perumal *et al.*, 1989; Decker *et al.*, 1993; Abad *et al.*, 1995; Kumar *et al.*, 1995; Choi *et al.*, 1999; Lotharius *et al.*, 1999). We examined a possible neuroprotective effect of *C. tschonoskii extract* on 6-OHDA-induced apoptosis in PC12 cells, which are rat pheochromocytoma cells, widely used for neurodegenerative diseases (Saito *et al.*, 2007; Shim *et al.*, 2009). As expected, *C. tschonoskii* extract used in the study, was found to contain quercetin, quercitrin, apigenin, genistein and kaempferol, which are known to be antioxidants (Fig. 1; Chang and Jeon, 2004; Jeon *et al.*, 2007). *C. tschonoskii* EtOH extract significantly prevented PC12 cell death usually induced by treatment with 6-OHDA (Fig. 2). Chloroform, butanol and ethylacetate fractions of *C. tschonoskii* EtOH extract markedly decreased PC12 cell death from 6-OHDA treatment (Fig. 2B). 6-OHDA significantly increased the levels of intracellular ROS and NO in PC12 cells, while *C. tschonoskii* EtOH extract was able to attenuate 6-OHDA-induced oxidative stress of PC12 cells (Fig. 3A and B). 6-OHDA increased the apoptotic morphological changes, including chromatin condensation and cell shrinkage, as detected by Hoechst 33342 nuclear staining, as well as DNA fragmentation *via* an increase of Bax, decrease of Bcl-2, activation of caspase-3, and cleavage of PARP (Fig. 4). However, *C. tschonoskii* EtOH extract attenuated the apoptotic characteristics (Fig. 4A and B). In addition, *C. tschonoskii* EtOH extract reduced the expression of Bax, whereas the levels of Bcl-2, procaspase-3 and PARP were increased in a dose-dependent manner (Fig. 4C). These results suggest that *C. tschonoskii* extract pre-treatment shifted the balance between pro-apoptotic regulators and anti-apoptotic regulators to favor cell survival. The results appear to be due to the anti-oxidant effects of flavonoid compounds in *C. tschonoskii* EtOH extract and are also directly supported by previous studies which have shown both cytoprotective and ROS scavenging activities of *C. tschonoskii* extract against H_2O_2 induced oxidative stress (Zhang *et al.*, 2007). Moreover, quercetin, a flavonoid component of *C. tschonoskii* extract, has been reported to protect dopamine neurons from oxidative insults and apoptosis (Mercer *et al.*, 2005; Bournival *et al.*, 2009). On the other hand, catechins of green tea have been also shown to inhibit 6-OHDA-induced apoptosis in PC12 cells (Nie *et al.*, 2002).

The MEF2D transcription factors play critical roles in neuronal cell survival (Black and Olson, 1998; Mao *et al.*, 1999; Mao and Wiedmann, 1999; Okamoto *et al.*, 2000; Li *et al.*, 2001; Gong *et al.*, 2003; Liu *et al.*, 2003). Degradation of MEF2D was involved in the death of cortical neurons caused by glutamate and MPP^+ (Gong *et al.*, 2003; Tang *et al.*, 2005; Smith *et al.*, 2006). We also observed that down-regulation of MEF2D might be involved in 6-OHDA-induced PC12 cell death (Kim *et al.*, in revision). *C. tschonoskii* EtOH extract reduced 6-OHDA-induced MEF2D down-regulation (Fig. 5), indicating that *C. tschonoskii* extract protects PC12 cells from 6-OHDA-induced death by enhancing the expression of MEF2D.

MAP kinase and PI3K/Akt pathways play central roles in cell death and survival (Veeranna *et al.*, 2000; Brunet *et al.*, 2001; Chong *et al.*, 2005). We also found that 6-OHDA increased the phosphorylation of MAP kinases such as ERK1/2 (Fig. 6; Kulich *et al.*, 2007), whereas the phosphoylation of Akt was decreased in a dose-dependent manner compared to the control (Fig. 7; Jiang and Yu, 2005). Several studies have shown that Akt signaling promotes MEF2 activity (Subramaniam and Unsicker, 2006; Sako *et al.*, 2009). We investigated whether *C. tschonoskii* extract could reduce the activation of the MAP kinase pathway induced by 6-OHDA. The *C. tschonoskii* EtOH extract decreased the activation of ERK1/2 and JNK, while phosphorylation of p38 was not affected (Fig. 6). Thus, the neuroprotective effect of *C. tschonoskii* EtOH extract might arise from inhibiting the activation of ERK1/2 and JNK pathways. *C. tschonoskii* EtOH extract did increase Akt phosphorylation (Fig. 7), indicating that C. tschonoski extract protected against PC12 cell death by enhancing the activation of Akt.

In summary, *C. tschonoskii* attenuates dopaminergic neuronal cell death induced by 6-OHDA via inhibiting the generation of ROS and NO. As a result, the following neuroprotective effects are observed: up-regulation of MEF2D; inactivation of ERK1/2 and JNK pathways; activation of the Akt pathway. The net result is a reduction of apoptotic signaling. Consequently, so as to develop new strategies for treating Parkinson's disease, it may be valuable to es-

tablish the detailed functional roles of *C. tschonoskii* extract during 6-OHDA-induced PC12 cell death.

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