Neuroprotective Effects of Phenylethanoid Glycosides from *Cistanches salsa* against 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-Induced Dopaminergic Toxicity in C57 Mice

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The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been employed to create a Parkinson's disease-like model in both rodents and primates based primarily on its ability to create a striatal dopamine deficit due to the loss of dopaminergic neurons in the substantia nigra compacta. The present study was carried out to determine the possible effects of phenylethanoid glycosides (PhGs) from *Cistanches salsa* (C. A. MEY, G. BECK) on attenuating the serious behavioral disorder and increasing dopamine (DA) levels in the striata of MPTP-lesioned C57 mice. MPTP (30 mg/kg i.p. for 4 d) induced serious behavioral disorders and significantly reduced striatal DA levels in C57 mice. In spontaneous motor activity and rotarod tests, obvious behavioral differences were seen between control and model groups. PhGs (10, 50 mg/kg) significantly increased the spontaneous movement number and latent period of mice on the rotating rod ($p < 0.01$). Injections of MPTP 30 mg/kg for 4 d caused a significant reduction in DA, 3,4-dihydroxyphenyl acetic acid, and homovanillic acid in striata analyzed by HPLC-electrochemistry ($p < 0.01$). The neurotoxic effects of MPTP were attenuated by pretreatment with PhGs (10, 50 mg/kg) in a dose-dependent fashion. The apparent neuroprotective effects of PhGs on nigral dopaminergic neurons were also confirmed by the results of immunohistochemical staining. The present *in vivo* data clearly demonstrate that PhGs can protect dopaminergic neurons against dopamine neurotoxicity induced by MPTP, as suggested by an earlier *in vitro* study. The neuroprotective effects of PhGs were the first reported for a natural product.

Key words neuroprotective effect; phenylethanoid glycosides; *Cistanche salsa*; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

Parkinson's disease (PD) is a neurodegenerative disease characterized by bradykinesia, resting tremor, rigidity, and gait disturbance. Numerous studies have demonstrated that PD is the consequence of substantial loss of substantia nigra neurons and depletion of the transmitter dopamine (DA). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes selective degeneration of the nigrostriatal dopaminergic neurons and is widely used to create an animal model of PD.¹⁾ MPTP is converted by monoamine oxidase B to the active metabolite MPP⁺ which is accumulated intracellularly in neurons via the dopaminergic transporter. MPP⁺ produces neurotoxic effects by inhibiting the complex I of the respiratory chain in DA neurons, which ultimately results in cell death. Because of the similarity between the MPTP mouse model and PD, it is tempting to propose that a similar scenario applies to the pathogenesis of PD.²⁾

Cistanche salsa (C. A. MEY) G. BECK is a parasitic plant native to the northwest of China. The stem of this plant is an important traditional Chinese medicine and used for the treatment of kidney deficiency and neurasthenia. The major active constituents of this herb are phenylethanoid glycosides (PhGs).³⁾ We found that acteoside of PhGs from *C. salsa* (C. A. MEY) G. BECK inhibited apoptosis by MPP⁺ in cerebellar granule neurons.⁴⁾ However, it is not clear whether PhGs affect MPTP-induced dopaminergic neuron damage *in vivo*. In this study, we investigated the effects of PhGs on MPTP-induced dopaminergic neuron damage in C57 mice and found PhGs significantly attenuated the serious behavioral disorder and increased the DA levels in the striata. The apparent neuroprotective effects of PhGs on substantia nigra neurons were

also confirmed using an immunohistochemical method.

MATERIALS AND METHODS

Source of PhGs PhGs from the stems of *C. salsa* were extracted by the method of Lei *et al.*⁵⁾ They were identified as 40% echinacoside, 10% acteoside and 50% 10 other trace PhGs on the basis of chemical evidence and spectral data.

Animals Male C57BL/6 mice weighing 20–25 g were purchased from the Laboratory Animal Center of Peking University Health Science Center (Beijing, China) and were housed in a temperature-controlled room with a 12-h day and night light cycle and free access to food and water. They were housed for 1 week prior to the experiments. Mice were randomly divided into 5 groups. Drugs for each group were administered as indicated in the treatment design (Table 1): group A (vehicle control, the same volume of saline); group B (model, MPTP 30 mg/kg, 120 mg/kg cumulative); group C (positive control, amantadine 40 mg/kg); and groups D and E (pretreatment with PhGs 10 and 50 mg/kg). One day after the last administration, the mice were subjected to behavioral experiments. Three days after the last administration, the mice were decapitated and DA determination and immunohistochemical assay were performed.

Behavioral Experiments Spontaneous Motor Test. The spontaneous activity test was started by placing the mice in a transparent plexiglass cylinder (23 cm × 30 cm, diameter × height) in an activity monitor (Experimental Factory of Chinese Academy of Medical Sciences, Beijing, China). The number of activity counts produced by interrupting consecu-

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Table 1. Design of Drug Administration

Group	n	Administration route	Dose (mg/kg per day)	
			d 1—7	d 8—11
Control	18	Saline <i>p.o.</i>	Same volume	Same volume
Model	18	Saline <i>i.p.</i>	Same volume	Same volume
		Saline <i>p.o.</i>		Same volume
Amantadine+MPTP	18	MPTP <i>i.p.</i>	40	30
		Amantadine <i>p.o.</i>		40
PhGs10+MPTP	18	MPTP <i>i.p.</i>	10	30
		PhGs <i>p.o.</i>		10
PhGs50+MPTP	18	MPTP <i>i.p.</i>	50	30
		PhGs <i>p.o.</i>		50
		MPTP <i>i.p.</i>		30

When two drugs were given, the each administration intermission was 1 h. PhGs, phenylethanoid glycosides 10 and 50 mg/kg, respectively.

tive infrabeams was expressed as counts per 5 min.⁶⁾

Rotarod Test. The mice were trained twice daily on 2 successive days to stay on a rotating rod (Experimental Factory of Peking University Health Science Center, Beijing, China) for 120 s (speed 12 rpm). The rotarod unit consists of a rotating spindle (diameter 7.3 cm) and individual compartments for each mouse. Subsequently, the mice were tested on the rotarod three times (speed 25 rpm). The duration from the beginning of rotation to mice leaving the rotating rod was used to indicate the endurance performance during 60 s. The mean endurance performance was the mean of three performances at the given speed. All values are expressed as mean $\pm$ S.E.M. and statistical significance was evaluated using the Mann–Whitney *U*-test.⁷⁾

HPLC Analysis HPLC with an electrochemical (EC) detector (Coularray, ESA, Inc., Chelmsford, MA, U.S.A.) was used to measure striatal levels of DA, 3,4-dihydroxyphenyl acetic acid (DOPAC), and homovanillic acid (HVA).⁸⁾ After the above tests, the mice were killed by decapitation, and the striata were dissected and assayed for DA, DOPAC and HVA using an HPLC-EC procedure. Striata were sonicated in 100 μ l of ice-cold solution A (0.4 M HClO₄) and after being stored for 1 h at 4 °C homogenates were centrifuged at 15000 $\times g$ for 15 min at 4 °C. Then 40 μ l of solution B (20 mM sodium citrate, 300 mM K₂HPO₄, and 2 mM Na₂EDTA) was added to the supernatant. After being mixed thoroughly, the solution was also stored for 1 h at 4 °C and centrifuged at 15000 $\times g$ for 15 min at 4 °C. The supernatant was filtered (pore size 0.22 μ m, Millipore filter) and 20 μ l of supernatant was autoinjected (ESA Model 542). The mobile phase consisted of 94% 150 mM citric acid and sodium citrate buffer (pH 4.3), 100 mM Na₂EDTA, 120 mM sodium octyl sulfate, and 6% methanol. The flow rate was 1.0 ml/min. The levels of DA and its metabolite were calculated and expressed as ng/mg tissue weight. Each group consisted of 6 animals.

Immunohistochemical Assay For immunohistochemical study, some of the animals were anesthetized with sodium pentobarbital (50 mg/kg, *i.p.*) 3 d after MPTP treatment, and the brains were perfusion-fixed with 10% buffered formalin for a minimum of 2 weeks following a normal saline flush. The brains were removed 1 h after perfusion fixation at 4 °C and immersed in 10% buffered formalin. Paraffin sections from the regions of the substantia nigra (5 μ m thick) were mounted and immunohistochemically stained with tyrosine

hydroxylase (TH). For TH immunohistochemistry, a polyclonal anti-TH antibody (Chemicon International, Temecula, CA, U.S.A.) and a Vectastain elite ABC kit (Vector Laboratories, CA, U.S.A.) were used. The paraffin sections were washed for 5 min in 10 mM phosphate-buffered saline (PBS, pH 7.4) and treated with 3% H₂O₂. The paraffin sections were then washed three times, for 5 min/each time in 10 mM PBS, followed by a 30-min preincubation with 10% normal goat serum. The brain sections were then incubated with anti-TH antibody (1:200) including 0.3% Triton X-100 overnight at 4 °C. After a 15-min rinse in fresh 10 mM PBS, the sections were incubated with a biotinylated second antibody for 2 h and then administrated with an avidin–biotin peroxidase complex for 30 min at room temperature. Signals of immunoreactions were visualized with diaminobenzidine (DAB) (Sigma Chemical).⁹⁾ Microphotographs were taken with an objective lens of 10 $\times$ magnification.

Western Blotting For Western blot analysis, after decapitation, brains were quickly removed and the stratum and substantia nigra were rapidly dissected out on an ice-cold glass Petri dish. Samples were immediately weighed, then frozen and stored at –80 °C until assay. The stratum and substantia nigra frozen at –80 °C were individually homogenized in 80 mM Tris–HCl buffer (pH 7.4) containing 0.1% sodium dodecyl sulfate (SDS), 0.4 mM dithiothreitol, 2 mM EDTA, and the protease inhibitor phenylmethanesulfonyl fluoride (0.1 mM). After incubation in buffer for 30 min at 4 °C, samples were centrifuged at 15000 $\times g$ for 10 min at 4 °C. Supernatants were collected and the protein concentration was determined. The Lowry protein assay was used with bovine serum albumin (BSA) as the standard for protein measurement. Protein was denatured at 100 °C for 5 min in sample buffer and electrophoresed onto a 7.5% SDS-PAGE. After transfer to a nitrocellulose membrane, the blot was washed in Tris-buffered saline containing 0.05% Tween-20, then blocked with 5% BSA and 5% skim milk for 1 h and incubated with a polyclonal anti-TH antibody (Chemicon International, Temecula, CA, U.S.A.) diluted 1:500 overnight at 4 °C. After washing, blot was incubated with goat anti-rabbit IgG linked with peroxidase diluted 1:1000 for 1 h. The reaction was developed with a chemiluminescence method (ECL; Amersham).

Statistical Analysis Values were expressed as mean $\pm$ S.E.M. To analyze the differences between groups, statistical analysis was conducted using *t*- and two-way

ANOVA tests, while the differences in the rotarod test were analyzed using the Mann–Whitney *U*-test. The level of significance was set at $p < 0.05$.

RESULTS

Behavioral Experiments The results of spontaneous motor activity and rotarod tests for MPTP lesions and PhGs treatment are shown in Figs. 1 and 2, respectively. These results strongly suggest that PhGs pretreatment reversed, at least in part, the spontaneous activity and coordinated movement impairments. The MPTP-treated group had less spontaneous motor activity and a shorter latent period in the rotarod test ($p < 0.01$) compared with the control group. A significant increase in these tests is also shown in Figs. 1 and 2 due to PhGs (10, 50 mg/kg) treatment compared with the MPTP model group ($p < 0.01$). Amantadine was used as a positive control and is a releaser or reuptake inhibitor of DA. Amantadine (40 mg/kg) significantly increased the spontaneous movement number and latent period on the rotating rod compared with the MPTP model group ($p < 0.01$). Furthermore, the protective effects of PhGs (50 mg/kg) on MPTP-lesioned C57 mice were similar to those of amantadine (40 mg/kg) although the protective effect of PhGs was stronger than those of amantadine in the spontaneous motor test and that of PhGs was weaker than that of amantadine in the rotarod test (Figs. 1, 2).

Level of DA The present results confirm that administration of MPTP can induce a marked decrease in DA (0.35 ± 0.13 ng/mg tissue), DOPAC (2.08 ± 0.75 ng/mg tissue) and HVA (1.03 ± 0.29 ng/mg tissue) in striata but not be induced in the control group (2.65 ± 0.73 ng/mg tissue, 6.23 ± 0.67 ng/mg tissue and 1.83 ± 0.09 ng/mg tissue, respectively, $p < 0.01$, Table 2). Pretreatment with PhGs (10, 50 mg/kg) markedly enhanced the level of DA and DOPAC ($p < 0.05$ or $p < 0.01$) in a dose-dependent fashion. Amantadine also markedly enhanced the level of dopamine ($p < 0.01$) and DOPAC ($p < 0.01$) in MPTP-lesioned C57 mice. Moreover, the effect was stronger than that of PhGs (10, 50 mg/kg). These results suggest that the effects of PhGs in behavioral experiments correlated with the increase in DA levels in the striata of MPTP-lesioned C57 mice. Furthermore, PhGs caused a significant increase in both DOPAC and the DOPAC/dopamine ratio (data not shown).

Immunohistochemical Assay Typical microphotographs of TH immunostaining in the substantia nigra are shown in Fig. 3. TH is a rate-limiting enzyme in DA synthesis. Dopaminergic neurons could easily be detected in the substantia nigra using TH antibody.

The cytoplasm and fiber of dopaminergic neurons were intensely stained with evident immunoreactive positive signals. Many dopaminergic neurons were lost in the substantia nigra following the administration of MPTP. No such pathologic changes were seen in the substantia nigra following the administration of PhGs plus MPTP. These *in vivo* results support and reconfirm prior results (Yanyun Li *et al.*, unpublished observations) showing that PhGs can protect dopaminergic neurons from injuries caused by toxic MPTP in the body and nerve terminal regions.

Western Blotting TH immunoreactivity in the striatum and substantia nigra was examined by Western blotting. Fig-

Fig. 1

T-bars represent the S.E.M. of movement number in 5 min. Values are mean $\pm$ S.E.M., $n = 10$. ** $p < 0.01$ compared with the model group.

Fig. 2

T-bars represent the S.E.M. of the latent period on the rotating rod. Values are mean $\pm$ S.E.M., $n = 10$. ** $p < 0.01$ compared with the model group. Statistical significance was evaluated using the Mann–Whitney *U*-test.

Table 2. Effects of PhGs Pretreatment on DA and Its Metabolites (DOPAC and HVA) in Striata

Treatment	Striata level (ng/mg of tissue)		
	Dopamine	DOPAC	HVA
Control	$2.65 \pm 0.73^{**}$	$6.23 \pm 0.67^{**}$	$1.83 \pm 0.09^{**}$
Model (MPTP 30 mg/kg)	0.35 ± 0.13	2.08 ± 0.75	1.03 ± 0.29
Amantadine + MPTP	$1.44 \pm 0.29^{**}$	$5.35 \pm 0.90^{**}$	$1.53 \pm 0.30^{*}$
PhGs (10 mg/kg) + MPTP	$0.98 \pm 0.34^{**}$	$3.63 \pm 0.87^{*}$	1.41 ± 0.30
PhGs (50 mg/kg) + MPTP	$1.36 \pm 0.44^{**}$	$4.44 \pm 0.76^{**}$	$1.48 \pm 0.22^{*}$

Values are mean $\pm$ S.E.M. from three independent experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$ compared with the model group.

ure 4 shows a band of apparent molecular weight of 60 kDa, which corresponds to the molecular weight of TH. The model group showed the lower staining intensity compared with the control group, and the intensity of the band was similar in the control group, amantadine+MPTP group, and PhGs (50 mg/kg)+MPTP group, in agreement with the above immunohistochemical results.

Fig. 3. Immunohistochemical Staining of Dopaminergic Neurons with Antibody for TH in the Substantia Nigra

(A) Control: numerous substantia nigra cells are seen. The cytoplasm is rich and the cellular processes are evident. There were strong immunoreactive positive signals in the cytoplasm. (B) Model (MPTP 30 mg/kg): only a few immunoreactive positive cells are seen and the cell processes disappeared in most cells. (C) PhGs (10 mg/kg)+MPTP: there were significantly more immunoreactive positive cells than in the injury model but the immunoreactive positive signals are faint. (D) PhGs (50 mg/kg)+MPTP: there were numerous immunoreactive positive cells in the substantia nigra. The cell processes are obvious and the immunoreactive positive signals were stronger than that of cells in (C).

Fig. 4.

Western blotting showed TH levels in the striatum and substantia nigra of each group. Molecular weight marker is shown on the left. 1, Control; 2, MPTP (30 mg/kg); 3, amantadine+MPTP; 4, PhGs (10 mg/kg)+MPTP; 5, PhGs (50 mg/kg)+MPTP. A comparative reduction in TH immunoreactivity was seen in band 2. Band 3, 4, and 5 show that amantadine and PhGs can significantly prevent the reduction in TH.

DISCUSSION

The present study demonstrates that PhGs can protect or rescue nigrostriatal neurons from MPTP-induced dopaminergic toxicity to the substantia nigra compacta neurons. The current *in vivo* data (Table 2, Fig. 3) are consistent with the results of an earlier *in vitro* study showing that the main active component acteoside of PhGs can protect dopaminergic neurons in primary culture against damage caused by MPP⁺ (Yanyun Li *et al.*, unpublished observations). Moreover, the present results (Figs. 1, 2) further support our current working hypothesis that PhGs can improve behavioral deficits in mice induced by MPTP, which causes a decrease in spontaneous motor activity and shortens the duration from the beginning of the rotation to mice leaving the rotating rod. The effects of PhGs are dose dependent (Figs. 1, 2). MPTP

causes an expressive lesion of the substantia nigra compacta and a significant reduction in DA levels in the striatum.¹⁰⁾ Our results suggest that PhGs can increase the levels of DA and its metabolites in the striata of MPTP-lesioned C57 mice. We conducted an additional experiment to observe the effect of PhGs in intact mice, and the DA level increased slightly but there was no significant difference compared with the control group. Amantadine, a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist known to increase DA release in the striatum, is frequently associated with L-DOPA in the treatment of PD. The effect of PhGs on increasing the DA level in the striatum is similar to that of amantadine, but that on the NMDA receptor remains to be determined. In addition, it is not known whether PhGs are releasers or reuptake inhibitors of DA. Moreover, the present data (Table 2) suggest that PhGs may alter striatal type B monoamine oxidase activity in our experimental paradigm since the ratio of DOPAC/dopamine was changed by PhGs.

It has been reported that cell death in the C57 mouse 5 or 10 d after MPTP treatment is dose dependent.^{11,12)} Using TH immunoreactivity on alternate sections to identify surviving neurons in the substantia nigra compacta, we found that MPTP caused cell death in the C57 mouse substantia nigra compacta (Fig. 3). The main component acteoside of PhGs significantly improved cell viability and protected cells from MPP⁺-induced apoptosis by inhibiting caspase activation in cerebellar granule neurons⁴⁾ and dopaminergic neurons (un-

published data). Our results show that PhGs can increase the number of dopaminergic neurons *in vivo* in MPTP-lesioned C57 mice. TH immunoreactivity in the striatum and substantia nigra was also examined by Western blotting (Fig. 4). The results showed a band of apparent molecular weight of 60 kDa, which corresponded to the molecular weight of TH. The model group showed lower staining intensity compared with the control group, but the intensity of the band in the PhGs (50 mg/kg)+MPTP group was similar to that in the control group, in agreement with the immunohistochemistry results (Fig. 3). However, the mechanisms involved in the effect still need to be clarified, and it is suggested that the mechanisms may contribute to facilitating striatal DA release. PhGs may be hydrolyzed into smaller molecules to play a part in the action *in vivo* because they have a glycoside chemical structure. Studies on the metabolites of PhGs *in vivo* and their neuroprotective effects are ongoing in our laboratory.

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