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Dangguijakyak-san, a medicinal herbal formula, protects dopaminergic neurons from 6-hydroxydopamine-induced neurotoxicity

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ABSTRACT

Aim of the study: Dangguijakyak-san (DJS) is a multi-herbal formula that has long been widely used in traditional Oriental medicine to treat gynecologic disorders, including neurological symptoms. Recent clinical and experimental studies have reported aging and anti-neurodegenerative effects of DJS. In this study, we evaluated the neuroprotective effects of DJS on dopaminergic (DA) neurons damaged by 6-hydroxydopamine (6-OHDA).

Materials and methods: To evaluate the protective effects of DJS, we analyzed viability in SH-SY5Y neuroblastoma cells and tyrosine hydroxylase (TH) staining in primary DA cells. To explore the possible mechanism(s) of neuroprotection, we assessed anti-oxidant activity by measuring reactive oxygen species (ROS) and glutathione (GSH) levels. To determine mitochondria-mediated apoptotic activity, we examined mitochondrial membrane potential, cytochrome *c* release, and caspase-3 activation.

Results: DJS at 0.05–5 μg/mL significantly protected SH-SY5Y cells from 6-OHDA toxicity, dose-dependently, and attenuated 6-OHDA damage in primary DA cells. DJS reduced 6-OHDA-induced intracellular ROS production and GSH depletion and inhibited mitochondrial membrane instability, cytosolic cytochrome *c* release, and caspase-3 activation.

Conclusions: These results demonstrate that DJS has neuroprotective effects in DA neurons against 6-OHDA-induced toxicity through anti-oxidant and anti-mitochondrial-mediated apoptotic activities.

1. Introduction

Dangguijakyak-san (DJS), also called Danggui-Shaoyao-san in China or Tokishakuyakusan in Japan, is a well-known traditional formula composed of six medicinal herbs. DJS has long been used to treat various conditions in women, such as abdominal pain, dizziness after labor, menstrual irregularities (Lee, 1996; Heo, 1999), and post-menopausal disorders with neurological symptoms (Park et al., 2007; Goto et al., 2009) in Asian traditional medicine. Studies of DJS have shown that is also has actions as an ovarian hormone (Usuki et al., 1995; Choi et al., 1998), anti-anemic (Akase et al., 2003), anti-hypertensive (Takei et al., 2004), and as an anti-aging agent (Kou et al., 2005).

Neurological associated functions of DJS have been the focus of several clinical and experimental studies. It attenuates mild cognitive impairment (Kitabayashi et al., 2007) and improves cerebral function for post-stroke patients (Goto et al., 2009). In animal studies, DJS improves spatial cognition impairment (Egashira et al., 2004), central cholinergic nervous system dysfunction (Itoh et al., 1996), and Aβ-induced spatial recognition deficits (Hu et al., 2010). DJS also has neuroprotective effects in hydrogen peroxide-induced toxicity in PC12 cells (Qian et al., 2008), $A\beta_{25-35}$-induced neuronal damage in cultured rat cortical neurons, and senile dementia in aged mice (Egashira et al., 2005).

The dopaminergic (DA) system is involved in not only motor symptoms in Parkinson's disease (Obeso et al., 2004) but also regulating mood disorders, sleep disorders, and depression (Diehl and Gershon, 1992; Dailly et al., 2004; Ruhe et al., 2007). DJS has been used to improve depression and sleep disorders in postmenopausal women (Park et al., 2007) which are related with DA neurons. However, no neuroprotective effect of DJS on DA cells has yet been reported. In this study, we evaluated the protective effects of DJS on DA neurons, rat primary dopaminergic and SH-SY5Y cells (Levites et al., 2001), damaged by the neurotoxin 6-hydroxydopamine (6-

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OHDA) which leads oxidative stress via the generation of reactive oxygen species (ROS) (Blum et al., 2001). We analyzed viability in SH-SY5Y and mesencephalic DA cells and investigated the mechanism(s) of neuroprotection by DJS by analyzing ROS, glutathione (GSH), mitochondrial membrane potential ($\Delta\Psi m$), cytochrome *c* release, and caspase-3 assays in SH-SY5Y cells.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), fetal bovine serum (FBS), and penicillin–streptomycin were purchased from Gibco Industries, Inc. (Auckland, New Zealand). TEMED, protein assay, skim milk, and Tween-20 were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Rabbit anti-cleaved caspase-3 and mouse anti-cytochrome *c* antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Rabbit anti-TH antibody was obtained from Chemicon International, Inc. (Temecula, CA). Albiflorin and paeoniflorin were purchased from Wako Chemical Co. (Tokyo, Japan). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Extract preparation

We purchased individual dried herbs from Jungdo Herbal Drug Co. Ltd. (Seoul, Korea). DJS consisted of 10 g Paeoniae Radix (*Paeonia lactiflora* Pallas, Paeoniaceae), 6 g Cnidii Rhizoma (*Cnidium officinale* Makino, Umbelliferae), 6 g Alismatis Rhizoma (*Alisma orientale* Juzepezuk, Alismataceae), 3 g Angelicae Gigantis Radix (*Angelica gigas* Nakai, Umbelliferae), 3 g Poria (*Poria cocos* Wolf, Polyporaceae), and 3 g Atractylodis Rhizoma Alba (*Atractylodes macrocephala* Koidzumi, Compositae). In total, 31 g of mixed dried herbs was boiled with 310 mL of distilled water for 2 h at 100 °C based on the way of decocting DJS in the clinical use. The suspension was filtered, lyophilized, yielding 20.16% of powder, and kept at −20 °C. Before each experiment, the extract was dissolved in serum-free media. DJS was standardized based on the contents of albiflorin and paeoniflorin, the major and active components of DJS (Hu et al., 2010) using reverse-phase HPLC (Nanospace SI-2 series system; Shiseido Co., Ltd., Tokyo, Japan) equipped with UV detector. Separation was carried out using a Kinetex™ 2.6 μm Core-Shell C18 column (100 mm × 2.0 mm; Phenomenex, Inc., Torrance, CA) at 30 °C. The mobile phase was 0.01% phosphoric acid in water containing 10% acetonitrile at a flow rate of 0.2 mL/min. The detector wavelength was set at 230 nm. The injection volume was 10 μL. DJS and reference to the calibration curve obtained with albiflorin and paeoniflorin were analyzed in triplicates. Albiflorin and paeoniflorin were found in DJS at a mean level of 3.45 ± 0.02 mg/g and 13.10 ± 0.08 mg/g, respectively.

2.3. SH-SY5Y culture and treatment

Human neuroblastoma SH-SY5Y cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM containing 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator under 5% CO_2 at 37 °C. All experiments were carried out 48 h after cells had been seeded in 96-well plates (2×10^5 cells/mL) and 60-mm dishes (1.5×10^6 cells/dish). At approximately 80% confluency, cells were treated for 24 h with DJS (0.01–5 μg/mL) with or without 150 μM 6-OHDA for the last 6 h.

2.4. MTT assay

We measured cell viability using a MTT assay. After the treatment of DJS with or without 6-OHDA, the supernatants removed and 1 mg/mL of MTT was added to the cells for 3 h. MTT medium was carefully removed and the formazan product subsequently dissolved in DMSO. Optical density at 570 nm was determined using a spectrophotometer (Versamax microplate reader; Molecular Devices, Sunnyvale, CA).

2.5. Intracellular ROS generation

Treated cells were incubated with 10 μM H_2DCF-DA, a fluorescent probe for intracellular ROS generation, at 37 °C for 30 min as previously described (Kim et al., 2010). After washing with PBS, we measured the fluorescence intensity of DCF at an excitation wavelength of 495 nm and emission wavelength of 530 nm using a fluorescence microplate reader (SpectraMax Gemini EM; Molecular Devices).

2.6. Measurement of total glutathione

We detected total GSH levels using the Total Glutathione Quantification kit according to the instruction manual (Dojindo Molecular Tech., Tokyo, Japan). Treated cells were lysed and treated with 5% 5-sulfosalicyclic acid. A co-enzyme working solution, buffer solution, and enzyme working solution were added to each well at 37 °C for 5 min. Then, a GSH standard solution, sample solution, and substrate working solution were added for 10 min each. Absorbance was measured using a spectrophotometer at a wavelength of 405 nm, and concentrations of GSH were determined in the sample solution using a GSH standard curve.

2.7. Measurement of $\Delta\Psi m$

We used the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1; Stratagene, La Jolla, CA) to measure mitochondrial membrane potential. Cells were incubated with JC-1 at 37 °C for 15 min after treatment. Cells were washed and transferred to 96-well plates. The ratio of red (585/590 nm) and green (510/527 nm) fluorescence was determined using a fluorescence plate reader.

2.8. Measurement of caspase-3 activity

We assessed caspase-3 activity using a fluorometric assay kit (BioVision, Inc., Mountain View, CA) according to the manufacturer's instructions. Treated cells were lysed in 50 μL chilled cell lysis buffer on ice for 15 min and centrifuged (14,000 × *g*, 4 min, 4 °C). The supernatant was incubated with a fluorometric substrate 7 amino-4-trifluoromethyl coumarin (DEVD-AFC) and fluorescence was measured with excitation and emission wavelengths of 400 nm and 505 nm, respectively.

2.9. Western blotting

Western blotting was used to determine cleaved caspase-3 and cytochrome *c* expression levels. Cell lysates were separated by 15% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically onto membranes. Membranes were incubated with 5% skim milk in TBST for 1 h. They were then incubated with rabbit anti-cleaved caspase-3 primary antibody (1:1000 dilution) or mouse anti-cytochrome *c* primary antibody (1:500 dilution) at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibodies for 1 h. Antibody detection was carried out using an

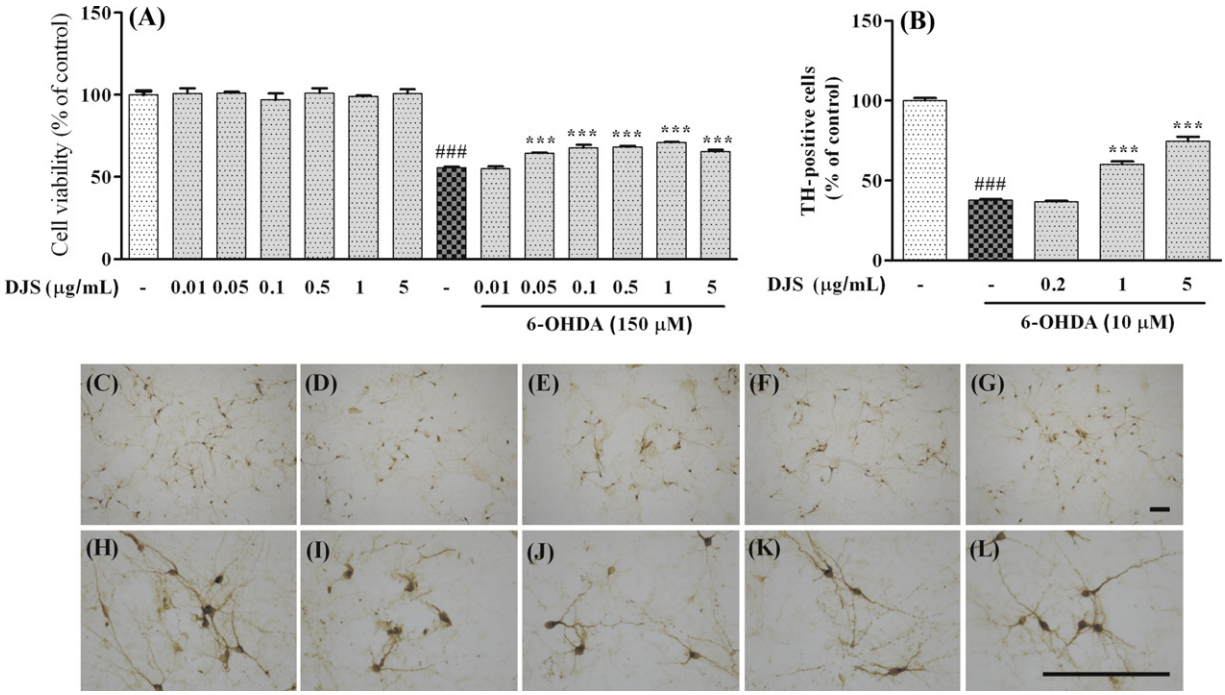

Fig. 1. Neuroprotective effects of DJS in SH-SY5Y and mesencephalic DA cells. Cell viability of DJS with or without 6-OHDA neurotoxicity was determined by MTT assay (A). The numbers of TH-positive cells were counted (B) and representative photomicrographs are shown (C–L): (C and H) control groups; (D and I) 6-OHDA only treated groups; (E and J) 6-OHDA + DJS 0.2 μg/mL; (F and K) 6-OHDA + DJS 1 μg/mL and (G and L) 6-OHDA + DJS 5 μg/mL. Scale bar = 100 μm. Each column represents the mean ± SEM of four replicates. Data are expressed as percentages relative to the untreated controls. $^{###}P < 0.001$ compared with the control group; $^{***}P < 0.001$ compared with the 6-OHDA-only treated group.

ECL detection kit and visualized using the LAS-4000 mini system (Fujifilm Corp., Tokyo, Japan).

2.10. Primary culture of DA cells and TH immunohistochemistry

Primary rat dopaminergic cell cultures were performed as previously described (Kim et al., 2010). Rat mesencephalons were obtained from Sprague–Dawley rat embryos at gestational day 14 (Orient Bio, Osan, Korea). Mesencephalons were dissected, collected, dissociated, and plated in 24-well plates with coverslips pre-coated with poly-L lysine. On the sixth day *in vitro*, the medium was changed to serum-free MEM and cells were treated with DJS at 0.5 or 1 μg/mL for 6 h, followed by treatment with or without 10 μM 6-OHDA for a further 18 h. Treated cells were fixed with 4.0% paraformaldehyde for 30 min, followed by treatment with H_2O_2 for 15 min. They were incubated overnight with an anti-TH antibody (1:2000 dilution) and for 60 min with biotinylated secondary antibody followed by avidin–biotin peroxidase complex solution for 60 min. Peroxidase activity was visualized with 3,3-diaminobenzidine for 5 min. After every incubation step, cover slips were washed three times with PBS. Coverslips were mounted onto gelatin-coated glass slides, air-dried, and photographed under a microscope (Axioskop 2; Carl Zeiss, Inc., Göttingen, Germany). To count visual TH-positive cells, four wells of each treatment group were selected and nine representative areas per well were counted at 100× magnification.

2.11. Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by least significant differences (LSD). We used SPSS 12.0K for Windows (SPSS Inc., Chicago, IL). Significance was accepted at $P < 0.05$.

3. Results and discussion

DJS is a widely used traditional herbal formula with neurological actions. In this study, we evaluated the protective effects of DJS on 6-OHDA-induced neurotoxicity. We initially examined the effects of DJS on viability of SH-SY5Y cells damaged by 6-OHDA using a MTT assay, which has been widely used in assessing neurotoxicity (Datki et al., 2003). Treatment with DJS alone at 0.01–5 μg/mL did not affect cell viability (Fig. 1A). Treatment with 150 μM 6-OHDA significantly decreased cell viability, by 55.7 ± 0.5%, versus the control. These reductions were significantly prevented by pretreatment with DJS at 0.05–5 μg/mL and the maximal effect was shown at 1 μg/mL (Fig. 1A). These results demonstrate that DJS exerts neuroprotective effects on SH-SY5Y cells following oxidative stress induced by 6-OHDA. We confirmed these results in primary mesencephalic DA cells. We determined that 10 μM 6-OHDA significantly reduced the number of TH-immunopositive cells, to 37.6 ± 0.6% of the control and morphologically altered these cells, causing shrinkage of cell bodies and withering of neurites. Treatment with DJS at 1 and 5 μg/mL protected DA cells to 60.2 ± 1.7% and 74.6 ± 2.9% of the control, respectively (Fig. 1B) and preserved the neuronal morphology of DA cells (Fig. 1C–L). These results demonstrate that DJS protects DA cells against 6-OHDA toxicity.

To explore the neuroprotective mechanism(s), we assessed the anti-oxidant and anti-mitochondria-mediated apoptotic effects of DJS in SH-SY5Y cells. Previous studies have determined that treatment with 6-OHDA leads to neuronal death by production of ROS and by affecting mitochondria (Saito et al., 2007). We measured ROS generation and total GSH level after treatment of 6-OHDA with or without DJS as a measure of oxidative stress. It is known that ROS can damage cell structures (Prokai et al., 2005) and GSH plays an important role in neuroprotection against oxidative stress in damaged DA neurons (Leret et al., 2002). Pre-treatment with DJS at 0.1, 0.5, and 1 μg/mL significantly reduced ROS production by 6-OHDA

(Fig. 2A). DJS at 0.5 and 1 μg/mL also inhibited total GSH decreases induced by 6-OHDA (Fig. 2B). In the present study, even though the inhibition effects of DJS on ROS production was not consistent with the effect on total GSH level, these results demonstrate that effects of DJS on protection from 6-OHDA are due to anti-oxidant actions.

It is generally accepted that cell apoptosis induced by 6-OHDA is mediated by mitochondrial dysfunction, characterized by changes in $\Delta\Psi m$, cytochrome *c* release, and caspase-3 (Liu et al., 2009), which we measured in this study. The effects of DJS on $\Delta\Psi m$ were evaluated using JC-1 solution where the $\Delta\Psi m$ is indicated by a decrease in the red/green fluorescence intensity ratio. Exposure to 6-OHDA considerably reduced the mean fluorescent intensity, to 59.6 ± 0.6% of the control. In contrast, pretreatment with DJS at 0.5 and 1 μg/mL significantly enhanced the mean fluorescent intensity, to 81.9 ± 3.1% and 92.1 ± 3.0%, respectively (Fig. 3A). This was coupled with an increase in cytochrome *c* release in SH-SY5Y cells treated with 6-OHDA that was inhibited by DJS treatment (Fig. 3B). Exposure to 150 μM 6-OHDA also enhanced the activity of caspase-3, to 223.7 ± 13.7% of control values. Treatment with DJS at 0.1, 0.5, and 1 μg/mL dose-dependently inhibited caspase-3 activity, to 179.7 ± 3.0%, 177.1 ± 1.6%, and 158.8 ± 6.6% of the control value (Fig. 3C); these results were confirmed by Western blotting analysis (Fig. 3D). These results indicated that DJS protected the cells by inhibiting the activation of caspase-3. Caspase-3 is the final executor of apoptotic DNA damage, so its activation is widely used as a marker of apoptosis (Ji and Gao, 2008). Our results were consistent with those of previous studies suggesting an inhibitory effect of DJS against mitochondria-mediated apoptosis (Qian et al., 2008). Furthermore we experimentally demonstrated that DJS protected against the neurotoxicity of 6-OHDA and this effect was related to its anti-oxidant and anti-mitochondria-mediated apoptotic activities.

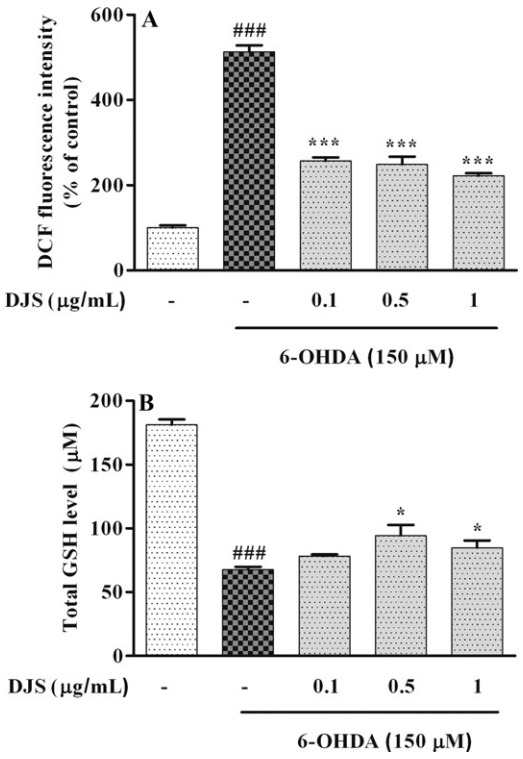

Fig. 2. Anti-oxidant effects of DJS in SH-SY5Y cells against 6-OHDA neurotoxicity. The ROS and GSH levels were measured using the fluorescence dye of DCF (A) and DTNB (B), respectively. Each column represents the mean ± SEM of four replicates. Data are expressed as percentages relative to untreated controls. $^{###}P < 0.001$ compared with the control group; $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the 6-OHDA-only treated group.

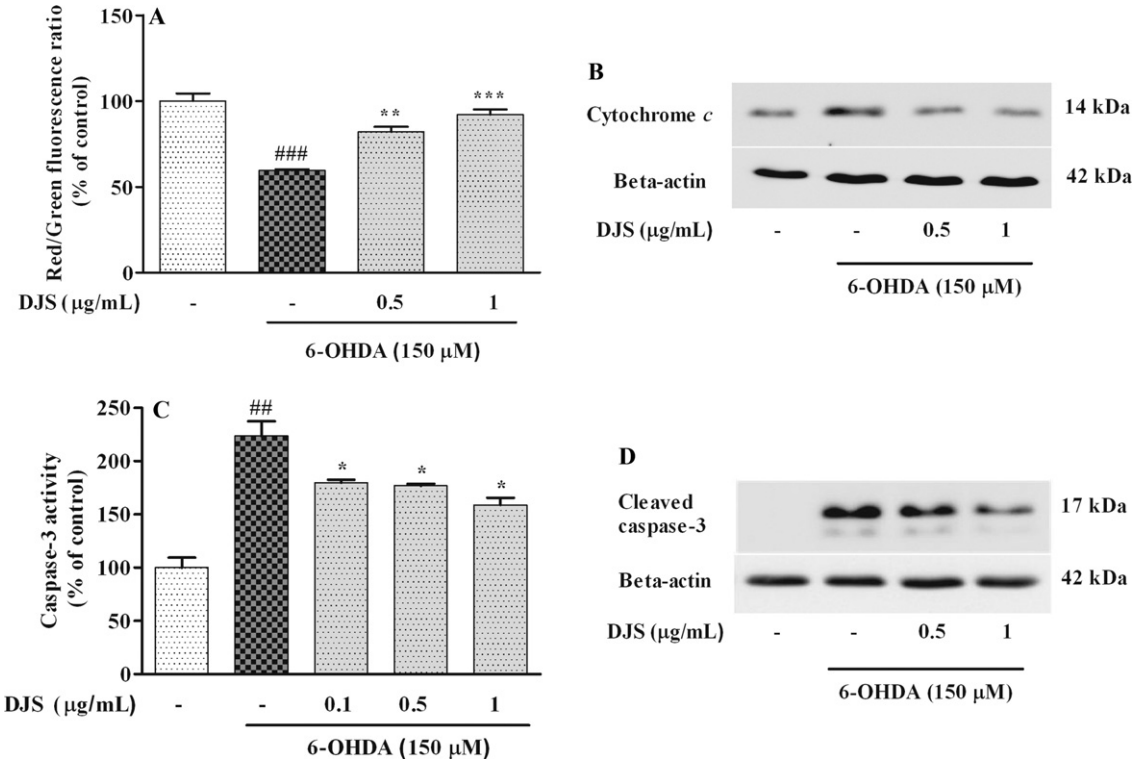

Fig. 3. Anti-mitochondria-mediated apoptotic effects of DJS in SH-SY5Y cells against 6-OHDA neurotoxicity. $\Delta\Psi m$ was measured as the ratio of red/green fluorescence intensity using JC-1 reagent (A). Caspase-3 activity was determined by fluorescence substrate, DEVD-AFC (C). Western blotting was performed to measure cytochrome *c* release (B) and cleaved caspase-3 expression (D). Each column represents the mean ± SEM of four replicates. Data are expressed as percentages relative to untreated controls. $^{###}P < 0.001$ compared to the control group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the 6-OHDA-only treated group.

Increased attention has been directed towards ethnic medicinal herbal extracts that have neuroprotective effects, because the various active entities and valuable combinations of herbal extracts have multimodal activities (Mandel et al., 2005; Weeks and Pere, 2006; Weeks, 2009). For example, Bak Foong Pill (Jia et al., 2005), Toki-to (Sakai et al., 2007), Cistanchis Herba (Zhao et al., 2010), Uncariae Ramulus et Uncus (Shim et al., 2009), Cyperi Rhizoma (Lee et al., 2010), Cassiae Semen (Ju et al., 2010), and Mori Fructus (Kim et al., 2010) have been reported to inhibit degeneration of DA neurons and attenuate symptoms caused by neurotoxins, such as 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine *in vitro* and/or *in vivo* models. In this study, we demonstrated that DJS also protected DA neurons *in vitro* from 6-OHDA toxicity. The representative chemical components of DJS are monoterpene glycosides, phenolic compounds, and phthalides, which exhibit anti-inflammation and anti-oxidant activities (Chen et al., 2009). Especially, albiflorin and paeoniflorin in Paeoniae Radix (Kim et al., 2009), atractylenolides in Atractylodis Rhizoma Alba (Lin et al., 2009), and ligustilide in Cnidii Rhizoma and Angelicae Gigantis Radix (Kuang et al., 2006) are known to have multiple bioactivities in neuro-associated diseases. Thus, they may be active components involved in protective effects against oxidative stress, mitochondrial dysfunction, apoptosis, and neuroinflammation.

In conclusion, DJS inhibits neuronal damage induced by 6-OHDA through its anti-oxidant and anti-mitochondria-mediated apoptotic actions in SH-SY5Y cells and primary DA cells. These results suggest that DJS could be a useful neuroprotective candidate for further *in vivo* studies.

Conflict of interest

The authors declare that there is no conflict of interest

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