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Effect of 4-AP on MPP+/ MPTP-induced Parkinson's disease model

Qiongwen Rong[1,2,3], Chang Zhou[1,2,3], Yuanyuan Ma[1,2,3*]

[1] Department of Neurology, The First Affiliated Hospital of Hainan Medical University, Haikou, Hainan Province, 570102, China
[2] Hainan Clinical Medical Center, Hainan Medical University, Haikou, Hainan Province, 570102, China
[3] Hainan Key Laboratory of Tropical Brain Research and Transformation, Haikou, Hainan Province, 570102, China

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ABSTRACT

To study the effect of 4-AP on Parkinson's disease (PD) cells and animal model. PD cells were pretreated with different concentrations of 4-AP for 24 hours, then PD cells were prepared by MPP+, and the cell activity was detected by CCK8 kit. PD mice were prepared by MPTP and then given 4-AP for 10 days. Finally, the behavioral changes of mice were detected by pole climbing test and open field test, and the expression of TH in the midbrain was detected by IHC and WB. 4-AP could increase the activity of PD cells induced by MPP+. In the field experiment, the total spontaneous activity distance of PD mice (1380.01 ± 151.84) cm was not different from that of 4-AP intervention (1228.65 ± 358.25) cm but was reduced than that of normal mice (2121.89 ± 235.95) ($P<0.05$). In the pole climbing test, the pole climbing time of PD mice was (7.95 ± 1.02) seconds, compared with that of PD mice treated with 4-AP, there was no difference between the two groups, but it was reduced than that of normal mice ($P<0.05$). IHC and Western blot showed that the mesencephalic TH of PD mice and drug-treated mice were reduced than that of normal mice ($P<0.05$), however, drug intervention could not reduce the expression of TH in mice with PD ($P>0.5$). 4-AP pretreatment can reduce the toxic and side effects of MPP+. 4-AP can not improve the motor function impairment of PD mice, nor can it reduce the toxic effect of MPTP on dopaminergic neurons. There are differences between pre-treatment and post-intervention in the treatment of MPP+/MPTP-induced PD. In order to better explore the drug treatment and target of PD, it is hoped that a cure for PD can be found in PD animals. The timing of intervention and cell and animal experiments should complement each other.

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Introduction

Parkinson's disease (PD) is a chronic progressive degenerative disease of the central nervous system, which cannot be cured at present. It is mostly based on its pathological changes to improve the symptoms of PD based on drugs. In order to fundamentally solve PD, the search and development of neuroprotective agents have always been a research hotspot of neurodegenerative diseases (1,2). 4-aminopyridine (4-AP) is a voltage-gated potassium (Kv) channel antagonist, which can reduce accumulation, oxidation, inflammation and Rho kinase activation in an in vitro model of α-synuclein PD. 4-AP can also improve visual function and motor skills in patients with multiple sclerosis, relieve fatigue and have neuroprotective effects (3). Studies have shown that 4-AP is an effective neurotransmitter regulator and has a wide range of beneficial effects on gait in patients with nervous system diseases (spinal cord injury and cerebellar ataxia) (4). In order to explore the effect of 4-AP on Parkinson's disease, we used MPP+/MPTP-induced PD cells and a mouse model to study the effect of 4-AP on PD.

Materials and Methods

Experimental cells and animals

Human neuroblastoma cell line (SH-SY5Y cell line, purchased from Wuhan Punosai Life Technology Co., Ltd.). Eighteen male SPF C57BL/6 mice, weighing (20.34g ± 0.58g), 8 weeks old, were provided by Changsha Tianqin Biotechnology Co., Ltd. The mice were fed with an artificial circadian rhythm for 12 hours at room temperature (20-24) ℃ and relative humidity (55-60)%.

Materials and instruments

MPTP is purchased from Sigma Company of the United States (the concentrated liquid prepared by MPTP is stored at-20 ℃); MPP+ is purchased from Sigma Company of the United States (the concentrated liquid of MPP+ mixed with 10mol/L is stored at-20 ℃); 4-AP (purchased from sigma Company of the United States and prepared into the solution of 0.1mg/ml to be used); CCK8 kit, rabbit anti-tyrosine hydroxylase antibody, rabbit anti-GAPDH antibody and goat anti-rabbit antibody are purchased from Abcam Company; PMSF, RIPA protein lysate biosharp. The gel image analysis system uses Tianneng Technology Co., Ltd.; the mouse open field experimental trajectory analysis instrument was purchased from Shanghai Xinsoft Information Technology Co., Ltd.

Cell grouping and model preparation

The SH-SY5Y cell line was divided into a blank control group, model group, drug intervention group and drug intervention + model group. Set the 4-AP of the

* Corresponding author. Email: 13907580113@163.com

concentration gradient (0.01mol/L, 0.03mol/L, 0.1mol/L, 0.3mol/L, 1mol/L, 3mol/L, 10mol/L). On the first day, the cells were pretreated with different concentrations of 4-AP for 24 hours in the drug intervention group and drug intervention + model group. On the second day: according to the experimental requirements, the model group and drug intervention + model group were incubated with MPP+ of 1mol/L for 24 hours to prepare the PD cell model. Finally, the cell viability was detected by the CCK8 kit.

Animal grouping and model preparation

Eighteen male C57 mice were randomly divided into 3 groups with 6 mice in each group after 1 week of adaptive feeding. Normal blank control group (intraperitoneal injection of 0.2ml saline, average once every 3.5 days for 5 weeks, a total of 35 days); intragastric administration of 0.2ml sterilized water for injection, once a day for 10 consecutive days. Total 45 days), PD model group (intraperitoneal injection of MPTP30mg/kg every 3.5 days for 5 weeks for 35 days), and 0.2ml sterilized water for injection once a day for 10 consecutive days. Total 45 days), drug intervention group (intraperitoneal injection of MPTP30mg/kg, averagely once every 3.5days for 5 weeks, total 35 days), and intragastric administration of 4-AP of 1mg/kg, once a day for 10 consecutive days. Total 45 days).

Behavioral assessment

Field experiment

The mice were placed separately in the open field (450mm × 450mm × 400mm) composed of white acrylic boards in the center of the site, and a camera was placed 1m above the range of movement of the mice. After the start, each mouse moved 5min in the mine. At the end of the experiment, each mouse was cleaned with 75% ethanol and water to eliminate the odor. The total distance, times and distance of crossing the central grid of mice were analyzed by Smart3.0 video tracking software.

Pole climbing experiment

A diameter 2.5cm foam ball is placed at the top of a wooden pole with a length of 50cm and a diameter of 1cm, and the surface of the pole is wound with gauze. The mice were trained for 3 days before the formal experiment. The mice were placed head-up on the top of the ball, and the contact time of their forelimbs to the platform was recorded. Repeat 3 times, take the average, the interval of each experiment is not less than 1min, and use alcohol to remove the residual odor.

Western Blot

After anesthesia, the substantia nigra was taken from the ice and stored at-80 ℃ after quick freezing with liquid nitrogen. The mesencephalic tissue was ground by homogenizer after PMSF and RIPA were put into the ice, and the total protein was extracted by centrifugation 15min at 4 ℃ for 15 minutes. After measuring the protein concentration, add a loading buffer at 4:1 and boil for 3-5min. 40 μ g protein was added into each well, and SDS-PAGE gel electrophoresis and PVDF membrane were transferred. Rabbit anti-TH antibody was incubated overnight on a shaker bed at 4 ℃. Goat anti-rabbits were incubated at room temperature for 2 hours. Using ImageJ to analyze the gray value of the stripe.

Immunohistochemistry (IHC)

After anesthesia, the brains of mice were perfused with ice PBS and paraformaldehyde, and fixed with paraformaldehyde for 24 hours, the paraffin blocks of substantia nigra were prepared. After dewaxing and rehydration, antigen repair, serum sealing and other steps, the anti-TH antibody was incubated overnight at 4 ℃. The sheep anti-rabbit secondary antibody was incubated at room temperature for 1 hour, stained with hematoxylin, and sealed with neutral resin after alcohol gradient dehydrated xylene. Under the microscope, ImageJ was used to count TH-positive neurons.

Statistical analysis

Through the analysis of SPSS20.0 software, the data were expressed by mean ±standard deviation (mean±SD). One-way ANOVA was selected for inter-group data statistics, and multiple comparisons between groups were considered to have statistical differences.

Results

4-AP can increase the activity of PD model cells induced by MPP+

SH-SY5Y cells were incubated with 0.01mmol/L to 10mmol/L 4-AP for 24h without affecting cell activity. When incubated with 4-AP+MPP+, compared with incubated with MPP+ alone, 4-AP pretreated cells could improve cell activity ($P<0.05$). The 4-AP concentration of 1mol/L was the most significant increase in cell activity at 66%.

Oral administration of 4-AP did not improve motor dysfunction in PD **mice**

After the establishment of the model, 4-AP of 1mg/kg was given continuously for 10 days, and then the behavior was tested. The behavior of mice was tested after administration. In the field experiment, the total spontaneous activity distance of PD mice (1380.01 ± 151.84) cm was not different from that of 4-AP intervention (1228.65 ± 358.25) cm, but it was significantly lower than that of normal mice (2121.89 ± 235.95) cm. In the pole climbing test,

Table 1. The total distance of the open field experiment, the number of times passing through the central grid, and the time of the pole climbing experiment.

Group	Total (cm)	Entry time	Pole (s)
Control	2121.89±235.95	16.8±3.7	3.89±0.35
PD Model	1380.01±151.84	3.7±3.4	7.95±1.02
AP+PD Model	1228.65±358.25	2.67±1.06	8.93±0.63
F	10.12	12.68	48.04
P	0.012*	<0.007	<0.001***

the pole climbing time of PD mice was (7.95 ± 1.02) seconds, compared with that of PD mice treated with 4-AP, there was no difference between the two groups, but it was significantly lower than that of normal mice (P<0.001) (Table 1 and Figure 1).

Oral administration of 4-AP can not increase the content of tyrosine hydroxylase in PD mice.

IHC and Western blot showed that TH in the midbrain of PD mice and drug-treated mice decreased significantly compared with normal mice, but drug intervention could not reduce the expression of TH in PD mice (P>0.5) (Figure 2).

Discussion

PD is a rapidly progressive neurological disease, and its incidence is increasing year by year. Treatment schemes include drug treatment such as levodopa and improved non-drug interventions. As no treatment can slow or stop the progression of PD, researchers hope to provide new insights into the treatment of PD through the study of drugs and its mechanism (5). The pathogenesis of PD involves mitochondrial dysfunction, oxidative stress, inflammation, apoptosis, neuroprotection and so on (6-8). Kv channels are widely expressed in the central nervous system and are the key mediators of neuronal excitability (9). The function of the Kv channel is regulated by ROS and corresponding reductase to meet the needs of cells (10). KV ion channel regulates apoptosis, proliferation and apoptosis through the function of mitochondria, and plays a neuroprotective role (11). Similarly, Kv participates in the decrease of apoptotic volume and regulation of apoptosis in lymphocytes, and the expression of the KV channel is related to the increase of sensitivity to apoptotic stimulation (12). In summary, KV is closely related to oxidative stress, mitochondrial function, apoptosis and proliferation. It is further suggested that the KV potassium channel may be a potential therapeutic target for the treatment of PD (13).

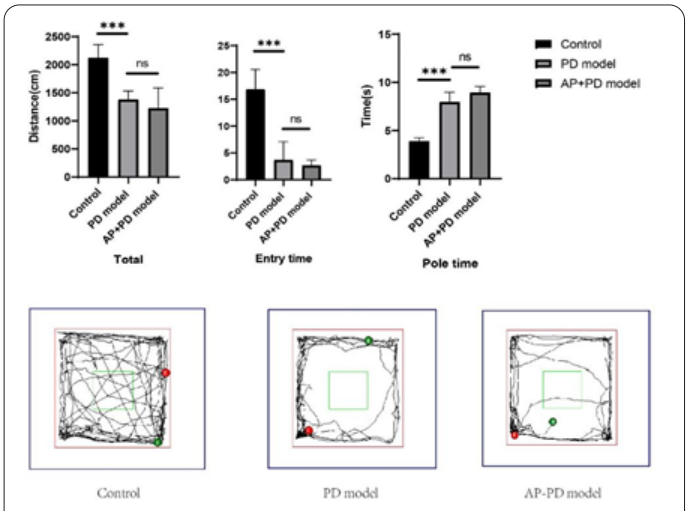

Figure 1. Behavior trajectories and statistical diagrams of open field test and pole climbing mice. Red is the outer frame of mouse activity, green is the center grid, B represents the starting position, and E represents the mouse position at the end of the record.

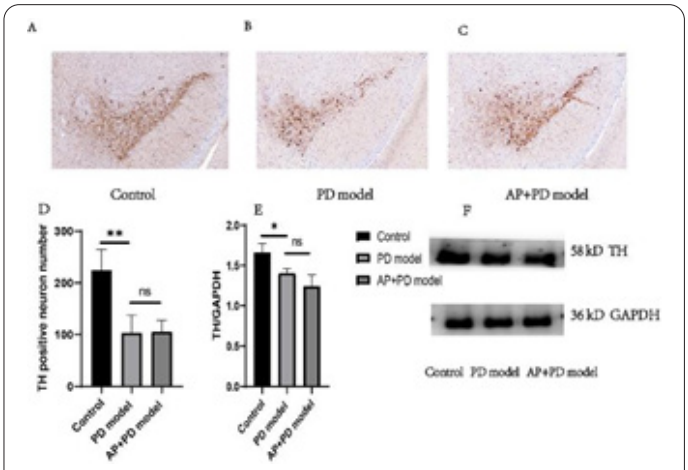

Figure 2. Expression of TH in the midbrain. Amurc shows the expression of TH-positive neurons in the substantia nigra of mice, enlarged 4Xtrait D for immunohistochemical statistical analysis, E for WB statistical analysis, and F for the WB band of midbrain TH.

4-AP is a KV ion channel blocker. As a new and effective voltage-gated potassium channel blocker for the treatment of neurodegenerative diseases, it participates in apoptosis volume reduction and apoptosis regulation in multiple systems. As a neuroprotective agent, it has high development potential (14,15). At the same time, 4-AP can improve the conduction of action potential in demyelinated nerve fibers, thereby increasing the release of neurotransmitters at synapses and neuromuscular junctions. In patients with multiple sclerosis, it can improve motor dysfunction, sensory abnormalities, optic neuritis and other symptoms. It has been used as a new drug for the study of various nervous system diseases (16). Therefore, in this experiment, we used the MPP+-induced SH-SY5Y cell line to observe the effect of MPP+-induced PD cell activity by 4-AP pretreatment. It was found that 4-AP preincubation of 1mol/L could indeed reduce the toxic and side effects of MPP+ on the SH-SY5Y cell line. Low and high concentrations of 4-AP did not affect the activity of MPP+-induced cells. Our results are consistent with previous experimental results for our follow-up study.

We gave each mouse 30mg/kg MPTP for 5 weeks to induce PD mice. After the establishment of the model, 1mg/kg 4-AP was given continuously to observe the behavior of mice and the changes of TH in the substantia nigra of the midbrain. The results showed that 4-AP did not improve the total distance of PD mice in the open field, nor did it increase the number of times to cross the central grid, nor did it improve the time of the pole climbing test. In the subsequent pathological examination, it was found that there was no change in mesencephalic TH in PD mice treated with 4-AP. Through the study, it is found that most of the existing studies on the improvement of functional function and the protective effect of dopaminergic neurons in mice with PD adopt pre-intervention or simultaneous intervention to explore the role and mechanism of drugs, receptors and their signal pathways (17,18). In PD mice, pretreatment with GTS-21, a selective α 7 nicotinic acetylcholine receptor (α 7nAChR) agonist, could restore MPTP-induced motor activity and dopaminergic neuronal death. The anti-inflammatory and neuroprotective effects were induced by inhibiting PI3K/Akt and NF- κ B signal pathways (19). Similarly, Quercetin (QCT) preconditioning can improve the motor behavior damage of PD mice induced by MPTP and protect against the loss of dopaminergic neurons by regulating the signal pathway of cell death (20). The regulation of neuroinflammation provides

a possible method for the treatment of PD-related diseases. Dietary morin can prevent MPTP-induced motor dysfunction and improve the damage of dopaminergic neurons in the striatum and substantia nigra by regulating inflammation and neuroprotection (21). Whether it is natural food, medicine, or traditional Chinese medicine, the existing studies are based on pre-intervention and then using MPTP to prepare PD mice, and then observe the behavioral and pathological changes of PD mice and their signal pathways (22-25).

MPP+/MPTP has the role of mitochondrial toxicity, pre-intervention with certain substances can be found to have neuroprotective effects in cells/animals in most cases, which is why we can find various signal pathways involved in the occurrence and development of PD in cell/animal experiments, but no drug or mechanism can completely improve the symptoms of PD and reverse the death of dopaminergic neurons. In the cell experiment, we used pre-intervention to find that 4-AP could partially reduce the toxicity of MPP+ to SH-SY5Y. In the animal experiment, we took the intervention after the preparation of the model, and no positive results were found in PD mice. This further verifies that the mechanism of pre-intervention may reduce the toxicity and side effects of MPP+/MPTP through some signal pathway, and the treatment after successful preparation of the PD model, except for the drug treatment based on levodopa, there is no effective treatment at present. The diagnosis of PD is based on clinical findings. When patients are diagnosed with PD, follow-up drugs or other treatments can improve the symptoms of PD, which is the focus of our solution. Improving the early diagnosis and treatment after diagnosis is the problem that we urgently need to solve (26).

Author's contribution

The newsletter authors Qiongwen Rong and Yuanyuan Ma are responsible for the design of the overall experiment. Yuanyuan Ma and Qiongwen Rong completed the experiment and article writing and subsequent revision and review.

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