Toxicity Induced by Madopar (L-DOPA) during Curing Parkinson’s Disease by Inhibiting Mitochondrial Function and AKT Pathway

ABSTRACT

Madopar is an effective drug being used clinically for curing Parkinson’s disease (PD) nowadays. However, a growing number of studies found that the Madopar has a falling trend in curative effect including dyskinesia and the reasons are still unknown very well. In order to invest the reasons, we constructed 6-OHDA-induced rat PD model including control group, 6-OHDA group and Madopar + 6-OHDA group, respectively. We detected the behaviour of rotation and roll-rod behaviours 2 weeks later, and the results showed no protection effect and appeared SD rats’ abnormal behaviours. L-dopa treated SH-SY5Y cells for 48 h, cell viability has been inhibited and level of ROS in mitochondria has increased. By detecting levels of apoptosis-related proteins, p-AKT/AKT and Bax/Bcl2 found L-dopa-induced cell apoptosis and death. The decreasing of Sirt3 in mitochondria with the treat of L-DOPA suggests a relationship with mitochondrial function. In a word, the inhibition function of mitochondria and apoptosis in DA neuron cell was the main reasons of L-dopa, which played a weak role in anti-PD.

KEYWORDS Madopar, L-dopa, ROS, p-AKT, AKT, Bax, Bcl2, Parkinson’s disease, PD

INTRODUCTION

Parkinson’s disease (PD) is one of the most common neurodegenerative diseases. Epidemiology show that the morbidity statistic is nearly 1% of PD in 55-year-old people1. PD etiology and pathogenesis are not yet clear. They may be related to social factors, drug factors, patients themselves and so on1. At present, the cause of PD is not yet clear. It is generally considered to be related to the genetic, age, environment, oxidative stress, as well as the generation of free radicals caused by loss of mitochondrial function, immune abnormalities, excitatory amino acids and other factors that caused the death of DA in neuron midbrain Ing4.1

Madopar is a major drug that cures PD in clinic. But, a growing number of studies found that the Madopar has a falling trend in curative effect including dyskinesia4. The curative effect is not very good due to its side effects1. Madopar major constituents are L-dopa and benserazide, while the positive component is L-dopa. Though the function of Madopar which as a dopamine supplement, it is still unknown the protective mechanism of madopar in vivo and vitro. The aim of this study is to find out the probable reasons and mechanism of madopar to PD in rats and SH-SYSY cells.

L-dopa and its compound is the effective drug most commonly used to relieve symptoms, however, its effect will be faded gradually 2–4 years later in the treatment of PD. The mechanism of toxicity and the mechanism of action are not very clear. The early experiments confirmed that L-dopa have toxic effects in vitro culture of dopamine neurons5. Later, animal experiments in vivo found that substantia Ingra dopamine neurons of rats with toxic effect of L-dopa in 6-OHAD processing procedure5. Low doses of L-dopa did not play a protection role in the model of rotenone which was an inhibitor on mitochondria complexes in the midbrain neurons. Besides, L-dopa can...
cause its dopamine neurons die without the obvious damage effect to the other neurons. It may be the reason to nulls the cure of PD, however, no study has reported. It is found that low dose of l-dopa can accelerate the death of dopamine neurons for patients with PD who have mitochondria metabolic disorders. These support the idea that l-dopa can aggravate the state of patients with PD in the long-term use, but is still not confirmed finally. So, we assume that l-dopa causes apoptosis of neuro cells and inhibit apoptosis caused by l-dopa be a new target to cure PD.

**CHEMICALS AND ANTIBODIES**

Madopar was purchased from Shanghai Roche Pharmaceuticals Ltd. and Apomorphine and l-dopa were purchased from American Sigma-Aldrich Chemical Co. Antibodies against p-AKT/AKT, Sirt3 and bcl-2/Bax were purchased from Cell Signalling Technology, Boston, USA.

**METHODS**

**PD model**

After 1 week of acclimatisation, SD rats were lesioned by 6-OHDA in the substantia nigra and 8 μg of 6-OHDA (4 μL in saline containing 0.2 mg/mL ascorbic acid) was administered into the left substantia nigra pars compacta (SNpc, AP: 5.2 mm; L: 1.8 mm; DV: 8.2 mm). Apomorphine was administered i.p. at a dose of 0.5 mg/kg and rotation was monitored for 30 min using the same experimental set-up as for apomorphine-induced rotation. Full 360° turns in the direction contralateral to the lesion were counted and only number of turns ≥210r in 30 min acts as the successful PD model leaded by 6-OHDA.

**Rota-rod test**

The rats were tested on the rod at an accelerating speed (from 2 to 20 rpm) over 5 min and yet stayed for another 2 min. And then the mean value of seconds on the rod was counted from the beginning.

**Cell culture assay**

SH-SY5Y cells were cultured in DMEM (life) supplemented with 10% FBS at 37°C in a chamber of humidified 5% of CO₂/95% of air.

**CCK-8 kit test**

CCK-8 kit is a toxicity test kit, which reflects cell proliferation survival. 5 × 10³ cells were cultivated in 96-well plates. Cells were disposed by 10 μL CCK-8 solution for another 4 h and finally measured at the 450 nm by Sartorius multimode detector. Cells morphology were observed and pictures were taken using Nikon SMZ18 microscope.

**Western blotting**

The whole proteins were subjected to 8 or 15% SDS polyacrylamide gel electrophoresis and were then transferred to polyvinylidene difluoride membranes that were blocked with TBST containing 5% bovine serum albumin (BSA). Membranes were probed with the appropriate primary antibodies for 12 h at 4°C temperature and washed with TBST for three times, then incubated with anti-mouse or anti-rabbit antibody (1:2000) for 3 h at room temperature, and then washed with TBST. Proteins were expressed by using Nikon DS-Ri2 digital instrument, and then edited by Quantity One and Photoshop CS6.

**STATISTICAL ANALYSIS**

The data are presented as means ± SD and analysed by the Graph pad prism 5 statistical software. One-way analysis of variance (ANOVA) was applied to analyse the differences in data of biochemical parameters among the different groups. Differences were considered as statistically significant at *p < 0.05.

**RESULTS**

**The PD model successful rate induced by 6-OHDA in rats**

Test rats’ rotation behaviour at the first week after 6-OHDA lesion, count was ≥210r in 30 min. As shown in Fig. 1, at the 1st week, the successful rate of PD model was 26%, and 2 weeks after lesion, the ratio was increased to 36%.

**The PD model induced by 6-OHDA and the activity of Madopar on evaluating rat’s behaviours**

As shown in Fig. 2, it is suggested that (A) the turns in 30 min of 6-OHDA group rats were nearly 360r and has a slight decline but without significant difference after l-dopa treated. (B) All the rats of these three groups were tested on the rod and the latency time has suggested that the rats of 6-OHDA group exhibited weak movement ability (p < 0.01). Compared to 6-OHDA group, rats of l-dopa group had no effect of protection.

![Fig. 1](https://example.com/figure1.png) The PD model successful rate induced by 6-OHDA in rats.
Fig. 2. The rotation behaviour of turns in 30 min and rota-rod test of the protection effect of L-dopa in PD induced by 6-OHDA. The data are expressed as mean ± SD. Control, n = 7; 6-OHDA, n = 7; L-dopa, n = 5. Significances were marked as **p < 0.01 vs 6-OHDA group.

Fig. 3. The morphology images and cell inhibition of L-dopa in cells of SH-SY5Y cells by CCK-8 assay. Significances were marked as **p < 0.01 vs control group, as bar = 100 μM.

Fig. 4. The ROS generation rate of L-dopa in SH-SY5Y cells. Significances were marked as **p < 0.01 vs control group.

The cell morphology images and cell inhibition ability led by L-dopa in SH-SY5Y cells

To further verify the probably reason of L-dopa had no protection function in SD PD model led by 6-OHDA, SH-SY5Y cells were treated with L-dopa for 48 h, SH-SY5Y cells were examined under a bright-field microscope (Fig. 3a). The inhibition effects and the cytotoxicity were determined by CCK-8 assay. The result shows that L-dopa affects cell viability and markedly induced cell death in a concentration-dependent manner. L-dopa (156, 312.5, 625, 1.25 and 2.5 μM) increased cell viability inhibition to 15.3, 16.5, 60.9, 96.3 and 96.9%, respectively (Fig. 3b).

The ROS generation of L-DOPA itself in cells of SH-SY5Y cells

As illustrated in Fig 4, the L-dopa (625 μM and 1.25 mm) treated for 24 h could significantly increase the fluorescence intensity of DCF, which representing increased ROS generation (p < 0.01) comparing to the control group.

The toxicity of L-dopa may relate with apoptosis

PI3Kinase (PI3K) is involved in the inhibition of apoptosis, and Akt is a critical mediator that regulates this PI3K-dependent neuronal cell survival responses. ROS can inhibit the activity of PI3K and inhibit the phosphorylation of Akt that decrease the amount of p-Akt. To determine whether the PI3K/Akt signalling pathway is regulated by L-dopa,
Mechanism of Madopar to PD in rats

**Fig. 5** The western blot results of proteins p-Akt and Akt of l-dopa in SH-SY5Y cell. Significances were marked as ***p < 0.001 vs control group.

SH-SY5Y cells were treated with different concentration of l-dopa for 24 h and then the phosphorylation of Akt (Ser473) and Akt was examined by Western blot analysis. As shown in Fig. 5a, l-dopa decreases the level of p-Akt compared with the control group.

Bcl-2 has been implicated in inhibiting apoptosis, whereas Bax promotes apoptosis. Therefore, the Bcl-2/Bax ratio is one of the main biomarkers for evaluating the l-dopa mediated apoptotic process. Western blot analysis showed that l-dopa treatment greatly decreased the expression of Bcl-2 and slightly increased the expression of Bax, resulting in a reduction in the Bcl-2/Bax ratio (Fig. 6a, b).

**l-DOPA can inhibit the expression of deacetylase Sirt3 in mitochondria**

Sirt3 is one of the seven mammalian Sir2 homologs of the yeast Sir2 gene, which mediates the effect of oxidative stress, mitochondria function and caloric restriction and life span extension in neurodegenerative diseases. Figure 6 suggests that l-dopa has an effect of regulating the Sirt3 expression. Western blot shows Sirt3 down-expression markedly in a concentration-dependent manner. The Sirt3/β-actin rate decreases significantly (p < 0.001) as the concentrate of l-dopa (156, 312.5, 625 and 1.25 μM).

**The toxicity mechanism of l-dopa in neurodegenerative disease**

As Fig. 7 suggests, l-dopa suppress mitochondria function by acting with Sirt3 and inhibiting the deacetylase Sirt3 expression. Further, a mass of ROS accumulated in mitochondria has attacked mitochondria respiratory chain and mitochondrial membrane to induce mitochondria function collapse. The phenomenon of p-Akt deceased resulted in apoptosis relative proteins Bcl2 reduction and Bax accumulation which contributes to apoptosis in dopaminergic cell of PD.

**DISCUSSION**

In this study, 6-OHDA acts as a lesion drug to induce PD model. We found that l-dopa has little protective
effects in rotation behaviour and rod test, the reasons may the toxicity of l-dopa itself in neurons. So, in this experimental research, we found the effects of l-dopa in SH-SY5Y cells. Results reflect that l-dopa damaged cells in viability and morphology, inhibiting cells growth in a concentration-dependent manner. l-dopa (156, 312.5, 625, 1.25 and 2.5 μM) increased cell viability inhibition to 15.3, 16.5, 60.9, 96.3 and 96.9%, respectively. Further, the second mechanism found that l-dopa led ROS generation and accumulation in mitochondria. As a result, l-dopa increases the ratio of Bax/Bcl2 and decreases the level of p-AKT/AKT. And the inhibition rate of protein Sirt3 treated with l-DOPA in SH-SY5Y cells increases gradually (Fig. 8).

P-AKT is the activity form of AKT, the target pathway of insulin, and the activity targets of phosphorylation are 473 and 308, respectively. Akt also known as PKB, plays an important role in cell survival and apoptosis. Growth and survival factors such as insulin can be activated Akt signalling pathways. PI3K-Akt signalling pathway is a classic signalling pathway, LY294002 etc. PI3K inhibitors inhibit PI3K; usually can inhibit Akt activation to lead Bcl2/Bax ratio downregulated resulting in cell apoptosis.

l-dopa and carbidopa usually share to eliminate the peripheral role, the use of levodopa alone, what is the impact of the worth studying? Neurons rely heavily on their mitochondria because of the constant high-energy demand. Dysfunction of mitochondrial energy metabolism leads to reduced ATP production, impaired calcium buffering and generation of reactive oxygen species. Sirt3 mediates the effect of oxidative stress, mitochondria function and caloric restriction and life span extension in neurodegenerative diseases. There is a strong evidence that mitochondrial dysfunction results in neurodegeneration and may contribute to the pathogenesis of PD. Here, we have attempted to discuss in a nutshell, the key findings on the role of mitochondrial dysfunction in PD and its potential as a therapeutic target to cure PD.

REFERENCES