

Research Paper

Neuroprotection by scorpion venom heat resistant peptide in 6-hydroxydopamine rat model of early-stage Parkinson's disease

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Abstract: Neuroprotective effect of scorpion venom on Parkinson's disease (PD) has already been reported. The present study was aimed to investigate whether scorpion venom heat resistant peptide (SVHRP) could attenuate ultrastructural abnormalities in mitochondria and oxidative stress in midbrain neurons of early-stage PD model. The early-stage PD model was established by injecting 6-hydroxydopamine (6-OHDA) (20 μ g/3 μ L normal saline with 0.1% ascorbic acid) into the striatum of Sprague Dawley (SD) rats unilaterally. The rats were intraperitoneally administered with SVHRP (0.05 mg/kg per day) or vehicle (saline) for 1 week. Two weeks after 6-OHDA treatment, the rats received behavior tests for validation of model. Three weeks after 6-OHDA injection, the immunoreactivity of dopaminergic neurons were detected by immunohistochemistry staining, and the ultrastructure of neuronal mitochondria in midbrain was observed by electron microscope. In the meantime, the activities of monoamine oxidase-B (MAO-B), superoxide dismutase (SOD) and content of malondialdehyde (MDA) in the mitochondria of the midbrain neurons, as well as the inhibitory ability of hydroxyl free radical and the antioxidant ability in the serum, were measured by corresponding kits. The results showed that 6-OHDA reduced the optical density of dopaminergic neurons, induced damage of mitochondrial ultrastructure of midbrain neurons, decreased SOD activity, increased MAO-B activity and MDA content, and reduced the antioxidant ability of the serum. SVHRP significantly reversed the previous harmful effects of 6-OHDA in early-stage PD model. These findings indicate that SVHRP may contribute to neuroprotection by preventing biochemical and ultrastructure damage changes which occur during early-stage PD.

Key words: scorpion venom; Parkinson's disease; mitochondria; antioxidation

蝎毒耐热肽对6羟多巴早期帕金森病大鼠的神经保护作用

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摘要: 本文旨在研究蝎毒耐热肽(SVHRP)是否可以缓解早期帕金森病(Parkinson's disease, PD)模型中脑神经元线粒体超微结构异常和氧化应激。将6羟多巴(6-OHDA, 20 μ g/3 μ L含0.1%抗坏血酸生理盐水)单侧注射到Sprague Dawley (SD)大鼠纹状体制备早期PD模型, PD大鼠腹腔注射SVHRP或相同体积对照溶液(生理盐水)处理1周。在6-OHDA注射2周后, 对大鼠进行行为学检测; 6-OHDA注射3周后, 用免疫组织化学法检测多巴胺能神经元的免疫反应活性, 用电子显微镜观察脑神经元线粒体的超微结构, 用试剂盒检测脑神经元线粒体的单胺氧化酶B活性、超氧化物歧化酶活性和丙二醛含量, 并进一步检测血清抑制羟自由基能力和抗氧化能力。结果显示, 早期PD大鼠多巴胺能神经元的光密度相对对照组明显降低, 脑神经元线粒体超微结构的损伤显著加重, 超氧化物歧化酶活性明显下降, 单胺氧化酶B活性和丙二醛含量显著升高, 血清抑制羟自由基能力和总的抗氧化能力显著下降。而SVHRP能够明显逆转6-OHDA的上述损伤作用。以上结果提示, SVHRP通过减轻早期PD的异常氧化应激和超微结构的损伤来发挥神经保护作用。

关键词: 蝎毒; 帕金森病; 线粒体; 抗氧化

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Parkinson's disease (PD) is one of most common neurodegenerative diseases with the progressive neurodegeneration of the nigrostriatal pathway. Numerous factors such as mitochondrial dysfunction, inflammation-mediated cell injury and reactive oxygen species have been implicated in the etiology of PD^[1]. Diagnosis of PD is based on typical motor symptoms accompanied by the signs that more than 60% dopaminergic neurons died in the substantia nigra and 20%–30% dopamine (DA) left in the striatum^[2,3]. Unfortunately, by the time of diagnosis, most of the dopaminergic neurons in the substantia nigra are dead, which is too late to be cured. In the elderly, PD is more common with most cases happening after the age of 50, and the inducement of PD occurred very early even from neonate^[4,5]. Moreover, the early-stage of PD without typical clinical symptom proceeds slowly^[2], and the most obvious symptoms are movement-related, which include rigidity, shaking, slowness of movement and so on.

In the late stages of PD, cognitive and behavioral problems may appear, with dementia commonly arising in the advanced stages of the disease^[6,7]. However, the mechanism of early-stage PD is still unclear, and the related study is limited as it is difficult to collect early PD cases without the exact clinical symptoms. Therefore, early PD animal and cell models are more ideal to be used in research^[8,9]. DA axon terminal lesions induced by injecting 6-OHDA, the catecholamine selective neurotoxin, into the striatum were used as tools for getting selective partial lesions of the nigrostriatal DA system in the rat^[8], and that model can mimic the nigrostriatal pathology in different stages of PD^[10]. Multifarious neurotoxic mechanisms are implicated in 6-OHDA-induced neuronal damage resulting in the development of an effective model for PD to test different drugs and formulations for their anti-parkinson activity^[1,11]. *L*-DOPA (*L*-3,4-dihydroxyphenylalanine) is used as a common drug in the clinical treatment of PD. Unfortunately, there are many serious side effects of chronic levodopa administration in the treatment of PD, including end-of-dose deterioration of function, on/off oscillations, and so on^[12]. Scorpion venoms and their toxins, which are composed of plentiful sources of fascinating neuropeptides binding with high specificity and affinity to multifold ion channels, have been used extensively as tools for clearing the pharmacological effects and the molecular basis of neurotransmission and electrical excitability^[13,14]. The Chinese scorpion *Buthus martensi* Karsch belongs to the Buthidae family and is used to

treat neurological symptoms such as mimetic paralysis and incomplete paralysis^[15]. Our previous study have shown that scorpion venom protects the dopaminergic neurons in the substantia nigra and improves the related behavior deficits in PD rats and mice^[16,17]. The mechanism of those effects are reversing the abnormal expression of proenkephaline^[18], altering neural nitric oxide synthesis^[19,20] and inhibiting the immuno-reactivity of microglia cells^[14]. However, the neuroprotection of scorpion venom heat resistant peptide (SVHRP) in early-stage PD is not completely clear until now. So in the present study, we focused on the point that whether SVHRP administration would attenuate ultrastructural abnormalities in mitochondria and abnormal expression of oxidative stress markers in early-stage PD rats.

1 MATERIALS AND METHODS

1.1 Preparation of early-stage PD animal model

All the experimental procedures were carried out according to the Animal Ethics Standards and Regulations for the Administration of Affairs Concerning Experimental Animals. Thirty six healthy, male, Sprague-Dawley (SD) rats, 6–8 weeks old, weighing 180–120 g, were got from the Animal Center of Dalian Medical University [No. SCXK (Liao) 2002-0002]. The rats were randomly divided into three groups: control group ($n = 12$), early PD group ($n = 12$) and SVHRP-treated group ($n = 12$). Being deeply anaesthetized with 4% chloral hydrate (400 mg/kg, i.p.), the rats were fixed on the stereotaxic instrument (Stoelting Company, USA), 3.4 mm far from tooth bar. The injection target location (striatum) was AP = +1.0 mm, R = 3.0 mm (right beside), H = -4.5 mm (subdural), based on George Paxinos & Charles Watson stereo rat's brain localization^[21]. The rats in both SVHRP-treated group and early PD group received 20 μ g of 6-OHDA (Sigma, USA) dissolved in vehicle saline (0.1% ascorbic acid in physiological saline, 3 μ L) by stereotaxic injection into striatum at a rate of 1 μ L/min. The control group was injected with the same volume of vehicle without 6-OHDA in the same target point. Two hours after the 6-OHDA injection, the rats in SVHRP-treated group and early PD group were given SVHRP (0.05 mg/kg per day, i.p.) or vehicle (saline) for 1 week, respectively. SVHRP was created by Department of Physiology, Dalian Medical University (China Invention Patent: ZL01 1 06116.9).

1.2 Behavioral assessment

All behavioral tests were conducted in a consistent manner by the same investigator in terms of technique and time of test, and the surroundings were kept quiet. Rotational behavior: 2 weeks after lesions, apomorphine (Apo, Sigma, 0.5 mg/kg, i.c.) dissolved in 0.4 mL physiological saline was given to the rat to evoke rotational behavior. Thirty minutes of rotational behavior of each rat was recorded. A successful early PD model was made only if the rotation of the rat was less than 7 r/min. Adjusting step test: An oblique 1.1-metre-long wooden plank was prepared to connect a start line and the home cage. Before the 6-OHDA lesion, all rats were habituated for three successive days. During the test, the hind limbs and one forelimb of the rat were immobilized when the rats moved slowly across the plank with the free forelimbs. Each forelimb was tested twice during a test session. The time and the steps taken from the starting line to the home cage were recorded. All these tests were started from the left side to the right side, and each test was repeated twice.

1.3 Immunohistochemistry staining

The rats were anesthetized deeply with 4% chloral hydrate (400 mg/kg, i.p.) and then perfused transcardially respectively with 1% and 4% paraformaldehyde. The brain was then placed into 4% paraformaldehyde for post-fixation and later submerged in phosphate buffer saline (PBS) containing 20% sucrose overnight at 4 °C. The brains were cut into 50 µm thick slices on the Microtome-Cryostat. The slices were rinsed three times in PBS for 10 min, incubated with 1% bovine serum albumin for 30 min and with primary antibody against tyrosine hydroxylase (TH, Sigma, 1:500) overnight at 4 °C. The sections were rinsed another three times in PBS for 10 min and further processed using biotinylated-second antibody (Wuhan Boster company, China, 1:400) at room temperature for 1.5 h. The sections were rinsed for three times in order to incubate with avidin-biotin complex A:B:PBS (1:1:400) at room temperature for 2 h. Then the sections were detected with diaminobenzidine. In the control group, PBS was used instead of primary antibody.

1.4 Measurement of ultrastructure by the electron microscope

The coronal brain slices (50 µm thick) of SD rats from each group were fixed with 0.1 mol/L hydrochloric acid buffer solution containing 2% paraformaldehyde and 1.25% glutaraldehyde. The slices were put into 2%

osmic acid for 1 h for post-fixation, and then were embedded by Epon812 plate. The area embedded in the tissue was selected through a light microscope. Double uranium-lead staining was performed on the ultra-thin section (50 nm), and the slices were observed by a transmission electron microscope. Two embedded blocks were selected to make the ultra-thin section in each animal randomly with 2 copper meshes selected. The copper mesh with better contrast was observed in the electron microscope with the 100 000 magnification. The ultrastructure of mitochondria in the lesioned side of midbrain was observed.

1.5 Biochemical estimation

After the behavioral tests, the animals were sacrificed by cervical decapitation. The brain was removed and kept on ice, then the midbrain was separated, blotted on filter paper, then weighed and homogenized in cold buffer A (250 mmol/L mannitol, 5 mmol/L EDTA, 5 mmol/L Hepes, 0.1% BSA, pH 7.4). The homogenates were centrifuged at 1 000 r/min for 5 min at 4 °C. The supernatant was again centrifuged at 10 000 r/min for 10 min at 4 °C. The sedimentation was dissolved in 5 mL buffer solution A, divided in centrifuge tubes containing 20 mL 30% Percoll (225 mmol/L mannitol, 1 mmol/L EDTA, 25 mmol/L Hepes, 0.1% BSA, pH 7.4), and centrifuged at 10 000 r/min for 30 min. The brown part at the bottom collected was mitochondria, which was used for further estimation of monoamine oxidase-B (MAO-B), superoxide dismutase (SOD), and malondialdehyde (MDA). The mitochondria were put on the ice after adding 50 mol/L PBS (40 times). The protein quantity was measured using the Lowry method. MAO-B is mainly located in the mitochondria (outer membrane of mitochondrial) of the neuron. MAO-B, SOD and MDA in the homogenate were estimated by the protocols of respective kit (Nanjing Jiancheng Bio-engineering Institute). Determination of hydroxyl radical and total antioxidative capacity level in serum: The blood was got from the angular vein and centrifuged at 3 500 r/min for 10 min at 4 °C. The supernatant was diluted 20 folds with physiology saline, and 0.2 mL serum was selected to determine the capacity in the inhibition of the serum hydroxyl radical using Fenton reaction and Griess reagent. The total antioxidant capacity was measured by ferroin colourimetry in serum.

1.6 Statistics and analysis

All data were expressed as mean ± standard deviation (SD). Differences between groups were assessed using

ANOVA in SPSS 13.1. Multiple comparisons among the groups were performed using SNK method. For all comparisons, values of $P < 0.05$ were considered significant statistically.

2 RESULTS

2.1 Behavioral assessment

The rotation of the rats in early PD was less than 7 r/min. In adjusting step test, there was no significant difference in the time and the steps taken from the starting line to the home cage between model and control groups.

2.2 Effect of SVHRP on TH-immunoreactivity (IR) positive dopaminergic neurons

Figure 1 showed the number and optical density of TH-IR positive dopaminergic neurons. The results showed that, there were no differences of number of TH-IR positive dopaminergic neurons among three groups. However, the optical density of TH-IR positive dopaminergic neuron of early PD group was lower than those of control and SVHRP-treated groups.

2.3 Effect of SVHRP on mitochondria ultrastructure

The results showed that the ultrastructure of mitochondria was integrate in the control, which showed that

mitochondrial cristae arrange orderly, whereas obvious damage occurred in the mitochondria of substantia nigra from the rat with early PD (shown by the arrows), which was evidenced by mitochondria swelling and mitochondrial cristae broken. In the meantime, SVHRP attenuated the damages in early PD rats (Fig. 2).

2.4 Effect of SVHRP on MAO-B, MDA and SOD activities in mitochondria

In order to provide more evidence for the neuroprotection of SVHRP on reversing the abnormal activities of MAO-B, SOD, and MDA in the mitochondria of the midbrain in the rats, we examined the effect of SVHRP on MAO-B, MDA and SOD activities in mitochondria. The results showed that SVHRP significantly reversed the increased MAO-B ($P < 0.01$, Fig. 3A) and MDA ($P < 0.01$, Fig. 3B) activities, and restored the decreased SOD activity ($P < 0.01$, Fig. 3C) of the mitochondria in the midbrain of early PD rats.

2.4 Effect of SVHRP on antioxidant ability in the serum

We also measured the effect of SVHRP on antioxidant ability. The results showed that SVHRP reversed the decreased inhibition ability of hydroxyl free radical ($P < 0.01$, Fig. 4A) and total antioxidant ability in the serum ($P < 0.01$, Fig. 4B) of early PD rats, which suggested

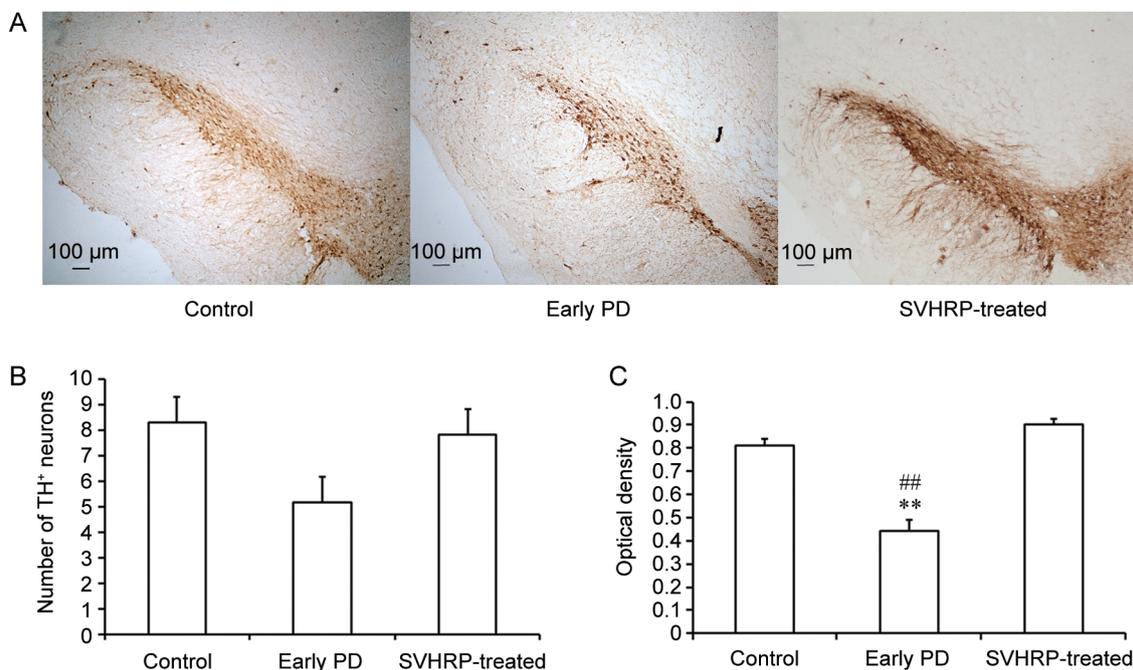


Fig. 1. Tyrosine hydroxylase (TH)-immunoreactivity (IR) positive dopaminergic neurons in different groups detected by immunohistochemistry staining. *A*: Representative images. Scale bar, 100 μm . *B*: The number of TH positive neurons. *C*: The optical density of TH positive neurons. Mean \pm SD, $n = 6$. ** $P < 0.01$ vs control; ## $P < 0.01$ vs SVHRP-treated.

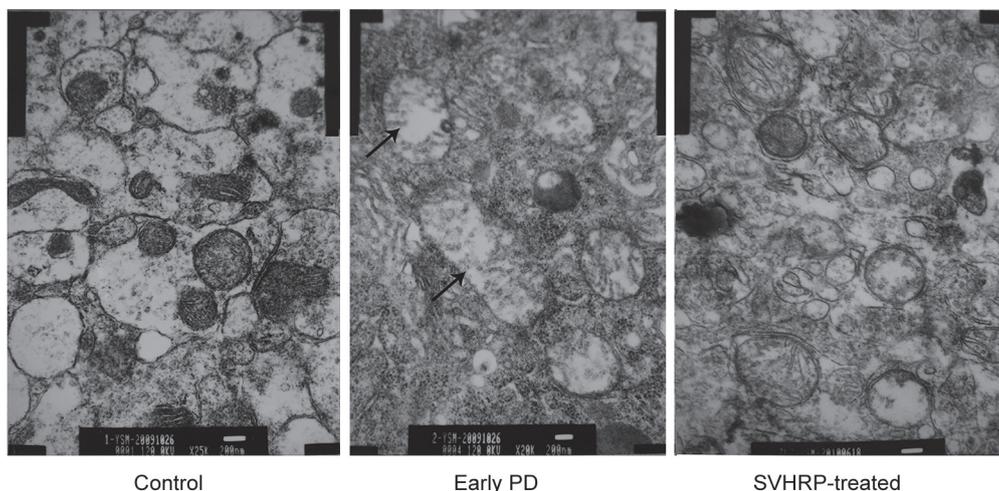


Fig. 2. Mitochondrial ultrastructures in the neurons of substantia nigra in the rats from different groups detected by electron microscopy. Scale bar, 200 nm.

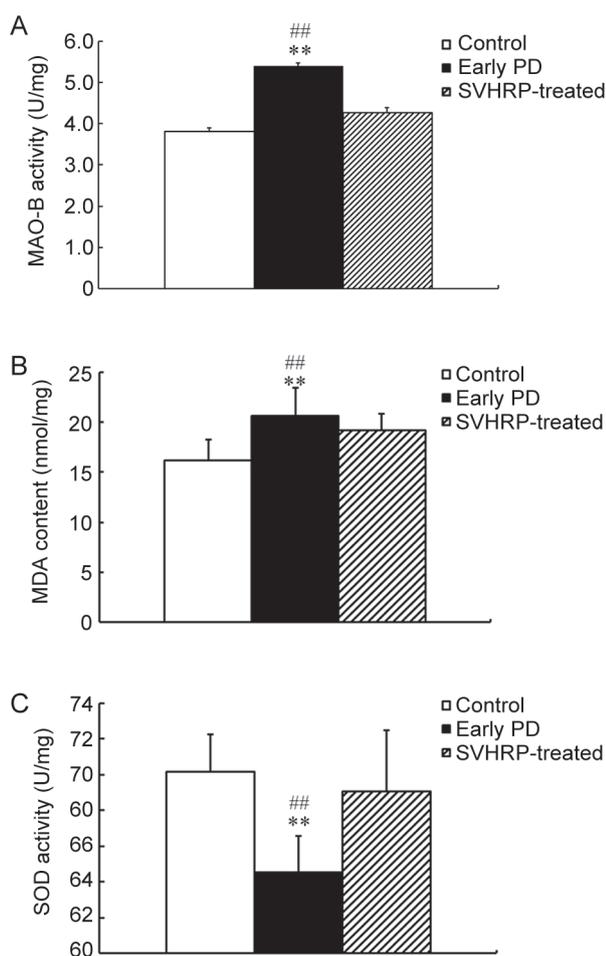


Fig. 3. Effects of SVHRP on antioxidative ability in mitochondria of neurons in midbrain. *A*: MAO-B activity. *B*: MDA content. *C*: SOD activity. Mean \pm SD, $n = 6$. $^{**}P < 0.01$ vs control; $^{##}P < 0.01$ vs SVHRP-treated.

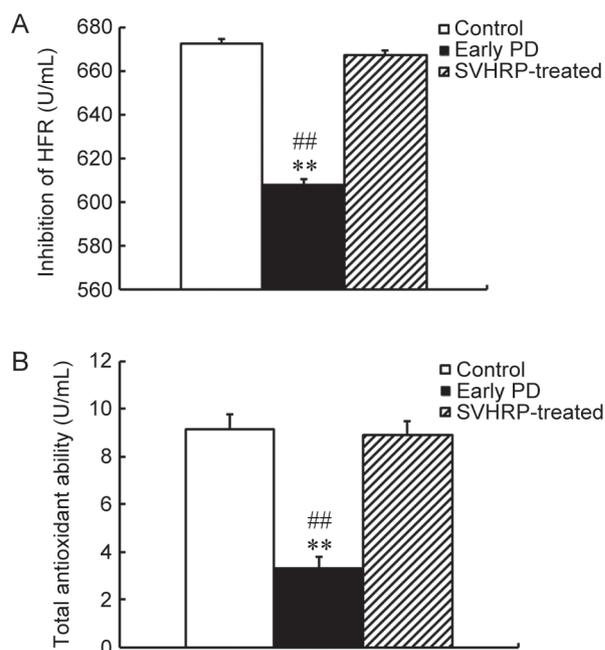


Fig. 4. Effects of SVHRP on antioxidative ability in the serum. *A*: Inhibition ability of hydroxyl free radical (HFR). *B*: Total antioxidant ability in the serum. Mean \pm SD, $n = 6$. $^{**}P < 0.01$ vs control; $^{##}P < 0.01$ vs SVHRP-treated.

that the serum antioxidant ability of SVHRP-treated rats was reversed significantly.

3 DISCUSSION

Studies in both post-mortem PD patient tissues and Parkinsonian animal models have provided strong evi-

dence supporting the involvement of oxidative stress in the progression of PD^[22].

The induction of 6-OHDA, an oxidative stress neurotoxin, which can increase oxidative damage and decrease antioxidant ability in midbrain have been proved in rodent and human models of PD^[23]. Therefore, PD model induced by 6-OHDA can be used to select the candidate used as an antioxidant that could be a promising therapeutic target for PD. Recent studies have shown that specific peptide from scorpion venom might be a good candidate agent for the therapy of PD^[16–20], however, underlying molecular mechanism for the neuroprotection of SVHRP in early-stage PD remains unclear.

Our previous work has proved that SVHRP can enhance the immunoreactivity of dopaminergic neurons in the substantia nigra^[17]. The possible reason is that after the damage, the remaining dopaminergic neurons have the strong compensatory capacity of synthesizing and releasing the DA, which keeps the content of DA in striatum in a stable condition and prevents the obvious behavior deficits syndromes^[10]. In this study, there was no significant difference of the number of dopaminergic neurons among these three groups, however, the optical density of TH-IR positive dopaminergic neurons was decreased in early PD rats, but reversed by SVHRP, which suggests that in the early PD the ability of composing DA decreases, while SVHRP can enhance that ability. The axons of dopaminergic neurons are much more vulnerable than the cell body. So, it's very senseful to make sure the early PD animal model is successfully established by observing the changes of axons. In the present study, we made the early PD animal model according to the previous paper^[8], and the behavior tests further proved the model was successful. As for the evaluation for the protection of SVHRP, the damaged state of axons is a sensitive indicator, and the results showed that SVHRP could protect the cell body by maintaining the function of axons. That can prove SVHRP could protect axons of dopaminergic neurons indirectly.

The antioxidant actions and the protection of mitochondria by SVHRP were studied by using the 6-OHDA rat model for early PD. Major findings from the present study suggest that SVHRP was found to be successful in reversing the abnormal activities of MAO-B, SOD, and MDA in the mitochondria of neurons in the midbrain, and improving the antioxidant ability of the serum in early-stage PD model rats. In the DA degradation

pathway, MAO-B is the principal catabolic enzyme. DA is preferentially deaminated by MAO-B in the human nigrostriatal dopaminergic system. One possible source of increased oxidative stress is the elevated brain MAO-B levels, which have been demonstrated to increase with age and be related to neurodegenerative disease both in mice and human beings^[22]. Increased oxidative stress in the Parkinsonian substantia nigra is believed to contribute to neurodegeneration, in part due to regionally elevated levels of MAO-B. In PD patients, platelet MAO-B activity was significantly higher^[23]. Selective MAO-B inhibitors can protect neuronal cells in cellular and animal models of neurodegeneration^[24]. MAO-B inhibitors increase DA levels, which should compensate for the nigrostriatal deficits in DA, so they are widely used as anti-PD drugs^[25]. Our finding that SVHRP reversed the increased activities of MAO-B in the midbrain of early-stage PD rats supports the antioxidant mechanism of SVHRP-involved protection in early PD. MDA inhibits the aldehyde biotransformation step of DA catabolism, causing elevated levels of the endogenous neurotoxin 3,4-dihydroxyphenylacetaldehyde (DOPAL), which may be involved in oxidative stress leading to selective neurodegeneration as seen in PD^[26].

Brain MDA levels significantly increased in 6-OHDA-lesioned rats^[27], including midbrain^[28], temporal lobe^[29], hippocampus and striatum^[30]. Significant correlations were found between the increased MDA levels and behavioral parameters in the rats with the spatial memory deficits by injecting 6-OHDA directly into the substantia nigra^[31]. Plasma MDA increased in PD patients' peripheral blood and peaked at early disease stages^[32,33]. In the present study, MDA levels increased significantly in early PD rats, which is in agreement with previous studies^[34–36] demonstrating that MDA is an early marker for PD. Furthermore, SVHRP reversed the increased levels of MDA, which helps to understand the mechanism of the protection of SVHRP in early-stage PD.

The activity of SOD increased in rats with unilateral lesion of right substantia nigra induced by 6-OHDA accompanied by cognitive impairment^[31]. The antioxidant defense enzyme SOD decreased in brain regions of 6-OHDA-lesioned rat^[8,27–29,37–39], such as temporal lobe^[29], striatum^[40]. There was significant increase of SOD activity in peripheral blood of PD patients^[33,41,42]. Patients with PD had significantly higher activity of SOD in red blood corpuscle (RBC). Endothelial SOD

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