

Anti-apoptotic effect of *Shudipingchan* granule in the substantia nigra of rat models of Parkinson's disease

Qing Ye¹, Xiao-lei Yuan¹, Jing He¹, Jie Zhou¹, Can-xing Yuan^{1,*}, Xu-ming Yang^{2,*}

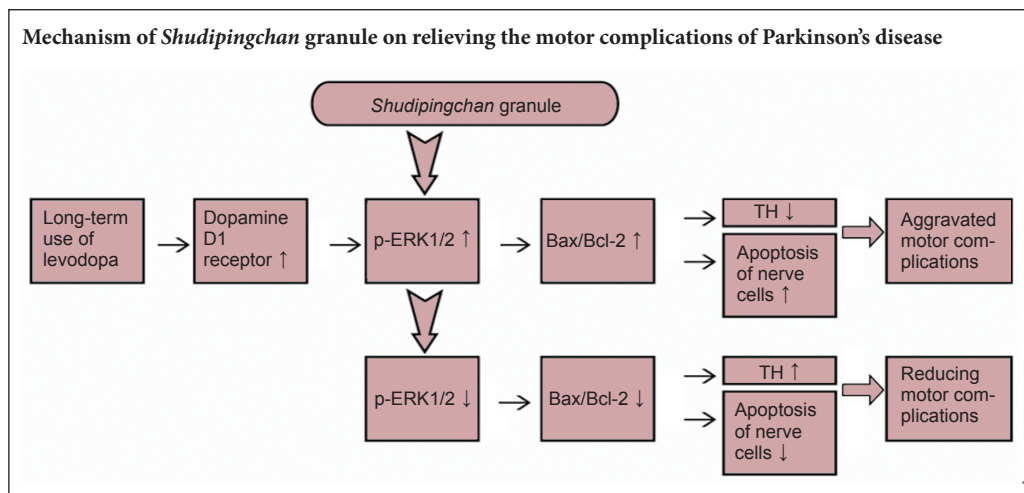
1 Department of Neurology, Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai, China
2 College of Acupuncture and Manipulation, Shanghai University of Traditional Chinese Medicine, Shanghai, China

How to cite this article: Ye Q, Yuan XL, He J, Zhou J, Yuan CX, Yang XM (2016) Anti-apoptotic effect of *Shudipingchan* granule in the substantia nigra of rat models of Parkinson's disease. *Neural Regen Res* 11(10):1625-1632.

Open access statement: This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

Funding: This study was financially supported by the National Natural Science Foundation of China, No. 81302926, 30472207; the Major Project of Shanghai Science and Technology Commission of China, No. 15401970100; the Shanghai Municipal Commission of Health and Family Planning of China, No. ZY3-RCPY-2-2005.

Graphical Abstract



*Correspondence to:
Can-xing Yuan or
Xu-ming Yang,
ycanxing@hotmail.com or
fslbest@126.com.

orcid:
0000-0001-6220-7590
(Can-xing Yuan)
0000-0002-2880-2609
(Xu-ming Yang)

doi: 10.4103/1673-5374.193242

Accepted: 2016-08-21

Abstract

Levodopa is the gold-standard treatment for Parkinson's disease. However, although it alleviates the clinical symptoms, it cannot delay the progressive apoptosis of dopaminergic neurons or prevent motor complications in the long term. In the present study, we investigated the effect of *Shudipingchan* granule on neuronal apoptosis in a rat model of Parkinson's disease, established by injecting 6-hydroxydopamine into the substantia nigra pars compacta and ventral tegmental area. We then administered levodopa (20 mg/kg intraperitoneally, twice daily) with or without *Shudipingchan* granule (7.5 mL/kg intragastrically, twice daily), for 4 weeks. The long-term use of levodopa accelerated apoptosis of nigral cells and worsened behavioral symptoms by activating the extracellular signal-regulated kinase pathway and downstream apoptotic factors. However, administration of *Shudipingchan* granule with levodopa reduced expression of phosphorylated extracellular signal-regulated kinase 1/2 and Bax, increased tyrosine hydroxylase and Bcl-2, reduced apoptosis in the substantia nigra, and markedly improved dyskinesia. These findings suggest that *Shudipingchan* granule suppresses neuronal apoptosis by inhibiting the hyperphosphorylation of extracellular signal-regulated kinase and downregulating expression of anti-apoptotic genes. *Shudipingchan* granule, used in combination with levodopa, can effectively reduce the symptoms of Parkinson's disease.

Key Words: nerve regeneration; Parkinson's disease; levodopa; substantia nigra; apoptosis; *Shudipingchan* granule; extracellular signal-regulated kinase pathway; behavior; neural regeneration

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by degeneration and loss of dopaminergic neurons in the substantia nigra. Apoptosis is strongly implicated in this neuronal loss, but the signal transduction pathway is unclear (Jiménez-Urbietta et al., 2015). The introduction of

levodopa (LD) was an important milestone in the history of PD treatment. However, patients with PD must be treated with LD-based drugs for a long time, and more than 50% patients will develop complications (Lipski et al., 2011) such as LD-induced dyskinesia. Furthermore, LD becomes less effective after 5–8 years of treatment. These issues seriously

affect patients' quality of life (Encarnacion and Hauser, 2008; Sharma et al., 2015).

The appearance of motor complications with LD may be associated with its activation of the extracellular signal-regulated kinase (ERK) pathway (Song et al., 2009; Cerovic et al., 2015). The phosphorylation (activation) of ERK1/2 and its translocation from the cytoplasm to the nucleus mediates the transcription of multiple apoptotic genes, leading to neuronal apoptosis (Song et al., 2009; Cerovic et al., 2015). Therefore, to delay the progression of PD, it is important to prevent or slow the progressive degeneration of dopaminergic neurons in the substantia nigra and reduce the toxic effects of LD.

LD-induced dyskinesia is caused mainly by the activation of the direct pathway, which is mediated by dopamine D1 receptors (Ye et al., 2014). *Shudipingchan* granule, a traditional Chinese medicine (TCM), can relieve LD-induced dyskinesia symptoms by decreasing the activity and affinity of the dopamine D1 receptor, downregulating gene expression, and inhibiting the overactivation of the direct pathway (Ye et al., 2014).

Here, in an attempt to prevent the complications associated with LD therapy, we investigated the effects of *Shudipingchan* granule in combination with LD in a rat model of PD. We evaluated behavior, nigral apoptosis, and the expression of apoptosis-related factors, to investigate whether this TCM can protect neurons by inhibiting activation of the ERK pathway, and prevent the progression of PD.

Materials and Methods

Ethics statement and animals

This study was approved by the Experimental Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine, China (approval No. 09047), and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Precautions were taken to minimize the suffering and the number of animals used in each experiment.

Seventy-two adult male specific-pathogen-free Sprague-Dawley rats, weighing 180–220 g, were provided by the Laboratory Animal Center, Shanghai University of Traditional Chinese Medicine, China (animal license No. SCXK (Hu) 2014-0005).

Preparation of PD models

Sixty male rats were anesthetized and placed in a stereotaxic frame (Narishige, Tokyo, Japan). We established the 6-hydroxydopamine (12 μ L) PD model using the two-point method (Teng et al., 2015). The coordinates of two points in the brain were determined according to the *Rat Brain Stereotaxic Atlas* (Bao and Shu, 1991), as follows: (1) substantia nigra pars compacta: 4.8 mm behind the anterior fontanelle, 2.0 mm from the right sagittal suture, and 8.0 mm below the dura; (2) ventral tegmental area: 4.8 mm behind the anterior fontanelle, 1.2 mm from the right sagittal suture and 8.2 mm below the dura. We punctured the skull using a dental drill and injected 6 μ g 6-hydroxydopamine

(Sigma, St. Louis, MO, USA) into each region at a rate of 1 μ L/min. The needle was retained in place for 10 minutes before being removed. The incision was closed and the animals allowed to recover. Rats were injected with apomorphine (0.5 mg/kg intraperitoneally; Sigma) on the 14th day after modeling. Rats that constantly rotated to the left more than seven times per minute were regarded as successful models of PD (Li et al., 2010). In total, 36 rats were successful models and used in the subsequent experiments. The remaining 12 naïve male rats were used as controls and underwent the same procedures but were injected with 6 μ L normal saline instead of 6-hydroxydopamine.

Drug administration

Shudipingchan granule was composed of 15 g prepared rehmannia root, 15 g Asiatic Cornelian cherry fruit, 20 g Chinese taxillus herb, 15 g tall gastrodis tuber, 10 g stiff silkworm, 15 g kudzu vine root, 15 g zedoray rhizome, 15 g danshen root, 15 g Jackinthe pulp tuber, and 6 g scorpion. It was made into granules by Jiangyin Tianjiang Pharmaceutical Co., Ltd., China (lot No. 1410302) and 19.2 g (equivalent to adult dosage, 128 g crude drug) was dissolved in 100 mL of normal saline, so that each mL contained 1.28 g crude drug.

The PD models were equally and randomly allocated to three groups: PD, LD, and LD + TCM ($n = 12$ per group). Rats in the LD group were given 20 mg/kg LD (Sigma) and 2.5 mg/mL benserazide (Sigma) dissolved in sterile normal saline containing 0.05% ethyl alcohol and 0.1% ascorbic acid (twice a day, intraperitoneally). Rats in the LD + TCM group received the same LD preparation in addition to 7.5 mL/kg TCM (twice a day, by gavage). Rats in the PD group and the 12 normal control rats received equivalent volumes of normal saline for gavage and intraperitoneal injection. Administration was twice daily for 4 weeks, in all groups.

Behavioral evaluation

Rotation number was measured by a rotation meter (Beijing Grand Technology Co., Ltd., Beijing, China). Number of turns, latency to turn, maximum rotation, and total duration of turning were recorded from the beginning of rotation for 30 minutes (Cao et al., 2012). Rats were observed in a separate cage once every 7 days after model establishment.

The abnormal involuntary movement scale (AIMS) score was evaluated in the LD and LD + TCM groups once a week from 14 days after model induction, as previously described (Cao et al., 2004). Assessments lasted 120 minutes, separated into 30 minute bins, and started immediately after the injection of LD. We scored four regions separately, investigating trunk, foreleg, orofacial region, and axial movement. Each region received a score of 0–4: 0, no abnormal movement; 1, occasional abnormal movement; 2, frequent abnormal movement; 3, continuous abnormal movement that stops upon stimulation (removal from cage); 4, continuous abnormal movement that does not stop upon stimulation. The

highest score for each assessment was 16, with a total possible score of 64 per day.

Tissue preparation

On day 28, 2 hours after the last dose, six rats from each group were anesthetized with 10% chloral hydrate and perfused through the heart with 4% paraformaldehyde. The brain was removed and postfixed in the same solution for 12 hours. Brain tissue was cut into blocks, dehydrated, dipped in wax and embedded in paraffin. The paraffin blocks were sliced into 5 μm -thick sections using a microtome (Leica RM2235, Wetzlar, Germany). Serial sections containing the substantia nigra were consecutively mounted onto slides for immunohistochemistry, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and Nissl staining, so that each slide contained every third section.

Immunohistochemistry

Mounted sections containing the substantia nigra were dewaxed and hydrated, and antigen retrieval was performed using a microwave. The slides were then incubated with 3% hydrogen peroxide, normal goat serum, and polyclonal rabbit antibodies against Bax, Bcl-2, tyrosine hydroxylase (TH), and phosphorylated (p)-ERK1/2 (all 1:1,000; Cell Signaling Technology, Danvers, MA, USA), at 4°C overnight. The sections were then incubated with biotinylated goat anti-rabbit IgG (1:1,000; Abcam, Cambridge, UK) at 37°C for 30 minutes and streptavidin/horseradish peroxidase solution at 37°C for 30 minutes. Proteins were visualized using 3,3'-diaminobenzidine, counterstained with hematoxylin, dehydrated, permeabilized and mounted. Immunopositive cells appeared as brownish-yellow granules in the cytoplasm under a light microscope (Nikon E100, Shanghai, China). Ten high-power fields were randomly selected for quantification, and the relative expression of Bax, TH, Bcl-2 and p-ERK1/2 was calculated from pixel dots (Hu and Chen, 2015), with one pixel dot equal to 0.095 μm^2 .

TUNEL

Mounted sections containing the substantia nigra were dewaxed, incubated in proteinase K (Cell Signaling Technology, Danvers, MA, USA) at 37°C for 20 minutes, and mixed with 50 μL of terminal deoxynucleotidyl transferase and 450 μL of fluorescein labeled deoxyuridine triphosphate at 37°C for 20 minutes. The slides were dried, and the sections were treated with 50 μL of TUNEL reaction mixture in a dark and wet box, at 37°C for 60 minutes in accordance with the instructions of a TUNEL reaction kit (TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, Liaoning Province, China). After three washes with PBS, the slides were viewed under a fluorescence microscope (Nikon BIOE342, Tokyo, Japan).

Nissl staining

Mounted sections containing the substantia nigra were dewaxed, hydrated and stained with 5% Dahlia aqueous solution (Chroma, Berlin, Germany) for 1–2 minutes. After

washing with deionized water, the sections were decolorized with 95% ethanol for 1–2 minutes, Nissl bodies were permeabilized with xylene, and the sections were dehydrated with anhydrous ethanol. The slides were coverslipped with resin-based mounting medium and viewed under a light microscope (Nikon E100) (Yu et al., 2007).

Western blot assay

The remaining six rats from each group were sacrificed using an overdose of anesthesia and their brains were collected rapidly. The substantia nigra was dissected out, homogenized by high-frequency oscillation in cell lysis buffer at 4°C for 30 minutes, and centrifuged at 12,000 r/min at 4°C for 15 minutes. The supernatant was collected and stored at –80°C until use. After protein quantification, four times the volume of sample buffer was added, and proteins were denatured at 95°C for 5 minutes. Next, samples containing 20 μg of protein were added to a 10% sodium dodecylsulfate polyacrylamide gel, separated using electrophoresis, and transferred to a nitrocellulose membrane. The membrane was incubated with rabbit monoclonal antibodies against rat Bcl-2, Bax, TH and p-ERK1/2 (all 1:1,000; Cell Signaling Technology), and β -actin (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), at 4°C overnight. After washing with Tris-buffered saline containing Tween 20 (TBST), the membrane was incubated with biotinylated goat anti-rabbit IgG (1:5,000; Cell Signaling Technology) for 2 hours at room temperature. After washing again in TBST, the membrane was incubated with sucrose phosphorylase at room temperature for 30 minutes and protein bands were visualized with 3,3'-diaminobenzidine. Average optical density values of specific protein bands were calculated using the CMIAS true color medical image analysis system (Mike Audi, Beijing, China).

Statistical analysis

All results are expressed as the mean \pm SD. Graphs were made in Excel and statistical analysis was performed using SPSS 19.0 (IBM, Armonk, NY, USA). For behavioral tests, Mauchly's test of sphericity was used to judge whether there were relations among the repeated measures data. If any ($P < 0.05$), multivariate analysis of variance should be taken next, or Greenhouse-Geisser corrected results should be taken. Then the Bonferroni *t*-test was used for pairwise comparisons of each group. Multivariate analysis was used to compare different groups at each time point. For immunohistochemistry, western blot assay, TUNEL and Nissl staining, data were normally distributed and were compared using a one-way analysis of variance and independent samples *t*-test. $P < 0.05$ was considered statistically significant.

Results

Shudipingchan granule improved PD-related behavior

The number of rotations per 30 minutes (Figure 1) did not differ significantly between groups before the start of treatment (day 0) ($P > 0.05$). In the LD group, the number of rotations on days 7, 14 and 21 was lower than on day 0 ($P <$

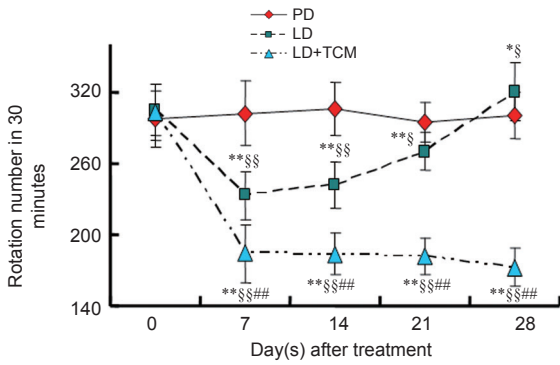


Figure 1 Effect of *Shudipingchan* granule on the number of apomorphine-induced rotations in rat models of PD. Rat models of PD received saline (PD), LD alone (LD), or LD and *Shudipingchan* granule (LD + TCM). Data are expressed as the mean \pm SD ($n = 12$ rats per group). Bonferroni t -test was used for pairwise comparisons between time points within each group. Multivariate analysis was for pairwise comparisons between groups at each time. * $P < 0.05$, ** $P < 0.01$, vs. day 0 (before treatment); § $P < 0.05$, §§ $P < 0.01$, vs. PD group; ### $P < 0.01$, vs. LD group. PD: Parkinson's disease; LD: levodopa; TCM: traditional Chinese medicine (*Shudipingchan* granule).

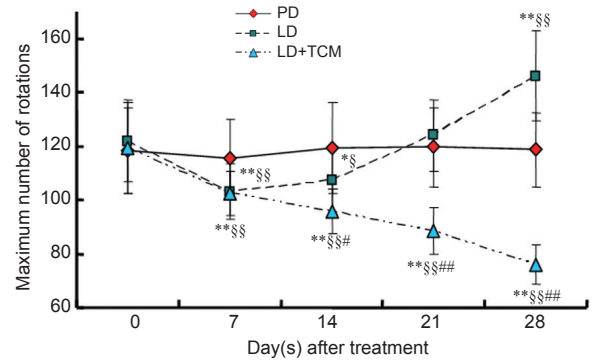


Figure 2 Effect of *Shudipingchan* granule on maximum number of rotations in 30 minutes in rat models of PD. Rat models of PD received saline (PD), LD alone (LD), or LD and *Shudipingchan* granule (LD + TCM). Data are expressed as the mean \pm SD ($n = 12$ rats per group). Bonferroni t -test was used for pairwise comparisons between time points within each group. Multivariate analysis was for pairwise comparisons between groups at each time. * $P < 0.05$, ** $P < 0.01$, vs. day 0 (before treatment); § $P < 0.05$, §§ $P < 0.01$, vs. PD group; # $P < 0.05$, ### $P < 0.01$, vs. LD group. PD: Parkinson's disease; LD: levodopa; TCM: traditional Chinese medicine (*Shudipingchan* granule).

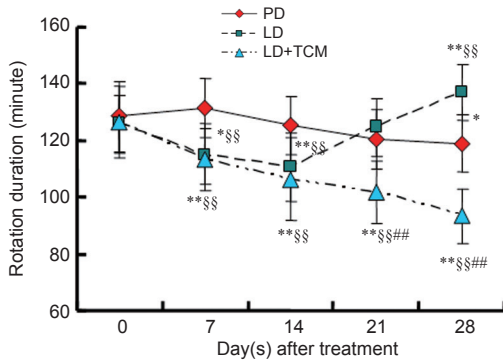


Figure 3 Effect of *Shudipingchan* granule on the duration of apomorphine-induced rotation in rat models of PD. Rat models of PD received saline (PD), LD alone (LD), or LD and *Shudipingchan* granule (LD + TCM). Data are expressed as the mean \pm SD ($n = 12$ rats per group). Bonferroni t -test was used for pairwise comparisons between time points within each group. Multivariate analysis was for pairwise comparisons between groups at each time. * $P < 0.05$, ** $P < 0.01$, vs. day 0 (before treatment); §§ $P < 0.01$, vs. PD group; ### $P < 0.01$, vs. LD group. PD: Parkinson's disease; LD: levodopa; TCM: traditional Chinese medicine (*Shudipingchan* granule).

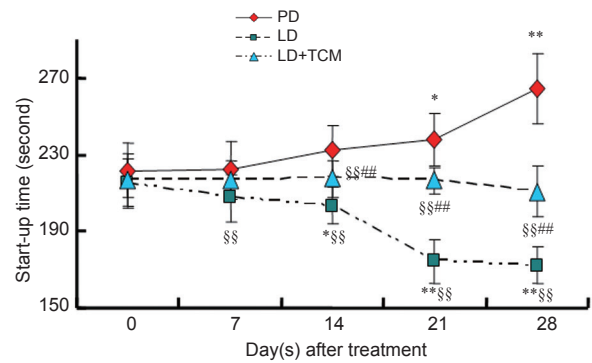


Figure 4 Effect of *Shudipingchan* granule on the latency of apomorphine-induced rotation in rat models of PD. Rat models of PD received saline (PD), LD alone (LD), or LD and *Shudipingchan* granule (LD + TCM). Data are expressed as the mean \pm SD ($n = 12$ rats per group). Bonferroni t -test was used for pairwise comparisons between time points within each group. Multivariate analysis was for pairwise comparisons between groups at each time. * $P < 0.05$, ** $P < 0.01$, vs. day 0 (before treatment); §§ $P < 0.01$, vs. PD group; ### $P < 0.01$, vs. LD group. PD: Parkinson's disease; LD: levodopa; TCM: traditional Chinese medicine (*Shudipingchan* granule).

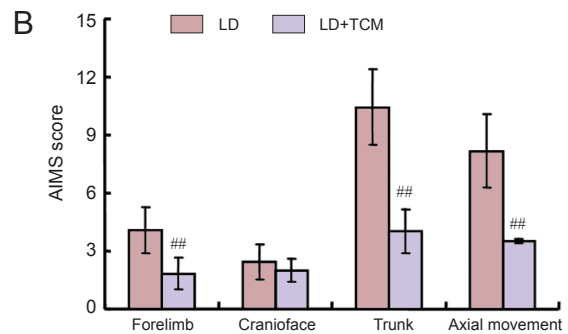
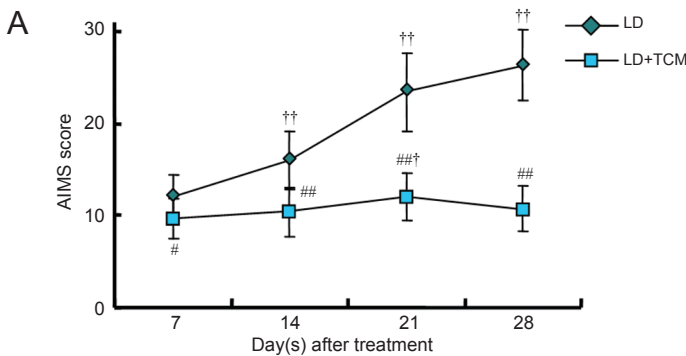


Figure 5 Effect of *Shudipingchan* granule on AIMS of PD rats. Rat models of PD received LD alone (LD) or LD with *Shudipingchan* granule (LD + TCM). (A) Total AIMS score; (B) AIMS scores of different body parts on day 28. Data are expressed as the mean \pm SD ($n = 12$ rats per group). Independent samples t -tests were used to compare groups. # $P < 0.05$, ### $P < 0.01$, vs. LD group; † $P < 0.05$, †† $P < 0.01$, vs. day 7. PD: Parkinson's disease; LD: levodopa; TCM: traditional Chinese medicine; AIMS: abnormal involuntary movement scale.

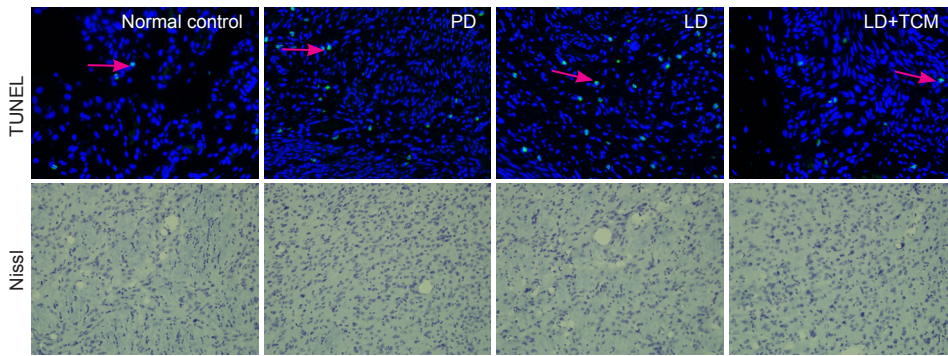


Figure 6 Effect of *Shudipingchan* granule on the number of apoptotic cells and Nissl bodies in the substantia nigra of rat models of PD ($\times 200$). Normal control group: Naïve Rats. Experimental groups: Rat models of PD received saline (PD), LD alone (LD), or LD and *Shudipingchan* granule (LD + TCM). Apoptotic cells (arrows) fluoresced green after TUNEL staining. The dark blue granules are Nissl bodies, which indicate surviving neurons after Nissl staining. There were fewer apoptotic cells in the substantia nigra of the LD + TCM group than in the LD and PD groups. PD: Parkinson's disease; LD: levodopa; TCM: traditional Chinese medicine (*Shudipingchan* granule); TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling.

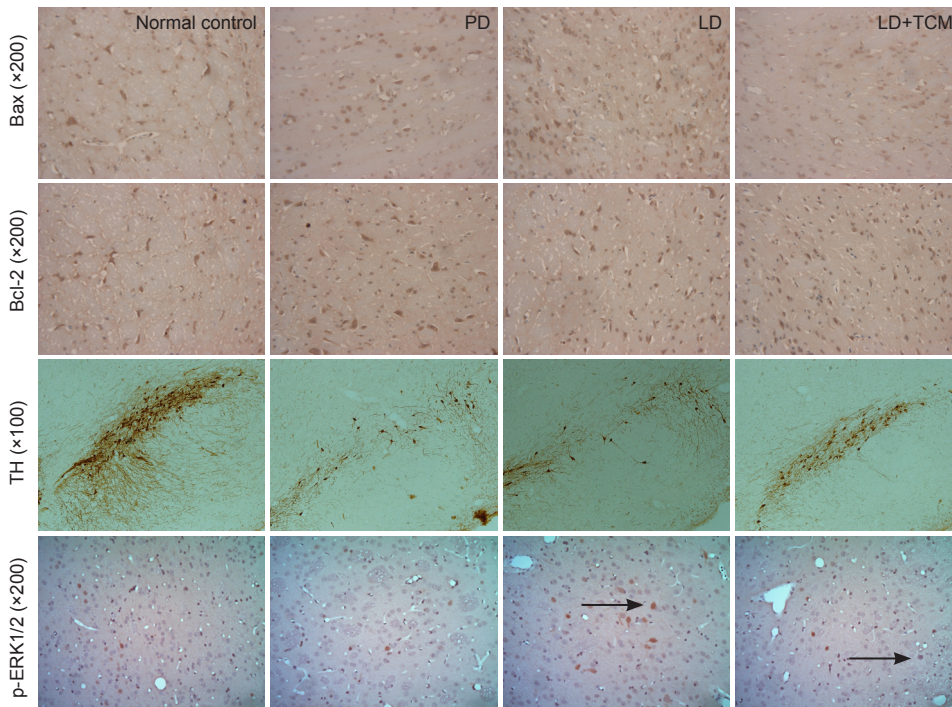


Figure 7 Effect of *Shudipingchan* granule on the expression of apoptosis-related factors and ERK in the substantia nigra of rat models of PD (immunohistochemical staining). Normal control group: Naïve rats. Experimental groups: Rat models of PD received saline (PD), LD alone (LD), or LD and *Shudipingchan* granule (LD + TCM). Immunoreactivity appeared as brown granules or filaments. Bax and Bcl-2 were both expressed in the cytoplasm. Tyrosine hydroxylase (TH) was mainly expressed in the cytoplasm, and appeared as brown filaments. p-ERK1/2 (arrows) appeared as brown granules. p-ERK1/2 was mainly expressed in the nucleus in the LD group, and in the cytoplasm in the LD + TCM group. PD: Parkinson's disease; LD: levodopa; TCM: traditional Chinese medicine (*Shudipingchan* granule).

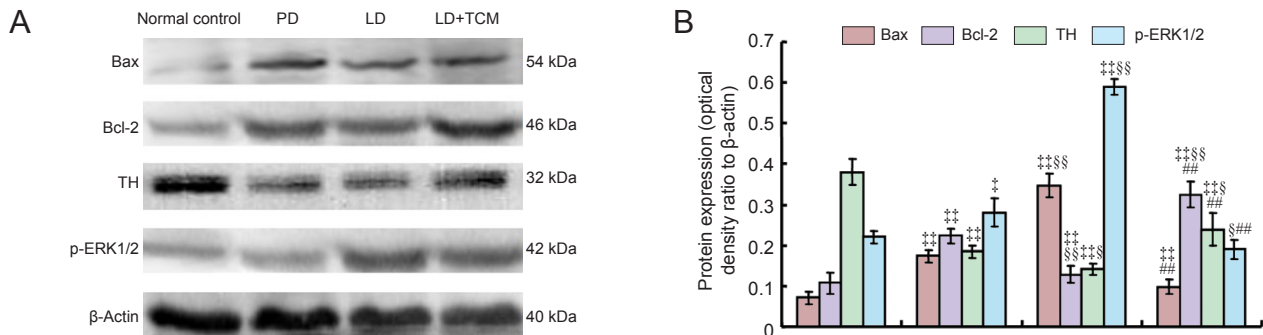


Figure 8 Effect of *Shudipingchan* granule on protein expression of apoptosis-related factors in the substantia nigra of rat models of PD (western blot assay). Normal control group: Naïve rats. Experimental groups: Rat models of PD received saline (PD), LD alone (LD), or LD and *Shudipingchan* granule (LD + TCM). Data are expressed as the mean \pm SD with six rats per group. One-way analysis of variance was used to identify differences among the four groups. $\ddagger P < 0.05$, $\ddagger\ddagger P < 0.01$, vs. normal control group; $\S P < 0.05$, $\S\S P < 0.01$, vs. PD group; $\#\#\# P < 0.01$, vs. LD group. PD: Parkinson's disease; LD: levodopa; TCM: traditional Chinese medicine (*Shudipingchan* granule); TH: tyrosine hydroxylase; p-ERK1/2: phosphorylated extracellular signal-regulated kinase 1/2.

0.01 or $P < 0.05$), but on day 28 it was significantly higher than before treatment ($P < 0.05$). In the LD + TCM group, a steady decrease in number of rotations was observed throughout the experiment ($P < 0.01$). The number of rotations was lower in the LD group than in the PD group on days 7, 14 and 21 ($P < 0.01$), but higher than in the PD group on day 28 ($P < 0.05$). In the LD + TCM group, the number of rotations was lower than that in the PD group from day 7 onwards ($P < 0.01$). Furthermore, the number of rotations in the LD + TCM group was significantly lower than in the LD group at all time points after treatment ($P < 0.01$; **Figure 1**).

The maximum number of rotations per 30 minutes in the PD group did not change significantly at any time point ($P > 0.05$). In the LD group, it was lower on days 7 and 14 ($P < 0.05$) and higher on day 28 ($P < 0.01$) than before treatment. The maximum number of rotations in the LD + TCM group reduced continually between days 7 and 28 ($P < 0.01$). In the LD group, it was lower than in the PD group on days 7 and 14, and higher on day 28. The maximum number of rotations per 30 minutes in the LD + TCM group was lower than that in the PD group from day 7 onwards ($P < 0.01$). Compared with the LD group, the maximum number of rotations per 30 minutes in the LD + TCM group was significantly lower on days 14, 21 and 28 after treatment ($P < 0.05$ or $P < 0.01$; **Figure 2**).

The duration of turning in the PD group was shorter on day 28 than on day 0 ($P < 0.05$). In the LD group, rotation lasted a shorter time on days 7 and 14 ($P < 0.05$), and longer on days 21 and 28 ($P < 0.01$) than on day 0. In the LD + TCM group, turning duration became steadily shorter over time ($P < 0.01$). The turning duration in the LD group was shorter than that in the PD group on days 7 and 14, but longer on day 28 ($P < 0.01$). In the LD + TCM group, rotation lasted a shorter time than in the PD group at all time points ($P < 0.01$), and was significantly shorter than in the LD group on days 21 and 28 ($P < 0.01$; **Figure 3**).

Compared with day 0, the rotation latency in the PD group increased on days 21 and 28 ($P < 0.05$ or $P < 0.01$). In the LD group, it was shorter on days 14, 21 and 28 than on day 0 ($P < 0.05$). The LD + TCM group showed no changes over time ($P > 0.05$). Rotation latency in the LD group was less than that in the PD group at all time points after treatment ($P < 0.01$). In the LD + TCM group, the latency was shorter than in the PD group from day 14 ($P < 0.01$). Compared with the LD group, latency in the LD + TCM group was significantly greater on days 14, 21 and 28 ($P < 0.01$; **Figure 4**).

The AIMS score of the LD group increased from day 7 to day 28 ($P < 0.01$), and was over 20 from day 21. In the LD + TCM group, however, a higher score was noted only on day 21 when compared with day 7 ($P < 0.05$), and the score was lower than that in the LD group at all time points examined ($P < 0.05$ or $P < 0.01$; **Figure 5A**).

Forelimb, trunk, and axial movement scores were significantly lower in the LD + TCM group than in the LD group on day 28 ($P < 0.05$), but there was no significant difference in craniofacial movement between the two groups ($P > 0.05$; **Figure 5B**).

Shudipingchan granule decreased apoptotic cells and Nissl bodies in the substantia nigra of PD rats

TUNEL and Nissl staining showed that there were more apoptotic cells in the substantia nigra of the PD, LD, and LD + TCM groups than in the normal control group ($P < 0.01$). There were also more apoptotic cells in the substantia nigra of the LD group than in the PD group ($P < 0.05$). However, there were fewer apoptotic cells in the LD + TCM group than in the LD and PD groups ($P < 0.01$; **Figure 6, Table 1**).

Effects of Shudipingchan granule on immunoreactivity of apoptosis-related factors and ERK in the substantia nigra of rat models of PD

Immunohistochemistry showed that the immunoreactivity of Bax and Bcl-2 in all experimental groups was significantly greater than in the normal control group ($P < 0.01$). Bax immunoreactivity was significantly greater, and Bcl-2 significantly lower, in the LD group than in the PD group ($P < 0.01$). Compared with the LD and PD groups, the immunoreactivity of Bax was lower and that of Bcl-2 was higher, in the LD + TCM group ($P < 0.01$; **Figure 7, Table 2**).

The immunoreactivity of TH in all three experimental groups was significantly lower than that in the control group ($P < 0.01$). Immunoreactivity of TH was significantly lower in the LD group than in the PD group ($P < 0.01$), and compared with the LD group, TH immunoreactivity was significantly greater in the LD + TCM group ($P < 0.01$; **Figure 7, Table 2**).

p-ERK1/2 immunoreactivity in the PD group was not significantly different from control ($P > 0.05$). However, compared with the PD and control groups, p-ERK1/2 immunoreactivity was significantly greater in the LD and LD + TCM groups ($P < 0.01$). Compared with the LD group, the immunoreactivity of p-ERK1/2 decreased significantly in the LD + TCM group ($P < 0.01$; **Figure 7, Table 2**).

Western blot assay showed that protein expression of Bax and Bcl-2 was significantly greater ($P < 0.01$) and TH significantly lower ($P < 0.05$) in the PD, LD and LD + TCM groups than in the control group. Compared with the PD group, protein expression of Bax and p-ERK1/2 was greater ($P < 0.01$) and Bcl-2 and TH expression was lower ($P < 0.01$) in the LD group. Compared with the LD group, protein expression of Bax and p-ERK1/2 was lower and Bcl-2 and TH higher ($P < 0.01$) in the LD + TCM group (**Figure 8**).

Discussion

PD is a common neurodegenerative disease in the elderly. It is characterized by the selective loss of dopaminergic neurons in the substantia nigra and significant reduction in striatal dopamine content (Burguillos et al., 2011). ERK1/2 is one of the main mitogen-activated protein kinases, and is part of a very important signal transduction pathway for the normal development and function of the central nervous system. It is involved in regulating functions of neurons, such as proliferation, differentiation, survival, and death (Lee et al., 2015; Wang et al., 2015).

Long-term LD treatment in mouse models of PD increases

Table 2 Effect of *Shudipingchan* granule on the immunoreactivities of apoptosis-related factors and ERK in the substantia nigra of rat models of PD (immunohistochemistry)

Group	Bax	Bcl-2	TH	p-ERK1/2
Normal control	8.63±1.30	12.67±2.21	93.88±12.85	5.25±0.34
PD	40.88±2.64 ^{‡‡}	24.17±4.84 ^{‡‡}	74.00±8.85 ^{‡‡}	4.76±0.23
LD	51.13±4.53 ^{‡‡‡§§}	18.67±3.86 ^{‡‡§§}	62.38±7.20 ^{‡‡§§}	8.09±0.65 ^{‡‡§§}
LD+TCM	33.25±3.91 ^{‡‡‡§§##}	35.83±5.56 ^{‡‡§§##}	71.13±8.48 ^{‡‡##}	4.33±0.47 ^{‡##}

Normal control group: Naïve rats. Experimental groups: Rat models of PD received saline (PD), LD alone (LD), or LD and *Shudipingchan* granule (LD + TCM). Immunoreactivity of a target protein was calculated as pixel points. Data are expressed as the mean ± SD with 12 rats in each group. One-way analysis of variance was used to identify differences between the four groups. ‡*P* < 0.05, ‡‡*P* < 0.01, vs. normal control group; §§*P* < 0.01, vs. PD group; ##*P* < 0.01, vs. LD group. PD: Parkinson's disease; LD: levodopa; TCM: traditional Chinese medicine (*Shudipingchan* granule); ERK: extracellular signal-regulated kinase; TH: tyrosine hydroxylase; p-ERK1/2: phosphorylated extracellular signal-regulated kinase 1/2.

Table 1 Effect of *Shudipingchan* granule on apoptotic cells and Nissl bodies in the substantia nigra of rat models of PD

Group	Apoptotic cells (/200 μm ²)	Nissl bodies (/100 μm ²)
Normal control	22.44±3.64	154.83±8.09
PD	75.56±4.95 ^{‡‡}	65.17±5.71 ^{‡‡}
LD	80.78±7.43 ^{‡‡§§}	46.17±3.43 ^{‡‡§§}
LD+TCM	49.89±4.17 ^{‡‡§§##}	67.67±4.23 ^{‡‡##}

Normal control group: Naïve rats. Experimental groups: Rat models of PD received saline (PD), LD alone (LD), or LD and *Shudipingchan* granule (LD + TCM). Data are expressed as the mean ± SD (*n* = 12 rats per group). One-way analysis of variance was used to compare the four groups. ‡‡*P* < 0.01, vs. normal control group; §*P* < 0.05, §§*P* < 0.01, vs. PD group; ##*P* < 0.01, vs. LD group. PD: Parkinson's disease; LD: levodopa; TCM: traditional Chinese medicine (*Shudipingchan* granule).

ERK1/2 phosphorylation in neurons in the substantia nigra. Therefore, the activation of ERK1/2 in the substantia nigra may participate in the occurrence of chronic LD-induced abnormal behavior (Chagniel et al., 2012; Cote and Kuzhikanathil, 2015; Yang et al., 2015). Recent studies have shown that the occurrence of motor complications in PD is strongly associated with the direct pathway, mediated by the expression of the dopamine D1 receptor in the nigrostriatal system and activation of the downstream ERK signal transduction pathway (Lindenbach et al., 2013; Mango et al., 2014). The phosphorylation of ERK occurs mainly in direct-pathway projection neurons (Lindenbach et al., 2013; Mango et al., 2014). Blocking D1 receptors with SCH23390 was shown to markedly reduce AIMS and p-ERK1/2 in rats with LD-induced dyskinesia (Xu et al., 2008). Such findings show that ERK1/2 is activated mainly through the direct pathway.

Apomorphine-induced rotation is a common behavioral evaluation method of rat models of PD. In addition, latency, maximum number of rotations within 30 minutes, and turning duration, can also be used to evaluate the degree of damage to the substantia nigra. In other words, the sooner the rat starts rotating, the faster it rotates, and the longer it keeps rotating, the more severe the damage (Ding et al., 2011). According to our results, LD had good therapeutic effects at the beginning of the treatment period, and each behavioral score was better than in the PD group. However, as administration of LD continued, rotations began to increase in number and duration, and latency to rotate reduced. The AIMS scores in

the LD group also increased, especially those for trunk and axial movement, and LD-induced dyskinesia was observed. In the LD + TCM group, each behavior score was better than in the PD group and there was no rebound in the later treatment period. AIMS scores were markedly lower in the LD + TCM group than in the LD group during the same period. Forelimb, trunk and axial movement scores in particular showed improvement across the whole treatment period, and LD-induced dyskinesia did not occur. The expression of p-ERK1/2 was not significantly different between the PD and normal control groups. p-ERK1/2 expression was higher in the LD group than in the PD group. ERK1/2 was mainly localized in the nucleus, and the ERK pathway was activated. ERK expression was mainly localized in the cytoplasm, whereas p-ERK1/2 was transferred into the nucleus. The activation of p-ERK could promote the expression of Bax protein, and the dimerization of Bad/Bcl-xL (Park et al., 2013). When Bcl-2 protein is in the form of a homodimer, the cells tend to survive. However, if Bax protein is highly expressed and is in the form of a homodimer or a heterodimer with Bcl-2, the cells tend to die (Kikuchi et al., 2015). The increase in Bax/Bcl-2 ratio can also activate the expression of downstream cytochrome c, and then activate the expression of the caspase family, especially caspase-3, which leads to cell death (Sanchez et al., 2012; Kavitha et al., 2013). In our study, the LD group showed a higher expression of Bax and more apoptotic cells in the substantia nigra, but lower expression of Bcl-2 and TH and fewer surviving dopaminergic neurons and Nissl bodies compared with the PD group. The degree of apoptosis in dopaminergic neurons of the substantia nigra is consistent with the behavioral change observed. The LD + TCM group had lower p-ERK1/2 expression than the LD group, and it was mainly located in the cytoplasm. The ratio of Bax/Bcl-2 was smaller, TH expression was higher, and there were markedly fewer apoptotic dopaminergic neurons. These results showed that *Shudipingchan* granule is anti-apoptotic and acts by inhibiting the activation of the ERK pathway.

In conclusion, long-term treatment of LD activated the direct pathway in the substantia nigra and striatum. It also activated the ERK signal transduction pathway, causing p-ERK1/2 to translocate from the cytoplasm to the nucleus, which in turn upregulated the expression of the downstream

apoptotic gene Bax, and downregulated the anti-apoptotic gene Bcl-2. This accelerated apoptosis in the substantia nigra, causing behavioral deterioration and producing LD-induced dyskinesia. Treatment with the TCM *Shudipingchan* granule in combination with LD synergistically attenuated PD-related behaviors, inhibited ERK hyperphosphorylation and downregulated the expression of apoptosis-associated proteins, thus reducing neuronal apoptosis in the substantia nigra.

Acknowledgments: We would like to thank all the doctors from Department of Neurology, Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine of China and all staffs from Laboratory Animal Center, Shanghai University of Traditional Chinese Medicine of China for providing technical support.

Author contributions: QY, CXY and XMY were responsible for study concept and design. QY, XLY, JZ and JH implemented the experiment. QY, XMY and XLY analyzed data, wrote the manuscript and conducted statistical analysis. QY and CXY were in charge of the funds. CXY and XMY supervised the paper and provided technical or information support. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Plagiarism check: This paper was screened twice using Cross-Check to verify originality before publication.

Peer review: This paper was double-blinded and stringently reviewed by international expert reviewers.

References

- Bao XM, Shu SY (1991) Rat Brain Stereotactic Atlas. Beijing: People's Health Publishing House.
- Burguillos MA, Hajji N, Englund E, Persson A, Cenci AM, Machado A, Cano J, Joseph B, Venero JL (2011) Apoptosis-inducing factor mediates dopaminergic cell death in response to LPS-induced inflammatory stimulus: evidence in Parkinson's disease patients. *Neurobiol Dis* 41:177-188.
- Cao F, Luo F, Zhang L, Wei GR, Chen L (2012) Experimental study of substantial nigral morphology and behavioral changes in rat models of Parkinson's disease. *Nao yu Shenjing Jibing Zazhi* 20:375-378.
- Cao XB, Sun SG, Xu Y, Wang L, Su Y (2004) Experimental study of treatment of levodopa on the dyskinesias induced by Parkinson's disease. *Zhonghua Yixue Zazhi* 84:505-507.
- Cerovic M, Bagetta V, Pendolino V, Ghiglieri V, Fasano S, Morella I, Hardingham N, Heuer A, Papale A, Marchisella F, Giampà C, Calabresi P, Picconi B, Brambilla R (2015) Derangement of Ras-guanine nucleotide-releasing factor 1 (Ras-GRF1) and extracellular signal-regulated kinase (ERK) dependent striatal plasticity in L-DOPA-induced dyskinesia. *Biol Psychiatry* 77:106-115.
- Chagniel L, Robitaille C, Lebel M, Cyr M (2012) Striatal inhibition of calpains prevents levodopa-induced neurochemical changes and abnormal involuntary movements in the hemiparkinsonian rat model. *Neurobiol Dis* 45:645-655.
- Cote SR, Kuzhikandathil EV (2015) Chronic levodopa treatment alters expression and function of dopamine D3 receptor in the MPTP/p mouse model of Parkinson's disease. *Neurosci Lett* 585:33-37.
- Ding HJ, He JC, Wang WB (2011) The dynamic study on the behavioral effect on rats with Parkinson's disease treated by Levodopa. *Chongqing Yike Daxue Xuebao* 36:1281-1284.
- Encarnacion EV, Hauser RA (2008) Levodopa-induced dyskinesias in Parkinson's disease: etiology, impact on quality of life, and treatments. *Eur Neurol* 60:57-66.
- Hu YY, Chen W (2015) Effect of Bushenzhichan prescription combined with needle embedding therapy on the expression of Bcl-2 and Bax in Parkinson's disease model rats. *Zhongguo Laonian Xue Zazhi* 35:1341-1343.
- Jiménez-Urbietta H, Gago B, de la Riva P, Delgado-Alvarado M, Marin C, Rodríguez-Oroz MC (2015) Dyskinesias and impulse control disorders in Parkinson's disease: From pathogenesis to potential therapeutic approaches. *Neurosci Biobehav Rev* 56:294-314.
- Kavitha M, Nataraj J, Essa MM, Memon MA, Manivasagam T (2013) Mangiferin attenuates MPTP induced dopaminergic neurodegeneration and improves motor impairment, redox balance and Bcl-2/Bax expression in experimental Parkinson's disease mice. *Chem-Biol Interact* 206:239-247.
- Kikuchi H, Kuribayashi F, Mimuro H, Imajoh-Ohmi S, Nakayama M, Takami Y, Nishitoh H, Nakayama T (2015) Lack of GCN5 remarkably enhances the resistance against prolonged endoplasmic reticulum stress-induced apoptosis through up-regulation of Bcl-2 gene expression. *Biochem Biophys Res Commun* 463:870-875.
- Lee Y, Chun HJ, Lee KM, Jung YS, Lee J (2015) Silibinin suppresses astroglial activation in a mouse model of acute Parkinson's disease by modulating the ERK and JNK signaling pathways. *Brain Res* 1627:233-242.
- Li D, Huang YX, Wang XJ, Luo WF, Liu CF (2010) Establishment and evaluation of the behavior of the Parkinson's disease rat model of levodopa induced dyskinesias. *Suzhou Daxue Xuebao: Yixue Ban* 30:236-239.
- Lindenbach D, Dupre KB, Eskow Jaunarajs KL, Ostock CY, Goldenberg AA, Bishop C (2013) Effects of 5-HT1A receptor stimulation on striatal and cortical M1 pERK induction by L-DOPA and a D1 receptor agonist in a rat model of Parkinson's disease. *Brain Res* 1537:327-329.
- Lipski J, Nistico R, Berretta N, Guatteo E, Bernardi G, Mercuri NB (2011) L-DOPA: a scapegoat for accelerated neurodegeneration in Parkinson's disease? *Prog Neurobiol* 94:389-407.
- Mango D, Bonito-Oliva A, Ledonne A, Cappellacci L, Petrelli R, Nistico R, Berretta N, Fisone G, Mercuri NB (2014) Adenosine A1 receptor stimulation reduces D1 receptor-mediated GABAergic transmission from striato-nigral terminals and attenuates L-DOPA-induced dyskinesia in dopamine-denervated mice. *Exp Neurol* 261:733-743.
- Park HJ, Park KH, Shin KS, Lee MK (2013) The roles of cyclic AMP-ERK-Bad signaling pathways on 6-hydroxydopamine-induced cell survival and death in PC12 cells. *Toxicol In Vitro* 27:2233-2241.
- Sanchez A, Tripathy D, Yin X, Luo J, Martinez J, Grammas P (2012) Pigment epithelium-derived factor (PEDF) protects cortical neurons in vitro from oxidant injury by activation of extracellular signal-regulated kinase (ERK) 1/2 and induction of Bcl-2. *Neurosci Res* 72:1-8.
- Sharma S, Singh S, Sharma V, Singh VP, Deshmukh R (2015) Neurobiology of L-DOPA induced dyskinesia and the novel therapeutic strategies. *Biomed Pharmacother* 70:283-293.
- Song L, Kong M, Ma Y, Ba M, Liu Z (2009) Inhibitory effect of 8-(3-chlorostyryl) caffeine on levodopa-induced motor fluctuation is associated with intracellular signaling pathway in 6-OHDA-lesioned rats. *Brain Res* 1276:171-179.
- Teng L, Hong F, He JC (2015) Oxidative stress in the rat models of Parkinson's disease with Yin deficiency stirring wind pattern, and the interventional effect of compound formula Rehmannia on the disease condition. *Zhongguo Shiyan Dongwu Xuebao* 23:26-27.
- Wang C, Nie X, Zhang Y, Li T, Mao J, Liu X, Gu Y, Shi J, Xiao J, Wan C, Wu Q (2015) Reactive oxygen species mediate nitric oxide production through ERK/JNK MAPK signaling in HAPI microglia after PFOS exposure. *Toxicol Appl Pharmacol* 288:143-151.
- Xu Y, Cao B, Huang RL (2008) The Role of ERK1/2 in the pathogenesis of levodopa induced dyskinesia. *Cuzhong yu Shenjing Jibing* 15:156-163.
- Yang X, Zhao H, Shi H, Wang X, Zhang S, Zhang Z, Zu J, Zhang W, Shen X, Cui G, Hua F (2015) Intranigral administration of substance P receptor antagonist attenuated levodopa-induced dyskinesia in a rat model of Parkinson's disease. *Exp Neurol* 271:168-174.
- Ye Q, Yuan XL, Zhou J, Yuan CX, Yang XM (2014) Effect of zishenpingchan granule on neurobehavioral manifestations and the activity and gene expression of striatal dopamine d1 and d2 receptors of rats with levodopa-induced dyskinesias. *Evid Based Complement Alternat Med* 2014:342506.
- Yu S, Li X, Liu G, Han J, Zhang C, Li Y, Xu S, Liu C, Gao Y, Yang H, Ueda K, Chan P (2007) Extensive nuclear localization of α -synuclein in normal rat brain neurons revealed by a novel monoclonal antibody. *Neuroscience* 145:539-555.

Copiedited by Slone-Murphy J, Yajima W, Yu J, Qiu Y, Li CH, Song LP, Zhao M