

Neuroprotective Effects of Herbal Butanol Extracts from *Gynostemma pentaphyllum* on the Exposure to Chronic Stress in a 6-Hydroxydopamine-Lesioned Rat Model of Parkinson's Disease Treated with or Without L-DOPA

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1. Introduction

Parkinson's disease (PD), which is characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta, is accompanied by symptoms of muscular rigidity, bradykinesia, rest tremor, and loss of postural balance (Fearnley & Lees, 1991). Mitochondrial dysfunction by reactive oxygen species (ROS)-induced oxidative stress has also been suggested to be important in the loss of dopaminergic neurons in PD (Ozawa et al., 1990). Therefore, the degeneration of the dopaminergic nigrostriatal tracts in PD results in a corresponding decrease in the levels of dopamine and its metabolites, including 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and norepinephrine (Hornykiewicz, 1982).

3,4-Dihydroxyphenylalanine (L-DOPA), the precursor of dopamine, is the most prescribed therapy for the symptomatic relief of PD (Neil & David, 2008; Marsden, 1994). However, chronic prolonged therapy for PD with L-DOPA results in a loss of drug efficacy and irreversible adverse effects, and subsequently leads to the development of motor complications, such as fluctuation and dyskinesia (Jankovic, 2005). L-DOPA and dopamine can accelerate the degenerative process in the residual cells in patients with PD and induce oxidative stress-induced neurotoxicity by generating ROS in primary dopaminergic neurons and dopaminergic cell lines (Cheng et al., 1996). ROS generation leads to neuronal damage and apoptotic or non-apoptotic cell death (Walkinshaw & Waters, 1995). Dopaminergic neurons are in a perpetual state of oxidative stress, and this imbalance may lead to reduced levels of endogenous antioxidants (Merad-Boudia, 1998). In addition, chronic treatment with L-DOPA

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leads to the production of a specific dopaminergic neurotoxin, 6-hydroxydopamine (6-OHDA), in the striatum of rodents (Borah & Mohanakumar, 2010). These results fuel the search for new agents for PD that are anti-oxidative substances or non-dopaminergic alternatives that can relieve the L-DOPA-induced cytotoxicity.

Various stressful stimuli can induce the production of many ROS and activate both the sympathetic nervous system and the hypothalamic-pituitary-adrenal-axis (Ganong, 2001), which increases the release of dopamine, norepinephrine, epinephrine, glucocorticoids, glutamate, and corticotropin releasing factor in the brain and peripheral circulation (Kandel et al., 2000). Chronic stress-induced adverse reactions are increased in neurodegenerative diseases, including anxiety disorders, depression, schizophrenia, stroke, Alzheimer's disease, and PD (Amanda et al., 2002). For example, the tremor in PD may be worsened by anxiety or anger (Schwab & Zieoer, 1965). In addition, a decrease in dopamine levels and an enhancement of dopamine turnover have been observed in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice after immersion immobilization stress, resulting in the mice being remarkably akinetic (Urakami et al., 1988). Repeated or relatively prolonged exposure to stress can also change central dopamine biosynthesis and extracellular dopamine levels in rat models (Ahmed et al., 1955), and changes in the cellular characteristics in the prefrontal cortex, such as dendritic atrophy and neuronal loss, have been found in response to stress (Rajkowska, 2000).

The stereotaxic injection of 6-OHDA into the substantia nigra, medial forebrain bundle, and striatum of the brain has been commonly used to produce experimental animal models of PD. These injections selectively injure dopaminergic neurons through the formation of various ROS (Perese et al., 1989). In addition, anti-oxidants, such as glutathione, catalase, and N-acetylcysteine, have been shown to be protective against 6-OHDA-induced cytotoxicity in PC12 and dopaminergic cells (Przedborski et al., 1995; Paxinos & Watson, 1986).

Gynostemma pentaphyllum (Cucurbitaceae; GP) is usually used as an herbal tea, and it is widely believed to result in various protective and functional improvements in diabetes, depression, anxiety, fatigue, hyperlipidemia, immunity, oxidative stress, and tumors (Razmovski-Naumovski et al., 2001). The major constituents of GP, which have been isolated, are a number of gypenoside derivatives (Razmovski-Naumovski et al., 2001). The gypenoside-rich fraction shows neuroprotective effects in the MPTP-induced mouse model of PD (Wang et al., 2010). The ethanol extract from GP has been found to have anti-stress and immunomodulatory functions in mice (Choi et al., 2008; Im et al., 2009). GP ethanol extract also exhibits protective effects against neurotoxicity by reducing tyrosine hydroxylase (TH) neuronal cell death and by normalizing dopamine levels in the 6-OHDA-lesioned rat model of PD (Choi et al., 2010). These results suggest that GP may function as a potential therapeutic and antioxidant in PD. The ethanol extract of GP was partitioned to obtain the butanol extract (GP-BX). GP-BX has been shown to have gypenoside-rich components, which were identified as gypenoside derivatives, and these include gynosaponin TN-1, gynosaponin TN-2, gypenoside XLV, and gypenoside LXXIV (Choi et al., 2010; Razmovski-Naumovski et al., 2005; Nagai et al., 1981; Takemoto et al., 1984; Yoshikawa et al., 1987).

The purpose of the present study was to investigate whether orally administered GP-BX obtained from the leaves of GP had protective effects against chronic stress in the 6-OHDA-

lesioned rat model of PD with or without long-term L-DOPA treatment. Dopaminergic neuronal cell death induced by chronic stress in 6-OHDA-lesioned rats was blocked by the coadministration of GP-BX, and this was shown by histochemical (the number of surviving TH-immunopositive neuronal cells) and neurochemical (dopamine, DOPAC, HVA, and norepinephrine levels) techniques.

2. Experimental methods

2.1 Chemicals

L-DOPA, 6-OHDA, dopamine, norepinephrine, DOPAC, HVA, benserazide hydrochloride, apomorphine, and L-ascorbic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). TH antibody was obtained from Millipore (Temecula, CA, USA). Anti-mouse IgG, Vectastain diaminobenzidine (DAB), and avidin/biotin complex (ABC) kits were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). All other chemicals were of analytical grade.

2.2 Preparation of GP-BX

GP was obtained from Geochang (Gyungnam, Korea), and a voucher specimen of the herbal leaves of GP was deposited at the herbarium of the College of Pharmacy, Chungbuk National University (Cheongju, Korea). The air-dried leaves of GP (10 kg) were extracted with ethanol (80%, v/v), and then the ethanol extracts were evaporated to dryness (GP ethanol extract, 1.05 kg; yield, 10.5%, w/w). The dry GP ethanol extracts (1 kg) were suspended in water and portioned subsequently with n-hexane, ethylacetate, and n-butanol. The final butanol extracts were evaporated to dryness under reduced pressure and temperature (GP-BX, 155 g; yield, 15.5%, w/w).

2.3 Animals

Rats (Sprague-Dawley, male, 200–250 g) were purchased from Samtako Co. (Animal Breeding Center, Osan, Korea). Animals were housed two per cage in a temperature-controlled environment with a 12-h light/dark cycle (lights on at 07:00) and with *ad libitum* access to standard rat food and water. All procedures were performed according to the guidelines of the Animal Ethics Committee of College of Pharmacy (Chungbuk National University).

2.4 Preparation of 6-OHDA-lesioned rats

The rats were anesthetized intraperitoneally with Zoletil 50 (100 mg/kg, Virbac, Carros, France) and placed in a stereotaxic stand (David Kopf Instruments, Tujunga, CA, USA). The coordinates for the striatum were measured accurately (antero-posterior, AP: -5.3 mm; lateral, ML: +1.9 mm; dorso-ventral, DV: -7.5 mm; relative to bregma). Next, 6-OHDA (8 µg/2 µL in saline solution containing 0.1% of L-ascorbic acid) was injected into the left substantia nigra pars compacta at 1 µL/min using a Hamilton syringe. After the injection, the needle was left in place for 5 min before being retracted in order to allow for complete diffusion of the medium. The rats were left until they had recovered from the anesthesia. Two weeks after the surgery, rats were challenged with apomorphine (0.5 mg/kg, s.c.), and the contralateral rotation was monitored. Rats showing fewer than 150 rotations per 30 min were excluded from further studies.

2.5 The exposure to chronic stress

Two weeks after the 6-OHDA lesions, the rats were placed individually in the electrified shock chamber for the exposure to chronic stress, and they received unavoidable electric footshock (EF) (intensity, 0.2 mA; duration, 10 s; interval, 10 min) at 14:00 every other day for 28 days using a shock generator (Seil Electric Co., Taejeon, Korea).

2.6 Drug treatment

Rats were divided into four groups with each group containing 7–10 rats. GP-BX (30 mg/kg), which was freshly prepared every day with water, was administered to 6-OHDA-lesioned rats orally (p.o.) once a day for 28 days. L-DOPA (10 mg/kg, i.p.) was treated with benserazide (15 mg/kg, i.p.) prepared in saline in order to prevent the peripheral decarboxylation of L-DOPA. The rats were sacrificed the day after the last exposure to stress and GP-BX administration. The experimental design was described as follows.

Experiment I:

Group I (normal rat groups): received 3 μ L of saline containing 0.1% L-ascorbic acid by stereotaxic injection into the substantia nigra.

Group II (6-OHDA-lesioned rat groups): received 6-OHDA (8 μ g/2 μ L in saline solution containing 0.1% of L-ascorbic acid) by stereotaxic injection into the left substantia nigra.

Group III (6-OHDA-lesioned rat groups + chronic EF stress): exposed to EF stress for 28 days two weeks after receiving 6-OHDA (8 μ g/2 μ L).

Group IV (6-OHDA-lesioned rat groups + chronic EF stress + GP-BX): administered GP-BX (30 mg/kg) for 28 days to EF stress-exposed 6-OHDA-lesioned rat groups (Group III).

Experiment II:

Group I (L-DOPA-treated 6-OHDA-lesioned rat groups): treated with L-DOPA (10 mg/kg) for 28 days two weeks after receiving 6-OHDA (8 μ g/2 μ L).

Group II (L-DOPA-treated 6-OHDA-lesioned rat groups + chronic EF stress): exposed to EF stress for 28 days in L-DOPA (10 mg/kg)-treated 6-OHDA-lesioned rat groups (Group I).

Group III (L-DOPA-treated 6-OHDA-lesioned rat groups + GP-BX): administered GP-BX (30 mg/kg) for 28 days in L-DOPA-treated 6-OHDA-lesioned groups (Group I).

Group IV (L-DOPA-treated 6-OHDA-lesioned rat groups + chronic EF stress + GP-BX): administered GP-BX (30 mg/kg) for 28 days in L-DOPA-treated 6-OHDA-lesioned groups exposed to chronic EF stress (Group II).

2.7 TH-immunohistochemistry staining

For the immunohistochemical study, the rats were sacrificed 28 days after 6-OHDA lesioning and then perfused intracardially with saline, which was followed by 4% paraformaldehyde of the fixative solution. The brain was removed from the skull and placed in 30% sucrose solution. Sections of 35- μ m thickness were cut with a Vibratome (Leica Microsystems GmbH, Wetzlar, Germany). The tissue sections were incubated with primary anti-TH antibody raised in rabbits and diluted in PBS containing 0.3% Triton X-100 (1:200, AB152, Millipore) overnight at 4°C. A 1:250 dilution of biotinylated anti-rabbit IgG was used as a secondary antibody, and the sections were then incubated with an ABC kit. TH immunoreactivity was visualized using a DAB kit (Vector Laboratories, Inc.). Photomicrographs of TH and digitized bright-field images were captured using a Zeiss Axiophot microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) (100X

magnification). Cell counting was done using a computerized image analysis system (Axiovision software, Carl Zeiss MicroImaging GmbH). Analysis values obtained on the ipsilateral side (6-OHDA-lesioned side) were expressed as a percentage of those on the intact contralateral side (intact side).

2.8 Biochemical analysis

The brains were removed quickly, and the striatum was dissected in cold conditions. The samples were homogenized in 300 μ L HClO₄. The homogenates were immediately centrifuged at 50,000 \times g at 4°C for 20 min, and then, the supernatants were filtered using pore filters (0.45 μ m). The levels of dopamine, DOPAC, HVA, and norepinephrine in the striatum were measured with a high-performance liquid chromatography (HPLC) system. The HPLC system consisted of a solvent delivery pump (Model 1525, Waters, Milford, MA, USA), an electrochemical detector (+0.85 V, Ag/AgCl reference electrode; Model 2465; Waters), and a Waters 120 ODS-BP column (5 μ m, 50 \times 4.6 mm). The mobile phase consisted of 10 mM citric acid, 0.13 mM Na₄EDTA, 0.58 mM SOS, and 10% methanol, and a flow rate of 1 mL/min. The results were expressed in terms of ng/g tissue.

2.9 Statistical analysis

All data were expressed as means \pm S.E.M. Data were analyzed with an one-way analysis of variance (ANOVA) followed by a Tukey's test. P values <0.05 were considered statistically significant.

3. Results

3.1 TH-immunopositive neuronal cell survival in the substantia nigra of 6-OHDA-lesioned rats exposed to chronic EF stress and administered GP-BX

TH-immunopositive neuronal cell death by 6-OHDA lesions in the substantia nigra was ameliorated by the administration of GP-BX at 30 mg/kg (p.o.) for 28 days (Figure 1). TH-immunopositive neurons were observed consecutively in both the substantia nigra compacta and lateralis. TH-immunostained nerve fibers in the substantia nigra were tangled into a net, and the cells were either poly- or ovoid-shaped in the normal areas (Figure 1, A-I). The substantia nigra regions near the 6-OHDA-lesioned areas displayed drastic reductions in TH-immunopositive neuronal cells, and the staining intensity was decreased compared with the intact sides of the control rat groups (Figure 1, A-II). After exposure to chronic EF stress, TH-immunopositive neuronal cells were decreased in the substantia nigra of both the normal and the 6-OHDA-lesioned rats, even though the color was uneven, compared to the 6-OHDA-lesioned rat groups without chronic EF stress (Figure 1, A-II and III). However, the administration of GP-BX at 30 mg/kg (p.o.) for 28 days ameliorated the loss of TH-immunopositive neuronal cells induced by the exposure to chronic EF stress in both the intact and 6-OHDA-lesioned sides of 6-OHDA-lesioned rats (Figure 1, A-IV).

The number of TH-immunopositive neuronal cells on the ipsilateral sides (6-OHDA-lesioned sides) was analyzed as a percentage of those in the intact contralateral sides (intact sides) of 6-OHDA-lesioned rat groups. In the 6-OHDA-lesioned rat groups, 6-OHDA lesions caused a marked decrease in the number of TH-immunopositive neuronal cells in the intact and 6-OHDA-lesioned sides to 79.1% and 35.8%, respectively, compared to the normal rat groups (Figure 1, B-I and II). In addition, the exposure to chronic EF stress in the 6-OHDA-lesioned rat groups further decreased the number of TH-immunopositive neuronal cells in

the intact and 6-OHDA-lesioned sides to 45.9% and 19.9%, respectively, compared to the 6-OHDA-lesioned rat groups (Figure, B-II and III). However, in the 6-OHDA-lesioned rat groups exposed to chronic EF stress, GP-BX administration (30 mg/kg) for 28 days showed a protective effect on the loss of the number of TH-immunopositive neuronal cells in the intact and 6-OHDA-lesioned sides to 63.0% and 38.1%, respectively (Figure 1, B-III and IV).

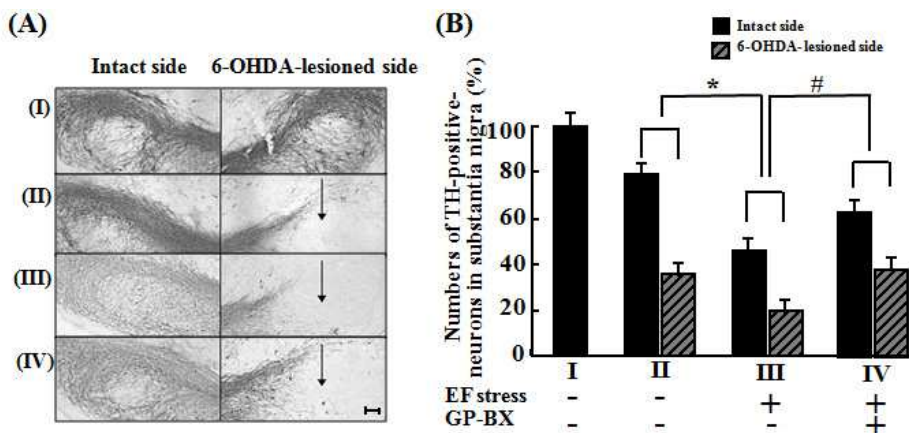


Fig. 1. Photomicrographs of tyrosine hydroxylase (TH) immunoreactivity on substantia nigra tissue sections from representative rats of each group (A), and the number of surviving TH-immunopositive neuronal cells in the ipsilateral substantia nigra [6-hydroxydopamine (6-OHDA)-lesioned side] was analyzed as a percentage of that in the intact contralateral side (intact side) (B). Normal rat groups (I), 6-OHDA-lesioned rat groups (II), 6-OHDA-lesioned rat groups + chronic electric foot (EF) stress (III), and 6-OHDA-lesioned rat groups + chronic EF stress + *Gynostemma pentaphyllum*-butanol extract (GP-BX) (IV). Rats were treated with GP-BX (30 mg/kg/day, p.o.) or vehicle (0.9% saline, p.o.) and then subjected to every-other-day sessions of EF stress (duration and interval of 10 s for 10 min, 2 mA). These data are representative of 7–10 animals per group, and the arrow indicates the 6-OHDA-lesioned side. TH-immunopositive neuronal cells were analyzed as a percentage of intact side. Scale bar is 100 μm . * $p < 0.05$ compared with 6-OHDA-lesioned rat groups; # $p < 0.05$ compared with 6-OHDA-lesioned rat groups + chronic EF stress (ANOVA followed by Tukey's test).

3.2 The levels of dopamine, DOPAC, HVA, and norepinephrine in the striatum of 6-OHDA-lesioned rats exposed to chronic EF-stress and administered GP-BX

The levels of dopamine, DOPAC, HVA, and norepinephrine in the striatum of GP-BX-administered normal rats (those without 6-OHDA lesions) were not altered compared to the GP-BX-untreated rat groups (data not shown). In addition, no differences were seen on the intact side of normal rats, 6-OHDA-lesioned rat groups, and 6-OHDA-lesioned rat groups administered GP-BX (30 mg/kg, 28 days).

A significant decrease in the levels of dopamine, DOPAC, HVA, and norepinephrine by 47.0%, 44.3%, 38.6%, and 40.5% in the 6-OHDA-lesioned sides of the 6-OHDA-lesioned rat groups, respectively, was observed (Figure 2, I and II). Chronic EF stress-exposed 6-OHDA-lesioned rat groups had a more marked decrease in the levels of dopamine, DOPAC, HVA, and norepinephrine to 71.7% and 28.2%, 66.9% and 28.3%, 61.0% and 25.3%, and 71.6% and

27.4%, respectively, in both the intact and 6-OHDA-lesioned sides, compared with 6-OHDA-lesioned rats without chronic EF stress (Figure 2, II and III). However, GP-BX administration (30 mg/kg) for 28 days resulted in an improvement in the reduced levels of dopamine, DOPAC, HVA, and norepinephrine by chronic EF stress to 84.6% and 47.8%, 79.7% and 47.9%, 72.8% and 46.0%, and 88.6% and 46.8%, respectively, in the intact and 6-OHDA-lesioned sides of the 6-OHDA-lesioned rat groups (Figure 2, III and IV).

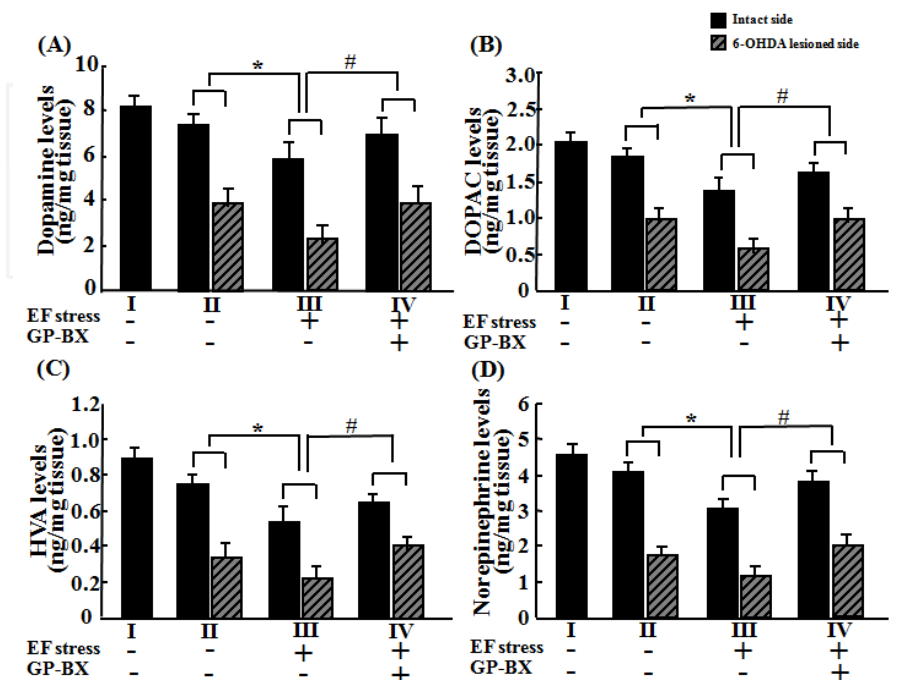


Fig. 2. Effects of GP-BX on the levels of dopamine (A), 3,4-dihydroxyphenylacetic acid (DOPAC; B), homovanillic acid (HVA; C), and norepinephrine (D) in the striatum of 6-OHDA-lesioned rats. Normal rat groups (I), 6-OHDA-lesioned rat groups (II), 6-OHDA-lesioned rat groups + chronic EF stress (III), and 6-OHDA-lesioned rat groups + chronic EF stress+ GP-BX (IV). Rats were treated with GP-BX (30 mg/kg/day, p.o.) or vehicle (0.9% saline, p.o.) and then subjected to every-other-day sessions of EF stress (duration and interval of 10 s for 10 min, 2 mA). After 4 weeks, the brains were removed, and the levels of dopamine, DOPAC, HVA, and norepinephrine were determined by a high-performance liquid chromatography (HPLC) method. Results represent means \pm S.E.M. for 7–10 animals per group. * $p < 0.05$ compared with 6-OHDA-lesioned rat groups; # $p < 0.05$ compared with 6-OHDA-lesioned rat groups + chronic EF stress (ANOVA followed by Tukey's test).

3.3 TH-immunopositive neuronal cell survival in L-DOPA-treated 6-OHDA-lesioned rats exposed to chronic EF stress and administered GP-BX

Treatment with L-DOPA (10 mg/kg) for 28 days in 6-OHDA-lesioned rats slightly increased the number of TH-immunopositive neuronal cells in the 6-OHDA-lesioned sides compared to the L-DOPA-untreated 6-OHDA-lesioned rats (Figure 1-II and 3-I), indicating that a low

dose of L-DOPA showed a protective and therapeutic activity. However, with exposure to chronic stress, the number of TH-immunopositive neuronal cells was significantly reduced in the substantia nigra in the L-DOPA-treated 6-OHDA-lesioned rat groups (Figure 3, A I and II). Furthermore, GP-BX administration (30 mg/kg) for 28 days protected against the loss of TH-immunopositive neuronal cells in L-DOPA-treated 6-OHDA-lesioned rat groups with or without chronic EF stress (Figure 3, A III and IV). Chronic EF stress induced the loss of a number of TH-immunopositive neuronal cells in both the intact and 6-OHDA-lesioned sides: the number of TH-immunopositive neuronal cells in the 6-OHDA-lesioned sides was decreased to 45.1% by the exposure to chronic EF stress in L-DOPA (10 mg/kg)-treated 6-OHDA-lesioned rat groups compared with those without chronic EF stress (Figure 3, A I and II). However, GP-BX administration (30 mg/kg) recovered the number of TH-immunopositive neuronal cells by 12.1% in the intact sides of L-DOPA-treated 6-OHDA-lesioned rats (Figure 3, B I and III) and also increased them by 18.6% and 36.7%, respectively, in the intact and 6-OHDA-lesioned sides of chronic EF stress-exposed 6-OHDA-lesioned rats compared with GP-BX-untreated groups (Figure 3, B II and IV).

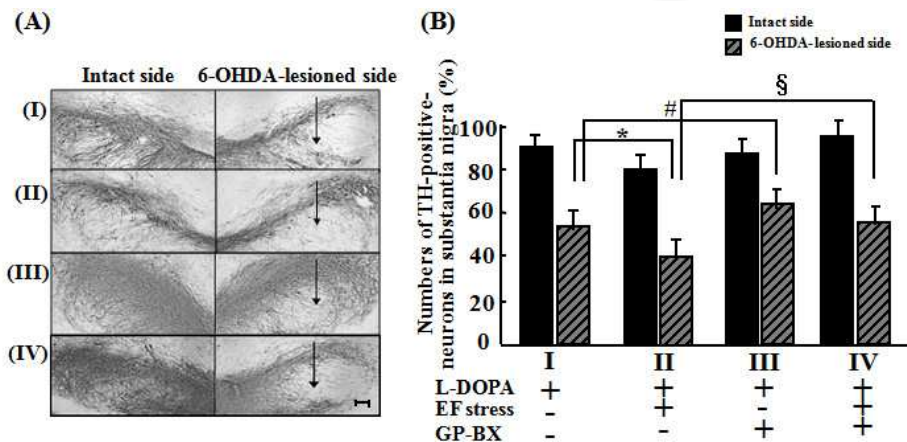


Fig. 3. Photomicrographs of TH immunoreactivity on substantia nigra tissue sections from representative rats of each group (A), and the number of surviving TH-immunopositive neuronal cells in the ipsilateral substantia nigra (6-OHDA-lesioned side) was analyzed as a percentage of that in the intact contralateral side (intact side) (B). L-DOPA-treated 6-OHDA-lesioned rat groups (I), L-DOPA-treated 6-OHDA-lesioned rat groups + chronic EF stress (II), L-DOPA-treated 6-OHDA-lesioned rat groups + GP-BX (III), and L-DOPA-treated 6-OHDA-lesioned rat groups + chronic EF stress + GP-BX (IV). Rats were treated with GP-BX (30 mg/kg/day, p.o.) or vehicle (0.9% saline, p.o.) and then subjected to every-other-day sessions of EF stress (duration and interval of 10 s for 10 min, 2 mA). L-DOPA (10 mg/kg/day, i.p.) was administered with benserazide (15 mg/kg/day, i.p.) prepared in saline. These data are representative of 7–10 animals per group, and the arrow indicates 6-OHDA-lesioned side. TH-immunopositive neuronal cells were analyzed as a percentage of intact side. Scale bar is 100 μ m. * $p < 0.05$ compared with L-DOPA-treated 6-OHDA-lesioned rat groups; # $p < 0.05$ compared with L-DOPA-treated 6-OHDA-lesioned rat groups; § $p < 0.05$ compared with L-DOPA-treated 6-OHDA-lesioned rat groups + chronic EF stress (ANOVA followed by Tukey's test).

3.4 The levels of dopamine, DOPAC, HVA, and norepinephrine in the striatum of L-DOPA-treated 6-OHDA-lesioned rats exposed to chronic EF stress and administered GP-BX

The levels of dopamine, DOPAC, HVA, and norepinephrine were slightly increased in the striatal regions of the 6-OHDA-lesioned rat groups treated with L-DOPA (10 mg/kg), compared with those of the L-DOPA-untreated groups (Figures 2 and 4), but they were still decreased by 6-OHDA lesions (Figure 4, A-D I). The exposure to chronic EF stress in the L-

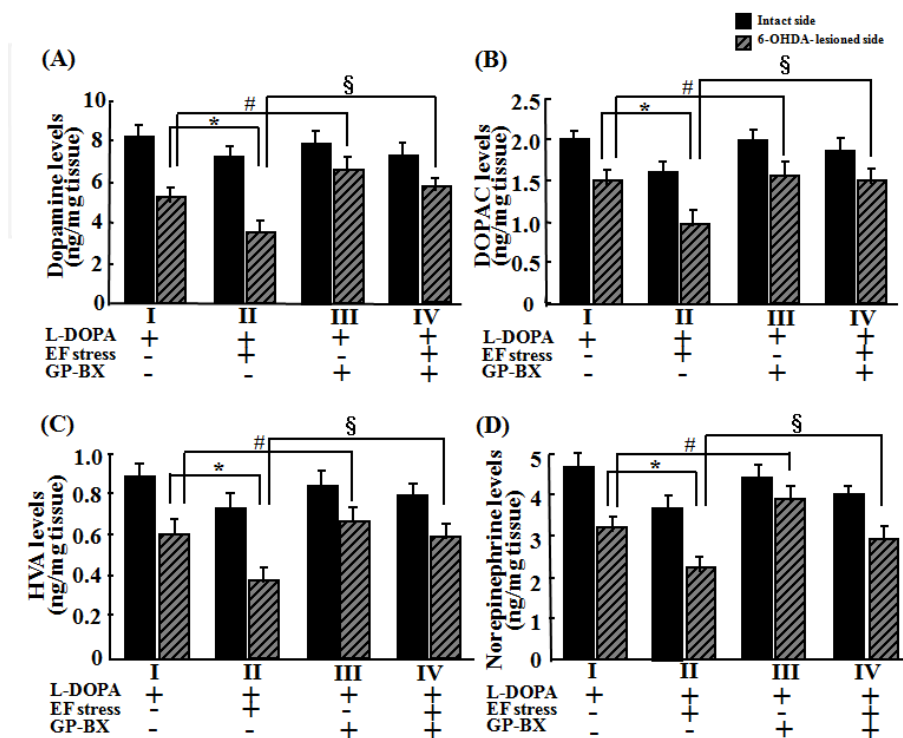


Fig. 4. Effects of GP-BX on the levels of dopamine (A), DOPAC (B), HVA (C), and norepinephrine (D) in the striatum of 6-OHDA-lesioned rats. L-DOPA-treated 6-OHDA-lesioned rat groups (I), L-DOPA-treated 6-OHDA-lesioned rat groups + chronic EF stress (II), L-DOPA-treated 6-OHDA-lesioned rat groups + GP-BX (III), and L-DOPA-treated 6-OHDA-lesioned rat groups + chronic EF stress + GP-BX (IV). Rats were treated with GP-BX (30 mg/kg/day, p.o.) or vehicle (0.9% saline, p.o.) and then subjected to every-other-day sessions of EF stress (duration and interval of 10 s for 10 min, 2 mA). L-DOPA (10 mg/kg/day, i.p.) was administered with benserazide (15 mg/kg/day, i.p.) prepared in saline. After 4 weeks, the brains were removed, and the levels of dopamine, DOPAC, HVA, and norepinephrine were determined by an HPLC method. Results represent means \pm S.E.M. for 7-10 animals per group. * $p < 0.05$ compared with L-DOPA-treated 6-OHDA-lesioned rat groups; # $p < 0.05$ compared with L-DOPA-treated 6-OHDA-lesioned rat groups; § $p < 0.05$ compared with L-DOPA-treated 6-OHDA-lesioned rat groups + chronic EF stress. (ANOVA followed by Tukey's test).

DOPA (10 mg/kg)-treated 6-OHDA-lesioned rat groups showed a further significant decrease in the levels of dopamine, DOPAC, HVA, and norepinephrine in the 6-OHDA-lesioned sides of the striatal regions by 43.0%, 47.9%, 43.1%, and 47.4%, respectively, compared with those of the unstressed groups (Figure 4, A-D I and II). This was significantly recovered by 30 mg/kg GP-BX administration for 28 days (Figure 4, A-D I and III). In addition, 30 mg/kg GP-BX administration for 28 days resulted in an improvement in the levels of dopamine, DOPAC, HVA, and norepinephrine to 62.5%, 54.5%, 55.1%, and 31.7%, respectively, in the L-DOPA (10 mg/kg)-treated 6-OHDA-lesioned rat groups (Figure 4, A-D I and IV).

4. Discussion

The neurotoxin 6-OHDA is commonly used for animal models of PD, and it is believed to cause dopaminergic cell death with a unilateral destruction of the nigrostriatal system (Schober, 2004). Among the various bioactive functions of GP, it has been known to have anti-oxidant, anti-inflammatory, and immunostimulatory actions (Razmovski-Naumovski et al., 2005). In addition, GP ethanol extract has been found to have an anti-stress function against chronic EF stress in mice (Choi et al., 2008; Im et al., 2009). In this study, the neuroprotective functions of GP-BX on the exposure to chronic EF stress in the 6-OHDA-lesioned rat model of PD with or without long-term L-DOPA treatment were investigated by determining the quantities of TH-immunopositive neuronal cells surviving in the substantia nigra and the levels of dopamine, DOPAC, HVA, and norepinephrine in the striatum.

GP ethanol extracts at doses of 10–50 mg/kg/day for 28 days did not show toxic effects, such as weight loss or death in rats (Choi et al., 2008; Im et al., 2009), and the water extract (750 mg/kg) of GP also did not produce any significant toxic effects in rats during a 6-month period of treatment (Attawish et al., 2004). GP-BX (30 mg) was selected in this study, and its administration for 28 days did not exhibit adverse effects, such as weight loss, diarrhea, vomiting, or death.

The infusion of 6-OHDA into the CNS resulted in decreased rotational movements, including stereotypic behavior, by the change of monoamine contents (Deumens et al., 2002), which was recovered by GP-BX administration (data not shown). These findings suggest that GP-BX showed a preventive activity against 6-OHDA-lesioned rats.

The number of TH-immunopositive surviving cells showed a large decrease in the ventrolateral side of the substantia nigra (intact side), and their numbers were well maintained in the ventral tegmental area (VTA) of the ventral side (intact side). These findings were very similar to the pathological findings of PD. However, the number of TH-immunopositive neuronal cells in the VTA decreased slightly due to the passage of time with 6-OHDA (Figure 1, A I and II). The chronic exposure to EF stress every other day for 28 days enhanced the 6-OHDA-induced dopaminergic neuronal cell death in the 6-OHDA-lesioned rat groups used as a PD model system (Figures 1 and 2). The chronic EF stress also inhibited the therapeutic effects of L-DOPA (10 mg/kg) in the 6-OHDA-lesioned rats (Figures 3 and 4). However, GP-BX administration (30 mg/kg) for 28 days ameliorated the enhanced neurotoxic effects induced by the exposure to chronic EF stress in 6-OHDA-lesioned rats with or without L-DOPA: the number of surviving TH-immunopositive

neuronal cells in the substantia nigra and the levels of dopamine, DOPAC, HVA, and norepinephrine in the striatum were recovered by GP-BX. In addition, GP-BX inhibited 6-OHDA-induced neurotoxicity in the brain regions of normal rats and 6-OHDA-lesioned rats (data not shown), which was similar to the findings with GP ethanol extract (Choi et al., 2010). These results indicate that oral administration of GP-BX exhibited a preventive and protective activity against the chronic EF stress- and/or 6-OHDA-induced dopaminergic neuronal cell death in rats.

Stressful stimuli induced the production of ROS and increased the release of catecholamines and glucocorticoids (Ganong, 2001; Kandel et al., 2000), which reduced the function of immune systems (Im et al., 2009). Immobilized stress inhibited the neuroprotective effects of free-running wheel exercise in a rat model of PD (Urakami et al., 1988). The exposure to chronically repetitive stress also reduced dopamine levels in the rat brain, leading to decreased ambulatory activity (Ahmed et al., 1995; Rajkowska, 2000). ROS, which are generated by 6-OHDA by autoxidation, directly destroyed DNA, essential proteins, and cell lipid membranes to cause necrosis (Schober, 2004). In addition, 6-OHDA was detected in rat brain after L-DOPA treatment due to the high levels of dopamine and hydrogen peroxide (Maharaj et al., 2005), which induced dopaminergic neuronal cell death by inflammatory processes and oxidative apoptosis (Blum et al., 2004). L-DOPA treatment in MPTP-induced PD rodents increased the striatal 6-OHDA levels, which may be sensitized by monoamine oxidase inhibitor (Borah & Mohanakumar, 2010). Long-term treatment with L-DOPA caused disabling motor side effects in PD and alleviated oxidative stress-induced neurotoxicity by ROS formation against striatal dopaminergic neurons and PC12 cells (Basma et al., 1995; Walkinshaw & Waters, 1995; Migheli et al., 1999). Subchronic or chronic L-DOPA treatment resulted in increased levels of dopamine and hydroxyl-free radicals in the striatum (Pandey et al., 2009). L-DOPA also showed treatment dose-dependent dual functions, including protection and neurotoxicity, in the 6-OHDA-lesioned rat model of PD (Cenci, 2009). In addition, L-DOPA at low concentrations (3–10 μ M) produced trophic or cell-protective effects on neuronal and differentiated PC12 cells (Mena et al., 1997). In this study, L-DOPA treatment of 10 mg/kg for 28 days showed a slightly protective effect by increasing TH-immunopositive surviving cells in 6-OHDA-lesioned rats. However, the TH-immunopositive surviving cells were decreased by chronic EF stress (Figures 1 and 3), suggesting that the function of L-DOPA in rat model of PD was aggravated by the exposure to chronic EF stress. Taken together, these results suggest that the formation of ROS by chronic stress can enhance dopaminergic neuronal cell death in 6-OHDA-lesioned rats with or without L-DOPA treatment. Therefore, it is proposed that antioxidants scavenging 6-OHDA- or L-DOPA-induced ROS are a key to the prevention and control of the symptoms of PD (Andrew et al., 1993).

Previously, we reported that GP ethanol extract had an anti-stress function by improving the loss of body weight and the reduction of grip strength in rodents, which was induced by chronic EF stress (Choi et al., 2008). The extract also showed an immunomodulatory activity by preventing dexamethasone-induced immunosuppression (Im et al., 2009). In addition, GP ethanol extract protected against 6-OHDA-induced neurotoxicity in 6-OHDA-lesioned rats (Choi et al., 2010). In this study, GP-BX exhibited a protective activity against chronic EF stress by reducing L-DOPA-induced neuronal cell death in 6-OHDA-lesioned rats treated with L-DOPA. These results suggest that the protective functions of GP-BX on chronic EF

stress- and L-DOPA-induced neurotoxicity could be mediated by the modulation of the ROS formation and immune system in rodents.

The gypenoside-rich fraction, gypenosides, protected against oxidative neurotoxicity involving glutamate in primary cultures of rat cortical cells (Shang et al., 2006) and showed anti-inflammatory activity (Lin et al., 1993). Gypenosides also showed a protective effect on dopaminergic neuronal cell death in the MPTP-induced rat model of PD (Wang et al., 2010). It has been shown that GP-BX has several gypenoside derivatives, including gynosaponin TN-1, gynosaponin TN-2, gypenoside XLV, and gypenoside LXXIV (Choi et al., 2010; Razmovski-Naumovski et al., 2005; Nagai et al., 1981; Takemoto et al., 1984; Yoshikawa et al., 1987). These data further support that GP-BX can be applied for the prevention of the symptoms of PD by scavenging the formation of ROS.

Besides herbal GP, black tea extract exhibited neuroprotective and neurorescue effects against 6-OHDA-induced degeneration of the nigrostriatal dopaminergic system (Chaturvedi et al., 2004), and Yeoldahanso-tang, which is a Korean herbal formula containing 10 herbs, also protected against neurotoxicity in a MPTP-induced mice model of PD (Bae et al., 2011). Therefore, the comparative functions for PD among these herbal extracts, including drug interactions, adverse effects, and toxicity may need to be studied further.

5. Conclusion

GP-BX showed protective functions for dopaminergic neurons from chronic stress- and L-DOPA-induced neurotoxicity in 6-OHDA-lesioned rat model of PD. Considering our results, GP-BX may be helpful in preventing the L-DOPA-induced adverse or oxidative toxic effects for PD, especially with chronic stress, as well as slow down the progression of PD symptoms. Clinical trials for patients with PD using herbal GP extract and its bioactive components need to be studied further.

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7. References

- Ahmed S.H.; Stinus, L., Moal, M.L. & Cador, M. (1995), Social deprivation enhances the vulnerability of male Wistar rats to stressor-and amphetamine-induced sensitization. *Psychopharmacology*, Vol.117, No.1, pp. 116-124, ISSN 0033-3158
- Amanda D. S.; Sandra, L., Castro, Michael, J. & Zigmond. (2002). Stress-induced Parkinson's disease: a working hypothesis, *Physiology & Behavior*, Vol.77, No.4-5, pp. 527-531, ISSN 0031-9384
- Andrew, R.; Watson, D.G., Best, S.A., Midgley, J.M, Wenlong, H. & Petty, R.K. (1993). The determination of hydroxydopamines and other trace amines in the urine of

- parkinsonian patients and normal controls. *Neurochemical Research*, Vol.18, No.11, pp. 1175-1177, ISSN 0364-3190
- Attawish, A.; Chivapat, S., Phadungpat, S., Bansiddhi, J., Techadamrongsin, Y., Mitrijit, O., Chaorai, B. & Chavalittumrong, P. (2004). Chronic toxicity of *Gynostemma pentaphyllum*. *Fitoterapia*, Vol.75, No.6, pp. 539-551, ISSN 0367-326X
- Bae, N.; Ahn, T., Chung, S., Oh, M.S., Ko, H., Oh, H., Park, G. & Yang, H.O. (2011). The neuroprotective effect of modified Yeoldahanso-tang via autophagy enhancement in models of Parkinson's disease. *Journal of Ethnopharmacology*, Vol.134, No.2, pp. 313-322, ISSN 0378-8741
- Basma, A.N.; Morris, E.J., Nicklas, W.J. & Geller, H.M. (1995). L-DOPA cytotoxicity to PC12 cells in culture is via its autoxidation. *Journal of Neurochemistry*, Vol.64, No.2, pp. 825-832, ISSN 0022-3042
- Blum, D.; Torch, S., Lambeng, N., Nissou, M., Benabid, A.L., Sadoul, R. & Verna, J.M. (2001). Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease. *Progress in Neurobiology*, Vol.65, No.2, pp. 135-172, ISSN 0301-0082
- Borah, A. & Mohanakumar, K.P. (2010). L-DOPA-induced 6-hydroxydopamine production in the striata of rodents is sensitive to the degree of denervation. *Neurochemistry International*, Vol.56, No.2, pp. 357-62, ISSN 0197-0186
- Cenci, M.A. (2007). L-DOPA-induced dyskinesia: cellular mechanisms and approaches to treatment. *Parkinsonism & Related Disorders*, Vol.13, pp. S263-S267, ISSN 1353-8020
- Cheng, N.; Maeda, T., Kume, T., Kaneko, S., Kochiyama, H., Akaike, A., Goshima, Y. & Misu. (1996). Differential neurotoxicity induced by L-DOPA and dopamine in cultured striatal neurons, *Brain Research*, Vol.16, No.743, pp. 278-283, ISSN 0006-8993
- Chaturvedi RK.; Shukla, S., Seth, K., Chauhan, S., Sinha, C., Shukla, Y. & Agrawal, AK. (2006). Neuroprotective and neurorescue effect of black tea extract in 6-hydroxydopamine-lesioned rat model of Parkinson's disease. *Neurobiology of Disease*, Vol.22, No.2, pp. 421-34, ISSN 0969-9961
- Choi, H.S.; Lim, S.A., Park, M.S., Hwang, B.Y., Lee, C.K., Kim, S.H., Lim, S.C. & Lee, M.K. (2008). Ameliorating effects of the ethanol extracts from *Gynostemma pentaphyllum* on electric footshock stress. *Korean Journal of Pharmacognosy*, Vol.39, No.4, pp. 341-346, ISSN 0253-3073
- Choi H.S.; Park M.S., Kim S.H., Hwang B.Y., Lee C.K. & Lee M.K. (2010). Neuroprotective effects of herbal ethanol extracts from *Gynostemma pentaphyllum* in the 6-hydroxydopamine-lesioned rat model of Parkinson's disease. *Molecules*, Vol.15, No.4, pp. 2814-2824, ISSN 1420-3049
- Deumens, R.; Blokland, A. & Prickaerts, J. (2002). Modeling Parkinson's disease in rats: an evaluation of 6-OHDA lesions of the nigrostriatal pathway. *Experimental Neurology*, Vol.175, No.2 pp. 303-317, ISSN 0014-4886
- Fearnley, M. & Lees, A.J. (1991). Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain*, Vol.114, No.5, pp. 2283-2301, ISSN 0006-8950

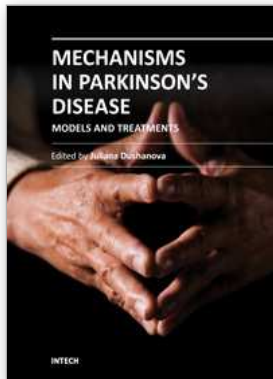
- Ganong, W. F. (2003). *Review of Medical Physiology*, 23th ed. pp. 359–384, McGraw-Hill, ISBN 0071287289, United States
- Hornykiewicz, O. (1982). Imbalance of brain monoamines and clinical disorders. *Progress in Brain Research*, Vol.55, pp. 419–429, ISSN 0079-6123
- Jankovic, J. (2005). Motor fluctuations and dyskinesias in Parkinson's disease: clinical manifestations. *Movement Disorders: Official journal of the Movement Disorder Society*, Vol.20, No.11, pp. S11–S16, ISSN 0885-3185
- Im, S.A.; Choi, H.S., Hwang, B.Y., Lee, M.K. & Lee, C.K. (2009). Augmentation of immune responses by oral administration of *Gynostemma pentaphyllum* ethanol extract. *Korean Journal of Pharmacognosy*, Vol.40, No.1, pp. 35–40, ISSN 0253-3073
- Kandel, E.R.; Schwartz, J. H. & Jessell, T.M. (2000) *Principles of Neural Science*, McGraw-Hill, ISBN 0071287289, United States
- Lin, J.M.; Lin, C.C., Chiu, H.F., Yang, J.J. & Lee, S.G. (1993). Evaluation of the anti-inflammatory and liver-protective effects of *Anoectochilus formosanus*, *Ganoderma lucidum* and *Gynostemma pentaphyllum* in rats. *The American Journal of Chinese Medicine*, Vol.21, No.1 pp. 59–69. ISSN 0192-415X
- Maharaj H.; Sukhdev Maharaj D., Scheepers M., Mokokong R. & Daya S. (2005). L-DOPA administration enhances 6-hydroxydopamine generation. *Brain Research*, Vol.1063, No.2, pp. 180-186, ISSN 0006-8993
- Marsden, C.D. (1994). Parkinson's disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, Vol.57, No.6, pp. 672–681, ISSN 0022-3050
- Mena, M.A.; Davila, V., & Sulzer, D. (1997). Neurotrophic effects of L-DOPA in postnatal midbrain dopamine neuron/cortical historicity cocultures. *Journal of Neurochemistry*, Vol.69, pp. 1398-1408, ISSN 0022-3042
- Merad-Boudia, M.; Nicole, A., Santiard-Baron, D., Saille, C. & Ceballos-Picot, I. (1998). Mitochondrial impairment as an early event in the process of apoptosis induced by glutathione depletion in neuronal cells: relevance to Parkinson's disease. *Biochemical Pharmacology*, Vol.56, No.5, pp. 645–655, ISSN 0006-2952
- Migheli, R.; Godani, C., Sciola, L., Delogu, M.R., Serra, P.A., Zangani, D., De Natale, G., Miele, E. & Desole, M.S. (1999). Enhancing effect of manganese on L-DOPA-induced apoptosis in PC12 cells: role of oxidative stress. *Journal of Neurochemistry*, Vol.73, No. 3, pp. 1155-1163, ISSN 0022-3042
- Nagai, M.; Izawa, K.; Nagumo, S.; Sakurai, N.; Inoue, T. (1981). Two glycosides of a novel dammarane alcohol from *Gynostemma pentaphyllum*. *Chemical & Pharmaceutical Bulletin*, Vol.29, pp. 779–783, ISSN 0009-2363
- Neil, A. & David, B. (2008). Parkinson's disease, *Medicine*, Vol.36, No.12, pp. 630–635, ISSN 0025-7974
- Ozawa, T.; Tanaka, M., Ikebe, S., Ohno, K., Kondo, T. & Mizuno, Y. (1990). Quantitative determination of deleted mitochondrial DNA relative to normal DNA in parkinsonian striatum by a kinetic PCR analysis. *Biochemical and Biophysical Research Communications*, Vol.172, No.2, pp. 483–489, ISSN 0006-291X

- Paxinos, G. & Watson, C. (1986). *The Rat Brain in Stereotaxic Coordinates*, 2nd ed.; Academic Press, ISBN 0126930198, Australia
- Pandey, A.; Borah, M., Varghese, P.K., Barman, K.P., Mohanakumar & R. Usha, R. (2009) Striatal dopamine level contributes to hydroxyl radical generation and subsequent neurodegeneration in the striatum in 3-nitropropionic acid-induced Huntington's disease in rats. *Neurochemistry International*, Vol.55, No.6, pp. 431-437, ISSN 0197-0186
- Perese, D.A.; Ulman, J., Viola, J., Ewing, S.E. & Bankiewicz, K.S. (1989). A 6-hydroxydopamine-induced selective parkinsonian rat model. *Brain Research*, Vol.494, No.2, pp. 285-293, ISSN 0006-8993
- Przedborski, S.; Levivier, M., Jiang, H., Ferreira, M., Jackson-Lewis, V., Donaldson, D. & Togasaki, D.M. (1995). Dose-dependent lesions of the dopaminergic nigrostriatal pathway induced by intrastriatal injection of 6-hydroxydopamine. *Neuroscience*, Vol.67, No.3, pp. 631-647, ISSN 0306-4522
- Rajkowska G. (2000). Postmortem studies in mood disorders indicate altered numbers of neurons and glial cells. *Biological Psychiatry*, Vol.48, No.8, pp. 766-777, ISSN 0006-3223
- Razmovski-Naumovski, V.; Huang, T.H.-W., Tran, V.H., Li, G.Q., Duke, C.C. & Roufogalis, B.D. (2005). Chemistry and pharmacology of *Gynostemma pentaphyllum*. *Phytochemistry Review*, Vol.4, pp. 197-219
- Schober, A. (2004). Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. *Cell and Tissue Research*, Vol.318, No.1, pp. 215-224, ISSN 0302-766X
- Schwab, R.S. & Zieoer, I. (1965) Effect of mood, motivation, stress and alertness on the performance in Parkinson's disease. *Psychiatria et Neurologia*, Vol.150, No. 6, pp. 345-357
- Shang, L.; Liu, J., Zhu, Q., Zhao, L., Feng, Y., Wang, X., Cao, W. & Xin, H. (2006). Gypenosides protect primary cultures of rat cortical cells against oxidative neurotoxicity. *Brain Research*, Vol.1102, No.1, pp. 163-174, ISSN 0006-8993
- Takemoto, T.; Arihara, S.; Yoshikawa, K.; Kawasaki, J.; Nakajima, T.; Okuhira, M. (1984). Studies on the constituents of Cucurbitaceae plants. XI. On the saponin constituents of *Gynostemma pentaphyllum* MAKINO. (7). *Yakugaku Zasshi*, Vol.104, pp. 1043-1049, ISSN 0031-6903
- Urakami, K.; Masaki, N., Shimoda, K., Nishikawa, S. & Takahashi K. (1988). Increase of striatal dopamine turnover by stress in MPTP-treated mice. *Clinical Neuropharmacology*, Vol.11, No.4, pp. 360-368, ISSN 0362-5664
- Walkinshaw, G. & Waters, C.M. (1995). Induction of apoptosis in catecholaminergic PC12 cells by l-DOPA. Implications for the treatment of Parkinson's disease. *The Journal of Clinical Investigation*, Vol.95, No.6, pp. 2458-2464, ISSN 0021-9738
- Wang, P.; Niu, L., Gao, L., Li, WX., Jia, D., Wang, XL. & Gao, G.D. (2010). Neuroprotective effect of gypenosides against oxidative injury in the substantia nigra of a mouse model of Parkinson's disease. *The Journal of International Medical Research*, Vol.38, No.3, pp. 1084-92.

Yoshikawa, K.; Arimitsu, M. Kuki, K. Takemoto, T.; Arihara, S. (1987). Studies on the constituents of Cucurbitaceae plants. XVIII. On the saponin constituents of *Gynostemma pentaphyllum* MAKINO. (13). *Yakugaku Zasshi*, Vol.107, pp. 361-366, ISSN 0031-6903

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Parkinson's disease (PD) results primarily from the death of dopaminergic neurons in the substantia nigra. Current PD medications treat symptoms; none halt or retard dopaminergic neuron degeneration. The main obstacle to developing neuroprotective therapies is a limited understanding of the key molecular mechanisms that provoke neurodegeneration. The discovery of PD genes has led to the hypothesis that misfolding of proteins and dysfunction of the ubiquitin-proteasome pathway are pivotal to PD pathogenesis. Previously implicated culprits in PD neurodegeneration, mitochondrial dysfunction, and oxidative stress may also act in part by causing the accumulation of misfolded proteins, in addition to producing other deleterious events in dopaminergic neurons. Neurotoxin-based models have been important in elucidating the molecular cascade of cell death in dopaminergic neurons. PD models based on the manipulation of PD genes should prove valuable in elucidating important aspects of the disease, such as selective vulnerability of substantia nigra dopaminergic neurons to the degenerative process.

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