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Effects of *Zhichan* powder on signal transduction and apoptosis-associated gene expression in the substantia nigra of Parkinson's disease rats

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Abstract

Previous studies have shown that *Zhichan* powder elevated immunity and suppressed oxidation in mice. Rat models of Parkinson's disease were induced by stereotaxically injecting 6-hydroxydopamine into the substantia nigra. The rat models were intragastrically treated with *Zhichan* powder, which is composed of milkvetch root, ginseng, bunge swallowwort root, himalayan teasel root, *Magnolia officinalis*, *Ligustrum lucidum* Ait. and szechwan lovage rhizome. Immunohistochemistry and reverse transcription-PCR results demonstrated that mRNA and protein expression of tumor necrosis factor receptor 1, Fas, caspase-8, cytochrome C, Bax, caspase-3, and p53 significantly increased, but Bcl-2 expression significantly decreased in the substantia nigra of rats with Parkinson's disease. Following *Zhichan* powder administration, mRNA and protein expression of tumor necrosis factor receptor 1, Fas, caspase-8, cytochrome C, Bax, caspase-3, and p53 diminished, but Bcl-2 expression increased in the rat substantia nigra. These results indicate that *Zhichan* powder regulates signal transduction protein expression, inhibits apoptosis, and exerts therapeutic effects on Parkinson's disease.

Key Words

Zhichan powder; Parkinson's disease; 6-hydroxydopamine; signal transduction; apoptosis; substantia nigra; traditional Chinese medicine; degenerative disease; neural regeneration

Research Highlights

(1) Protein and mRNA expression of signal transduction and apoptosis-associated factors (except Bcl-2) increased in the substantia nigra of Parkinson's disease rats.
(2) *Zhichan* powder negatively regulated the mRNA expression of signal tranduction and apoptosis-associated factors, but positively regulated Bcl-2 mRNA expression in the brain of Parkinson's disease rats.

Abbreviation

TNFR1, tumor necrosis factor receptor 1

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INTRODUCTION

The precise etiopathology and pathogenesis of Parkinson's disease remain unclear and are probably associated with heredity, environmental factors, excitatory toxins, excessive oxidative stress, the aging nervous system and signal transduction-associated gene expression[1].

Levodopa substitution therapy has been considered an effective method to treat Parkinson's disease[2], but this therapy can only improve patients' clinical symptoms, and cannot affect the Parkinson's disease process. Moreover, it is difficult to increase the therapeutic efficacy of levodopa and there are unpleasant side effects[3].

Many medicines are used to treat Parkinson's disease in China, such as the anti-oxidative stress drugs triptolide, fucoidan, ligustrazine and the anti-apoptosis drugs resveratrol, ginsenoside Rg1 and Rg2, daidzein and manyprickle acanthopanax root. The above-described medicines exert their effects by regulating the status of the whole body. These medicines are quite mild, do not have side effects, and can be used for a long period[4-6]. They are medicinal herbs, decoctions or monomers that were developed by clinical experience or as ancient Chinese herbal formulas. However, the drugs developed with specific anti-Parkinson's disease mechanisms of action are few, especially proprietary Chinese medicines *Zhichan* powder is composed of milkvetch root, ginseng, bunge swallowwort root, himalayan teasel root, *Magnolia officinalis*, *Ligustrum lucidum* ait and szechwan lovage rhizome. Crude milkvetch root tastes sweet, with a warming effect, and can increase energy and promote blood circulation and generation. Ginseng tastes sweet, is mildly bitter, strengthens *qi* and confers resistance to senility[7]. Bunge swallowwort root is dry, bitter, and acerbic, and nourishes the blood, tonifies the liver, and invigorates *qi* and blood[7].

A previous study confirmed that *Zhichan* powder elevated the immunity of Parkinson's disease mice, decreased the production of monoamine oxidase-B, and contributed to the secretion of superoxide dismutase[8]. The present study analyzed the effects of *Zhichan* powder on signal transduction and apoptosis-associated protein expression, explored the protective mechanism of *Zhichan* powder on dopaminergic neurons in the substantia nigra of Parkinson's disease rats, and provided theoretical evidence for the use of traditional Chinese medicine in the treatment of Parkinson's disease.

RESULTS

Quantitative analysis of experimental animals

Out of 90 Sprague-Dawley rats used in this study, 70 were randomly selected to undergo induction of a model of Parkinson's disease. Establishment of the model was successful in 42 rats (a success rate of 60%). The remaining 20 rats served as controls. A total of 42 Parkinson's model rats were randomly assigned to the model group (n = 20), the 7-week *Zhichan* powder group (n = 11) and the 14-week *Zhichan* powder group (n = 11). Rats in the 7- and 14-week tremor- stopping powder groups were administered 2 g/kg *Zhichan* powder intragastrically. Rats in the control and model groups were administered 2 mL/100 g saline. Three rats in the control group, eight in the model group, four in the 7-week *Zhichan* powder and four in the 14-week *Zhichan* powder group died during model induction and the rest of the study. After anatomical analysis, it was determined that 12 rats succumbed to esophageal mucosa injury and gastric bleeding and three rats died from emaciation (difficulty in taking food). A total of 17 rats in the control group, 12 in the model group, 7 in the 7-week *Zhichan* powder group and 7 in the 14-week *Zhichan* powder group were included in the final analysis.

Zhichan powder suppresses the expression of tumor necrosis factor receptor 1 (TNFR1), Fas, caspase-8 and cytochrome C in the substantia nigra of Parkinson's disease rats

Immunohistochemical staining results demonstrated that the expression of TNFR1, Fas, caspase-8 and cytochrome C was higher in the model group compared to the control group. The expression of TNFR1, Fas, caspase-8 and cytochrome C was lower following *Zhichan* powder treatment, compared to the model group, especially after 14 weeks of *Zhichan* powder treatment (Figure 1).

Effects of *Zhichan* powder on caspase-3, Bax, Bcl-2 and p53 expression in the substantia nigra of Parkinson's disease rats

Immunohistochemical staining results showed that caspase-3, Bax and p53 expression was greater, but Bcl-2 expression was lower in the model group compared to the control group. Caspase-3, Bax and p53 expression was lower, but Bcl-2 expression was greater following *Zhichan* powder treatment, compared with the model group. The effect was more significant following 14 weeks of *Zhichan* powder treatment (Figure 2).

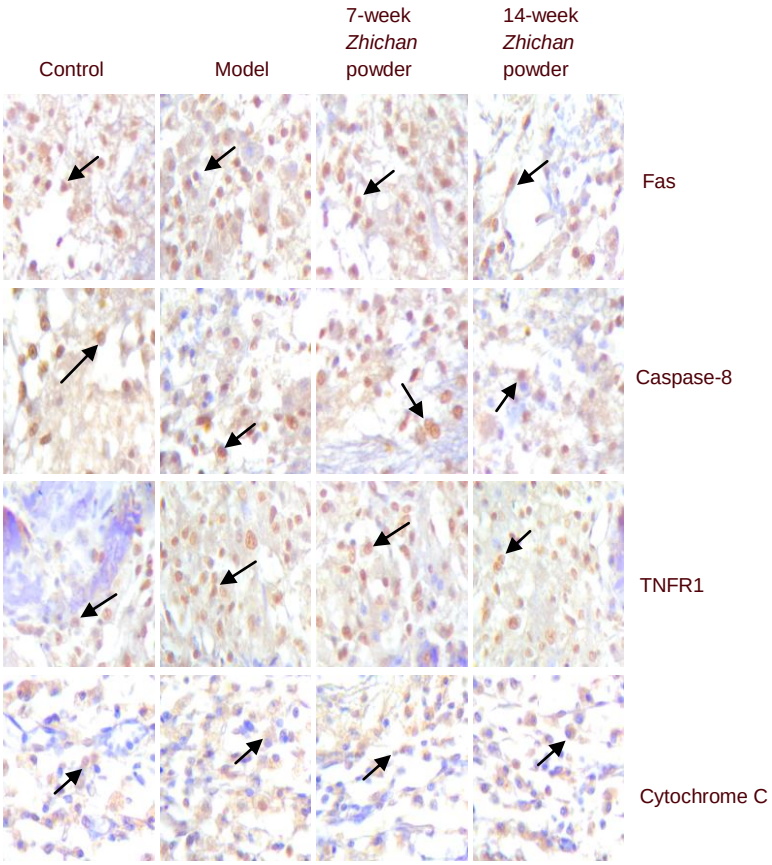

Figure 1 Expression of tumor necrosis factor receptor 1 (TNFR1), Fas, caspase-8 and cytochrome C in the rat substantia nigra (immunohistochemistry, × 400).

Expression of TNFR1, Fas, caspase-8 and cytochrome C was higher in the model group compared to the control group. The expression of TNFR1, Fas, caspase-8 and cytochrome C decreased in the 7-week and 14-week *Zhichan* powder groups. Signal transduction protein expression was lower in the 14-week *Zhichan* powder group than that in the 7-week *Zhichan* powder group. Arrows show positive cells.

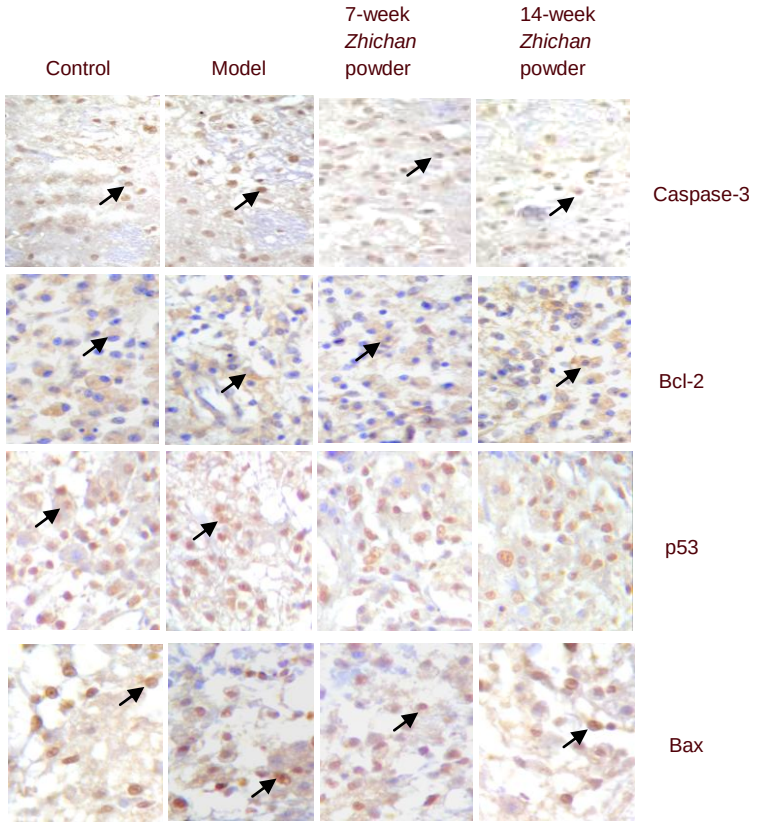

Figure 2 Caspase-3, p53, Bax and Bcl-2 expression in the rat substantia nigra (immunohistochemistry, × 400).

Caspase-3, Bax and p53 expression increased, but Bcl-2 expression diminished in the model group. However, caspase-3, Bax and p53 expression decreased, but Bcl-2 expression increased following *Zhichan* powder treatment. Arrows show positive cells.

Effects of *Zhichan* powder on caspase-3, Bax, Bcl-2, p53, TNFR1, Fas, caspase-8 and cytochrome C mRNA expression in the substantia nigra of Parkinson's disease rats (Figure 3)

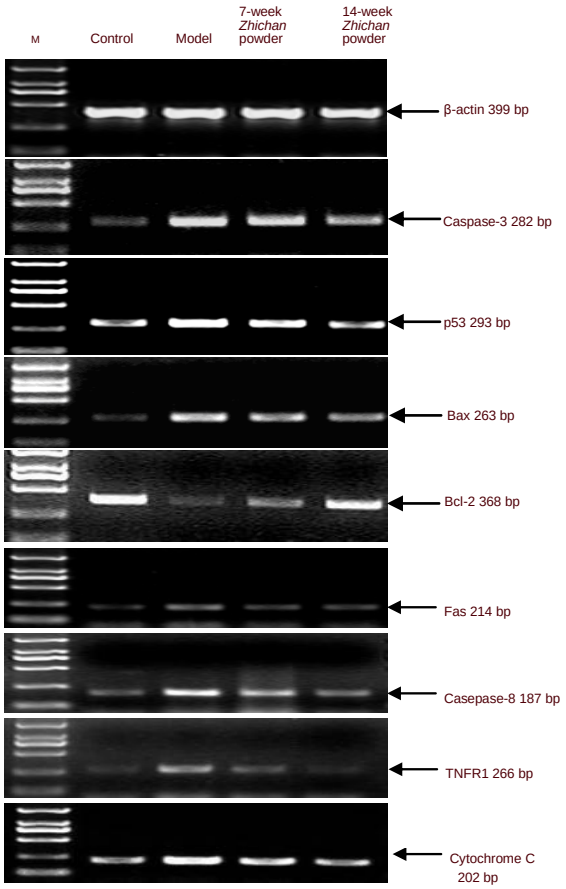

Figure 3 Caspase-3, p53, Bax, Bcl-2, Fas, caspase-8, tumor necrosis factor receptor 1 (TNFR1) and cytochrome C mRNA expression in the rat substantia nigra (reverse transcription-PCR).

M: DL2000 DNA Marker. Caspase-3, Bax, p53, TNFR1, Fas, caspase-8 and cytochrome C mRNA expression were significantly greater, but Bcl-2 mRNA expression was lower in the model group compared with the control group. However, caspase-3, Bax, p53, TNFR1, Fas, caspase-8 and cytochrome C mRNA expression were decreased, but Bcl-2 mRNA expression was increased following *Zhichan* powder treatment, especially following 14 weeks of *Zhichan* powder treatment.

Reverse transcription-PCR results showed that caspase-3, Bax and p53 mRNA expression was significantly higher, but Bcl-2 mRNA expression was lower in the model group compared to the control group ($P < 0.01$). Caspase-3, Bax and p53 mRNA expression was significantly lower, but Bcl-2 mRNA expression was greater following *Zhichan* powder treatment ($P < 0.01$). TNFR1, Fas, caspase-8 and cytochrome C mRNA expression was significantly greater in the model group than in the control group ($P < 0.01$). However, TNFR1, Fas, caspase-8 and cytochrome C mRNA expression was significantly decreased following *Zhichan* powder treatment ($P < 0.01$), especially after 14 weeks of *Zhichan* powder treatment ($P < 0.01$; Figure 3, Table 1).

Table 1 Caspase-3, p53, Bax, Bcl-2, Fas, caspase-8, tumor necrosis factor receptor 1 (TNFR1) and cytochrome C mRNA expression in the rat substantia nigra

Group	Caspase-3	p53	Bax	Bcl-2
Control	0.26±0.02	0.95±0.06	0.43±0.03	1.22±0.04
Model	0.90±0.02[a]	1.08±0.05[a]	0.90±0.08[a]	0.36±0.02[a]
Zhichan powder				
7-week	0.80±0.04[ab]	0.99±0.03[b]	0.77±0.06[ab]	0.47±0.02[ab]
14-week	0.59±0.03[abc]	0.93±0.04[b]	0.62±0.07[abc]	1.10±0.02[abc]
F	474.85	10.29	49.78	1 499.80
P	0.00	0.00	0.00	0.00

Group	Fas	Caspase-8	TNFR1	Cytochrome C
Control	0.48±0.05	0.19±0.05	0.17±0.02	0.77±0.04
Model	0.77±0.06[a]	0.56±0.01[a]	0.40±0.01[a]	1.01±0.02[a]
Zhichan powder				
7-week	0.63±0.03[ab]	0.49±0.01[ab]	0.23±0.01[ab]	0.96±0.03[ab]
14-week	0.54±0.02[bc]	0.30±0.05[abc]	0.18±0.01[bc]	0.81±0.02[bc]
F	42.97	114.41	321.11	80.96
P	0.00	0.00	0.00	0.00

Data are expressed as mean ± SD, with five rats in each group. [a]$P < 0.01$, *vs.* control group; [b]$P < 0.01$, *vs.* model group; [c]$P < 0.01$, *vs.* 7-week *Zhichan* powder group. The *q* test was used to compare the difference between groups.

DISCUSSION

Fas-induced apoptosis is mediated by various signaling pathways. FasL is predominantly expressed in activated natural killer cells, and interacts with Fas (CD95), resulting in target cell apoptosis[9]. Previous studies have found that caspase-3[10], caspase-8[11] and Fas[12-14] siRNAs effectively inhibit apoptosis. TNF plays an important role in inducing apoptosis. TNF combined with TNFR1 leads to a large increase in TNFR1-associated death domain protein, which simultaneously participates in the activation of two pathways[15]: one is proapoptotic pathway mediated by Fas associated death domain protein, another is antiapoptotic pathway mediated by TNFR1 correlation factor.

Results from this study showed that Fas and TNFR1 expression was greater in the substantia nigra of rats with Parkinson's disease induced by 6-hydroxydopamine injection compared to controls.

This indicates that Fas and TNFR1-mediated apoptosis signals were received by the cells, induced the upregulation of apoptosis-associated gene expression, promoted dopaminergic cell apoptosis, and resulted in the occurrence of Parkinson's disease in rats. Immunohistochemical results revealed that Fas, TNFR1 and caspase-8 protein expression increased in the model group, but decreased following treatment with *Zhichan* powder, especially after 14 weeks of *Zhichan* powder treatment. The above-described results suggest that *Zhichan* powder regulates the activity of signaling proteins and delays the transmission of Parkinson's disease damage signals from cell membranes to nuclei. The Bcl-2 protein, encoded by the Bcl-2 gene, suppresses cell apoptosis, so increased Bcl-2 gene expression could reduce dopaminergic neuron apoptosis[16]. The Bax protein plays an important role in apoptosis in the substantia nigra, and its increase can upregulate the apoptosis of dopaminergic neurons[17]. The Bcl-2 and Bax genes are a pair of apoptosis-regulated genes[18]. Bcl-2 blocks the release of cytochrome C, inhibits caspase-3 activation, and effectively suppresses the occurrence of apoptosis. Bax is a pro-apoptotic factor that promotes the release of cytochrome C[19-21]. In our study, apoptosis was further confirmed by caspase-3 activation[22]. Overexpression of Bcl-2 can cause changes in redox equilibrium in the nucleus and decrease caspase-3 activity[23]. Bcl-2, a substrate of caspase-3, can be hydrolyzed by caspase-3[24]. Bcl-2 expression was reduced, but Bax expression was remarkably increased in the substantia nigra of Parkinson's disease rats, which suggested that Bcl-2 and Bax participated in an apoptosis-inducing effect. Following *Zhichan* powder treatment, Bax expression decreased, but Bcl-2 expression increased, suggesting that *Zhichan* powder suppressed Bax expression in the rat substantia nigra and inhibited neuronal apoptosis. These results also verified that the relative ratio of pro-apoptotic and anti-apoptotic proteins exerts an important effect in apoptosis. Bcl-2 forms a heterodimer with Bax to terminate apoptosis, but Bax/Bax homodimer formation can promote apoptosis, consistent with previously published results[25-26].

The p53 content was low in normal cells, but high in injured cells. The increased p53 levels led to an increased Bax/Bcl-2 ratio, resulting in apoptosis. The p53 gene indirectly regulates cytochrome C expression, and promotes apoptosis[25-27]. P53 expression in the model group was persistently increased, which indicated that excessive p53 activation probably participates in the pathological process of Parkinson's disease. Nevertheless, p53 expression was significantly reduced in the rat substantia nigra following 7 weeks of *Zhichan* powder treatment. P53 target gene expression was downregulated following 7 weeks of *Zhichan* powder treatment, and still downregulated at 14 weeks. This indicated that cytochrome C release caused the secretion of apoptotic factors and contributed to apoptosis following mitochondrial injury. Our results show that, *Zhichan* powder suppresses p53 and cytochrome C expression, enhances the tolerance of dopaminergic neurons to 6-hydroxydopamine, and promotes the recovery of neurological function.

In summary, *Zhichan* powder inhibits signal transduction and apoptosis in the brain of Parkinson's disease rats. This provides novel theoretical evidence for clinical treatment and prevention of Parkinson's disease using *Zhichan* powder.

MATERIALS AND METHODS

Design

A randomized controlled animal study.

Time and setting

Experiments were performed at the Experimental Center, Norman Bethune College of Medicine, Jilin University, China from March 2009 to March 2010.

Materials

Animals

A total of 90 clean, purebred, Sprague-Dawley rats, aged 8 weeks, of both genders, weighing 150–160 g, were supplied by the Experimental Animal Center, Jilin University, China (animal license No. SCXK (Ji) 2007-0003). The rats were housed in separate cages at 20–25°C, 40–70% humidity and illumination intensity of 15–20 Lux, with free access to food and water. The experimental protocols were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by Ministry of Science and Technology of China[28].

Drugs

Zhichan powder was prepared by Professor Guozhong Gai from Changchun University of Chinese Medicine, and composed of milkvetch root, ginseng, bunge swallowwort root, himalayan teasel root, *Magnolia officinalis*, *Ligustrum lucidum* ait and szechwan lovage rhizome (provided by the Pharmacy of Changchun University of Chinese Medicine). The above-mentioned drugs were crushed separately, mixed in a certain

proportion, steeped in boiling water overnight, and decocted three times with water. The decoctions were merged and filtered. The filtrate was dried at 60–80°C. After trituration, a brown powder was obtained, and 1 g powder was equal to 5.36 g crude drug.

Methods

Preparation of 6-hydroxydopamine-induced Parkinson's disease models

The rats were anesthetized and placed on a stereotaxic apparatus (Ruiwode Life Science Co., Ltd., Shenzhen, Guangdong Province, China). The coordinates of the substantia nigra were identified[29-30]: 1.1 mm lateral to the median line, 4.4 mm posterior to the anterior fontanelle, 7.5 mm below the dura mater. After injection of 6-hydroxydopamine (Sigma, St. Louis, MO, USA) 12 μg at 10 μL/min, the needle was maintained in place for 1–5 minutes. Anti-infective therapy was conducted for 5 days[31-33]. Two weeks later, 0.25 mg/kg apomorphine (Shenyang First Pharmacy Co., Ltd., Northeast Pharmaceutical Group, Shenyang, Liaoning Province, China) was subcutaneously injected into the rats. The times and direction of rotation within 40 minutes were recorded, once a week, for 6 consecutive weeks. The mean value of three consecutive sessions was used as the rotation time. An average velocity > 7 rotations/min was considered a successful model induction.

Intragastric administration of Zhichan powder

The rats in the 7-week and 14-week *Zhichan* powder groups were administered 2 g/kg *Zhichan* powder (200 mg/mL) intragastrically twice for 7 and 14 weeks. The rats in the control and model groups were daily given 2 mL/100 g saline intragastrically for 14 weeks.

Sample collection

After gavage, the rats were anesthetized for blood collection from the abdominal aorta, and then sacrificed. The substantia nigra was obtained from the brain on a super clean bench. One half was fixed in 10% formalin, and another half was stored at –80°C for further use.

Expression of TNFR1, Fas, caspase-8, cytochrome C, Bcl-2, Bax, caspase-3 and p53 in the rat substantia nigra, determined by immunohistochemical staining

The rat substanitia nigra fixed in 10% formalin was embedded in paraffin and sectioned into 2-mm thick slices. These sections were subjected to immunohistochemical staining with streptavidin and peroxidase. Antigens were retrieved at 60°C for 120 minutes. These sections were deparaffinized, hydrated and immersed three times in PBS, each for 2 minutes. The sections were incubated in 3% H_2O_2 (Beijing Zhongnuo Taian Technology Co., Ltd., Beijing, China) for 10 minutes at room temperature to eliminate the activity of endogenous peroxidase, washed in PBS, blocked in 5% normal goat serum (Xinran Biotechnology, Shanghai, China) for 10 minutes at room temperature. After removal of serum, the sections were incubated with rabbit anti-TNFR1, Fas, caspase-8, cytochrome C, Bcl-2, Bax, caspase-3 or p53 polyclonal antibody (1: 50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The sections were incubated in biotin-labeled goat anti-rabbit secondary antibody (Beijing Biosynthesis Biotechnology, Beijing, China) 1:500 in PBS at 37°C for 30 minutes, and then in horseradish peroxidase-conjugated streptavidin (Beijing Biosynthesis Biotechnology) 1:1 000 at 37°C for 30 minutes. A PBS wash was performed between each step. The sections were developed with 3, 3'-diaminobenzidine (Beijing Bole Life Science, Beijing, China), counterstained, dehydrated, permeabilized, mounted with Citifluor (Citifluor Ltd., London, United Kingdom), observed under a light microscope (Nikon, Tokyo, Japan), and photographed.

Caspase-3, Bax, Bcl-2, p53, TNFR1, Fas, caspase-8 and cytochrome C mRNA expression in the rat substantia nigra, measured by reverse transcription-PCR

Total RNA was extracted with a Trizol kit (USA Life Technologics, Shanghai, China). PCR primers were synthesized by Shanghai Bioengineering Co., China. In accordance with the instructions of a GenAmpRNAPCR kit (Bioer Technology Co., Ltd., Hangzhou, China), the total reaction volume was 20 μL in a mixture containing 1 μL total RNA, 4 μL 5 × PCR buffer, 2 μL dNTPs (10 mM), 1.0 μL RNase inhibitor, 1 μL OligodT, 1 μL AMV reverse transcriptase and 10 μL sterile double distilled water. Following centrifugation, the reaction tube was incubated in a water bath at 37°C for 10 minutes, at 42°C for 1 hour, at 94°C for 5 minutes, and then cooled in an ice bath for 1 minute. The cDNA products were stored at –20°C. In accordance with the instructions of a GenAmpRNAPCR kit, 5 μL 10 × Taq enzyme buffer, 2 μL dNTPs (10 mM), 10 μL upstream target gene primer, 10 μL downstream target gene primer, 5 μL upstream internal reference primer, 5 μL downstream internal reference primer, 5 μL cDNA, 3 μL Taq enzyme, 5 μL diethyl pyrocarbonate-treated water were added in each 0.2 mL PCR reaction tube, for a total reaction volume of 50 μL.

PCR primer sequences are listed in Table 2.

PCR reaction conditions are shown in Table 3.

Table 2 PCR primer sequences

Gene	Primer	Length (bp)
β-actin	Sense: 5'-CTG TGC CCA TCT ATG AGG GTT ACG-3' Antisense: 5'-CAT AGA GGT CTT TAC GGA TGT CAA CG-3'	399
Caspase-3	Sense: 5'-TGC TTA CTC TAC CGC ACC CG-3' Antisense: 5'-AAC ATG CCC CTA CCC CAC TC-3'	282
Bax	Sense: 5'-TTT CAT CCA GGA TCG AGC AG -3' Antisense: 5'-CAA AGT AGA AGA GGG CAA CCA C-3'	263
Bcl-2	Sense: 5'-TGG GAT ACT GGA GAT GAA GAC T-3' Antisense: 5'-CCA CCG AAC TCA AAG AAG G-3'	368
p53	Sense: 5'-ATG GAG GAT TCA CAG TCG G-3' Antisense: 5'-TGA GAA GGG ACG GAA GAT G-3'	293
TNFR1	Sense: 5'-AGG TAC TGC CGT GCT GTT GC-3' Antisense: 5'-CAG GAT GAC TGA AGC GTG GG-3'	266
Fas	Sense: 5'-GAC TGA TAG CAT CTC TGA GGG TTT-3' Antisense: 5'-AGA ATA GTG TTT CCT GTC CGT GTA-3'	214
Caspase-8	Sense: 5'-GTT TCT G TTT TGG ATG AGG TG-3' Antisense: 5'-TGT TGC TGA GTT TGG GTA TGT-3'	187
Cytochrome C	Sense: 5'-GAG GAG GGT TCA GCT CGT ATT TG-3' Antisense: 5'-GAG GAT TGT GGA AGA GGG TAT GG-3'	202

TNFR1: Tumor necrosis factor receptor 1.

Table 3 PCR reaction conditions

Gene	Predenaturation	Denaturation	Annealing	Extension	Final extension	Cycle number
β-actin	94°C 5 minutes	94°C 30 seconds	55°C 30 seconds	72°C 1 minute	72°C 10 minutes	35
Caspase-3	94°C 5 minutes	94°C 30 seconds	60°C 30 seconds	72°C 1 minute	72°C 10 minutes	35
Bcl-2	94°C 5 minutes	94°C 30 seconds	55°C 30 seconds	72°C 1 minute	72°C 10 minutes	35
Bax	94°C 5 minutes	94°C 30 seconds	58°C 30 seconds	72°C 1 minute	72°C 10 minutes	35
p53	94°C 5 minutes	94°C 30 seconds	58°C 30 seconds	72°C 1 minute	72°C 10 minutes	35
TNFR1	94°C 5 minutes	94°C 30 seconds	61°C 30 seconds	72°C 1 minute	72°C 10 minutes	35
Fas	94°C 5 minutes	94°C 30 seconds	59.4°C 30 seconds	72°C 1 minute	72°C 10 minutes	35
Caspase-8	94°C 5 minutes	94°C 30 seconds	54°C 30 seconds	72°C 1 minute	72°C 10 minutes	35
Cytochrome C	94°C 5 minutes	94°C 30 seconds	61°C 30 seconds	72°C 1 minute	72°C 10 minutes	35

TNFR1: Tumor necrosis factor receptor 1.

Using tris-acetate-ethylenediamine tetraacetic acid buffer, the samples were electrophoresed in 1.5% agarose (Japan Takara, Dalian, China) containing ethidium bromide (1 μg/μL final concentration) (Sangon, Shanghai, China) at 100 V for 25 minutes at room temperature. The gels were observed with a gel imaging system (Tianneng Biological Science Co., Ltd., Hangzhou, China), photographed, and the absorbance value was recorded. The expression of the target gene is shown as the absorbance ratio of target gene/β-actin.

Statistical analysis

The data were expressed as mean ± SD, and analyzed using SPSS 17.0 (SPSS, Chicago, IL, USA). One-way analysis of variance was used to compare the differences among groups. There were significant differences. A paired *q*-test was used to compare the difference between indices.

Author contributions: Yongmao Liu, Jinshu Ma and Yafei Qiu provided the data. Qingwei Zhou integrated the data. Yongmao Liu participated in the study concept and design. Jiajun Chen, Qingwei Zhou and Yongmao Liu analyzed the data. Jiajun Chen, Qingwei Zhou, Yongmao Liu, Ye Kuang, Shihong Yi, Pengguo Zhang and Quan Wan wrote the manuscript. Jiajun Chen and Yongmao Liu were in charge of manuscript authorization. Jinshu Ma and Yafei Qiu participated in the statistical analysis.
Conflicts of interest: None declared.
Ethical approval: This study was approved by the Animal Ethics Committee, Norman Bethune College of Medicine, Jilin University, China.

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