

# Effects of pramipexole treatment on the $\alpha$ -synuclein content in serum exosomes of Parkinson's disease patients

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**Abstract.** Advances approaches in the treatment of Parkinson's disease are needed. The study was aimed to evaluate the therapeutic value of the new dopamine receptor agonist pramipexole. The effects of pramipexole on serum exosomes were investigated, and the possible mechanisms of action of the drug were explored. Initially, 68 patients were included in the study, of whom 3 cases did not complete the study. The remaining 65 patients were administered pramipexole at increasing doses starting at 0.25 mg twice a day for the 1st week, and reaching 1.5 mg three times daily at the 8th week. The doses were tapered during the course of the following 4 weeks. The total scores of the motor examination of the unified Parkinson's disease rating scale III (UPDRS III) and the total scores of the daily life activity in UPDRS II were compared before and after treatment. The relative expression amounts of  $\alpha$ -synuclein in serum exosomes were then calculated by western blot analysis. Scores of UPDRS III and UPDRS II following treatment were significantly lower than the scores prior to treatment, and the difference was statistically significant ( $P < 0.05$ ). The relative expression of  $\alpha$ -synuclein in serum exosomes was also found to be significantly lower after treatment ( $P < 0.05$ ). The relative expression of  $\alpha$ -synuclein in the effective treatment group was significantly lower than that in the ineffective treatment group, and the difference was statistically significant ( $P < 0.05$ ). The relative expression of  $\alpha$ -synuclein in serum exosomes was significantly correlated with treatment effects ( $P < 0.05$ ). In conclusion, pramipexole was effective and safe as a treatment for Parkinson's disease. The therapeutic effect of pramipexole may be associated with its reducing effect on the relative expression of  $\alpha$ -synuclein in serum exosomes.

## Introduction

Parkinson's disease, also known as tremor paralysis, is the second largest neurodegenerative disease, with morbidity rates second only to Alzheimer's disease (1). The etiology and pathogenesis of Parkinson's disease are not clear. The most important pathological changes are degeneration and loss of dopaminergic neurons in the substantia nigra of the midbrain, and the formation of  $\alpha$ -synuclein-based acidophilic inclusion bodies (lewy bodies) (2) in the remaining dopaminergic neurons. In 2010, it was reported that  $\alpha$ -synuclein was secreted out of the cells through exosomes in a calcium-dependent mechanism (3). The phenomenon was confirmed by a group of independent researchers (4). The finding of that study emphasized the importance of exosomes in the transcellular transport of  $\alpha$ -synuclein, and it strengthened the correlation between the amount of secreted exosomes and the pathogenesis of Parkinson's disease. It has also laid a theoretical basis for the early diagnosis and targeted treatment of Parkinson's disease.

In the present study, the therapeutic effects on Parkinson's disease of the new dopamine receptor agonist pramipexole and its relationship with serum exosomes and inclusion bodies were examined. The possible underlying therapeutic mechanisms were also investigated.

## Patients and methods

**Patients.** Initially, 68 patients diagnosed with primary Parkinson's disease were selected from Xianyang Central Hospital during the period October, 2012 to October, 2014. The diagnoses were confirmed according to the UK Brain Banks Network. The inclusion criteria for the study were: i) Age,  $\geq 18$  and  $< 75$  years. ii) First time diagnosis. Absence of history of Parkinson's disease drug use. iii) Patients with normal intelligence, good compliance and complete clinical data. The exclusion criteria for the study were: i) Symptomatic Parkinson's disease or Parkinson's plus a syndrome with severe peak dose dyskinesia. History of brain surgery and trauma, or malignant tumor. ii) Patients required to stay in bed for long periods of time. Patients with severe heart, liver and kidney dysfunctions. Patients allergic to pramipexole. iii) Pregnant and lactating women, patients with mental disorders, and any patients that refused to participate.

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Three patients did not complete the study, while the remaining 65 cases successfully completed the study. There were 38 men and 27 women, with an age range of 57-74 years, and an average age of  $67.8 \pm 9.2$  years. The course of disease at the time of diagnosis ranged from 1 week to 3 months, with an average of  $1.2 \pm 0.3$  months.

**Methods.** After obtaining the approval of the ethics committee of the Xianyang Central Hospital and the consent of patients and their relatives, the patients were administered oral pramipexole using the following dosing schedule: 0.25 mg twice a day for the 1st week, followed by 0.25 mg 3 times daily for the 2nd week. From the 3rd to 8th week, the doses were increased gradually starting at 0.25 mg 3 times daily and adding 0.25 mg to each dose every 3 days until 1.5 mg 3 times daily was achieved and until the end of the 8th week. Subsequently, the doses were tapered, and were reduced by 1.125 mg/week, during the course of the following 4 weeks. Liver and kidney function, and electrocardiograph indices were periodically checked and the patients' clinical symptoms were evaluated often.

**Observation indices.** Total scores of motor examination of the unified Parkinson's disease rating scale III (UPDRS III) and total scores of the daily life activity in UPDRS II, before and after treatment, were compared. To evaluate the result of the treatment, a reduction of  $\geq 50\%$  in the score of UPDRS II/III was defined as effective.

The exosomes were separated and purified from sera using an ExoQuick-TC kit (System Biosciences, Mountain View, CA, USA). The morphology of the exosomes was detected using a transmission electron microscope (JEOL, Tokyo, Japan). The presence of  $\alpha$ -synuclein in the exosomes was confirmed by western blot analysis of the relative expressions.

#### Detection methods

**Isolation of serum exosomes.** Elbow venous blood (5 ml) was drawn and placed into a serum separation gel tube (Becton-Dickinson and Company, Franklin Lakes, NJ, USA). After mixing, the tubes were maintained at  $37^\circ\text{C}$  for 30 min. Centrifugation was performed at  $2,000 \times g$  at  $4^\circ\text{C}$  for 15 min to achieve separation of the contents of each tube into three layers from top to bottom: serum, separation and blood cell layers. The serum layer was placed on ice and then stored in 500  $\mu\text{l}$  aliquots in 1.5 ml Eppendorf tubes (Becton-Dickinson and Company) at  $-80^\circ\text{C}$ . An ExoQuick-TC kit was used to purify the exosomes according to the manufacturer's instructions.

**Morphology of exosomes under transmission electron microscope.** Serum samples were thawed and mixed with 200  $\mu\text{l}$  phosphate-buffered saline (PBS) solution. The electron microscope copper mesh grid was dipped into the suspension and kept there for  $\geq 20$  min. Then, 2-3 PBS solution washes (50  $\mu\text{l}$  each) lasting 1 min were performed. The suspension was fixed to the copper mesh by the addition of 10  $\mu\text{l}$  4% glutaraldehyde for 5 min, followed by filter paper seeping and a PBS solution rinse. After drying at room temperature, the rinsed copper mesh was placed under a Hitachi-7500 transmission electron microscope (Lincoln, NE, USA) and images were captured.

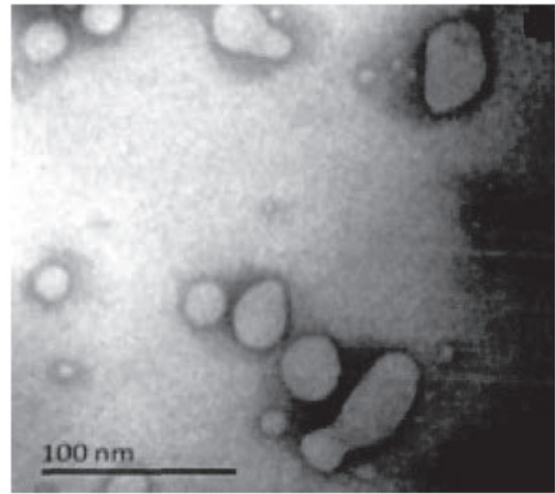


Figure 1. Electron microscope image showing vesicle-like structures consistent with isolated exosomes.

**Western blot analysis.** Total proteins were extracted from exosomes. The protein concentration was quantified by the bicinchoninic acid (BCA) method (Bio-Rad, Berkeley, CA, USA). Electrophoresis was run on SDS-PAGE gels. Western blotting was performed using rabbit anti-CD63 polyclonal antibody (dilution, 1:1000) (LifeSpan Biosciences, Seattle, WA, USA; cat. no.: LS-C387289) and rabbit anti-CD9 polyclonal antibody (dilution, 1:1000) (LifeSpan Biosciences; cat. no.: LS-C382578), rabbit polyclonal anti-GAPDH antibody (dilution, 1:2000) (Abcam, Cambridge, MA, USA; cat. no.: ab8245), rabbit anti- $\alpha$ -synuclein polyclonal antibody (dilution, 1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, cat. no.: sc-10717), and horseradish peroxidase-labeled goat anti-rabbit secondary antibody (dilution, 1:5000) (Sigma, St. Louis, MO, USA, cat. no.: A0545).

**Statistical analysis.** SPSS 20.0 software (IBM SPSS, Armonk, NY, USA) was used for statistical analysis. Data were presented as mean  $\pm$  standard deviation, the t-test was used for inter-group comparisons, and enumeration data were presented as a percentage (%), Spearman correlation analysis was used for  $\alpha$ -synuclein in exosomes and curative effects.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**General data.** Of the initial 68 patients, 3 cases did not complete the study (2 cases had aggravated symptoms and required change of medication or a combination of several drugs, and 1 case with severe liver and kidney damage). The remaining 65 cases successfully completed the study. There were 38 men and 27 women, with an age range of 57-74 years, and an average of  $67.8 \pm 9.2$  years. The course of disease at the time of diagnosis ranged from 1 week to 3 months, with an average of  $1.2 \pm 0.3$  months.

**Identification of exosomes.** Several vesicle-like structures of uniform size, with an average diameter of  $\sim 50$  nm were observed under electron microscopy (Fig. 1). Exosome-labeled proteins CD63 and CD9 were detected by western blotting in

Table I. UPDRS III and UPDRS II scores before and after treatment.

Items	Pre-treatment	Post-treatment	t	P-value
UPDRS II	12.6±3.3	7.7±1.5	5.624	0.034
UPDRS III	30.8±4.4	19.5±2.1	5.768	0.032

UPDRS, unified Parkinson's disease rating scale.

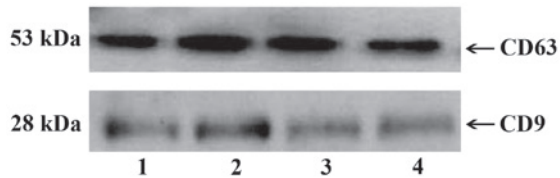


Figure 2. Western blotting showing exosome-labeled proteins CD63 and CD9 in 4 samples.

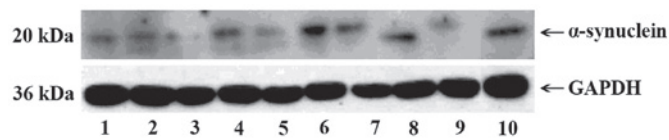


Figure 3. Western blotting showing  $\alpha$ -synuclein expressed in exosomes isolated from 10 samples.

4 samples (Fig. 2). Furthermore,  $\alpha$ -synuclein was expressed in exosomes isolated from 10 samples (Fig. 3).

**UPDRS III and UPDRS II scores before and after treatment.** After treatment, UPDRS III and UPDRS II scores were significantly lower than those before treatment, and the difference was statistically significant ( $P < 0.05$ ; Table I).

**Relative expression of  $\alpha$ -synuclein in serum exosomes before and after treatment.** The relative expression of  $\alpha$ -synuclein in serum exosomes after treatment was significantly lower than that before treatment, and the difference was statistically significant [(0.27±0.04),  $t = -0.523$ ,  $P = 0.017$ ] (Fig. 4).

**Correlation between the relative expression of  $\alpha$ -synuclein in serum exosomes and therapeutic effects.** From the 65 cases studied, 52 cases had what was considered effective treatment and 13 cases had an ineffective one. The relative expression of  $\alpha$ -synuclein in the effective treatment group was significantly lower than that in the ineffective treatment group, and the difference was statistically significant [(0.15±0.02 vs. 0.22±0.05),  $t = -0.603$ ,  $P < 0.01$ ]. The relative expression of  $\alpha$ -synuclein in serum exosomes was significantly correlated with treatment effects ( $r = 0.426$ ,  $P = 0.029$ ).

## Discussion

Exosomes are a membranous microvesicle with a 30-100 nm diameter and 1.13-1.19 g/ml of density (5). They are revealed

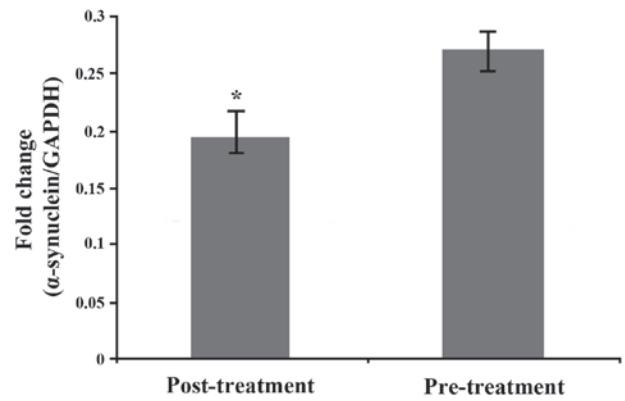


Figure 4. Relative expression of  $\alpha$ -synuclein in serum exosomes before and after treatment. \* $P < 0.05$ .

by a concave disk or cup mouth shape under the electron microscope (6). As a type of nanomembrane vesicle, it was firstly identified in the late 1980s in the culture and differentiation process of sheep reticulocytes (7). In 2005, Caby *et al* (8) identified the existence of exosomes in healthy human blood, indicating exosomes could act as a molecular medium and promote the exchange of information between cells or organs. Exosomes can be secreted from live cells cultured *in vitro* and *in vivo* (9). This phenomenon has been observed in neurons (10) and microglia (11) in nerve cells, and lymphocyte and mononuclear cells in non-neuronal cells. Exosomes also exist in various body fluid environments, such as serum (12). Detailed information for the biosynthetic pathway of exosomes has been previously published, including a useful graph diagram (13).

Exosomes play an important role in nerve development and regeneration as well as in the deformability of the cortex and hippocampal neuron synapses (14). In the case of pathological stimulation or loss, exosomes have immunostimulatory functions, activating and spreading the inflammatory response (12). This inflammatory response has been demonstrated to play an important role in the pathogenesis of Parkinson's disease. It has been confirmed to be able to accelerate the degenerative changes of the substantia nigra (15).

The identification of exosomes requires morphological observation and protein analysis. In the present study, the ExoQuick reagent method was used to separate and purify exosomes. In our experience, compared with the differential centrifugation method, the output of exosomes obtained from ExoQuick reagent method was higher and also purer. The transmembrane 4 superfamily CD63 and CD9 are expressed in exosomes but not in cytoplasmic membranes; they are, therefore, reliable exosome markers (16). CD63 and CD9 were abundant in exosome extractions.

$\alpha$ -synuclein is widely expressed in the neuronal presynaptic membrane and cytoplasm. Mutations on the gene encoding  $\alpha$ -synuclein are closely related to familial Parkinson's disease. The pathological abnormal aggregation of the protein is thought to adversely affect synaptic function and signaling, and to promote neuronal degeneration, playing an important role in the pathogenesis of Parkinson's disease. At first,  $\alpha$ -synuclein was only thought to be a form of cytoplasmic protein involved in the dopamine metabolism pathway.



However,  $\alpha$ -synuclein was also found in extracellular environments, such as serum (17), cerebrospinal fluid (18) and neuron cell culture (19). In the early onset of Parkinson's disease, where there is an  $\alpha$ -synuclein genic mutation, this protein was found to play a role in the pathogenesis of the disease. In 2012, it was confirmed (20) that an  $\alpha$ -synuclein oligomer exists in the exosomes of neuron cells and that the oligomer inside exosomes exhibits higher toxicity than the sporadic  $\alpha$ -synuclein oligomer outside of exosomes.  $\alpha$ -synuclein can be transported outside cells through the exosome channel. Such discoveries lead to the proposal that exosomes can transmit potential toxic proteins and cause disease in neighboring cells. Therefore, exosomes were dubbed the 'Trojan Horse' of neurodegenerative disease (21).

Dopamine receptor agonists are the first choice of treatment for the early functional decompensation seen in Parkinson's disease. As the disease progresses and drug doses are increased, the efficacy of levodopa decreased, and other medications are needed in the case of motor affectation. Pramipexole (2,6-diamino-4,5,6,7-tetrahydrobenzothiazole) is a non-ergotine D2 receptor agonist with unique pharmacological effects and exhibits high affinity for the D3 receptor sub-type (22). In the present study, scores of UPDRS III and UPDRS II after treatment were significantly lower than the scores prior to treatment, and the relative expression of  $\alpha$ -synuclein in serum exosomes was also significantly lower than that prior to treatment, and differences were statistically significant. The effective therapeutic rate was 80% (52/65), and there were no serious complications of the treatment. The relative expression of  $\alpha$ -synuclein in the effective therapy group was significantly lower than that in the ineffective therapy group with statistically significant differences. Furthermore, the relative expression of  $\alpha$ -synuclein in serum exosomes was significantly correlated with treatment effects.

In conclusion, pramipexole was effective and safe in treating patients with Parkinson's disease. Results obtained in the present study indicate that pramipexole reduces the relative expression level of  $\alpha$ -synuclein in serum exosomes.

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