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Zhichan powder regulates nigrostriatal dopamine synthesis and metabolism in Parkinson's disease rats☆

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Abstract
In this study, rat models of Parkinson's disease induced by substantia nigra injection of 6-hydroxydopamine were intragastrically administered *Zhichan* powder daily for 50 days. Reverse transcription PCR results showed that tyrosine hydroxylase mRNA expression in the rat substantia nigra was significantly increased, while monoamine oxidase B mRNA expression was significantly decreased in the *Zhichan* powder group, compared with the model group. In addition, the levels of striatal dopamine and homovanillic acid, the ratio of dopamine to homovanillic acid, and the activity of blood superoxide dismutase were all higher in the *Zhichan* powder group than in the model group, but the content of malondialdehyde in blood was lower. Our experimental findings indicate that *Zhichan* powder has an antioxidant effect, it can regulate the expression of monoamine oxidase B and tyrosine hydroxylase in the substantia nigra of Parkinson's disease rats, and it can facilitate the secretion of striatal dopamine and its metabolite homovanillic acid.

Key Words
Chinese herbal compound; *Zhichan* powder; Parkinson's disease; tyrosine hydroxylase; monoamine oxidase B; dopamine; homovanillic acid; superoxide dismutase; malondialdehyde; neurodegenerative diseases; neural regeneration

Research Highlights
(1) This is the first demonstration of the bioactive molecules associated with Parkinson's disease in rat striatum, substantia nigra and blood using modern molecular biology technology, and the first exploration of *Zhichan* powder's role in the treatment of Parkinson's disease.
(2) *Zhichan* powder, a Chinese herbal compound, has an antioxidant effect, can regulate the expression of monoamine oxidase B and tyrosine hydroxylase in the substantia nigra of Parkinson's disease rats, and facilitate the secretion of striatal dopamine and its metabolite homovanillic acid, resulting in a neuroprotective effect on substantia nigra neurons in Parkinson's disease rats.

Abbreviations
TH, tyrosine hydroxylase; MAO-B, monoamine oxidase B

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INTRODUCTION

A low level of nigrostriatal dopamine neurotransmitters is one of the main mechanisms underlying Parkinson's disease[1]. Tyrosine hydroxylase (TH) is the rate-limiting enzyme for the synthesis of dopamine and it regulates dopamine biosynthesis; in particular, TH levels in the nigrostriatal system may directly affect dopamine biosynthesis, so TH is closely related to the pathogenesis of Parkinson's disease[2-3]. Monoamine oxidase B (MAO-B) is one of the key enzymes in the dopamine catabolic process. It can increase the dopamine effective concentration through the inhibition of MAO-B activity, thus improving the symptoms of Parkinson's disease[4]. Superoxide dismutase, malondialdehyde and the dopamine metabolite homovanillic acid also contribute to Parkinson's disease[5-7]. Levodopa, a commonly used drug for Parkinson's disease, is associated with a number of adverse reactions[8-11]. Increasing attention has been paid to Chinese herbal neuroprotective treatment because it is associated with fewer adverse reactions and it has shown efficacy in the treatment of Parkinson's disease[12]. However, the mechanism associated with the effects of Chinese herbal medicine in the treatment of Parkinson's disease are not well understood[13]. *Zhichan* powder, a Chinese herbal medicine compound, can apparently improve the clinical symptoms of Parkinson's disease patients, especially their gait disturbances, tremor, speech disorder, poor self-care ability, reduced bimanual movements and writing disorders, and it has been reported to have an improvement rate of over 56%[14].

In this study, TH and MAO-B mRNA expression in the substantia nigra of Parkinson's disease rats was determined using reverse transcription PCR and biochemical assays after intragastric administration of *Zhichan* powder. The levels of striatal dopamine and its metabolite homovanillic acid, as well as the levels of superoxide dismutase and malondialdehyde in blood, were also analyzed in a broader attempt to understand the neuroprotective effect of *Zhichan* powder and its underlying mechanism in the treatment of Parkinson's disease.

RESULTS

Quantitative analysis of experimental animals

A total of 150 Sprague-Dawley rats were involved in this study. Among them, 30 rats were randomly selected as a normal control group (normal feeding + intragastrical administration of saline), and the remaining 120 rats were used for the establishment of Parkinson's disease models through intracerebral injection of 6-hydroxydopamine. Then, 64 successful models were randomly assigned into the model group (intragastrical administration of saline) and *Zhichan* powder group (intragastrical administration of *Zhichan* powder), with 32 rats in each group. Finally, the normal control group (n = 26; four rats died of esophageal mucosa injury), the *Zhichan* powder group (n = 26; six rats died of esophageal mucosa injury) and the model group (n = 25; five rats died of esophageal mucosa injury and two rats died of emaciation) entered the final analysis, which included 24 rats from each group.

Effect of *Zhichan* powder on TH gene expression in the substantia nigra of Parkinson's disease rats

Reverse transcription-PCR results showed that TH gene expression in the rat substantia nigra was significantly increased in the *Zhichan* powder and model groups compared with the normal control group at 10 days after administration (P < 0.05). The expression level reached a peak in the *Zhichan* powder group at 20 days after administration (P < 0.01). TH expression was maintained at a high level over a period of 50 days post-administration in the *Zhichan* powder group, whereas it gradually decreased in the model group to a level that was close to that in the normal control group at 50 days (Figure 1).

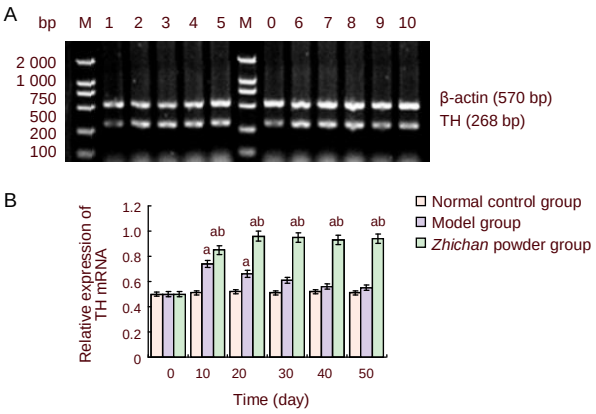

Figure 1 Effect of *Zhichan* powder on tyrosine hydroxylase (TH) mRNA expression in the substantia nigra of Parkinson's disease rats.

(A) Electrophoresis. M: DL2000 DNA marker; 0: normal control group at day 0; 1–5: *Zhichan* powder group at 10, 20, 30, 40, 50 days; 6–10: model group at 10, 20, 30, 40, 50 days.

(B) TH mRNA expression in the substantia nigra of Parkinson's disease rats.

[a]P < 0.05, *vs*. normal control group; [b]P < 0.01, *vs*. model group. Data are expressed as absorbance ratio of TH mRNA to β-actin (mean ± SD); there were four rats from each group in triplicate experiments, and results were analyzed by one-way analysis of variance and Student Newman Keuls test.

Effect of *Zhichan* powder on MAO-B gene expression in the substantia nigra of Parkinson's disease rats

Reverse transcription PCR results showed that the level of MAO-B gene expression in the rat substantia nigra was significantly increased in the *Zhichan* powder group and model group compared with the normal control group at 10 days after administration ($P < 0.01$). The expression levels reached a peak in the *Zhichan* powder group at 10 days ($P < 0.01$). Then, the TH expression level began to decrease ($P < 0.01$) in a time-dependent manner until the expression level was close to that in normal control group at 50 days (Figure 2).

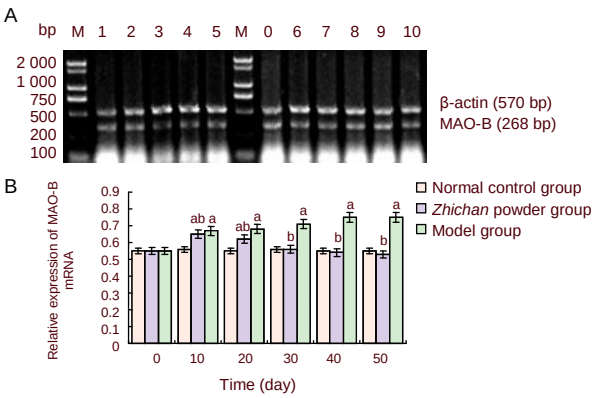

Figure 2 Effect of *Zhichan* powder on monoamine oxidase B (MAO-B) mRNA expression in the substantia nigra of Parkinson's disease rats.

(A) Electrophoresis. M: DL2000 DNA marker; 0: normal control group at day 0; 1–5: *Zhichan* powder group at 10, 20, 30, 40, 50 days; 6–10: model group at 10, 20, 30, 40, 50 days.

(B) MAO-B mRNA expression in the substantia nigra of Parkinson's disease rats.

[a]$P < 0.01$, *vs*. normal control group; [b]$P < 0.01$, *vs*. model group. Data are expressed as absorbance ratio of MAO-B mRNA to β-actin (mean ± SD); there were four rats from each group in triplicate experiments, and results were analyzed by one-way analysis of variance and Student Newman Keuls test.

At 10 days after *Zhichan* powder administration, the MAO-B content in the rat substantia nigra was significantly lower than that in the model group ($P < 0.01$), accounting for a 35.8% inhibition rate. With prolonged administration time, the inhibition of MAO-B activity was significantly increased and peaked at 50 days at an inhibition rate of nearly 48.9%. During the period 10–50 days post-administration, the MAO-B activity in the model group was significantly higher than that in the normal control group ($P < 0.01$; Table 1).

Effect of *Zhichan* powder on the levels of striatal dopamine and its metabolite homovanillic acid in Parkinson's disease rats

Fluorescence spectrophotometric analysis results revealed significant differences in the levels of striatal dopamine and its metabolite homovanillic acid in the normal control group compared with the model group and the *Zhichan* powder group after 50 days of *Zhichan* powder administration ($P < 0.05$). In addition, the *Zhichan* powder group had higher levels of dopamine and homovanillic acid, as well as a higher dopamine/ homovanillic acid ratio, than did the model group ($P < 0.05$; Table 2).

Table 1 Effect of *Zhichan* powder on monoamine oxidase B activity in the substantia nigra of Parkinson's disease rats

Time post-administration (day)	Monoamine oxidase B activity			Inhibition rate (%)
	Normal control group	Model group	*Zhichan* powder group	
0	35.35±0.00	35.35±0.00	35.35±0.00	0
10	35.26±0.60	86.37±1.21[b]	55.44±1.01[ab]	35.8
20	36.61±1.34	89.28±1.64[b]	53.38±0.99[ab]	40.2

[a]$P < 0.05$, *vs*. model group; [b]$P < 0.01$, *vs*. normal control group. Data are expressed as mean ± SD; there were four rats in each group, and results were analyzed by one-way analysis of variance and Student Newman Keuls test.

Brain protein serves as the source of monoamine oxidase B enzyme. The absorbance (*A*) value of reaction solution was determined using an ultraviolet spectrophotometer from the beginning of reaction to 3 hours after reaction, and the enzyme activity was calculated in an active unit that was 0.01 *A* value changes within 3 hours (U/mg protein/h).

Enzyme activity = $100A_{242\ nm}$ U/mg protein/h.

Inhibition rate = (enzyme activity of the model group – enzyme activity of the *Zhichan* powder group)/enzyme activity of the model group × 100%.

Table 2 Effect of *Zhichan* powder on striatal dopamine and homovanillic acid levels in Parkinson's disease rats

Group	Dopamine (ng/g brain wet weight)	Homovanillic acid (ng/g brain wet weight)	Dopamine and homovanillic acid ratio
Normal control	674.43±48.53	52.84±4.51	13.96±0.54
Model	125.21±21.12[a]	15.86±2.99[a]	8.05±5.32[a]
Zhichan powder	224.80±19.26[ab]	18.07±2.96[ab]	12.01±3.16[b]

[a]$P < 0.05$, *vs*. normal control group; [b]$P < 0.01$, *vs*. model group. Data are expressed as mean ± SD; there were four rats in each group, and results were analyzed by one-way analysis of variance and Student Newman Keuls test.

Effect of *Zhichan* powder on superoxide dismutase activity and malondialdehyde level in Parkinson's disease rats

At 50 days of *Zhichan* powder administration, blood

superoxide dismutase activity was significantly increased while malondialdehyde levels were significantly decreased ($P < 0.05$, $P < 0.01$; Table 3).

Table 3 Effect of *Zhichan* powder on superoxide dismutase and malondialdehyde levels in Parkinson's disease rats

Group	Superoxide dismutase (NU/mL)	Malondialdehyde (μM)
Normal control	190.61±21.14	3.89±0.96
Model	178.09±25.36[a]	6.01±1.19[a]
Zhichan powder	210.19±37.45[ab]	3.64±0.78[b]

[a]$P < 0.05$, *vs*. normal control group; [b]$P < 0.01$, *vs*. model group. Data are expressed as mean ± SD; there were four rats in each group, and results were analyzed by one-way analysis of variance and Student Newman Keuls test.

DISCUSSION

Parkinson's disease is a complex neurodegenerative disease, the main pathological changes of which are the degeneration and apoptosis of midbrain dopaminergic neurons[15]. Therefore, restoring dopaminergic function and promoting dopamine secretion are key in the treatment of Parkinson's disease.

An increasing amount of work has tried to identify why dopaminergic neurons selectively decrease in the substantia nigra, but no definitive reason has been accepted. Proposed theories include mitochondrial dysfunction, oxidative stress, apoptosis, neuronal excitability toxicity, environmental factors and genetic factors such as aging of the nervous system[16-19]. Among them, oxidative stress is considered to be a common pathological process in a variety of mechanisms. Any pathogenic factor in the substantia nigra may result in oxidative stress, thus inducing apoptosis or death of dopaminergic neurons, so oxidative stress is a key factor in Parkinson's disease[20]. Dopaminergic neurons are rich in midbrain but dopamine beta hydroxylase is absent, and most TH-immunoreactive neurons in the midbrain are dopaminergic neurons[21], thus, abnormal changes of intracellular TH content contribute to the clinical symptoms of Parkinson's disease and indirectly reflect the degree of oxidative stress. MAO-B is mainly distributed in 5-serotoninergic neurons, aminergic neurons and glial cells, and the enzyme content in many brain regions gradually increases with age[22]. In addition, dopamine synthesis, metabolism and turnover rate becomes more rapid in the residual dopaminergic neurons of Parkinson's disease patients. Intracellular dopamine can be degraded into 3,4-dihydroxy benzene acetic acid through MAO-B catalysis, and then generate large amounts of hydrogen peroxide, superoxide anion and hydroxyl radicals during the process of catecholamine-O-methyl-transferase metabolism for homovanillic acid; these free radicals oxidize cell membranes and organelle membrane systems, thus leading to Parkinson's disease. Therefore, inhibition of MAO-B activity can decrease the rate of dopamine degradation and metabolism, raise dopamine concentrations, and delay cell death in the substantia nigra[23-24]. Benserazide, a compound used to treat Parkinson's disease, acts together with the dopamine precursor levodopa to induce dopamine formation in the brain after decarboxylation[25]. However, its efficacy gradually weakens as treatment progresses, and long-term use of large doses can accelerate the death of dopaminergic neurons, thus limiting its clinical application[8-11]. Recent work has raised a major concern about the potential effect of Chinese herbal medicine in the treatment of Parkinson's disease, which is a multiple-factor, multiple-pathological-target disease[26-27].

The preliminary work of our research group has confirmed that *Zhichan* powder can regulate the signal transduction and apoptosis of dopaminergic neurons in Parkinson's disease rats. To further study the protective effect and underlying mechanism associated with *Zhichan* powder in Parkinson's disease treatment, we analyzed the levels of TH, MAO-B, dopamine, homovanillic acid, superoxide dismutase and malondialdehyde after *Zhichan* powder administration in Parkinson's disease rats.

Our experimental results showed that TH gene expression in the substantia nigra began to increase at 10 days after the administration of *Zhichan* powder and reached a peak at 20 days. The expression level in the *Zhichan* powder group was significantly higher than that in the model group and the normal control group, with a high level of expression being maintained over a period of 50 days. On the contrary, MAO-B gene expression initially increased and then began to decrease as treatment time proceeded, until the level was close to that in the normal control group at 50 days. This evidence suggested dependence of the effect on time and dose. An inhibition rate of 48.9% against rat serum MAO-B activity at 50 days also indicated the time-dependent manner of *Zhichan* powder's effect. In addition, there were significant differences in the levels of dopamine and homovanillic acid, and the dopamine/homovanillic acid ratio, between the *Zhichan* powder group and the model group. The above evidence shows that *Zhichan* powder increases TH gene expression and

reduces MAO-B gene expression in the rat substantia nigra, accordingly raising dopamine content and promoting a functional recovery of dopaminergic neurons. We speculate that the mechanism underlying the effect of *Zhichan* powder is transforming TH proteins produced in the substantia nigra into levodopa, which can be decarboxylated to neurotransmitter dopamine, thus decompensating the dopaminergic deficiency in the substantia nigra pathway induced by the damage of dopaminergic neurons. On the other hand, *Zhichan* powder probably inhibits MAO activity and then reduces dopamine degradation and reuptake, improves dopamine concentrations, and decreases the levels of hydrogen peroxide hydroxyl radicals and other neurotoxins, thus delaying nigral neuronal death and exhibiting protective effects on neurons.

Under normal physiological conditions, free radicals produced by human metabolism can be inactivated by the free radical system. Human free radical scavenging enzymes include superoxide dismutase, catalase, and antioxidant non-enzymes small molecule glutathiones[28]. When the nigrostriatal projection system is impaired in Parkinson's disease patients and oxidative stress is induced, free radicals damage biofilms and produce malondialdehyde, thus altering biofilm structure and permeability, leading to cell death[29-32]. At 50 days after *Zhichan* powder treatment, the malondialdehyde content was significantly lower in the *Zhichan* powder group than in the model group, while superoxide dismutase activity was increased significantly, which indicates that *Zhichan* powder can regulate superoxide dismutase activity, improve the capacity for clearing free radicals, reduce malondialdehyde secretion, and exert protective effects on neurons.

In this study, we analyzed the expression of Parkinson's disease-related active molecules in rats and the results confirmed the neuroprotective effect of *Zhichan* powder in a broader attempt to further investigate the action mechanism of *Zhichan* powder in the treatment of Parkinson's disease and provide novel evidence for Chinese herbal intervention.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

Experiments were performed from March 2008 to July 2011 at Experiment Center, Norman Bethune College of Medicine, Jilin University, China.

Materials

Animals

One-hundred and fifty clean, pure, Sprague-Dawley rats, aged 8 weeks, male or female, weighing 150–160 g, were provided by the Experimental Animal Center of Jilin University, China with license No. SCXK (Ji) 2007-0003. Animals were fed in separate cages at 20–25°C, with free access to water and food. The experimental disposals were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[33].

Drugs

Zhichan powder was produced by Professor Guozhong Gai, from Changchun University of Chinese Medicine, China. The prescriptions consisted of Astragalus root, Radix Ginseng, Radix Cynanchi bungei, Radix Dipsaci, Magnolia, Glossy Privet Fruit and Rhizoma Chuanxiong (Radix Dipsaci and Magnolia, Jinda Pharmaceutical Co., Ltd., Enshi Prefecture, Hubei Province, China; Glossy Privet Fruit, Hubei Northwest Chinese Herbal Medicine Production Base, Hubei Province, China; Astragalus root, Astragalus Membranaceus Production Demonstration Bases, Linjiang, Jilin Province, China; Radix Ginseng, Fusong Ginseng Production Base, Jilin Province, China; Rhizoma Chuanxiong and Radix Dipsaci, Maoze Chinese Herbal Medicine Processing Factory, Pengzhou City, Sichuan Province, China; Radix Cynanchi bungei, Yongqiang Shouwu Supply and Marketing Cooperatives, Binhai city, Jiangsu Province, China). All crude drugs were completely crushed and mixed; then, the mixture was immersed in boiling water overnight and decocted three times. The decoction solution was filtered and dried at 60–80°C, before being smashed into brown powder. Each gram of powder contained 5.36 g of crude drugs.

Methods

Establishment of a rat model of Parkinson's disease

The rat model of Parkinson's disease was established according to the methods described by Bao *et al* [34]. In brief, rats were anesthetized and fixed in a stereotactic instrument (RWD Life Science Company Limited, Shenzhen City, Guangdong Province, China), to define the coordinates of the substantia nigra pars compacta: 1.1 mm lateral to the midline, 4.4 mm behind the bregma, and subdural 7.5 mm. Then 6-hydroxy dopamine (Sigma, St. Louis, MO, USA) solution (3 mg/mL) was injected at a speed of 10 μL/min and a dose of 40 μg/kg. After injection, the needles were maintained for an additional 5 minutes and the wound was sutured, followed by penicillin antibiotic therapy for 5 days. Two weeks later, rats were subcutaneously injected with 0.25 mg/kg

apomorphine (Apomorphine, Northeast Pharmaceutical Group Shenyang First Pharmaceutical Company Limited, Shenyang City, Liaoning Province, China), to induce rotation. The total number and direction of rotations within 40 minutes were recorded, once per week, for 6 consecutive weeks. The average value of three measurement values was considered as the rotation times, and the model was successful upon the appearance of a rotation speed over 7 rotations per minute.

Rats in the *Zhichan* powder group received daily intragastric administration of *Zhichan* powder (200 g/L powder + saline) at 1 mL/100 g, twice per day. Rats in the normal control group and model group were given equal volumes of saline for 50 days. Four Sprague-Dawley rats were randomly selected from each group at each time point (once every 10 days for a total of five time points), blood samples were collected from the abdominal aorta, and brain tissue was sterilely harvested on an ultra-clean table and stored at –80°C.

TH and MAO-B gene expression in rat substantia nigra, as detected by reverse transcription PCR

The primers were designed and synthesized in Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China. The sequences are shown in Table 4.

Table 4 Sequences of PCR reaction primers

Gene	Sequences (5'–3')	Product size (bp)
β-actin	Sense: TCC GGC ATG TGC AAG GCC GG Antisense: AGA GGA TGC GGC AGT GGC CA	570
TH	Sense: TCG CCA CAG CCC AAG GGC TTC AGA A Antisense: CCT CGA AGC GCA CAA AAT AC	387
MAO-B	Sense: AGA AGC TCC AGT TGC CTA CAC G-5' Antisense: AGA GAA ATC TGA GAG TGT TCA T-5'	268

MAO-B: Monoamine oxidase B; TH: tyrosine hydroxylase.

Extraction of total RNA

Total RNA was extracted from the substantia nigra according to the instructions of the Trizol Reagent Kit (Invitrogen, Carlsbad, CA, USA). (1) Substantia nigra tissue (100 mg) was placed in a DEPC water-pretreated glass homogenizer, fully homogenized with 1 mL of Trizol, and stored at room temperature for 5 minutes. (2) Tissue homogenates were transferred to EP tubes and centrifuged at 12 000 × *g*, 4°C for 10 minutes. (3) The supernatants were transferred to fresh EP tubes, mixed with 0.2 mL of chloroform, stored at room temperature for 2–3 minutes, and centrifuged at 4°C and 12 000 × *g* for 15 minutes. (4) The colorless supernatants were transferred to clean EP tubes and mixed with equal volume of isopropanol, stored for 10 minutes at room temperature and centrifuged at 4°C and 12 000 × *g* for 10 minutes. (5) The supernatants were discarded, and 1 mL of 75% ice ethyl alcohol was added to each tube, then centrifuged at 4°C and 7 500 × *g* for 5 minutes. (6) After the supernatants had been removed and samples dried for 3–5 minutes at room temperature, 0.2 mL of RNase free water was added to make a total RNA solution. Finally, 2% agarose gel electrophoresis analysis was performed to analyze the quality of total RNA.

Total RNA reverse transcription into cDNA

Reverse transcription was performed in 0.5-mL EP tubes, according to the instructions of the GenAmpRNA PCR Kit (Hangzhou Bioer Technology Co., Ltd., Hangzhou city, Jiangsu Province, China). To each tube was added total RNA solution (1 μL), 5 × PCR buffer (4 μL), dNTPs (10 mM; 2 μL), RNase inhibitor (1 μL), oligodT (1 μL), AMV reverse transcriptase (1 μL) and sterile double-distilled water (10 μL), so that the total reaction volume was 20 μL. After mixing and centrifugation, reaction tubes were incubated at 37°C in a water bath for 10 minutes, at 42°C in a water bath for another 1 hour, and then at 94°C in a water bath for another 5 minutes. The reaction tube was immediately cooled for 1 minute after being taken out of the water bath, and the cDNA solution was stored at –20°C.

PCR reaction

To 0.2-mL PCR reaction tubes was added cDNA solution (2 μL), 10 × Taq enzyme buffer (5 μL), dNTPs (10 mM; 2 μL), target gene upstream and downstream primers (10 μL), internal reference upstream and downstream primers (5 μL), Taq enzyme (3 μL), and DEPC water (3 μL), so that the total reaction volume was 50 μL. The PCR reaction conditions used are shown in Table 5.

Table 5 PCR reaction conditions for tyrosine hydroxylase and monoamine oxidase-B genes

Item	β-actin	Tyrosine hydroxylase	Monoamine oxidase-B
Predegeneration	94°C 5 min	94°C 5 min	94°C 5 min
Degeneration	94°C 30 s	94°C 1 min	94°C 1 min
Annealing	55°C 30 s	58°C 1 min	56°C 1 min
Extension	72°C 1 min	72°C 2 min	72°C 2 min
Postextension	72°C 10 min	72°C 10 min	72°C 10 min
Cycles	35	35	35

Min: Minute; s: second.

Analysis of PCR products

Using TAE buffer solution, 1.5% agarose containing

ethidium bromide was produced at final concentration of 1 μg/μL. To each lane of a 1.5 agarose gel was added 10 μL of sample for electrophoresis analysis at room temperature and 100 V constant voltage for 25 minutes. After electrophoresis, the agarose gel was removed and observed under a gel imaging system (Hangzhou Tianneng Biotechnology Co., Ltd., Hangzhou city, Zhejiang Province, China). The absorbance value was recorded, and the ratio of target gene absorbance to β-actin (reference gene) absorbance was considered the expression level of the target gene.

Determination of MAO-B activity

The enzyme activity and inhibition rate were calculated according to Wang's methods[35].

Enzyme activity = $100A_{242\ nm}$ U/mg protein/h.

Inhibition rate = (enzyme activity in the model group – enzyme activity in the *Zhichan* powder group) / enzyme activity in the model group × 100%.

Determination of striatal dopamine and homovanillic acid contents

The contents of dopamine and its metabolite homovanillic acid were detected using fluorescence spectrophotometry[36]. Rat brain tissue was homogenized, successively extracted with n-butyl alcohol and heptane, oxidized with iodide, reduced with alkaline sodium sulfite, and re-adjusted with phosphoric acid to pH 7.8. The intensity of fluorescent bands was determined at a 310-nm excitation wavelength and 390-nm absorption wavelength. The dopamine content was measured. After homovanillic acid solution was mixed with ammonium hydroxide and potassium ferricyanide, the absorbance was detected using a fluorescence spectrometer at $A_{320\ nm/440\ nm}$. After the measured fluorescence value for the standard sample was subtracted from the measured fluorescence value for the blank tube, a standard curve was generated and the regression equation was obtained. Then, the contents of dopamine and homovanillic acid in each gram of brain were calculated by regression analysis.

Determination of serum superoxide dismutase levels

Superoxide dismutase activity was detected using the xanthine oxidase method[23], according to the kit instructions (Nanjing Jiancheng Biological Engineering Company Limited, Nanjing, Jiangsu Province, China). The activity of superoxide dismutase was measured based on the formula: Activity (NU/mL) = ($A_{550\ nm}$ of control tube − $A_{550\ nm}$ of detected tube) / $A_{550\ nm}$ of control tube × 50% × dilution time.

NU is the nitrite unit, referring to the quantity of superoxide dismutase per milliliter of reaction liquid when the inhibition rate is 50%. The detection mechanism is as follows: through the xanthine and xanthine oxidase reaction system, superoxide anion free radical (O_2^-) is produced and oxidized with hydroxylamine to form nitrite, which is stained red by the chromogenic agent. The absorbance value was detected using a visible light spectrophotometer. When the detected sample contained superoxide dismutase, it had specific inhibition effects on superoxide anion free radicals, allowing the reduction of nitrite, the absorbance value of detected tube was lower than that of the control tube, and the activity of superoxide dismutase could be measured using the formula.

Determination of serum malondialdehyde levels

The serum malondialdehyde levels were detected spectrophotometrically according to the kit instructions (Nanjing Jiancheng Biological Engineering). Serum malondialdehyde level = ($A_{550\ nm}$ of detected tube – $A_{550\ nm}$ of blank tube) / ($A_{550\ nm}$ of standard tube – $A_{550\ nm}$ of standard blank tube) × standard sample concentration × dilution time.

Statistical analysis

Quantitative data are expressed as means ± SD using SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). Multiple group comparisons were performed using one-way analysis of variance and the Student Newman Keuls test. A $P < 0.05$ value was considered to represent a statistically significant difference.

Author contributions: Yongmao Liu, Yongwei Lou and Weimin Tang provided experimental data. Qingwei Zhou integrated experimental data. Yongmao Liu conceived and designed the study. Jiajun Chen, Qingwei Zhou and Yongmao Liu analyzed experimental data. Jiajun Chen, Qingwei Zhou, Yongmao Liu, Pengguo Zhang and Shihong Yi participated in the paper writing. Jiajun Chen and Yongmao Liu supervised the paper. Yongwei Lou and Weimin Tang performed the statistical analysis.

Conflicts of interest: None declared.

Ethical approval: The experiment was approved by Animal Ethics Committee, Norman Bethune College of Medicine, Jilin University, China.

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