

Original Article

Protective effects of octacosanol on 6-hydroxydopamine-induced Parkinsonism in rats via regulation of ProNGF and NGF signaling

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Aim: To investigate the protective effects of octacosanol in 6-hydroxydopamine-induced Parkinsonian rats and find whether octacosanol has effects on pro nerve growth factor (pro-NGF), NGF and the downstream effector proteins.

Methods: Behavioral tests, enzymatic assay, tyrosine hydroxylase immunohistochemistry, TUNEL and Western blot were used to investigate the effects of octacosanol in this rat model of PD.

Results: Oral administration of octacosanol (35–70 mg/kg, *po* for 14 d) significantly improved the behavioral impairments in rats induced by 6-OHDA and dose-dependently preserved the free radical scavenging capability of the striatum. Octacosanol treatment also effectively ameliorated morphological appearances of TH-positive neuronal cells in nigrostriatal systems and decreased the apoptotic cells induced by 6-OHDA in striatum. In addition, octacosanol strikingly blocked the 6-OHDA-induced increased expression of proNGF-p75NTR-sortilin death signaling complex and its downstream effector proteins. Meantime, octacosanol prevented the decreased levels of NGF, its receptors TrkA and p-Akt which together mediated the cell survival pathway.

Conclusion: The findings implicated that the anti-parkinsonism effects afforded by octacosanol might be mediated by its neuro-microenvironment improving potency through retrieving the ratios of proNGF:NGF and the respective receptors p75NTR:TrkA *in vivo*. Due to its excellent tolerability and non-toxicity, octacosanol may be a promising agent for PD treatment.

Keywords: Parkinson's disease; oxidative stress; proNGF; nerve growth factor; neuroprotection; apoptosis; octacosanol

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Introduction

Parkinson's disease (PD) is a progressive and age-related neurodegenerative disease characterized by a selective loss of dopaminergic neurons originating in the substantia nigra pars compacta (SNpc) and projecting to the dorsal striatum^[1]. To date, the most widely used and effective treatment for PD is dopamine replacement therapy via oral supplementation of the DA precursor levodopa (L-dopa). Treatment with L-dopa can alleviate major symptoms of PD. However, long-term treatment with levodopa is often complicated by the development of adverse effects. There have been additional anti-parkinsonian drugs, such as dopamine agonists, but the available

therapies do not protect against dopaminergic neurodegeneration. The prevalence of PD is likely to increase in the coming decades as the number of elderly people increases. Therefore, it is of utmost importance to develop new drugs that show or halt the rate of progression of PD.

Although the etiology of idiopathic PD remains incompletely understood, an increasing body of evidence suggests that a range of factors, such as oxidative stress, mitochondrial dysfunction, and environmental toxins, have been implicated in its pathogenesis^[2,3]. In order to elucidate the intracellular events leading to the cell death of dopaminergic neurons, 6-OHDA, a hydroxylated analog of the natural neurotransmitter dopamine, has been widely used to generate PD models *in vitro* and *in vivo*, which is accepted to induce a toxicity that mimics the neuropathological and biochemical characteristics of PD^[4,5].

Growing evidence has shown that the neurotrophins, a

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family of growth factors, play important roles in controlling neuronal survival or cell apoptosis within the central nervous system in the survival, development, and in pathological or neural injury. It has been shown that, proNGF, an unprocessed precursor form of neurotrophin family members (pro-neurotrophins), can act through a co-receptor system of p75NTR and sortilin to mediate cell apoptosis^[6-9] while NGF induces neuronal survival with TrkA and low-affinity binding p75NTR. Thus, as a “molecular signal switch”, p75NTR can determine cell death or survival by these two processes. On the one hand, p75NTR-dependent apoptosis is associated with an increase in Jun kinase (JNK) activity^[10-14] and JNK-dependent mitochondrial pathway including cytosolic accumulation of cytochrome c and caspase activation^[15, 16] *in vitro* or *in vivo*. On the other hand, mediated by p75NTR and TrkA receptors, NGF has been shown to promote survival and induction of neurite outgrowth by activation of pro-survival signaling pathway particularly that of phosphatidylinositol 3-kinase/Akt^[17]. Thus both the proNGF-p75NTR-sortilin signalling complex and NGF mediated PI3-K/Akt pathway may thus provide new targets for neuroprotection of nigrostriatal systems and the therapeutic treatment of PD.

Octacosanol ($\text{CH}_3[\text{CH}_2]_{27}\text{OH}$, molecular weight: 410.77), a low-molecular-weight primary aliphatic alcohol, is the main component of a natural product wax extracted from plants. Most studies have used a wheat-germ oil extract, or policosanol, a natural mixture of primary alcohols isolated from sugar cane wax, of which octacosanol is the main component^[18]. It has a number of indications for its use, many of which are currently being researched. In particular, its cholesterol-lowering effects, anti-aggregatory properties, cytoprotective use, and ergogenic properties have been widely investigated^[19]. Moreover, a small controlled study showed that some patients with mild Parkinsonism claimed of benefit from octacosanol^[20]. However this clinical trial was preliminary and the mechanism of octacosanol was not investigated. In the present study, we sought to systematically investigate the effects of octacosanol on the activities of ProNGF, NGF, and their downstream effector agents which play a critical role in determining the fate of neural cell in rat model of Parkinson's disease.

Materials and methods

Reagents

Octacosanol ($\text{CH}_3[\text{CH}_2]_{27}\text{OH}$) was synthesized by Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, and its purity was more than 99%.

6-OHDA was purchased from Sigma Chemical (St Louis, Missouri). Assay kits for glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) were purchased from Jiancheng Co (Nanjing, China). Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling assay (TUNEL) kit was purchased from Roche Applied Science (Penzberg, Germany). Rabbit anti-proNGF and mouse monoclonal anti-tyrosine hydroxy-

lase (TH) antibodies were purchased from Chemicon International, Inc (Temecula, CA). Rabbit antibodies for sortilin and NGF were purchased from Abcam plc (Cambridge, UK). Rabbit anti-p75NTR antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-phospho-Ser¹²⁸ Bad was purchased from Invitrogen Corporation (Carlsbad, CA). Rabbit antibodies for phospho-Thr¹⁸³/Tyr¹⁸⁵ JNK, phospho-Ser¹⁵ p53, phospho-Tyr^{674/675} TrkA were purchased from Cell Signaling Technology (Beverly, MA). Rabbit antibodies for Bcl-2, Bax, TrkA, phospho-Ser⁴⁷³ Akt, β -actin were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Secondary goat anti-rabbit IgG and rabbit anti-mouse IgG, SP immunohistochemical staining kit and 3,3'-diaminobenzidine (DAB) were purchased from Zhongshan Biotechnology Co (Beijing, China).

Animals and treatment protocol

Animal treatment and maintenance were carried out in accordance with the guidelines established by the National Institutes of Health for the care and use of laboratory animals and were approved by the Animal Care Committee of the Peking Union Medical College and Chinese Academy of Medical Sciences. Adequate measures were taken to minimize pain or discomfort. Seventy two adult male Sprague-Dawley rats (Weitong Lihua Experimental Animal Central, Beijing) weighing 210–220 g (3 months old) were maintained in a constant temperature ($22\pm 1^\circ\text{C}$) and humidity ($60\%\pm 10\%$) environment under a 12 h light/dark cycle. Food and water were available *ad libitum*. Rats were divided randomly into six groups: Normal, Sham (saline only), Model (6-OHDA), Ocs-Low (Ocs-L, 6-OHDA+17.5 mg/kg octacosanol), Ocs-Medium (Ocs-M, 6-OHDA+35 mg/kg octacosanol), and Ocs-High (Ocs-H, 6-OHDA+70 mg/kg octacosanol) groups.

Unilateral striatal lesions were performed as previously described^[21]. For stereotaxic lesion surgery, 6-OHDA was dissolved in 0.9% saline with 0.2% L-ascorbic acid ($5\ \mu\text{g}/\mu\text{L}$), and two injections of $3\ \mu\text{L}$ each were made into two sides of the right striatum. Stereotaxic lesions were performed under sodium pentobarbital (50 mg/kg, *ip*) anesthesia using a $10\ \mu\text{L}$ microsyringe. Lesion coordinates were: (1) AP: 0.5, L: -2.5, and V: -5 mm, (2) AP: -0.5, L: -4.2, and V: -5 mm relative to bregma and ventral from dura, with the tooth bar set at 0 mm. After injection of 6-OHDA at a rate of $1\ \mu\text{L}/\text{min}$, the cannula was left in place for 10 min before slowly retracting at $1\ \text{mm}/\text{min}$.

Four weeks after surgery, octacosanol (dissolved in saline containing 0.5% carboxymethylcellulose sodium) at the doses of 17.5, 35, and 70 mg/kg was intragastrically administered once daily for subsequent 14 days. The normal, sham and model groups received the same volume saline (vehicle only) containing 0.5% carboxymethylcellulose sodium (CMC) as that of the octacosanol.

Rotational behavior

Six weeks after surgery, rats were placed in circular bowls and allowed 15 min for habituation. The total number of contralateral turns was then counted over 30-min period following

subcutaneous apomorphine (Sigma) administration (sc, 0.5 mg/kg).

Narrow beam test

The narrow beam task was performed on the 44th day after 6-OHDA-injection^[22]. The narrow beam 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. 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 cancelled and maximum time was recorded for that trial. A fall was also recorded as a maximum time. Each of the rats was trained for three trials on the beam, including the tasks to initiate crossing the beam and to finish the total beam. Three pretrainings were performed before the behavioral testing and then the fourth trial was regarded as the probe test.

Tissue processing

After behavioral tests, animals ($n=7$ in each group) for biochemistry and western blot analysis were decapitated, and the brains were immediately taken out and rinsed in ice-cold isotonic physiologic saline. Lesioned (right) side and unlesioned (left) side striatum tissues were separately isolated and kept frozen in liquid nitrogen before analysis. Unless otherwise indicated, the right side striatum tissues from every group were used in all the experiments of the present study. For immunohistochemistry, the remaining animals were anesthetized via pentobarbital sodium overdose and perfused transaortically first with 0.1 mol/L phosphate buffered saline (PBS) followed by 4% paraformaldehyde (pH 7.4). The brains were removed and postfixed in the same 4% paraformaldehyde solution at 4 °C, after which they were equilibrated in 0.1 mol/L PBS containing 15%, 20%, and 30% sucrose at 4 °C, respectively. Coronal sections (14 μ m or 35 μ m) were cut using a cryostat.

Measurement of activities of GSH-Px, SOD, CAT, and levels of MDA

Activities of GSH-Px, SOD, CAT, and levels of MDA were measured with assay kits following the manufacturer's manual (Jiancheng Co, Nanjing, China)

TUNEL assay

To demonstrate internucleosomal DNA fragmentation *in situ*, sections were processed using a modified endlabeling technique according to the manual of Roche TUNEL kit. Briefly, sections (35 μ m) were rinsed in PBS for 30 min, and then

incubated with 3% H₂O₂ in methanol for 10 min at room temperature to inactivate endogenous peroxidase. Subsequently, sections were incubated in permeabilization solution for 30 min at room temperature. Sections were then transferred to a buffer containing TdT and fluorescein-dUTP and incubated in a humid chamber for 1 h at 37 °C. After a 5-min wash in PBS, sections were incubated in anti-fluorescein antibody conjugated with peroxidase for 30 min at 37 °C. Reaction product was visualized with a DAB substrate kit. After a final set of washes in PBS, the slices were mounted on slides, dehydrated, cleared, and coverslipped with mounting medium.

Tyrosine hydroxylase (TH) immunohistochemistry

Free-floating brain slices (35 μ m) were treated for TH immunohistochemistry. Sections were washed in PBS to remove cryopreservative and subsequently incubated for 10 min at room temperature in 3% H₂O₂ solution to reduce endogenous peroxidase activity, and then washed in PBS. The samples were placed in goat serum for 1 h at room temperature (SP immunohistochemical staining kit). Incubation overnight at 4 °C was performed with rabbit polyclonal anti-TH antibody. After incubation, the slices were washed with PBS and incubated with biotinylated goat anti-rabbit IgG for 1 h at room temperature, again washed, and then incubated in avidin-biotin horseradish peroxidase macromolecular complex for 1 h at room temperature. To develop color, the slices were incubated briefly in DAB substrate kit. After a final set of washes in PBS, the slices were mounted on slides, dehydrated, cleared, and coverslipped with mounting medium.

Western blot analysis

The whole protein lysates were processed as described in the literature^[23]. After protein content was determined by Bradford's method, proteins were separated by a SDS-polyacrylamide gel electrophoresis and then transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk or 5% BSA (when antibodies against phosphoproteins) in Tris buffer saline. The membrane was then incubated at 4 °C overnight with primary antibodies. After washing, the membranes were incubated with a horseradish peroxidase conjugated secondary antibody (goat anti-rabbit IgG or rabbit anti-mouse IgG, 1:10 000) for 1 h at room temperature. The antibody-reactive bands were visualized on X-ray film using superECL plus detection reagent.

Statistical analysis

To count TH-positive cells in the substantia nigra (SN), we chose 3 animals from each group and 2 coronal sections per animal. And all Western blot experiments were done at least in triplicate from six animals in each group. The density of positive cells in histochemical analysis was calculated with Image-Pro Plus image analysis software and the band intensity in Western blot analysis was calculated by AlphaEase®FC Software. Data were presented as mean \pm SD. Differences between mean values were evaluated by analysis for statistical significance by One-Way ANOVA (Tukey's HSD multiple

comparison). Statistical significance was set at $P < 0.05$.

Results

Effects of octacosanol on the rotational behavior

Six weeks after 6-OHDA injection, apomorphine-induced contralateral rotations were tested and pharmacologic treatment affected the number of rotations. As shown in Table 1, 6-OHDA treated animals exhibited more rotational numbers than those of the Sham ($P < 0.001$). The octacosanol (35 mg/kg and 70 mg/kg) significantly shortened the rotational numbers ($P < 0.05$), suggesting a relative sparing of the nigrostriatal neurons.

Table 1. Effects of treatment with octacosanol 17.5, 35, or 70 mg/kg separately for 2 weeks on behaviors of 6-OHDA-induced rats. $n = 12$. Data represent mean \pm SD. ^a $P < 0.01$ vs Sham group; ^b $P < 0.05$, ^c $P < 0.01$ vs Model group.

Groups	<i>n</i>	Dose (mg/kg)	Apomorphine-induced rotation	Narrow beam test	
				Latency (s)	Total time(s)
Normal	12	Vehicle only	-	0.67 \pm 0.54	6.84 \pm 5.17
Sham	12	Vehicle only	11.2 \pm 8.8	0.89 \pm 0.56	7.98 \pm 4.66
Model	12	Vehicle only	262.2 \pm 60.3 ^c	2.64 \pm 0.95 ^c	30.16 \pm 11.28 ^c
Ocs-L	12	17.5	200.3 \pm 37.9	1.76 \pm 0.83	20.55 \pm 10.38
Ocs-M	12	35	181.8 \pm 48.4 ^a	1.26 \pm 0.96 ^f	14.92 \pm 8.52 ^f
Ocs-H	12	70	160.3 \pm 42.3 ^f	1.25 \pm 0.78 ^f	11.32 \pm 7.55 ^f

Effects of octacosanol on narrow beam test

The narrow beam test was used to assess deficits in locomotion, and the ability to initiate locomotion in 6-OHDA treated rat model of PD^[22]. In this test, the time taken to initiate movement when placed on the beam was recorded as an index of akinesia in parkinsonian animals while the time taken to cross the beam was measured as a reflection of bradykinesia, balance and postural instability in parkinsonian animals. Treatment of rats with octacosanol for two weeks attenuated impairments in motor functions. As shown in Table 1, two weeks of treatment with octacosanol (35 and 70 mg/kg) reduced the 6-OHDA-induced increase of latencies in narrow beam test ($P < 0.01$). In Table 1, model rats showed an increase in total time compared with Sham ($P < 0.01$) while the octacosanol (35 mg/kg and 70 mg/kg) significantly shortened the total time in the narrow beam test ($P < 0.001$).

Effects of octacosanol on the activities of antioxidant defense system and MDA levels in 6-OHDA-lesioned striatum

The burden of reactive oxygen species (ROS) production induced by 6-OHDA is largely counteracted by intricate antioxidant enzymes that include the enzymatic scavengers SOD, CAT, and GSH-Px. In addition to these well characterized antioxidant enzymes, GSH, a small non-enzymatic molecule, is also very important in scavenging ROS. Therefore, we examined the effects of octacosanol on the 6-OHDA induced

oxidative stress. The enzymatic scavengers SOD, CAT, and GSH-Px were observed. As shown in Figure 1A and 1B, the activities of SOD and GSH-Px in the striatum tissues from rat treated by 6-OHDA alone decreased compared with those of the sham groups ($P < 0.05$ or $P < 0.01$). Although the activity of CAT in Model group tends to decline, there was no significant difference between the Model and Sham group (Figure 1C). The most significant improvement of SOD, GSH-Px, and CAT activities in the striatum tissue were observed in the Ocs-H groups ($P < 0.05$). Contrary to the function of antioxidants, MDA levels revealed an index of lipid peroxidation induced by ROS. In Figure 1D, quantitative measurement of MDA levels in striatum indicated a significant increase in the Model group ($P < 0.01$). While 6-OHDA alone increasing the MDA level in striatum, octacosanol treatment significantly reduced it, particularly in the octacosanol group at a dose of 70 mg/kg ($P < 0.01$).

Octacosanol increases the numbers of the TH-positive dopamine neurons and striatonigral projective fibers in the 6-OHDA-lesioned nigrostriatal system

In our study, TH immunohistochemical staining was per-

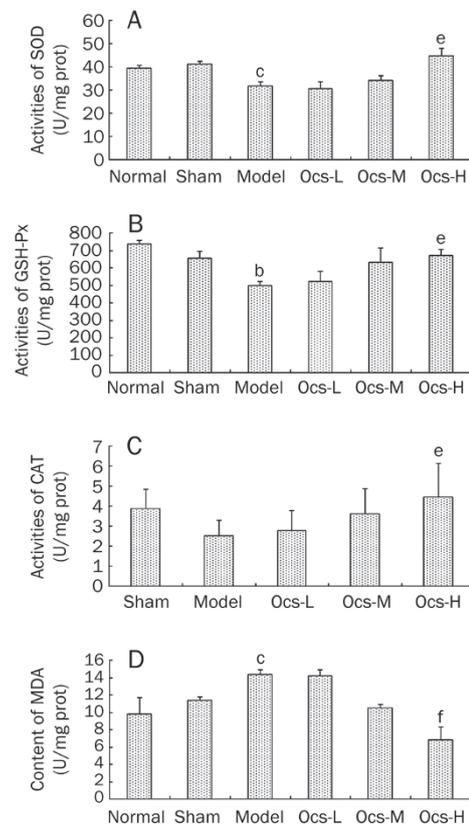


Figure 1. Octacosanol treatment (17.5, 35, and 70 mg/kg, 2 weeks) reversed the reduction of activities of SOD, GSH-Px, and CAT while reduced elevated MDA levels in 6-OHDA-lesioned striatum. (A) SOD activity, (B) GSH-Px activity, (C) CAT activity and (D) MDA level in each group. $n = 7$ in each group. Data represent mean \pm SD. ^b $P < 0.05$, ^c $P < 0.01$ vs Sham group; ^a $P < 0.05$, ^f $P < 0.01$ vs Model group.

formed to assess the neurotoxicity of 6-OHDA and the protection conferred by octacosanol in this model. TH is a rate limiting enzyme in DA synthesis and a specific marker for DA neurons. The results showed that the numbers of both TH-positive neurons and striatonigral projective fibers were significantly decreased in the substantia nigra of 6-OHDA-treated rats compared with those in the sham and normal groups (Figure 2A and 2B, $P < 0.05$). In contrast, rats receiving octacosanol treatment, particularly at the dose of 70 mg/kg, displayed significant preservation of TH-positive neurons and their projective fibers (Figure 2C, $P < 0.05$).

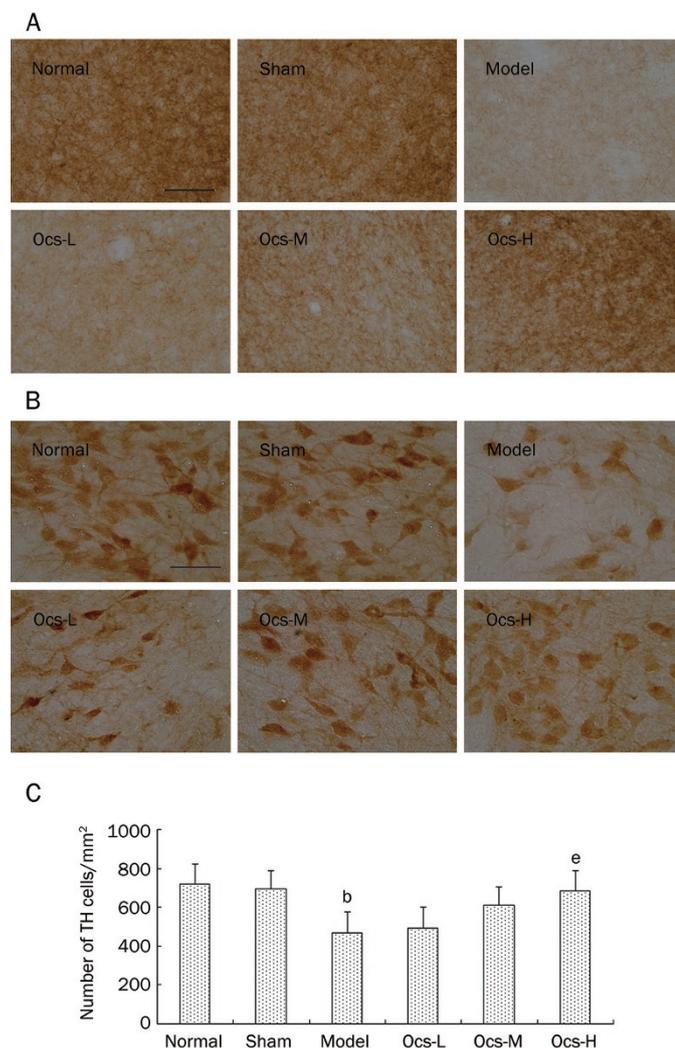


Figure 2. Effects of octacosanol treatment (17.5, 35, and 70 mg/kg, 2 weeks) on loss of dopamine neurons and their terminal fibers induced by 6-OHDA. (A) Representative photomicrographs of TH-positive striatal terminal fibers in every group. (B) Representative photomicrographs of TH-positive neurons immunohistochemistry in SN. (C) Quantitative analysis of TH positive cells in SN. Scale bar=50 μ m. $n=6$ in every group. Data represent mean \pm SD. The results indicated at least two independent experiments in each animal. ^b $P < 0.05$ vs Sham group; ^e $P < 0.05$ vs Model group.

Octacosanol attenuates the apoptosis of striatal neurons induced by 6-OHDA

We employed the TUNEL assay and examined effects of octacosanol on the 6-OHDA-induced apoptosis of striatal neurons *in situ* (Figure 3A and 3B). The TUNEL-positive cells in the striatum of model rats were markedly increased compared with those in the striatum of sham rats. And the TUNEL positive cells were rarely seen in striatum of rats treated with octacosanol, especially in the middle and high dose groups (Ocs-M, 35 mg/kg and Ocs-L, 70 mg/kg).

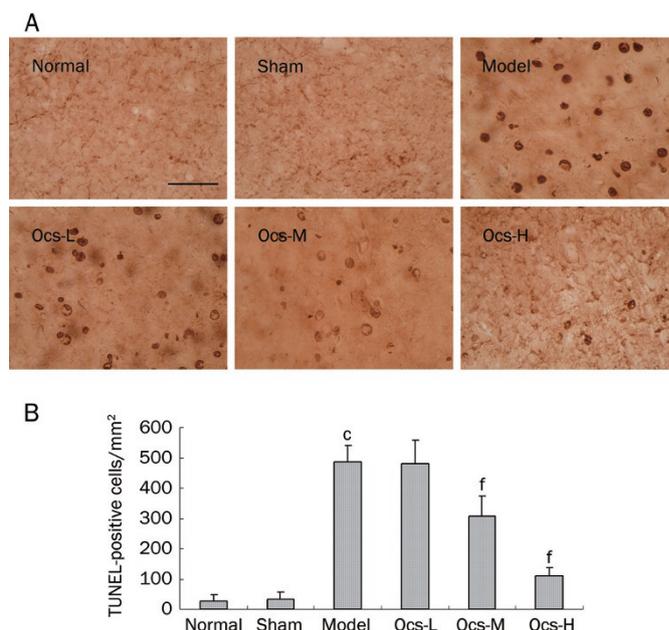


Figure 3. Effects of octacosanol treatment (17.5, 35, and 70 mg/kg, 2 weeks) on 6-OHDA-induced apoptosis in striatum. (A) Representative photomicrographs of TUNEL labeling in striatum. Scale bar=50 μ m. (B) Quantitative analysis of TUNEL-positive cells in striatum. $n=4$ in every group. ^c $P < 0.01$ vs Sham group; ^f $P < 0.01$ vs Model group.

Octacosanol suppresses the 6-OHDA-induced upregulation of ProNGF and the p75NTR/sortilin co-receptors

ProNGF is an important redox-sensitive signaling factor, which can bind a multi-component receptor complex comprising sortilin and p75NTR and promote apoptosis in different kinds of neuronal and non-neuronal cells^[6, 7, 9, 24]. To test whether the protection by octacosanol against 6-OHDA-induced cell death involves the ProNGF pathway, levels of proNGF and its downstream co-receptor system of p75NTR and sortilin were analyzed. Western blot analysis demonstrated that administration of octacosanol, especially at the dose of 70 mg/kg, significantly suppressed the 6-OHDA-induced upregulation of proNGF ($P < 0.01$) (Figure 4A and 4B). Similarly, treatment with octacosanol at the dose of 35 and 70 mg/kg significantly inhibited the 6-OHDA-induced accumulation of p75NTR and sortilin ($P < 0.05$ or $P < 0.01$) (Figure 4C-4E).

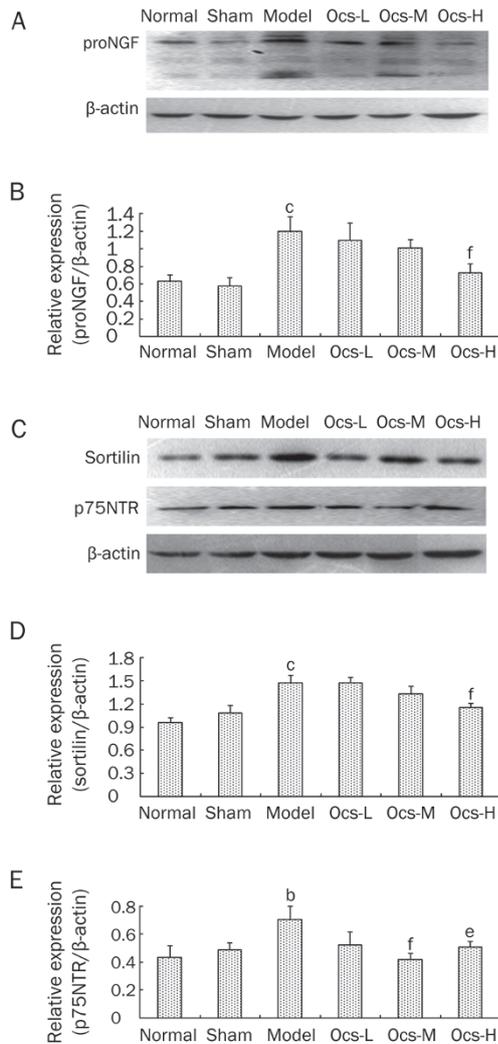


Figure 4. Inhibitory effects of octacosanol treatment (17.5, 35, and 70 mg/kg, 2 weeks) on 6-OHDA-induced elevated levels of proNGF and its p75NTR/sortilin co-receptor system upregulation. (A) Western blotting for proNGF in striatum in every group. (B) Percentage values of optical density ratios from proNGF immunoblot. (C) Western blotting for striatal sortilin and p75NTR in every group. Percentage values of optical density ratios from sortilin immunoblot (D) and p75NTR immunoblot (E). Relative optical density was normalized to β -actin. $n=6$ in every group. ^b $P<0.05$, ^c $P<0.01$ vs Sham group; ^e $P<0.05$, ^f $P<0.01$ vs Model group.

Octacosanol reduces the 6-OHDA-induced and p75NTR-dependent apoptosis via inhibiting the JNK activation and p53 phosphorylation

It has been also reported that p75NTR-induced JNK activation is a consistent feature of p75NTR-responsive cell types^[25] and that, under stress conditions, JNK stabilizes p53 and induces neuronal death^[26, 27]. Since our results showed that octacosanol inhibited the 6-OHDA-induced p75NTR accumulation and cell death, it is possible that it also could inhibit the JNK activation and, consequently, p53 phosphorylation. As shown in Figure 5, while, the phospho-Thr¹⁸³/Tyr¹⁸⁵ JNK expression

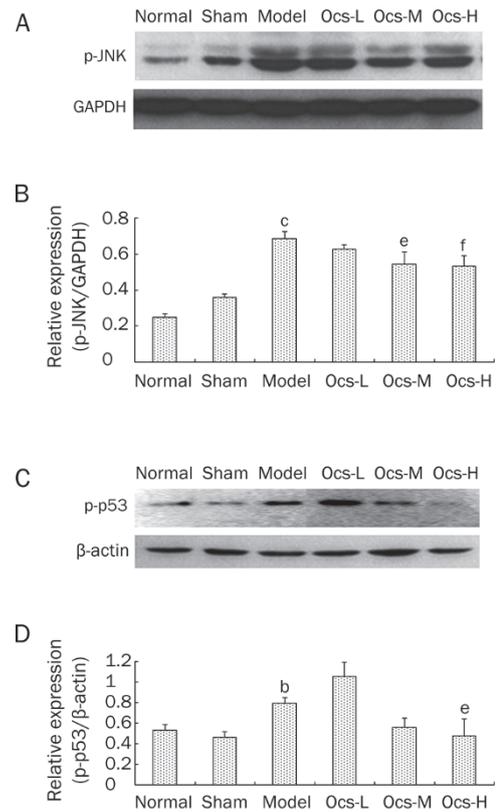


Figure 5. Inhibitory effects of octacosanol treatment (17.5, 35, and 70 mg/kg, 2 weeks) on 6-OHDA-induced activation of phospho-Thr¹⁸³/Tyr¹⁸⁵ JNK and its substrate phospho-Ser¹⁵ p53 in striatum. Western blotting for phospho-Thr¹⁸³/Tyr¹⁸⁵ JNK (A) and phospho-Ser¹⁵ p53 (C) in every group. Percentage values of optical density ratios from phospho-JNK (B, both isoforms were calculated) and phospho-Ser¹⁵ p53 immunoblot analysis (D). Relative optical density was normalized to GAPDH or β -actin. $n=6$ in every group. ^b $P<0.05$, ^c $P<0.01$ vs Sham group; ^e $P<0.05$, ^f $P<0.01$ vs Model group.

was significantly elevated by 6-OHDA-induction in the model rats ($P<0.001$, A and B), treatment of rats with octacosanol (35 and 70 mg/kg) markedly inhibited the JNK activation in striatum ($P<0.05$ or $P<0.01$, Figure 5B). In addition, treatment with octacosanol at the dose of 70 mg/kg significantly inhibited the p53 phosphorylation at the Ser¹⁵ site induced by the JNK activation in the model rats ($P<0.05$, Figure 5C and 5D).

Octacosanol inhibits the 6-OHDA-induced phosphorylation of Bad and increase in the ratio of Bax/Bcl-2

It has been established that cdc2 or JNK directly activates the cell death machinery by phosphorylating Bad at Serine 128^[28, 29]. In addition, the ratio of the expression of the pro-apoptotic Bax to the anti-apoptotic Bcl-2 determines the cellular response to p53. Consistent with the effects of octacosanol on JNK and p53, treatment with octacosanol at the dose of 70 mg/kg ($P<0.05$) significantly reduced the level of phospho-Ser¹²⁸ Bad in model rats induced by 6-OHDA (Figure 6A, 6B, $P<0.01$). Moreover, administration with Ocs-M and Ocs-H

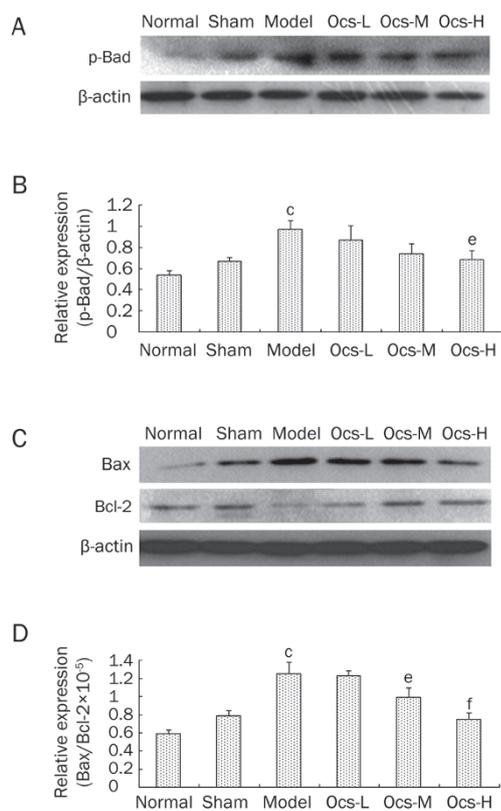


Figure 6. Octacosanol treatment (17.5, 35, and 70 mg/kg, 2 weeks) blocked phosphorylation of Bad and increased ratio of Bax/Bcl-2 in 6-OHDA-lesioned striatum. Western blotting for phospho-Ser¹²⁸ Bad (A) and Bax and Bcl-2 (C) immunoblot analysis. Percentage values of optical density ratios from phospho-Ser¹²⁸ Bad (B) and ratios of Bax and Bcl-2 (D). Relative optical density was normalized to β -actin. $n=6$ in every group. ^c $P<0.01$ vs Sham group; ^e $P<0.05$, ^f $P<0.01$ vs Model group.

(35 mg/kg and 70 mg/kg) for 14 days markedly restored the altered Bax/Bcl-2 ratio in the striatum of the 6-OHDA-induced model rats (Figure 6C, 6D).

Octacosanol decreases the 6-OHDA-induced expression of NGF and its receptor, TrkA

The discovery that NGF may be secreted as proNGF, which preferentially activates p75NTR, or as mature NGF, which preferentially activates TrkA, suggests that the balance between cell death and cell survival may be determined by the ratio of proNGF and mature NGF secreted by biosynthetic cells. And the protective effects of NGF are mediated through specific TrkA receptors, which lead to activation of pro-survival signalling pathways, particularly that of phosphatidylinositol 3-kinase/Akt^[17] which can inhibit apoptosis. In this view, we evaluated the expressions of NGF and its downstream effector proteins, TrkA and p-TrkA (Figure 7A and 7C). Western blot analysis indicated that octacosanol, especially at the dose of 35 and 70 mg/kg ($P<0.01$ or $P<0.001$), significantly inhibited the 6-OHDA-induced NGF reduction and increased the activated forms of TrkA (Figure 7B and 7D, $P<0.01$).

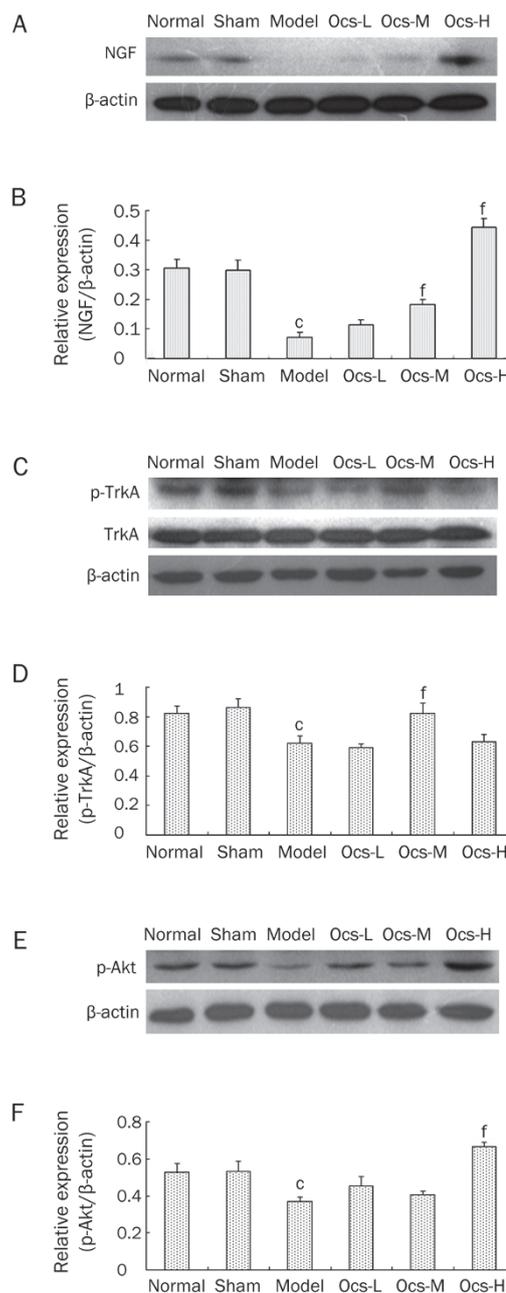


Figure 7. Octacosanol treatment (17.5, 35, and 70 mg/kg, 2 weeks) inhibited 6-OHDA toxicity induced decreased activities of NGF-mediated TrkA neurotrophic signaling. Western blotting for NGF (A), phospho-Tyr^{674/675} TrkA and total TrkA (C) and phospho-Ser⁴⁷³ Akt immunoblot analysis (E). Percentage values of optical density ratios from NGF (B), phospho-Tyr^{674/675} TrkA (D) and phospho-Ser⁴⁷³ Akt immunoblot (F). Relative optical density was normalized to β -actin. $n=6$ in every group. ^c $P<0.01$ vs Sham group; ^f $P<0.01$ vs Model group.

Effects of octacosanol on expression of striatal p-Akt against 6-OHDA toxicity

It is now well established that upon binding to TrkA receptors, mature neurotrophins stimulate multiple signaling pathways including the extracellular signal-regulated kinase 1/2

(Erk1/2) and the phosphatidylinositol 3/Akt kinase pathways, which promote neuronal survival^[30]. So we also investigated the protein levels of p-Akt in striatum induced by 6-OHDA. As shown in Figure 7E and 7F, 6-OHDA caused decreased expression of p-Akt ($P<0.01$) and this effect could be significantly suppressed by treatment with octacosanol at the dose of 70 mg/kg ($P<0.001$).

Discussion

In the present study, we examined the effects of octacosanol on 6-OHDA induced behavioral impairments and confirmed that octacosanol, especially at the doses of 35 and 70 mg/kg, can significantly ameliorate 6-OHDA induced motor impairments in rats. Moreover, the data suggested that octacosanol, especially at the doses of 70 mg/kg, decreased the activities of oxidative stress indicators (MDA, SOD, CAT, and GSH-Px) and attenuated the apoptotic neuronal death induced by 6-OHDA. Furthermore, we have revealed that the protection conferred by octacosanol was related to the regulation of death and survival signals mediated by ProNGF and NGF, especially via inhibition of several key proapoptotic signalling pathways mediated by JNK, Bad, p53, and the Bax/Bcl-2 ratio. Octacosanol has been used for treating diseases as a health agent and it is the predominant component of policosanol, comprising approximately 63% of the mixture (policosanol). The most widely studies of octacosanol are its cholesterol-lowering properties, and many studies have shown that octacosanol is very effective in lowering LDL and increasing HDL. Studies on humans and rats found that policosanol could significantly decrease LDL oxidation such as macrophage-mediated oxidation measured by thiobarbituric-acid-reactive-substances (TBARS) production^[31, 32]. In addition, it has been shown that policosanol seems to be an efficient inhibitor of platelet aggregation. Octacosanol also offers cytoprotective effects. For example, octacosanol attenuated disrupted hepatic reactive oxygen species (ROS) metabolism associated with acute liver injury progression in CCl₄-intoxicated rats^[33]. Additionally, Policosanol showed a protective effect on the myocardial necrosis induced by isoprenaline in a rat experimental model^[34]. Results of another study proved an anti-ischemic effect of policosanol administered after induction of cerebral ischemia, in two different experimental models in Mongolian gerbils, suggesting a possible therapeutic effect in cerebral vascular disorders^[35]. The effects of octacosanol mentioned above made a foundation for the present study design since the reaction mediated by reactive oxygen species was the common feature of those pathological changes including PD.

The pathogenesis of PD is still not fully clarified, but studies including animal models, human postmortem material, and genetic analyses have offered us important clues. For example, analyses of postmortem brain tissue from PD patients and of brain tissues from animal models have shown that the possible involvement of lipid peroxidation^[36], oxidative DNA damage^[37], and protein carbonylation^[38, 39] induced by oxidative stress may be attributed as a causative factor in PD.

6-OHDA is a selective catecholaminergic neurotoxin that has been used widely to produce PD models *in vitro* and *in vivo*. Interestingly, although 6-OHDA is thought to be an exogenous neurotoxin, some evidence shows that 6-OHDA can be formed *in vivo* from dopamine^[40], indicating that endogenous 6-OHDA may constitute one of the pathological metabolites of PD pathogenesis. In fact, it can be detected in brain and urine of PD patients^[40]. Thus, our investigation of the effects of octacosanol on 6-OHDA induced rats and its molecular signaling events should be helpful in determining whether octacosanol is a therapeutic agent for the treatment of PD patients.

The neurotrophins are initially synthesized as proforms which are then cleaved to release C-terminal mature forms of neurotrophins, which regulate neuronal differentiation, survival, neurite outgrowth, synaptic formation and plasticity^[41, 42]. For example, nerve growth factor (NGF), converted from proNGF, selectively binds to its high affinity TrkA receptors and subsequently promotes survival and induction of neurite outgrowth of target neurons during development^[43] or various stressful conditions such as trophic factor deprivation^[44], endoplasmic reticulum stress^[45], and ischemia^[46]. It has also been shown that the NGF level is decreased in PD patients and experimental parkinsonian rats^[47]. In addition, previous studies have indicated that NGF elicits a protective effect against oxidative stress including H₂O₂, MPP⁺, and 6-hydroxydopamine^[48-50]. Taken the above discussion relating to proNGF-p75NTR/sortilin mediated apoptosis signals together, all these findings suggest that the balance between cell death and cell survival may be determined by the ratios of proNGF: NGF and their respective receptors p75NTR: TrkA. In our study, octacosanol (35 and 70 mg/kg) was found to effectively prevent 6-OHDA induced the decrease of NGF and the activated forms of TrkA. Moreover, as well known, the PI 3-kinase/Akt pathway is particularly the downstream pro-survival signals of NGF mediated cell survival through TrkA receptors^[17]. The decreased expression of p-Akt caused by 6-OHDA in our research could be significantly removed by treatment with octacosanol at the dose of 70 mg/kg. However, our findings could not be confirmed by some groups who announced that proNGF was suggested to have a neurotrophic activity^[51, 52] and even mediate TrkA signaling under the condition of prior endocytosis and cleavage to mature NGF^[53]. With regard to these controversial findings, Sobottka *et al*^[54] pointed out that none of the previous studies have combined proNGF and mature NGF in the same cellular context and showed that in PC12 cells, with a lower maximum activity than NGF (58.3% as NGF did), proNGF can partially activate TrkA-mediated neurite outgrowth but impair NGF-mediated TrkA neurotrophic signaling.

Consistent with previous literature^[48-50], in our Parkinsonian rats, 6-OHDA orderly activated the molecular signals from proNGF-p75NTR/sortilin mediated apoptosis pathway while simultaneously blocked the cascade from NGF-TrkA mediated cell survival. Therefore, the ratios of proNGF:NGF and their respective receptors p75NTR:TrkA increased, next the balance between neurotrophic and neurotoxic activities may be

ruined and the fate of neural cells from nigrostriatal systems finally went to the apoptosis. Through improvement of the oxidative-stress microenvironment and inhibition of apoptotic cell death induced by 6-OHDA, octacosanol showed its anti-parkinsonism effects in 6-OHDA induced rats and these protective effects might be associated with its inhibition of proNGF-p75NTR/sortilin mediated cell death and activation of NGF-TrkA mediated cell survival. Together our present data, similar evidence of neuroprotective effects of octacosanol in MPTP-treated mouse models of PD (our unpublished data) and the results of only one small scale clinical trial conducted in 1984^[20] further support the protective potential of octacosanol in PD. To our knowledge, this is the first basic research on the pharmacodynamic action of octacosanol in neurodegenerative diseases such as PD and our studies provide strong evidences for the novel theory that the proNGF-p75NTR-Sortilin signalling complex may provide a new molecular target for the therapeutic treatment of Parkinson's disease. And furthermore, because of the non-toxicity in animal studies^[55] and its excellent tolerability in clinical trials^[56], it would be feasible and beneficial for the healthy general population to use octacosanol without major side effects or regular physician monitoring. Thus, octacosanol may be a neuroprotective and promising agent for PD treatment.

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Author contribution

Ping-ping ZUO, Hai-bo ZHU, Xin WANG, Tao WANG designed research; Tao WANG, Yan-yong LIU, Nan YANG performed research; Hai-bo ZHU contributed new reagents or analytic tools; Tao WANG, Yan-yong LIU, Ping-ping ZUO analyzed data; Tao WANG wrote the paper.

Abbreviations used

6-OHDA, 6-hydroxydopamine; AD, Alzheimer's disease; BSA, bovine serum albumin; CMC, carboxymethylcellulose sodium; CAT, catalase; DA, dopamine; DAB, 3,3'-diaminobenzidine; GSH-Px, glutathione peroxidase; JNK, Jun kinase; L-dopa, levodopa; MDA, malondialdehyde; NGF, nerve growth factor; Ocs, Octacosanol; PBS, phosphate buffered saline; PD, Parkinson's disease; ProNGF, precursor of nerve growth factor; prot., protein; ROS, reactive oxygen species; SN, substantia nigra; SOD, superoxide dismutase; TH, tyrosine hydroxylase; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling assay.

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