Universe Scientific Publishing

DOI: 10.18686/ahe.v6i17.5452

Study on the Mechanism of Liuwei Dihuang Pill Improving the Symptoms of Parkinson's Disease

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Abstract: Aim of the study: The progressive loss of dopaminergic neurons in the midbrain substantia nigra pars compacta (SNpc) is the main pathological feature of Parkinson's disease(PD), the motor dysfunction of PD are also related to it, and the reason is that the neuron-specific loss of autophagy leads to the accumulation of misfolded α-synuclein(α-syn) protein aggregates and cell apoptosis. The experiment made by the research group is aimed at obtaining the results of the α-syn， autophagy-inducing, and anti-apoptosis effects of Liuwei Dihuang Pill(LDP) to evaluate any potential antiparkinsonian properties to provide the favorable effects on motor symptoms in rotenone-induced PD rat models.Materials and methods: rotenone-induced PD rat models were treated with LDP, the behavioral changes of the rats were detected by the bar test and the narrow beam test, the protein levels of α-syn， autophagy-related proteins（ LC3B-II/LC3B-I, P62 ）, and apoptosis-related proteins (Bcl-2/ Bax) were measured using western blot assays.Results and conclusions: It was observed in the model group compared with the control group that LDP improved the behavioral winch including the bar test and the narrow beam test, reduced the content of α-syn, increased the induction of autophagy, and reduced the cell apoptosis. This study suggests that LDP effectively improved the behavioral and dopaminergic neuron damage in rotenone-induced PD rat models, and the mechanism of this action may be increased the induction of autophagy and the relief of apoptosis.

Keywords: Parkinson's disease; Liuwei Dihuang Pill; Motor symptoms; Autophagy; Apoptosis.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease which epidemiologically shows affects 1% of the population above 60 years of age[1]. With the increase in life, the incidence and prevalence of PD will increase substantially, which will be a significant economic and social burden[2]. However, no therapy can slow down or arrest the progression of Parkinson's disease at present[3]. The traditional Chinese medicine（ TCM ） has achieved good results in the treatment of Parkinson's disease through the theory of kidney-brain synergy[4,5], and the Liuwei Dihuang Pill（ LDP ）is a classical TCM formula, with the function of nourishing kidney, has been widely used for PD in clinic[6]. However, the underlying mechanism is not fully understood, to further evaluate the anti-Parkinsonian effect of LDP and explore its potential molecular mechanism, the rotenone-induced Parkinson's rat model was established. After the treatment of LDP, the Parkinson's disease-related behaviors were observed, and the potential neurobiological mechanisms were detected by observing autophagy and apoptosis in the midbrain substantia nigra pars compacta (SNpc).

1. Materials and Methods

1.1 Experimental Animals

Thirty male Sprague-Dawley(SD) rats (aged 12-16 weeks; weighing between 220-260g) were taken. They were fed in the specific pathogen-free (SPF) laboratory and kept in separated cages. The temperature was maintained at 22-25°Cwith relative humidity of 50-70%. Water and feed were supplied ad libitum. Rotenone (purchased from Tokyo Chemical Industries (TCI), Japan) was suspended in sunflower oil at a concentration of 2 mg/ml[7], and vortexed thoroughly just before injection to ensure a uniform suspension. Rotenone was injected daily at a concentration of 2 mg/kg. 30 rats were randomly and equally assigned to three groups as follows: control group (CON), model group(MOD) and treatment group (TRE). Each received subcutaneous injections daily for five weeks.

1.2 Drug Preparation

A certain amounts of LDP was dissolved in distilled water (Beijing Tongrentang Technology Co., Ltd.) to prepare an aqueous solution with a concentration of (0.675 mg/mL). CON without any treatment; CON and MOD were given distilled water (1 mL/100 g) by intragastric route; TRE was given an aqueous solution (1 mL/100 g) by intragastric route. All groups were administered at the beginning of the animal model and stopped at the end.

1.3 Behavioral Study

Animals were handled daily for 6 days before rotenone injections to minimize the effects of fear during manipulations. The behavioral tests were performed 24 hours after the last injections (Day 35) and were carried out between 10:00 AM and 3:00 PM by blinded investigators.

1.3.1 Bar Test

The bar test[8] was set to a height of 12 cm. Rats were placed gently with their forelimbs on the bar and their hindlimbs on the floor of the apparatus. The timer starts once the rat's front paw is on the stick. When the rat falls from this posture, the broken beam sensor stops the timer, and the time is displayed on the LCD, which is the freezing time. Each rat is tested five times, and the average value is recorded.

1.3.2 Beam Test

The narrow beam[9] used for the present experiments was a 105 cm long wooden beam, 4 cm wide and 3 cm tall. The beam was suspended 80 cm from the ground by wooden supports at either end. The wooden supports at the "starting" end of the beamformed a sheer drop while a platform was located at the other end, next to which was placed the home cage of the rat being tested. Beneath the beam was placed 1 m wide foam padding, approximately 12 cm thick to prevent injury to the animals in case of a fall. At the start end of the beam, a line was drawn 20 cm from the end of the beam. During a test, the rat was placed entirely within this 20 cm starting zone facing its home cage and a stopwatch started immediately upon release of the animal. The time was recorded when the animal placed a weight-bearing step entirely over the start line. This time represented the latency to begin the task. The stopwatch was then stopped when all four feet were placed entirely upon the finishing platform at the opposite end of the beam. The maximum time allowed for the task was 2 min. The start line must be crossed within 1 min from release or the test was canceled and maximum time was recorded for that trial. A fall was also recorded as a maximum time.

1.4 Western Blot Analysis

To assess the α-synuclein, LC3, p62, Bax, and Bcl-2 protein expression in the substantia nigra, The method of western blots was performed as follows. The rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g). When it loses its resistance and muscle tension is significantly reduced, The brain was obtained by decapitation on the ice. Residual blood was washed away with a cold phosphate-buffered solution (PBS). Substantia nigra was dissociated on ice with RIPA buffer containing protease inhibitor and PMSF for 30 mins and centrifuged at 12,000g (4°C, 30 mins). The final protein concentration was determined from the supernatant with a BCA kit (Beyotime Biotechnology, China). Similar amounts of protein samples (20-40 μg) were subjected to electrophoresis and transferred onto a PVDF membrane (Millipore, China). The membranes were blocked with 5% non-fat milk in TBS with 0.1% Tween 20 for 1 h and then incubated with primary antibodies [Rabbit anti-α-syn monoclonal antibody (1:500, Abcam， USA), anti-P62 antibody (1:1000, Thermofisher Scientific， USA), Rabbit Anti-LC3 antibody (1:1000, Novus, USA), Anti- Bax antibody (1:1000, Cell Signaling, USA), Anti- Bcl-2 antibody (1:1000, Cell Signaling, USA),] at 4°C overnight. the PVDF were washed 3 times in TBST (TBS with 0.05% v/v Tween-20) at room temperature and then incubated with horseradish peroxidase-conjugated secondary antibody diluted in TBST (1:2,000) for 1 h at room temperature followed by washing, signal detection was performed with an enhanced chemiluminescence kit(Millipore, China), and β-actin as an internal reference, finally analysis of the integral value of the optical density using ImageJ Software (NIH).

2. Statistical Analysis

All data were presented as the mean ± standard deviation (SD) and analyzed by the SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA). Differences in the data among each group were analyzed by one-way analysis of variance with Tukey's test. $P<0.05$ was considered statistically significant.

3. Results

3.1 Behavioral Study

The MOD showed a significant increase in both latencies to initiate crossing and total time on beam compared with the CON in the beam test. Latency was significantly increased from （1.23±0.28 s）to（28.62±2.05 s）. Total time was significantly increased

from （12.19±2.86 s） to （63.86±5.53 s）. In the bar test, freezing time also increased in the MOD compared with that in the CON from(1.80±0.53 s) to (39.72±2.00 s). By contrast, the TRE showed a significantly shorter time to latency to initiate crossing (10.62±0.36 s) and total time (32.32±1.23 s) on the beam than those for the MOD. The TRE also showed a significantly shorter freezing time (20.05±1.05 s) on the bar compared with that in the MOD. The above results are shown in Figure 1.

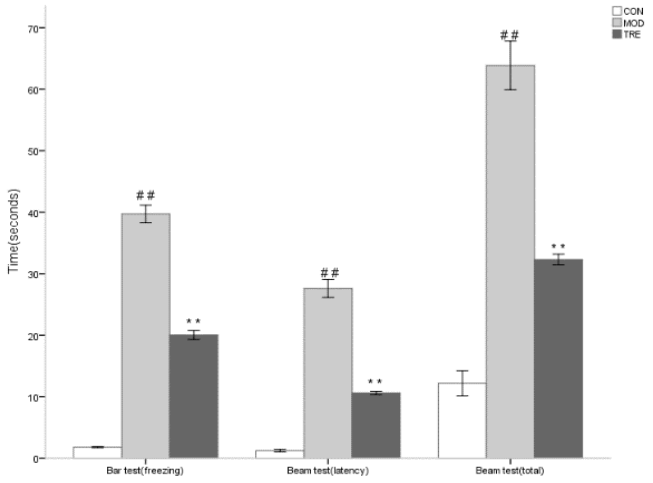

Fig 1. Compare the behavior of each group

Results were shown as the mean ± SD. # #P < 0.01, compared with the CON; * * P < 0.01, compared with the MOD.

3.2 Western-Blotting Study

Compared with the CON, the MOD showed a significant increase in the protein level of α-Syn, while the TRR showed a significant reduction compared with the MOD. Compared with the CON, the protein levels of LC3B-II/LC3B-I increased, while the P62 was reduced in the MOD group， and compared with the MOD, the protein levels of LC3B-II/LC3B-I increased significantly, while the P62 was reduced significantly in the TRE group. Compared with CON, the protein level of MOD is significantly reduced, while that of TRE is significantly increased compared to MOD. Compared with CON, the protein level of Bcl-2/Bax in MOD was significantly lower, while TRE was significantly higher than that in MOD. The above results are shown in Figure 2.

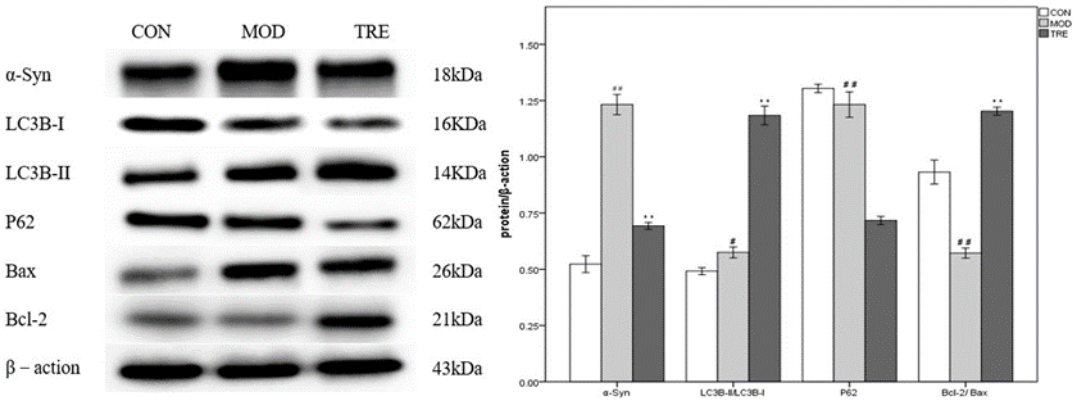

Fig 2. The protein level of α-Syn, LC3B-II/LC3B-I, P62 , and Bcl-2/ Bax were measured with western-blotting.

Results were shown as the mean ± SD. #P < 0.05, # #P < 0.01, compared with the CON; * P < 0.05, * * P < 0.01, compared with the MOD.

4. Discussion

PD is a rapidly developing neurodegenerative disease characterized by the loss of nigrostriatal dopamine cells leading to a gradient of striatal dopamine depletion, leading to an imbalance between the direct (promoting) and indirect (inhibitory) pathway through the basal ganglia, and eventually arising motor symptoms clinically[10]. Currently, the diagnostic criteria for PD are mainly identified by classical motor symptoms, including rest tremor, bradykinesia, rigidity, and postural instability [11]. In the behavioral tests we performed, The time is taken when the two forepaws left the bar served as a measure of Parkinson's rigid states[8]. the time required for the balance rod test which the rats need to start moving when placed on the beam served as an indicator of parkinsonian movement inability, and the total time required to cross the beam was used to measure bradykinesia, balance, and postural instability

in parkinsonian animals[9]. In the experimental results, we can see that LDP partly improves the motor symptoms of PD.

It has been shown that the motor symptoms of PD begin to manifest when 40 – 60% of the neurons in the SNpc are lost[12]. The main cause of cell death in dopamine neurons is the accumulated accumulation of α -synaptic nucleoprotein (α-Syn) [13]. It is indicated by the determination of the-Syn levels that the LDP reduces the deposition of the-Syn. While the removal of beta-synaptic nuclear proteins from cells depends mainly on the efficiency of autophagy, the dysregulation of the autophagy pathway favors the accumulation of beta-synaptic nuclear proteins and leads to neuronal death, thus promoting the pathogenesis of PD[14]. And in vitro and animal models, the stimulation of autophagy plays an otherwise positive role in neurodegeneration processes preventing or reversing PD[15]. Autophagy is a biological process involving a range of autophagy-related proteins, and LC3, a key protein during autophagy, has been intensively studied in autophagy[16]. The LIR domain interacts with LC3 to facilitate the promotion of autophagosome formation and delivery to autophagy-degrading [17]. The expression level of p62 protein is considered an indicator of the state of autophagic flux [18]. Therefore, we examined the autophagy-associated proteins LC3 and P62 in various rat groups. As a result, we can see that LDP significantly increased the autophagy levels in parkinsonian rats.

Pathologically, PD is defined by the loss of dopaminergic neurons in the nigra-dense nigra, located in the midbrain and associated with the Lewy body[19]. Apoptosis is the programmed elimination of cells during the normal development of eukaryotes and during the maintenance of body homeostasis, and this pathway is controlled by the BCL-2 protein family[20]. In contrast, if the relative amount of Bcl-2 is higher than that of Bax, it inhibits apoptosis[21]. Finally, we examined the expression of the apoptosis-related proteins Bcl-2 and Bax in various rats, and the results indicated LDP inhibits apoptosis, The electron microscope also reached the same conclusion.

To sum up, it can be speculated that LDP can alleviate Parkinson's symptoms possibly by enhancing autophagy to reduce the misfolded synaptic protein content and in the reduction of apoptosis. However, this study was only a pilot animal trial, and no single animal model can perfectly replicate all the pathogenic and clinical features of PD, which needs further studies to confirm the clinical significance of the study.

5. Conclusion

LDP can alleviate PD symptoms, possibly by the upregulation of LC3-I to LC3-II and the downregulation of P62 expression to enhance autophagy in nigra dopamine neurons and reduce synaptic protein content and Bax / Bcl-2 reduces apoptosis.

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