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Original Article

Neuroprotection of Dopaminergic Neurons by *Hominis Placenta* Herbal Acupuncture in *in vitro* and *in vivo* Models of Parkinson's Disease Induced by MPP+/MPTP Toxicity

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[Abstract]

Objectives : This study was designed to investigate the neuroprotective effects of *Hominis-Placenta*(HP)on dopaminergic neurons.

Methods : We examined the effect of i*nvitro* administration of HP against 1-methyl-4-phenylpyridinium(MPP+)-induced dopaminergic cell loss in primary mesencephalic culture and also used behavioral tests and performed analysis in the striatum and the substantia nigra of mouse brain, to confirm the effect of HP on dopaminergic neurons in an i*nvivo* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP)-induced PD mouse model. Animals were assigned to four groups: (1) Group 1(vehicle-treatedgroup), (2) Group 2(MPTPonlytreated group), (3) Group 3(MPTP+ saline-treated/ST_{36} group), and (4) Group 4(MPTP+HP-treated/ST_{36} group). HP at 20 μL of 48 mg/kg dose was injected at ST_{36} for 4 weeks at 2-day intervals. MPTP in saline was injected intraperitoneally each day for 5 days from the 8_{th} treatment of HP. We performed the pole test and rota-rod test on the first and seventh day after the last MPTP injection. To investigate the effect of HP on dopaminergic neurons, we performed analysis in the striatum and the substantia nigra of mouse brain after treatment with HP and/or MPTP.

Results : Treatment with HP had no influence on cell proliferation and caused no cell toxicity in PC_{12} and HT_{22} cells. Our study showed that HP significantly prevented cell loss and protected neurites against MPP+ toxicity. Although the *invivo* treatment of HP herbal acupuncture at ST_{36} showed a tendency to improve movement ability and protected dopaminergic cells and fibers in the substantia nigra and the striatum, it did not show significant changes compared with the MPTP treated group.

Conclusions : These data suggest that HP could be a potential treatment strategy in neuro-degenerative diseases such as Parkinson's disease.

Key words :
Hominis Placenta pharmacoacupuncture;
Parkinson's disease;
MPP+;
MPTP;
Behavioral test;
TH-immunohistochemistry

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Ⅰ. Introduction

Parkinson's disease(PD) is a common neurodegenerative disease characterized by motor dysfunctions such as tremor, rigidity and bradykinesia, resulting from the progressive loss of dopaminergic neurons in the substantia nigra pars compacta[1].

Oriental medicine embodies comprehensive principles and methods for maintaining personal health in daily life, which helps prevent disease[2]. In spite of extensive basic and clinical research on PD, no preventive or long-term effective treatment strategies to delay onset or to slow progression of PD are available[3,4]. Various pharmacological and surgical treatments have been used to treat patients with PD, but some of these have significant adverse effects such as motor fluctuations and dyskinesia[5]. Due to the limitations in these therapies, many PD patients seek oriental medicines such as acupuncture and herbal medications[6]. Approximately 40 % of patients with PD in the United states, 39 % of PD patients in the United Kingdom, and 76 % of PD patients in Korea have used complementary and alternative medicine(CAM)[7,8]. Moreover, oriental medicine such as acupuncture, electro-acupuncture, bee venom acupuncture, and herbal medicine have been demonstrated to possess neuroprotective properties that may be useful in preventing various forms of neuronal cell loss, including the nigrostriatal degeneration seen in PD[9-13]. Herbal acupuncture therapy which has been used many years ago in oriental medicine, is to inject extracted herbal fluid into acupoint to achieve both efficacy of acupuncture and herbal medicine[14]. Recent experimental reports suggested that bee venom acupuncture, a kind of herbal acupuncture, might have neuroprotective effects in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP)-induced mouse model of PD[6,15].

Hominis placenta(HP) is the dried placenta of healthy pregnant females which has been used to tonify or regulate the blood , tonify the essence, and warm kidney for thousands of years in oriental medicine[16,17]. It is the rich resources of various bioactive substances suchas polydeoxyribonucleotides (PDRN), ribonucleic acid(RNA), deoxyribonucleic acid (DNA), peptides, amino acids, enzymes, and trace elements[18,19]. According to recent studies, it was reported that herbal formulations comprised of herbs, including HP, showed anti-thrombotic effect and inhibitory effect on bone resorption[20-23].

In addition, HP herbal acupuncture, using the water extracts of HP as an herbal component, showed anti-inflammatory responsesin subchondral bone of a murine rheumatoid arthritis model, and neuroprotective effects in case of Alzheimer's disease mouse model[24,25].

In order to investigate the preventive and neuroprotective effects of HP on dopaminergic neurons, we examined the effect of *in vitro* administration of HP against toxin-induced dopaminergic cell loss in primary mesencephalic culture. We also used behavioral tests and performed analysis in the striatum and the substantia nigra of mice brain, to confirm the effect of HP on dopaminergic neurons in an *in vivo* MPTP-induced PD mouse model.

Ⅱ. Materials and methods

A. Materials

Roswell Park Memorial Institute medium(RPMI), Dulbecco's Modifid Eagle's Medium(DMEM), minimal essential medium(MEM), fetal bovine serum, horse serum, and penicillin-streptomycin were purchased from Gibco Industries, Inc(Auckland, New Zealand). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliu m bromide(MTT), 6-hydroxydopamine(6-OHDA), MPTP, 1-methyl-4-phenylpyridinium(MPP+), collagen, glucose, L-glutamine, dimethyl sulfoxide(DMSO), phosphate buffer(PB), phosphate-buffered saline(PBS), paraformaldehyde(PFA), glycerin, ethylene glycol, Tri0 ton X-100, 3,3-diaminobenzidine(DAB), sodium chloride, sucrose, and glucose were purchased from Sigma-Aldrich(St Louis, MO, USA). A rabbit anti-tyrosine hydroxylase(TH) antibody was purchased from Chemicon International Inc.(Temecula, CA, USA). Biotiny-

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lated anti-rabbit antibody, normal goat serum, and avidin-biotin peroxidase complex(ABC) standard kit were purchased from Vector Lab(Burlingame, CA, USA). The extract of Hominis placenta(HP) was obtained from Korean Pharmacopuncture Institute(Seoul, Korea). The HP extract was manufactured by the processes including extraction and sterilization, which were always under the quality controls of over 60 mg-soluble substances/mL. The amino acid compositions of the HP extract were as follows: aspartic acid (0.81 mg/mL), serine(0.23 mg/mL), glutamic acid(0.63 mg/mL), arginine(0.43 mg/mL), threonine(0.23 mg/mL), alanine(0.36 mg/mL), proline(0.43 mg/mL), valine(0.26 mg/mL), lysine(0.36 mg/mL), leucine(0.40 mg/mL). The HP extract was passed the germ-free, antihistamine, and endotoxinfree tests, and thus accepted as an injection in Korea.

B. Cell cultures and treatement

The PC_{12} cell line, a rat adrenal gland pheochromocytoma, was maintained in RPMI, supplemented with 5 % heat-inactivated fetal bovine serum, 10 % horse serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator of 5 % CO_2 at 37 °C. PC_{12} cells were seeded in a collagen pre-coated 96-well plate at a density of 2.0×10^4 cells/well, and 24 h later, various concentrations(15.625~250 μg/mL) of HP extract in RPMI were added to the cells for 24 h at 37 °C.

The HT_{22} cell line, a mouse hippocampal cell line, was maintained in DMEM, supplemented with 10 % heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a same incubator. HT_{22} cells were seeded in a 96-well plate at a density of 0.5×10^4 cells/well, and 24 h later, various concentrations(31.25~500 μg/mL) of HP extract in DMEM were added to the cells for 24 h at 37 °C.

C. Measurement of cell viability

PC_{12} and HT_{22} cell viability were measured by the colorimetric MTT assay. The treated cells were incubated with 0.5 mg/mL of MTT at 37 °C for 3 h. MTT medium was carefully aspirated and the formazan dye was eluted using DMSO. The plate was shaken and the absorbance was measured using a spectrophotometer(Versamax microplate reader, Molecular Device; Sunnyvale, CA, USA) at a wavelength of 570 nm. Cell viability was expressed as a percentage of the value in the vehicle-treated normalgroup.

D. Cultures of rat mesencephalic dopaminergic cells

Cell cultures were prepared from the mesencephalons of 14-day embryos of timed pregnant Sprague-Dawley rats(Daehan Biolink Co, Ltd, Eumseong, Korea). Mesencephalons were dissected, collected, dissociated, and plated in 24-well plates with cover slips pre-coated with PLL, at a density of 1.0×10^5 cells/well. Cultures were maintained in MEM, supplemented with 6.0 g/L glucose, 2 mM glutamine, and 10 % fetal bovine serum in a humidified incubator of 5 % CO_2 at 37 °C. On the 6_{th} day *in vitro*, cells were treated with HP extract at 0.1 and 1 μg/mL in medium for 1 h or 18 h, and stressed with 12 μM MPP^+ for a further 23 h or with 10 μM 6-OHDA for a further 6 h. Then, the cells were fixed with 4 % PFA at room temperature for 30 min. The cells were stored in PBS at 4 °C for immunohistochemistry.

E. Animals and treatment

Animal maintenance and treatment were carried out in accordance with the Principles of Laboratory Animal Care(NIH publication No. 85-23, revised 1985) and the Animal Care and Use guidelines of Kyung Hee University, Seoul, Korea. Male C57BL/6 mice(8 weeks old) were purchased from Daehan Biolink. Animals were assigned to four groups; (1) Group 1(vehicle-treated group, n=9), (2) Group 2(MPTP only treated group, n=10), (3) Group 3(MPTP + saline-treated/ST_{36} group, n=9), and (4) Group 4(MPTP + HP-treated/ST_{36} group,

n=10). The mice housed at an ambient temperature of 23 ± 1 ℃ and a relative humidity of 60 ±10 % under a 12-h light/dark cycle and were allowed free access to water and food. HP pharmacoacupuncture at 20 μL of 48 mg/kg dose was injected at ST_{36} for 4 weeks at 2-day intervals using 0.5 mL-syringe with 31 gauge-needle. The dose of HP pharmacoacupuncture was derived from the reported dosage of Yeom et al[24] and Lee et al[25] and the mouse metabolic rate in the previous studies. MPTP(MPTP base form; 30 mg/kg) in saline was injected intraperitoneally per day for 5 days from 8th treatment of HP pharmacoacupuncture(Fig. 1).

F. Behavioral tests

We performed the pole test, on the first and seventh day after the last MPTP injection. The mice were placed head upward near the top of a vertical rough-surfaced pole(diameter 8 mm, height 55 cm). The time taken for the mice to turn completely downward(time to turn, T-turn) and the time taken to reach the floor(locomotion activity time, T-LA) were recorded with a cut-off limit of 30 sec.

We also performed the rota-rod test, on the first and seventh day after the last MPTP injection. The mice were given a prior training session before initialization of any therapy to acclimate them to rota-rod apparatus. Mice were placed on the rotating rod(speed, 20 rpm). The length of time on the rod was taken as the measure of competency. The cut off time was taken as 300 sec and each mouse performed two separate trials. The two results were averaged and recorded(Fig. 1).

G. Brain tissue preparation

After the behavioral tests, the mice were anesthetized with 50 mg/kg Zoletil(intramuscularly) and were rapidly perfused transcardially with PBS, followed by 4 % PFA in 0.1 M PB. Then, brains were rapidly taken out, post-fixedin 4 % PFA and processed for cryoprotection in 30 % sucrose at 4 ℃. Frozen brains were cut into 30 μm coronal sections using a cryostat microtome(CM3000; Leica, Wetzlar, Germany). Then, the tissues were stored in storing solution containing glycerin, ethylene glycol, and PB at 4 ℃ for immunohistochemistry.

H. Immunohistochemistry

Fixed mesencephalic cells on the cover slips and free-floating brain sections were rinsed in PBS at room temperature before immunostaining. They were pre-treated with 1 % H_2O_2 in PBS for 15 min. For dopaminergic neuron detection, they were incubated with a rabbit anti-TH antibody(1:2,000 dilution) overnight at 4 ℃. They were then incubated with a biotinylated anti-rabbit IgG for 1 h followed by incu-

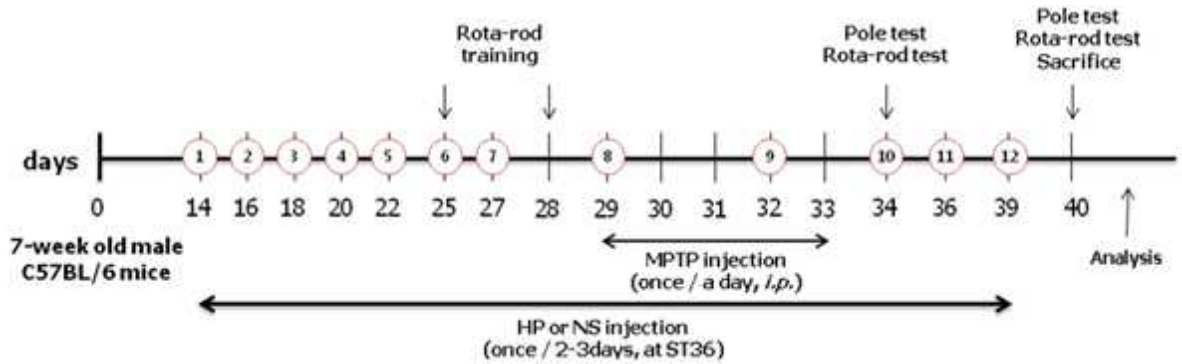

Fig. 1. Schedule of MPTP injections, HP pharmacoacupuncture treatment and behavioral tests
HP : Hominis Placenta. MPTP : 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. NS : normal saline.

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bation in ABC solution for 1 h atroom temperature. The activity was visualized with DAB for 3 min. After every incubation step, the cells and tissues were washed three times with PBS. Finally, the mesencephalic cells on the cover slips were mounted on gelatin-coated glass slides, air dried, and photographed with an optical light microscope(BX51T-32F01; Olympus Co, Tokyo, Japan). The free-floating brain tissues were mounted on gelatin-coated slides, dehydrated, cleared with xylene, and cover slipped using histomount medium. They were photographed with an optical light microscope. For quantification of the effect of HP extract in the mesencephalic dopaminergic cells, TH-positive cells were counted and TH-positive neurite lengths were measured using an ImageJ software (Bethesda, MD, USA) on at least four cover slips from independent experiments for each condition. Quantification of the effect of HP extract in brain tissues was performed by counting the TH-positvie cell numbers in the substantia nigra pars compacta at × 100 magnification under a microscope and by measuring the optical density of TH-positive fibers in the striatum at × 40 magnification using an ImageJ software. Data were expressed as a percentage of the value in the vehicle-treated normal group.

I. Statistical analysis

The data were expressed as mean ±standard error of the mean(SEM). Statistical significance was determined by one way analysis of variance, followed by a Tukey's multiple comparison test, using GraphPad Prism software. In all analyses, $p < 0.05$ was deemed to indicate statistical significance.

Ⅲ. Results

A. Effects of HP extract on neuronal cell viability in neuronal culture

We performed MTT assay in PC_{12} and HT_{22} neuronal cells. MTT, used commonly to measure cell viability, produces purple formazan in response to mitochondrial enzymes in live cells. Treatment with various concentrations of HP extract at 31. 25, 62.5, 125, 250, and 500 μg/mL for 24 h had no influence on cell proliferation and caused no cell toxicity in PC_{12} and HT_{22} cells, showing 98.8 ± 1.3 - 106.7 ± 2.6 % and 86.6 ± 5.4 - 91.8 ± 3.2 %, respectively, compared with the normal value(Fig. 2).

B. Effects of HP extract on toxin-induced dopaminergic cell damage in primary mesencephalic culture

We measured TH-immunoreactive(IR) neuron numbers and neurite lengths. Treatment with 6-OHDA and

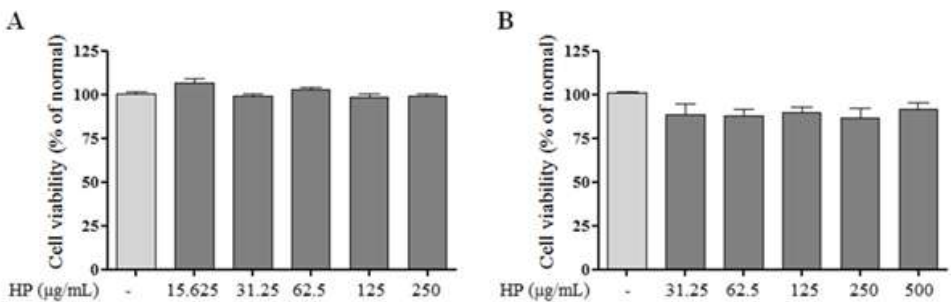

Fig. 2. Effects of HP extract on cell viability in PC_{12} and HT_{22} cells. PC_{12} and HT_{22} neuronal cells were treated with HP extract(15.625-500 μg/mL) for a total 24 h(A and B, respectively)
Cell viabilities were determined using MTT assay and were expressed as a percentage of the normals. Values are indicated as the mean ± SEM of six replicates.

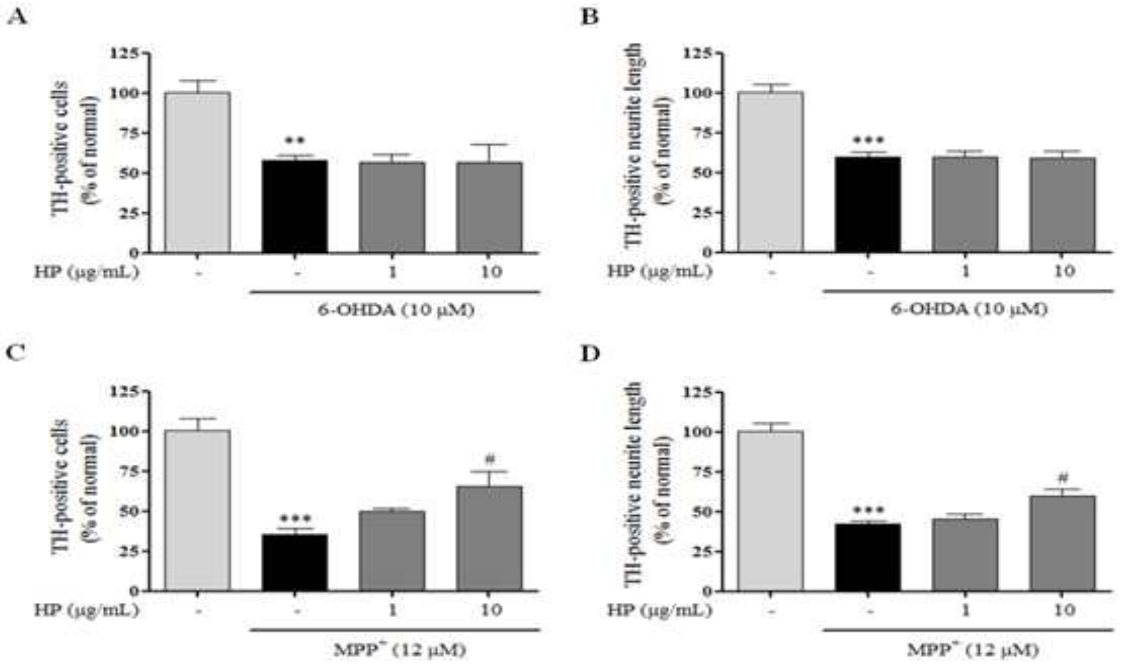

Fig. 3. Effects of HP extract on toxin-induced neuronal damage in rat primary dopaminergic cells

The rat primary dopaminergic cells were treated with HP extract(1 or 10 μg/mL) for 6 h and with 10 μM 6-OHDA for an additional 18 h(A and B), or with HP extract for 1 h and with 12 μM MPP+ for an additional 23 h(C and D). Fixed cells were stained with anti-TH antibody and visualized with DAB. The numbers of TH-IR neurons were counted(A and C) and the neurite lengths of them were measured(B and D). Data were expressed as a percentage of the normals.
Values are indicated as the mean ± SEM of four replicates.
*** : $p < 0.001$ compared with the normal group. ** : $p < 0.01$ compared with the normal group.
# : $p < 0.05$ compared with the MPP+-only treated group.

MPP+ reduced the number of TH-IR neurons by 57.8 ± 3.1 % and 35.6 ± 3.4 % compared with the normal value, respectively. Pre-treatment with 1 and 10 μg/mL HP extract did not prevent dopaminergic cell loss induced by 6-OHDA, but HP pharmacoacupuncture at 10 μg/mL prevented the cell loss induced by MPP+ by 65.2 ± 9.4 % compared with the normal value. In addition, 6-OHDA and MPP+ induced morphological changes of dopamine neurons including the shortening of neurite lengths by 59.5 ± 3.0 % and 42.0 ± 2.1 % compared with the normal group, respectively. However, HP extract at 10 μg/mL significantly protected neurites against MPP+ toxicity by showing an elongation of 59.8 ± 4.0 % compared with the normal value whereas it did not protected shortened neurites induced by 6-OHDA(Fig. 3). The representative microphotographs of TH-immunostaining are shown in Fig. 4 and Fig.5.

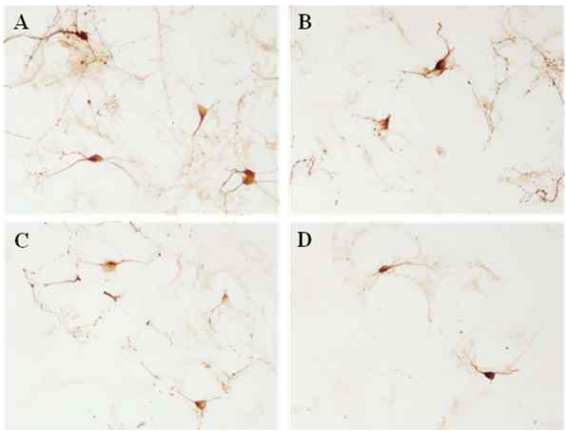

Fig. 4. The representative images of TH-IR cells for the effect of HP extract on 6-OHDA-induced toxicity in rat primary dopaminergic cells

The rat primary dopaminergic cells were treated with HP extract(1 or 10 μg/mL) for 6 h and with 10 μM 6-OHDA for an additional 18 h. Fixed cells were stained with anti-TH antibody, visualized with DAB, and photographed with an optical light microscope at × 200 magnification. Normal group(A), 6-OHDA-only treated group(B), 6- OHDA + HP 1 μg/mL treated group(C), and 6-OHDA + HP 10 μg/mL treated group(D).
Scale bar = 100 μm.

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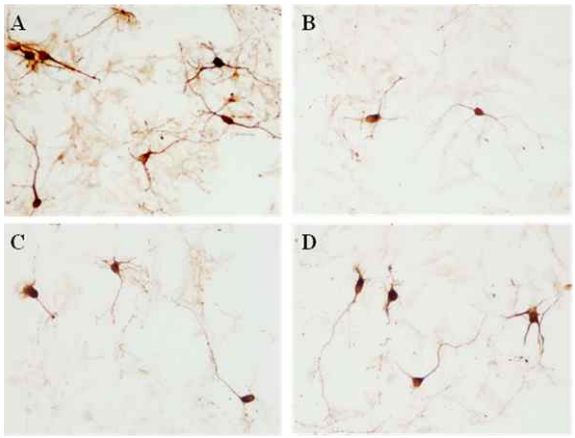

Fig. 5. The representative images of TH-IR cells for the effect of HP extract on MPP+-induced toxicity in rat primary dopaminergic cells
The rat primary dopaminergic cells were treated with HP extract(1 or 10 μg/mL) for 1 h and with 12 μM MPP+ for an additional 23 h. Fixed cells were stained with anti-TH antibody, visualized with DAB, and photographed with an optical light microscope at × 200 magnification. Normal group(A), MPP+-only treated group(B), MPP+ + HP 1 μg/mL treated group(C), and MPP+ + HP 10 μg/mL treated group(D). Scale bar = 100 μm.

C. Effects of HP pharmacoacupuncture on MPTP-induced behavioral changes in mice

To confirm the effect of HP pharmacoacupuncture on dopaminergic neurons in an *in vivo* Parkinson's disease model, we treated mice with HP pharmacoacupuncture and/or MPTP and analysed the results of a behavioral tests: the rota-rod test and the pole test. The rota-rod test and the pole test are the most commonly used tests to measure motor coordination in animals. One day and seven days after MPTP treatment, the time on the rod was significantly decreased by 92.9 ± 17.9 s and 103.8 ± 4.7 s, respectively, when mice of the normal group were on the rod for averagely 251.0 ± 15.4 and 198.5 ± 16.7 s, respectively. However, the treatment of saline or HP pharmacoacupuncture at ST_{36} for 4 weeks at 2-day-intervals did not protect significant movement impairment induced by MPTP toxicity(Fig. 6). In addition, T-turn and T-LA were significantly prolonged to 2.59 ± 0.15 s and 7.35 ± 0.20 s, respectively, on the first day after

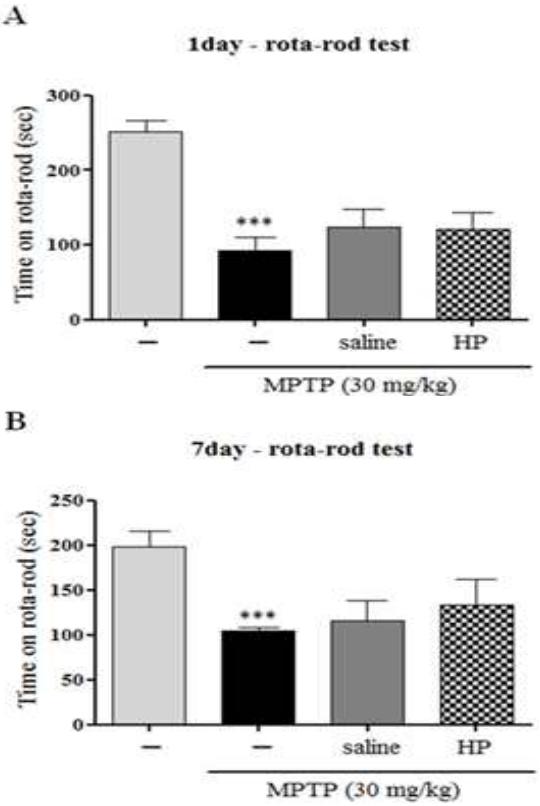

Fig. 6. Effect of HP pharmacoacupuncture on MPTP-induced hypokinesia in a mouse PD model
Mice were treated with saline or HP pharmacoacupuncture at ST_{36} for 4 weeks at 2-day intervals and with MPTP(30 mg/kg, by intraperitoneal injection) for 5 consecutive days on the 8_{th} treatment of HP pharmacoacupuncture. We conducted the rota-rod test after one day(A) and seven days(B) of the last MPTP injection. The time taken for the mice on the rod was recorded.
Values are indicated as the mean ± SEM of seven replicates.
*** : $p < 0.001$ compared with the normal group.

MPTP treatment and to 2.45 ± 0.15 s and 5.43 ±0.15 s, respectively, on the seventh day after MPTP treatment. However, saline or HP pharmacoacupuncture at ST_{36} treated groups did not show significant changes compared with the MPTP treated group but, HP pharmacoacupuncture treated group showed improvement tendency on movement ability(Fig. 7).

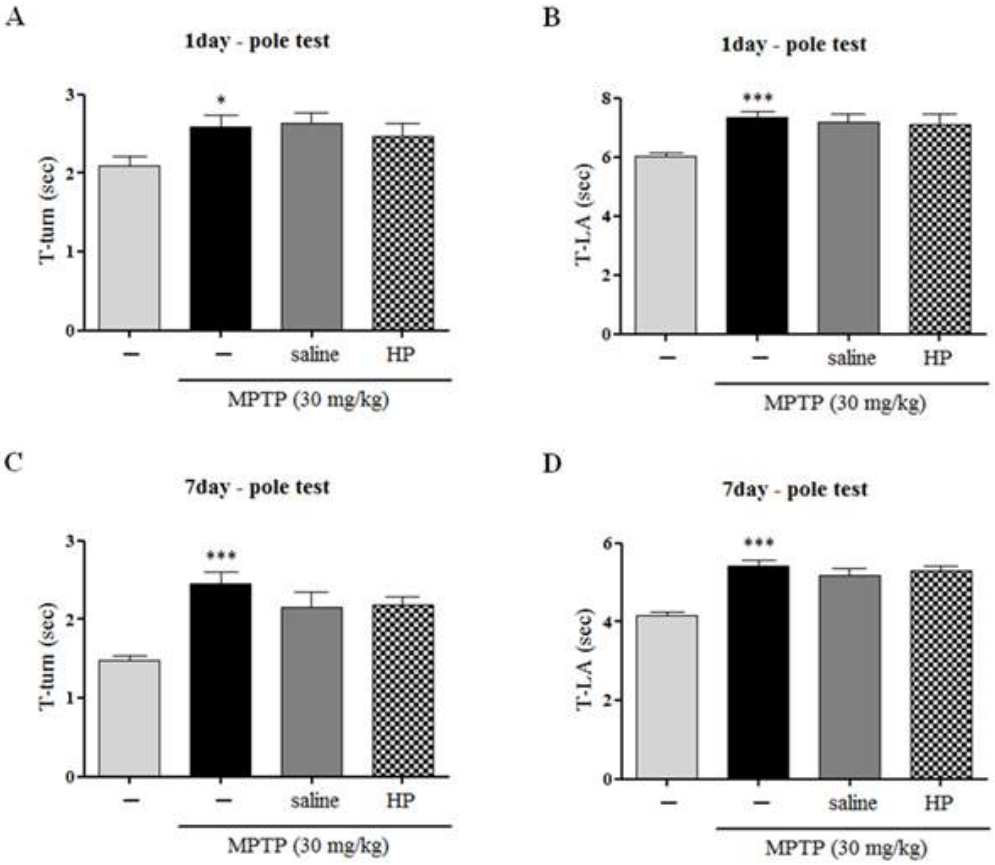

Fig. 7. Effect of HP pharmacoacupuncture on MPTP-induced bradykinesia in a mouse PD model

Mice were treated with saline or HP pharmacoacupuncture at ST_{36} for 4 weeks at 2-day intervals and with MPTP(30 mg/kg, by intraperitoneal injection) for 5 consecutive days on the 8th treatment of HP phar- macoacupuncture. We conducted the pole test after one day(A and B) and seven days(C and D) of the last MPTP injection. The time to turn completely downward(T-turn A and C) and to arrive at the floor(T-LA B and D) were recorded.

Values are indicated as the mean ± SEM of seven replicates.

*** : $p < 0.001$ compared with the normal group. * : $p < 0.05$ compared with the normal group.

D. Effects of HP pharmaco- acupuncture on MPTP-induced striatal dopaminergic neuronal degeneration in mice

We performed stereological analysis in the striatum and the substantia nigra of mice brain after treatment of HP pharmacoacupuncture and/or MPTP. Seven days after MPTP treatment, the number of TH-IR neurons in the substantia nigra was decreased by 36.6 ± 4.5 % compared with the normal value and the optical den- sity of TH-IR fibers in the striatum was decreased by 58.5 ± 2.2 % compared with the normal value. The treatment of HP pharmacoacupuncture at ST_{36} for 4 weeks at 2-day-intervals protected dopaminergic cells by 55.1 ± 3.2 % and dopaminergic fibers by 71.9 ±1.8 % in the substantia nigra and the striatum, respectively (Fig. 8). However, saline treatment on the same days did not show protective effects on dopaminergic neurons against MPTP toxicity in mice. The repre- sentative microphotographs of TH-immunostaining in the substantia nigra and the striatum are shown in Fig. 9 and Fig. 10, respectively.

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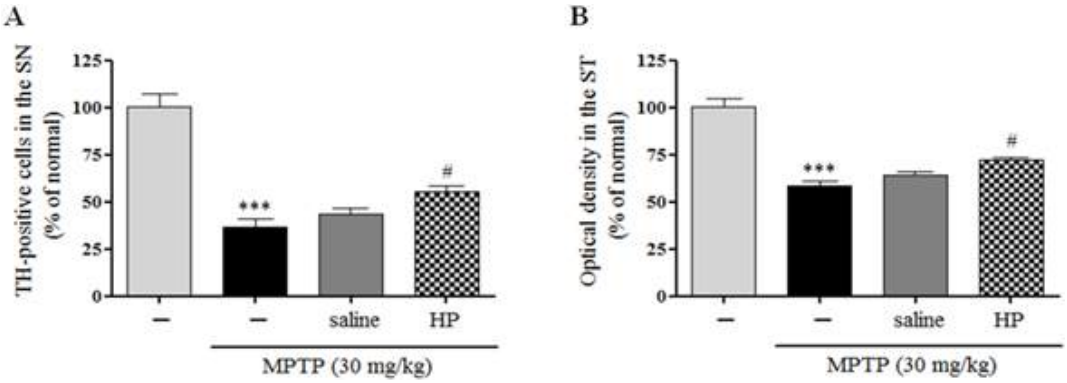

Fig. 8. Effect of HP pharmacoacupuncture on MPTP-induced dopaminergic neuronal damage in a mouse PD model

Mice were treated with saline or HP pharmacoacupuncture at ST_{36} for 4 weeks at 2-day intervalsand with MPTP(30 mg/kg, by intraperitoneal injection) for 5 consecutive days on the 8th treatment of HP. Dopaminergic neurons and fibers in the substantia nigra and the striatum, respectively, were visualised with TH-staining. Quantification of the TH-IR cells was performed by counting the number of TH-IR cells in the substantia nigra(A) and by measuring the optical density in the striatum(B). Data were expressed as a percentage of the normals. Values are indicated as the mean ± SEM of four replicates.

*** : $p < 0.001$ compared with the normal group.
# : $p < 0.05$ compared with the MPP+-only treated group. SN : substantia nigra. ST : Striatum.

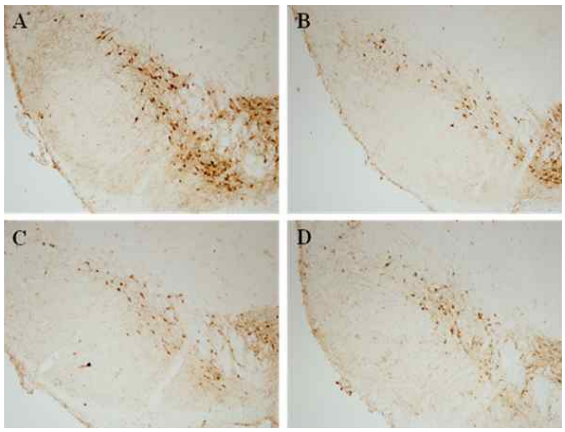

Fig. 9. The representative images of TH-IR cells in the substantia nigra for the effect of HP pharmacoacupuncture on MPTP-induced toxicity in a mouse PD model

Mice were treated with saline or HP pharmacoacupuncture at ST_{36} for 4 weeks at 2-day intervalsand with MPTP(30 mg/kg, by intraperitoneal injection) for 5 consecutive days on the 8th treatment of HP pharmacoacupuncture. Free-floating tissues were stained with anti-TH antibody, visualized with DAB, and photographed with an optical light microscope at × 100 magnification. Normal group(A), MPTP-only treated group(B), MPTP + saline-treated/ ST_{36} group(C), and MPTP + HP pharmacoacupuncture- treated/ST_{36} group(D).
Scale bar = 200 μm.

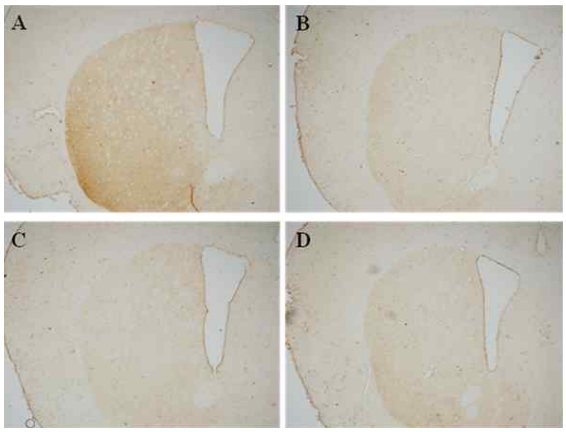

Fig. 10. The representative images of TH-IR fibers in the striatum for the effect of HP pharmacoacupuncture on MPTP-induced toxicity in a mouse PD model

Mice were treated with saline or HP pharmacoacupuncture at ST_{36} for 4 weeks at 2-day intervals and with MPTP(30 mg/kg, by intraperitoneal injection) for 5 consecutive days on the 8th treatment of HP pharmacoacupuncture. Free-floating tissues were stained with anti-TH antibody, visualized with DAB, and photographed with an optical light microscope at × 40 magnification. Normal group(A), MPTP-only treated group(B), MPTP + saline-treated/ST_{36} group(C), and MPTP + HP pharmacoacupuncture-treated/ ST_{36} group(D).
Scale bar = 200 μm.

Ⅳ. Discussion

Various types of acupuncture treatment have been used to prove the effects of neuroprotection against nigrostriatal dopaminergic neuronal cell loss. In animal studies, acupuncture and electro-acupuncture protected dopaminergic neurons against toxin-induced mouse/rat model[10-12]. Recently, review studies to assess the evidence for or against acupuncture as a treatment for PD are in progress[26,27]. In China, scalp acupuncture in combination with Madopa showed potential capability to improve cerebral glucose in PD patients[28]. In addition, many studies have found the neuroprotective effects of herbal acupuncture, especially bee venom acupuncture in MPTP-induced mouse model of PD[6,15].

In oriental medicine, HP acts on the Lung meridian, Liver meridian, Kidney meridian and is mainly used for the treatment of deficiency syndrome, including steaming bone disorder, dyspnea with coughing, nocturnal emission, night sweating, hemoptysis, impotence, hypogalactia. Since HP has been considered to be the very potent substance for replenishing vital energy, the blood and the essence, it has been used to strengthen the body resistence[16]. HP has been known to have various therapeutic effects including anti-inflammatory, antiviral, anti-oxidative, anti-mutagenic, analgesic properties, anticoagulant, anti-osteoporotic, and protection against radiation-induced apoptotic changes[29-33].

In the present study, treatment with various concentrations of HP extract had no influence on cell proliferation and caused no cell toxicity in PC_{12} and HT_{22} cells. *In vitro* and *vivo* procedures were utilized to investigate the neuroprotective effects of HP extract and pharmacoacupuncture against toxin-induced dopaminergic cell loss in primary mesencephalic culture and a mouse model of PD. Our study showed that HP extract significantly prevented the cell loss and protected neurites against MPP+ toxicity whereas it did not against 6-OHDA(Fig. 3).

Neurotoxins, namely 6-OHDA and MPTP, cause the degeneration of nigro-striatal dopamine neurons with the subsequent loss of striatal dopamine and has been used in animal models to develop antioxidant neuroprotective treatment methods[34]. MPTP and its metabolite MPP+ induce TH nitration, that is associated with the degeneration of dopaminergic neurons[35].

Based on these data, we assumed that HP pharmacoacupuncture would be more effective in MPTP-induced mouse model PD model than in other toxin-induced models. Therefore we performed behavioral tests and TH-immunohistologocal analysis in the striatum and the substantia nigra of mice brain, to investigate the effect of HP pharmacoacupuncture on dopaminergic neurons in an *in vivo* MPTP-induced PD mouse model. Since it is important to prevent disease in oriental medicine, we aimed this study to determine preliminarily if HP pharmacoacupuncture would prevent and reduce neuronal injury in an MPTP-induced mouse model of PD.

Although the *in vivo* treatment of HP pharmacoacupuncture at ST_{36} showed improvement tendency on movement ability and protected dopaminergic cells and fibers in the substantia nigra and the striatum, it did not show significant changes compared with the MPTP treated group(Fig. 6~8).

PD is characterized by akinesia, tremor, rigidity and postural instability[36]. To detect therapeutic effects and to evaluate behavioral outcome with the degree of nigral cell loss in individual mice, the pole test and the rota-rod test have been commonly used[37-41].

In our study, we could not confirm neuroprotective effects of HP pharmacoacupuncture in an *in vivo* Parkinson's disease model.

Although the etiology of PD remains unknown, several pathogenic factors play important roles in promoting the degenerative processes in the nigrostriatal system including oxidative stress, mitochondrial dysfunction, excitotoxicity, inflammatory processes, and apoptosis[42].

A number of studies have shown that medicinal herbs such as the hook of Uncaria rhynchophylla, Thuja orientalis leaves, *Cyperi Rhizoma*, and *Polygalae radix*, mainly by inhibiting generation of reactive oxygen species(ROS) and nitric oxide(NO), reduction of mitochondrial membrane potential, and caspase-3 activity, had protective effects on dopa-

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minergic neurons via their anti-oxidant and anti-apoptotic activity[43-46].

In China, Liu et al reported that electro-acupuncture at 100 Hz inhibited the upregulation of the level of necrosis factor α(TNF-α), interleukin 1β (IL-1β), and its neuroprotective role is likely to be mediated by by anti-inflammatory action[10].

Several reports have suggested MPTP induced microglial activation and enhanced, progressive dopaminergic degeneration[47-49]. Excessive activation of microglia, a major component of neuroinflammation, could be a driving force of the PD progression[47]. Accumulation of ROS, inflammatory-associated factors including cycloxygenase-2(COX-2) and inducible-nitirc oxide synthase(iNOS)-derived NO, and pro-inflammatory cytokines(including TNF-α, IL-1β) in the substantia nigra of PD patients further support that a state of chronic inflammation characterizes PD brain[50-55].

According to previous studies, HP pharmacoacupuncture was found to be effective to alleviate the expression profiles of inflammatory cytokines such as TNF-α, IL-1β, and interleukin 6(IL-6) in subchondral bone region of adjuvant-induced polyarthritic rat[24], and suppressed the over-expression of TNF-α, IL-1β in βamyloid peptide-induced mouse model of Alzheimer's disease[25].

From these results, we presumed that HP extract could protect neurons against neurotoxins by its anti-inflammatory effects. To demonstrate a more clear mechanism on HP extract effects against toxin-induced dopaminergic cell loss, further studies would be necessary.

ST_{36}, used in the present study, was selected according to previous studies that examined the anti-inflammatory effects of BV on MPTP-induced model of PD[15], and those of HP on rheumatoid arthritis model[24]. The other study suggested that acupuncture at ST_{36} suppressed nigrostriatal dopaminergic neuronal cell death in a rodent model of PD[11]. However, most of studies that examined HP as a therapeutic model showed effects through oral administration and intraperitoneal injection[33,56,57]. Although HP pharmacoacupuncture injection at CV_{12} showed positive effects on Alzheimer's disease model, virtually there's no difference between injection at CV_{12} and intrapenitoneal injection[25].

In our study, HP pharmacoacupuncture at 20 μL, according to the previous study that examined the anti-inflammatory effects of HP on rheumatoid arthritis model[24], was injected at ST_{36}. In primary mesencephalic culture, HP extract at 10 μg/mL significantly prevented the cell loss and protected neurites against MPP+ toxicity, but not at 1 μg/mL, in addition, many studies have suggested that HP showed effects dose-dependently[20,58,59].

Therefore we should verify neuroprotective effects of HP pharmacoacupuncture using more various acupoints, administration methods, and a proper dose to find out the most effective manners.

Further research is needed to identify the mechanism that mediate the action of HP, and additional *in vivo* studies should provide further evidence that HP pharmacoacupuncture is a promising alternative to current therapeutic agents for the prevention of dopaminergic neuronal degeneration.

Ⅴ. Conclusions

We found that HP extract protect MPP+-induced dopaminergic cell loss *in vitro*, and neuroprotective effects of HP pharmacoacupuncture on MPTP-induced striatal dopaminergic neuronal degeneration in an *in vivo* mouse model. However, it did not show significant changes compared with the MPTP treated group. HP pharmacoacupuncture could be a potential preventive treatment strategy in neurodegenerative diseases such as Parkinson's disease.

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