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

Jiajun Chen¹, Jinshu Ma², Yafei Qiu², Shihong Yi², Yongmao Liu², Qingwei Zhou³, Pengguo Zhang⁴, Quan Wan⁵, Ye Kuang³

¹ Department of Neurology, China-Japan Union Hospital, Jilin University, Changchun 130031, Jilin Province, China
² Department of Immunology, Norman Bethune College of Medicine, Jilin University, Changchun 130021, Jilin Province, China
³ Department of Biochemistry and Molecular Biology, Institute for Regenerative Medicine, Jilin University, Changchun 130021, Jilin Province, China
⁴ Department of Image, Second Clinical Hospital, Jilin University, Changchun 130021, Jilin Province, China
⁵ Department of Biotechnology, Life Science College, Jilin University, Changchun 130012, Jilin Province, China

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 transduction 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
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[3]. Levodopa substitution therapy has been considered an effective method to treat Parkinson’s disease[3], 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 acahanpanax 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[4,5]. 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 groups 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](image)

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Caspase-3</th>
<th>p53</th>
<th>Bax</th>
<th>Bcl-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.26±0.02</td>
<td>0.95±0.06</td>
<td>0.43±0.03</td>
<td>1.22±0.04</td>
</tr>
<tr>
<td>Model</td>
<td>0.90±0.02</td>
<td>1.08±0.05</td>
<td>0.90±0.08</td>
<td>0.36±0.02</td>
</tr>
<tr>
<td>Zhichan powder</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-week</td>
<td>0.80±0.04</td>
<td>0.99±0.03</td>
<td>0.77±0.06</td>
<td>0.47±0.02</td>
</tr>
<tr>
<td>14-week</td>
<td>0.59±0.03</td>
<td>0.93±0.04</td>
<td>0.62±0.07</td>
<td>1.10±0.02</td>
</tr>
<tr>
<td>$F$</td>
<td>474.85</td>
<td>10.29</td>
<td>49.78</td>
<td>1 499.80</td>
</tr>
<tr>
<td>$P$</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD, with five rats in each group. *$P < 0.01$, vs. control group; **$P < 0.01$, vs. model group; ***$P < 0.01$, 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\(^1^{16}\). The Bax protein plays an important role in apoptosis in the substantia nigra, and its increase can upregulate the apoptosis of dopaminergic neurons\(^1^{17}\). The Bcl-2 and Bax genes are a pair of apoptosis-regulated genes\(^1^{18}\). 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\(^1^{19-21}\). In our study, apoptosis was further confirmed by caspase-3 activation\(^2^{22}\). Overexpression of Bcl-2 can cause changes in redox equilibrium in the nucleus and decrease caspase-3 activity\(^2^{23}\). Bcl-2, a substrate of caspase-3, can be hydrolyzed by caspase-3\(^2^{24}\). 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\(^2^{25-26}\).

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\(^2^{25-27}\). 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\(^2^{28}\).

**Drugs**

Zhichan powder was prepared by Professor Guozhong Gai from Changchun University of Chinese Medicine, and composed of milkvetch root, ginseng, burdock root, 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 and crushed again. The mixture was sieved through a 100-mesh sieve, and the powder was dried at room temperature. The above steps were completed by Professor Guozhong Gai from Changchun University of Chinese Medicine.
The sections were incubated in 3% H_{2}O_{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 Technologies, Shanghai, China). PCR primers were synthesized by Shanghai Bioengineering Co., China. In accordance with the instructions of a GenAmpR.NA PCR kit (Bior 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 Oligo dT, 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 GenAmpRNA PCR 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.
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 t-test was used to compare the difference between indices.

Using tris-acetate-ethylenediamine tetraacetic acid buffer, the samples were electrophoresed in 1.5% agarose gel 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 (Tianeng 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 t-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.

**REFERENCES**


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