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Mechanism of Catgut Point Embedding Therapy on Parkinson's Disease Caused by Impaired Autophagy and Apoptosis

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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 is 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 PET 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 PET for 10 weeks, the behavioral changes of the rats were detected by the bar test and the narrow beam test observe the morphological changes of SNpc, the protein levels of α-synautophagy-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 treatment group compared with the modle group that PET improved the behavioral winch including the bar test and the narrow beam test, preserved the Cell morphology of SNpc, reduced the content of α-syn, increased the induction of autophagy and reduced the cell apoptosis. This study suggests that PET 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.

Key words: Parkinson's Disease · Point Embedding Therapy · 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, 3]. However, no therapy can slow down or arrest the progression of Parkinson's disease at present [4]. But in traditional Chinese medicine Point Embedding Therapy(PET) is being tested for disease-modifying potential [5]. Baihui (GV 20), Shenshu (BL 23), Taixi (KI 3) and Chorea-tremble controlled area are commonly used as acupuncture points for the treatment of PD [6, 7], Its underlying mechanism has not been completely understood. To further evaluate the anti-Parkinsonian effect of PET and explore its potential molecular mechanism, the rotenone-induced Parkinson's rat model was established. After the treatment of PET, 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).

MATERIALS AND METHODS

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 with relative humidity of 50-70%. Water and feed were supplied ad libitum.

Experimental Design: Rotenone (purchased from Tokyo Chemical Industries (TCI), Japan) was suspended in sunflower oil at a concentration of 2 mg/ml [8] and

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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, each received subcutaneous injections daily for five weeks, as follows: (1) control group (CON) (healthy rats without any treatment);(2) model group(MOD) (rotenone-induced PD rat models without any treatment); (3) treatment group (TRE) (rotenone-induced PD rat models treated with PET).

Embedding the Rat: Covered the heads of the rats with a hood [9] removed the hair at the site for thread embedding and then a collagen thread (K3-0 Longteng, Jiangxi China) cut into 10 mm using a disposable thread embedding needles (7# Essica Jiangsu, China) was embedded in the Baihui (GV 20), bilateral Shenshu (BL 23), bilateral Taixi (KI 3) and bilateral Chorea-tremble controlled area. All acupuncture points were following the acupoint positioning method provided by Chang *et al*.[10].

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.

Bar Test: The bar test [11] 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 rate 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.

Beam Test: The narrow beam [12] 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.

Light Microscopy: The midbrain was prepared by embedding it in paraffin after dehydrating it, making it transparent and fixing it with 10% buffered formalin. For histopathological evaluation, the tissues were cut into 4 μm thick serial sections and stained with Harris hematoxylin and eosin (HE). Sections were observed under a BH-2 light microscope (Olympus, Tokyo, Japan) to determine degenerated neurons' number and neuronal structure. The central third of each slice was selected and five randomly selected fields were counted at 400X magnification.

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).

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.

RESULTS

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.

Histopathological Effects of SNpc: The CON cells are arranged neatly and densely with normal morphology. The cell membrane of MOD showed obvious shrinkage, part of it was slightly swollen, the volume increased, the intercellular space was widened and it was on one side, the cytoplasm was obviously reduced and the nuclear membrane was partially incomplete and the number of cells was significantly reduced. The number of TRE cells was significantly more than that of MOD, the cell membrane was slightly shrunken, the cell volume was reduced, the cytoplasm was reduced and the nuclear membrane was intact. The above results are shown in Figure 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 3.

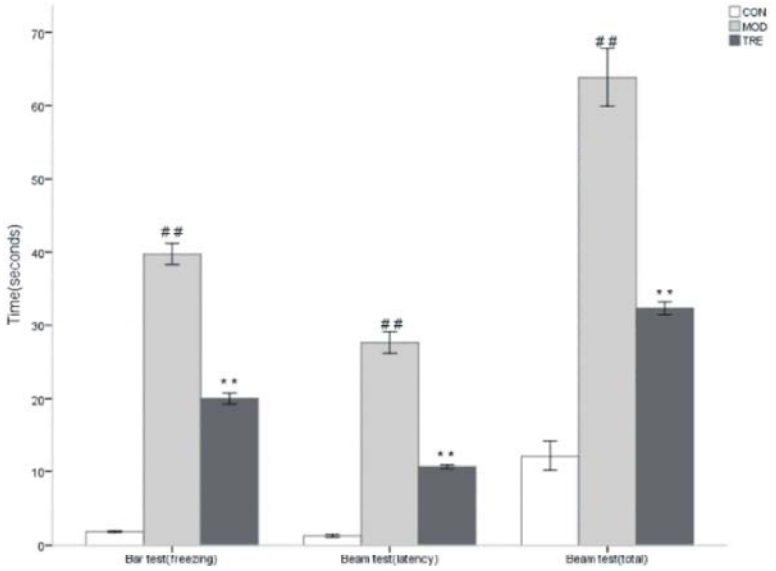
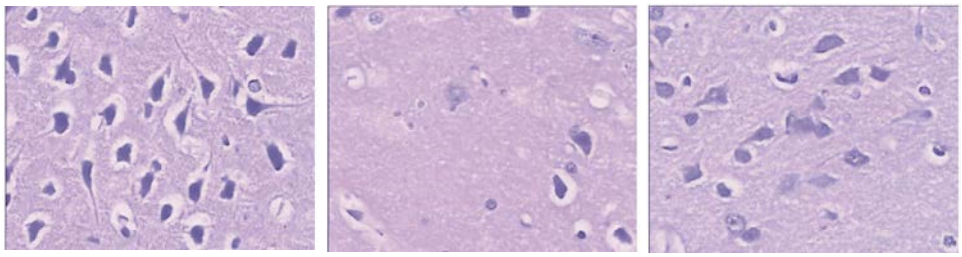


Fig. 1: Compare the behavior of each group. Results were shown as the mean ± SD. # #P < 0.01, compared with the CON; * * P < 0.01, compared with the MOD



CON MOD TRE

Fig. 2: Morphological analysis of nerve cells. Sections were examined under a light microscope to assess the number and neuronal structure of degenerating neurons

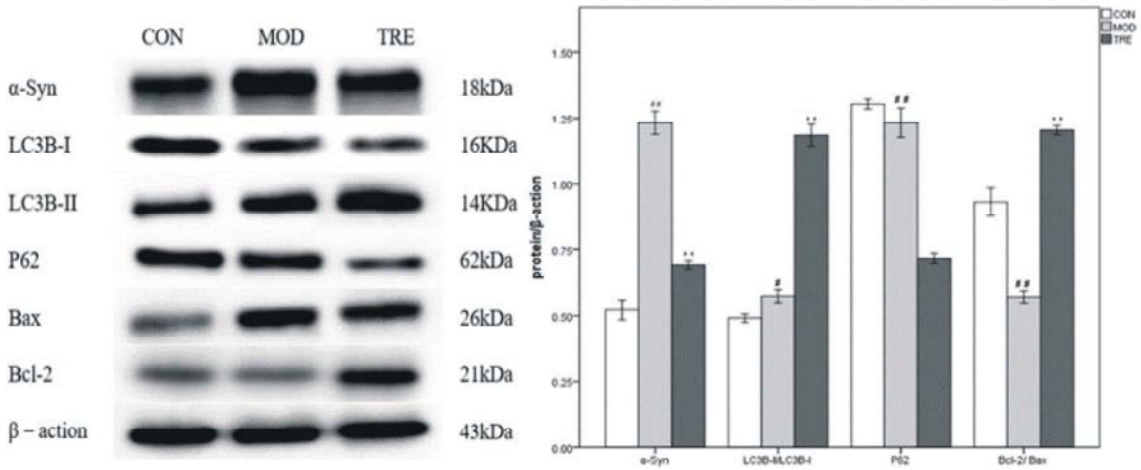


Fig. 3: The protein level of α-Syn, LC3B-II/LC3B-I, P62 and Bcl-2/ Bax were measured with western-blotting. Results were shown as the mean ± SD. #P < 0.05, # #P < 0.01, compared with the CON; *P < 0.05, **P < 0.01, compared with the MOD

DISCUSSION

PET has become an important branch of Acupuncture which originated in the 1950s [13]. It was developed based on traditional Acupuncture. The buried objects are absorbable threads. Because it forms a complex, lasting and non-specific stimulation on acupoints, this therapy is widely used in various clinical departments in China [14]. Modern research shows that PET can restore nerve function and regulate nerves. Reflect, enhance human immunity, improve local circulation, inhibit the release of inflammatory factors, reduce apoptosis, regulate cytokines and improve body metabolism [15]. The acupoints Baihui, Shenshu and Taixi used in this experiment were developed according to the theory of "combination of brain and kidney" and they are the main acupoints commonly used in the treatment of PD [16, 17], while the chorea tremor area is One of the primary areas of stimulation often used by clinicians to control tremors, it has been used since the early 1970s to treat involuntary limb movements and tremors [18].

In the selection of experimental animals [19], rodents exhibit a significant degree of human homology in brain tissue and their corresponding behavioral function. In addition, rodents can produce complex movements similar to humans and they exhibit functionally similar motor deficits after nigrostriatal dopamine (DA) lesions and similar motor responses to dopamine (DA) replacement therapy. WE SELECTED the SD rats commonly used rodent experimental models [20]. In terms of animal model selection, rotenone is a broad-spectrum insecticide extracted from natural plants, consisting of a lipophilic isoflavonoid that can easily bypass the blood-brain barrier (BBB) when consumed or administered in different ways[21]. When rotenone was administered for a long term at low doses, it induced selective cellular

degeneration of the substantia nigra pars compacta [22]. This study using rotenone-induced PD modeling caused the emergence of characteristic PD symptoms with the smallest consequences for animals while avoiding high animal mortality [8].

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 [23]. Currently, the diagnostic criteria for PD are mainly identified by classical motor symptoms, including rest tremors, bradykinesia, rigidity and postural instability [24]. In the behavioral tests we performed, 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 [12]. The time is taken when the two forepaws left the bar served as a measure of Parkinson's rigid states [11]. In the experimental results, we can see that PET 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 [25]. The main cause of cell death in dopamine neurons is the accumulated accumulation of α-synaptic nucleoprotein (α-Syn) [26]. It is indicated by the determination of the-Syn levels that the PET 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 [27, 28]. Autophagy is the "self-phagocytosis" of the cellular that protects cells from dying through the selective degradation of useless organelles and proteins [29]. The basal activity of autophagy in PD patients may be low and changing basal autophagy may provide potential therapeutic strategies for PD [30, 31]. And in vitro and in animal models, the stimulation of autophagy plays an otherwise positive role in neurodegeneration processes preventing or reversing PD [32]. 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 [33]. During the autophagosome membrane formation, LC3 is cleaved by the autophagy-associated gene protease 4 (Atg4) to produce LC3-I, which is located in the cytoplasm [34]. The LC3-II wound is made once bound to phosphatidylethanolamine (PE) through the synergistic activity of the autophagy-associated gene protease 7 (Atg4) [35]. LC3-II is required for autophagosome formation and corresponds to the number of autophagosomes. Thus, the amount of LC3-II or the conversion rate of LC3-I to LC3-II is generally recognized as a marker of mammalian autophagosomes [36]. The Sequestosome-1, also known as the ubiquitin-binding protein p62, is an autophagosome cargo protein p62 that has a specific domain with the LC3 interaction region (LIR), which ensures that the autophagy receptor binds targeted to LC3 on the phagocytic membrane [37-40]. The LIR domain interacts with LC3 to facilitate the promotion of autophagosome formation and delivery to autophagy-degrading [41]. p62 is degraded by lysosomal acidic enzymes after the fusion of lysosomes and autophagosomes [42]. The expression level of p62 protein is considered an indicator of the state of autophagic flux [43]. Therefore, we examined the autophagy-associated proteins LC3 and P62 in various rat groups. As a result, we can see that PET 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 [44]. While human autopsy studies show that apoptosis eventually loses dopaminergic neurons [45]. 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 [46]. BCL-2 family protein levels regulate pro-apoptotic or anti-apoptotic expression through a balance of dynamic processes [47]. If the relative amount of Bax is higher than in Bcl-2, acting as an apoptotic activator would promote cell death. In contrast, if the relative amount of Bcl-2 is higher than that of Bax, it inhibits apoptosis [48]. As an evolutionary and conserved mechanism determining cell fate, there is a complex relationship between apoptosis and autophagy and previous PD-related model studies showed that activation of autophagy can inhibit apoptosis in Parkinson model nigra neurons [49], rotenone-induced increased α-synaptic nuclear protein aggregation in dopaminergic neurons in PD rats, accompanied by decreased BCL-2 levels and arrest of autophagy flux. Inhibition of BCL-2 exacerbates autophagy and increases rotenone-induced

beta-synaptic nuclear protein aggregation, suggesting that BCL-2 dysregulation enhances rotenone-induced neurotoxin[50]. And BAX upregulation is an essential step for apoptosis in SNpc dopaminergic neurons [51]. Finally, we examined the expression of the apoptosis-related proteins Bcl-2 and Bax in various rats and the results indicated PET inhibits apoptosis, The electron microscope also reached the same conclusion.

To sum up, it can be speculated that PET 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 its clinical significance of the study.

CONCLUSION

PET 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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