Effect of Oleracein E, a Neuroprotective Tetrahydroisoquinoline, on Rotenone-Induced Parkinson’s Disease Cell and Animal Models

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ABSTRACT: Oleracein E (OE), a tetrahydroisoquinoline possessing potent antioxidant activity, was first isolated from a traditional Chinese medicine, Portulaca oleracea L., and is hypothesized to be a neuroprotectant. In the present study, we evaluated the effects of racemic OE on rotenone-induced toxicity in Parkinson’s disease (PD) cell and animal models. Pretreatment with OE (10 μM, 2 h) decreased lactic acid dehydrogenase (LDH) release and the apoptosis rate in rotenone (5 μM, 24 h)-treated SH-SYSY human neuroblastoma cells. Further mechanistic study indicated that OE reduced reactive oxygen species (ROS) levels, inhibited extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, reduced rotenone-induced up-regulation of the proapoptotic protein Bax, and prevented cytochrome C release and caspase-3 activation. In a rotenone-treated (intragastric 30 mg/(kg·d), 56 d) C57BL-6J mouse model, OE (intragastric 15 mg/(kg·d), 56 d) improved motor function, as indicated by an increased moving distance in the spontaneous activity test and sustained time on the rota-rod test. OE also elevated superoxide dismutase (SOD) activity, decreased malonaldehyde content, and reduced ERK1/2 phosphorylation in the midbrain and striatum of mice treated with rotenone. Furthermore, OE preserved tyrosine hydroxylase-positive neurons and maintained the density of dopaminergic (DAergic) fibers in the substantia nigra pars compacta (SNpc). Some of the effects of OE on PD models were similar to those of the positive control selegiline hydrochloride. Our results demonstrated that OE protects DAergic neurons against rotenone toxicity through reducing oxidative stress and down-regulating stress-related molecules. OE is worth exploring further for its neuroprotectant properties in the prevention and treatment of PD.

KEYWORDS: Tetrahydroisoquinoline, oleracein E, neuroprotectant, Parkinson’s disease, oxidative stress, ERK phosphorylation

Parkinson’s disease (PD) is an age-related neurodegenerative disease that affects more than 1% of the population worldwide. PD is clinically characterized by motor dysfunctions such as bradykinesia, resting tremor, and muscle rigidity, and is pathologically characterized by degeneration of DAergic neurons in the SNpc, coupled with intracytoplasmic inclusions known as Lewy bodies and reduction of dopamine in the substantia nigra and striatum. The etiology of PD is associated with genetic and environmental factors such as environmental toxins. Oxidative stress, mitochondrial dysfunction, and excitotoxicity are all implicated in the pathogenesis of sporadic PD. Although the dopamine replacement strategy using the dopamine precursor levodopa provides benefits to virtually all patients with PD, it also brings some adverse effects such as dyskinesia and fluctuation of motor responses after 5–10 years of treatment. Neuroprotectants are greatly needed for slowing or preventing disease progression.

Tetrahydroisoquinolines (TIQs) have been found as endogenous metabolites of dopamine in the brain of patients with PD. Some TIQs, such as 1-benzyl-TIQ, 1,3-diethyl-1-methyl-TIQ protected the rat mesencephalon against the toxicity induced by a variety of toxins, including MPTP, 6-hydroxydopamine, rotenone, and 1-benzyl-TIQ. In contrast, other TIQs, especially 1-methyl-TIQ, protect DAergic neurons. It was reported that 1-methyl-TIQ protected the rat mesencephalon against the toxicity induced by a variety of toxins, including MPTP, 6-hydroxydopamine, rotenone, and 1-benzyl-TIQ. Oleracein E (OE), possessing a tetrahydroisoquinoline and a pyrrolidine skeleton, was first isolated from Portulaca oleracea L. This plant has the nickname “longevity vegetable”; its aerial
part is recorded in Chinese pharmacopeia with the name Ma-Chi-Xian, possessing heat-clearing, detoxification, blood-cooling, blood-stanching, and antidysenteric functions.13 As a TIQ derivative, whether OE is a neuroprotectant or neurotoxicant remains to be determined. Recently, P. oleracea was reported to reduce oxidative stress, protect DAergic neurons, increase dopamine content in the striatum, and improve motor performance in rotenone-induced PD animal models.14–16 However, to date, constituents responsible for the anti-PD activity of P. oleracea are not yet known. Our previous in vitro experiments revealed that OE possessed potent DPPH radical-scavenging activity and inhibited lipid peroxidation induced by H2O2 in rat brain homogenate.17 Through oral administration of racemic OE at 3 and 15 mg/(kg·d) for 8 weeks, further in vivo experiments demonstrated that OE (15 mg/(kg·d)) exerted a neuroprotective effect on a senescent mouse model induced by chronic administration of large dose of α-galactose (1250 mg/(kg·d)) and NaNO2 (90 mg/(kg·d)), through alleviation of oxidative stress, amelioration of hippocampal neuronal damage, inhibition of neuronal apoptosis, and enhancement of spatial memory capacity.18 In the present study, we evaluated the pharmacological effects of racemic OE on rotenone-induced neurotoxicity in both cell and mouse models of PD and investigated the underlying molecular mechanisms.

RESULTS

Effects of OE on Rotenone-Induced Toxicity in SH-SY5Y Cells. LDH Assay. Cell necrosis results in destruction of the cell membrane, leading to LDH release from damaged cells into the medium. The amount of LDH released reflects the degree of injury in cells, and this assay is widely used for evaluating cell viability.19 Figure 1 demonstrates that treatment of SH-SY5Y cells with OE for 24 h at 0.1, 1, and 10 μM did not affect the LDH release rate. However, when the OE concentration was elevated to 20 and 50 μM, the LDH release rate increased significantly (p = 0.017 and p < 0.001, respectively), indicating that high concentrations of OE could induce toxicity to the cells, while low concentrations within 0.1–10 μM had no effects on cell viability. When cells were treated with 5 μM rotenone for 24 h, the LDH release rate was 3 times more elevated than that in the control group (p < 0.001) (Figure 2). In contrast, pretreatment of cells with OE for 2 h decreased the LDH release rate in a dose-dependent manner within the concentration range of 0.1–10 μM (p < 0.001), indicating that OE at these concentrations exerts a protective effect against rotenone toxicity.

Figure 1. Effects of OE on cell viability in SH-SY5Y cells as determined by the LDH release rate (n = 3) (*p < 0.05, ***p < 0.001, vs vehicle control).

Figure 2. Effects of OE on rotenone (5 μM)-induced toxicity in SH-SY5Y cells as determined by the LDH release rate (n = 3) (***p < 0.001 vs vehicle control; ###p < 0.001 vs rotenone model).

TUNEL Staining. Activation of endonucleases that cleave chromosomal DNA preferentially at internucleosomal sections is a hallmark of apoptosis. DNA strand breaks in situ can be detected through labeling 3’-OH with fluorochrome-tagged dUTP using exogenous terminal deoxynucleotidyl transferase. The assay is called TUNEL, the acronym of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.20 Figure 3 shows that some SH-SY5Y cells changed from short-spiny dendrite-like shapes to round after rotenone treatment for 24 h, and TUNEL staining showed that the apoptosis rate, that is, the ratio of the number of red-stained cells to the number of blue-stained cells, in the rotenone model significantly increased in comparison with the control group (p < 0.001). Pretreatment of cells with OE (10 μM, 2 h) had no effect on the control group; however, it significantly inhibited the rate of apoptosis induced by rotenone (p < 0.001).

ROS Assay. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) can diffuse into cells and is then hydrolyzed to 2′,7′-dichlorofluorescin (DCFH) by intracellular lipase, which results in it being trapped within the cells. The intracellular DCFH, a nonfluorescent fluorescein analog, can be oxidized to highly fluorescent 2′,7′-dichlorofluorescein (DCF) by ROS. The fluorescence intensity of DCF reflects the intracellular ROS level.21 Figure 4A,B shows that the fluorescence intensity clearly increased after rotenone-treatment for 1 and 24 h, but the treatment was more potent at 24 h. Further flow cytometric analysis indicated that fluorescence intensity induced by rotenone-treatment for 24 h was elevated approximately 50% more than that of the control group (p < 0.001), indicating that rotenone increases ROS production over time. Pretreatment of cells with OE (10 μM, 2 h) decreased fluorescence intensity by approximately 10% in rotenone-treated cells (p < 0.001), indicating that OE reduces ROS production and alleviates oxidative stress induced by rotenone.

Western Blot Assay. The expression of total ERK1/2 (t-ERK1/2), phosphorylated ERK1/2 (p-ERK1/2), phosphorylated MEK1/2 (p-MEK1/2) (an upstream kinase of ERK1/2 involved in ERK signaling pathways), cytochrome C (involved in mitochondrial membrane injury), and Bax, Bcl-2, and cleaved caspase-3, which are all involved in cell apoptosis, was assayed in this study. As shown in Figure 5A, after cells were treated with 5 μM rotenone for 1, 12, and 24 h, total ERK1/2 did not change or decreased only slightly; however, p-ERK1/2 expression after each treatment clearly increased. Further statistical analysis (Figure 5B) indicated that rotenone-treatment for 24 h induced a significant increase (p < 0.001) in the p-ERK1/2/ERK1/2 ratio in SH-SY5Y cells. Pretreatment of
cells with OE (10 μM, 2 h) significantly decreased the p-ERK1/2/ERK1/2 ratio (p = 0.002) in rotenone-treated cells. This inhibitory effect of OE against rotenone-induced ERK1/2 activation may be associated with its inhibition of phosphorylation of the upstream kinase p-MEK1/2 at 1 h, as shown in Figure 5A. Figure 6A–6D shows that rotenone-treatment for 24 h did not alter the expression of the antiapoptotic protein Bcl-2 (p > 0.05) in SH-SY5Y cells. However, expression of the
pro-apoptotic protein Bax ($p = 0.005$), mitochondrial cytochrome C ($p < 0.001$), and the apoptosis executor cleaved caspase-3 ($p < 0.001$) was significantly up-regulated, indicating that rotenone induces mitochondrial membrane damage and cell apoptosis. In contrast, pretreatment of cells with OE (10 μM, 2 h) remarkably down-regulated the expression of Bax ($p = 0.005$), cleaved caspase-3 ($p = 0.003$), and cytochrome C ($p = 0.012$) in rotenone-treated cells, indicating that OE inhibits cell apoptosis.

**Effects of OE on Rotenone-Induced Toxicity in C57BL-6J Mice. Survival Rate.** One mouse in the Rot+OE(H) group died at the end of the 56th day for unknown reasons, but no deaths occurred in the other five groups. There was no significant difference in the survival rate among the five groups.

**Body Weight.** Rotenone significantly reduced the body weights of C57BL-6J mice from day 7 to day 56, as shown in Supplementary Figure S1. Compared with the Rot group, there was no difference in the body weights in the Rot+SE or Rot+OE treated groups.

**Behavioral Performance.** The spontaneous locomotor activity test showed that the moving distance within 30 min in the Rot group was significantly less than that in the Con group ($p = 0.028$) (Figure 7A,B), indicating that rotenone (30 mg/(kg·d), 56 d) impaired the motor function of the mice and slowed down their moving speed. High-dosage OE significantly improved the motor function in the rotenone-induced mouse model of PD, as indicated by the increase in the moving distance ($p = 0.001$) (Figure 7B). Moreover, the rota-rod test
Table 1. Effects of OE on SOD Activity and MDA Levels in the Midbrain, Striatum, and Blood Plasma of Rotenone-Induced PD Mice**

<table>
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<th>biochemical parameters</th>
<th>Con</th>
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<th>Rot+SE</th>
<th>Rot+OE(L)</th>
<th>Rot+OE(H)</th>
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<td>midbrain SOD (U/mg prot)</td>
<td>250.14 ± 10.74</td>
<td>239.72 ± 19.49</td>
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<td>MDA (nmol/mg prot)</td>
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<td>16.84 ± 2.78**</td>
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<td>striatum SOD (U/mg prot)</td>
<td>269.78 ± 17.04</td>
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<td>251.12 ± 18.02</td>
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<td>12.91 ± 1.95</td>
<td>14.85 ± 2.22**</td>
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<td>plasma SOD (U/mL)</td>
<td>89.22 ± 12.07</td>
<td>70.13 ± 9.43***</td>
<td>84.31 ± 9.64**</td>
<td>77.00 ± 5.61</td>
<td>97.05 ± 10.53***</td>
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<td>MDA (nmol/mL)</td>
<td>4.85 ± 0.46</td>
<td>7.09 ± 1.10****</td>
<td>5.64 ± 0.89***</td>
<td>7.32 ± 1.01</td>
<td>5.57 ± 0.63***</td>
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**n = 9–10; *p < 0.05, **p < 0.01, ***p < 0.001 vs vehicle control; #p < 0.05, ##p < 0.01, ###p < 0.001 vs rotenone model.

Figure 8. Effect of OE on p-ERK1/2 expression in the midbrain and striatum of rotenone-induced PD mice. (A) Western blots for p-ERK1/2 and t-ERK1/2. (B) p-ERK/t-ERK expression ratio in the midbrain and striatum (n = 5) (*p < 0.05, ***p < 0.001 vs vehicle control; #p < 0.05 vs rotenone model).

![Western Blots](image)

Figure 9. Effects of OE on TH expression in the midbrain SNpc of rotenone-induced PD mice. (A) Immunohistological staining image. (B) Optical density of TH-positive fibers (n = 3–5) (***p < 0.001 vs vehicle control; #p < 0.05, ##p < 0.01 vs rotenone model).

![Immunohistological Staining](image)

showed that the sustained time on the rod in the Rot group was significantly decreased (p = 0.033) (Figure 7C) in comparison with the Con group. In contrast, both SE and high-dosage OE increased (p = 0.029 and 0.024, respectively) the sustained times on the rod, indicating that OE improves motor coordinating ability in rotenone-induced PD mice.

Biochemical Parameters. Table 1 shows that rotenone treatment significantly decreased SOD activity in the striatum (p = 0.031) and plasma (p < 0.001), while it significantly increased MDA content in the midbrain (p = 0.005), striatum (p = 0.045), and plasma (p < 0.001), indicating that rotenone decreases the antioxidant defense capacity of mice and leads to lipid peroxidation and oxidative stress. The positive control, SE, up-regulated SOD activity in the plasma (p = 0.002) and down-regulated the MDA content in the midbrain, striatum, and plasma in rotenone-treated mice (p < 0.001, p = 0.005, p < 0.001, respectively). The low dosage of OE (3 mg/(kg·d), S6 d) had no significant effect on these biochemical parameters (p > 0.05), whereas high-dosage OE (15 mg/(kg·d), S6 d) significantly increased SOD activity (p = 0.007, p = 0.022, p < 0.001, respectively) and decreased the MDA content (p = 0.005, p = 0.015, p < 0.001, respectively) in the midbrain, striatum, and plasma in rotenone-induced PD mice. These results indicated that both SE and OE(H) reduced lipid peroxidation in PD mice. However, in contrast to OE(H), SOD activity in the midbrain and striatum was not affected by SE, implying that SE may motivate other antioxidant enzymes or nonenzyme systems to alleviate oxidative stress in the brain of rotenone-induced PD mice.

Western Blot Assay. Figure 8A shows that there is no notable difference in total ERK1/2 expression in the midbrain and striatum among the five groups. However, rotenone induced clear up-regulation in p-ERK1/2 expression in C57BL/6j mice. Further statistical analysis indicated that the p-ERK1/2/t-ERK1/2 expression ratio was significantly increased in the midbrain (p = 0.021) and striatum (p < 0.001) in the Rot group (Figure 8B) in comparison with the Con group, demonstrating that rotenone induces ERK1/2 activation. SE and low-dosage OE had no effects (p > 0.05) on ERK phosphorylation in rotenone-treated mice; however, high-dosage OE significantly decreased the p-ERK1/2/t-ERK1/2 expression ratio in the midbrain (p = 0.043) and striatum (p = 0.037) of rotenone-

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induced PD mice, indicating that OE(H) inhibits ERK1/2 activation induced by rotenone. Western blots for the pro-apoptotic protein Bax and the antiapoptotic protein Bcl-2 in the midbrain and striatum are shown in Supplementary Figure S2A. Statistical analysis indicated there was no significant difference in the Bax/Bcl-2 expression ratio in the midbrain among the five groups (p > 0.05) (Supplementary Figure S2B). Because Bcl-2 expression in the striatum of some mice was not clear, statistical analysis of the Bax/Bcl-2 ratio in the striatum was not performed.

**Immunohistochemical Staining.** Tyrosine hydroxylase (TH) catalyzes the formation of L-dopa, which is the rate-limiting step in the biosynthesis of the neurotransmitter dopamine. TH is therefore regarded as an important biomarker for DAergic neurons. Figure 9A shows that rotenone induced changes in the shape of DAergic neurons. For example, the neurons became round, dendrites became shorter or disappeared, and neuronal fibers clearly decreased. Further statistical analysis by Image-Pro plus 6.0 software indicated that rotenone caused a significant reduction in the density of TH-positive fibers (Figure 9B) (p < 0.001). In contrast, SE (p = 0.009), low-dosage OE (p = 0.012), and high-dosage OE (p = 0.001) all increased the density of TH-positive fibers in rotenone-induced PD mice, indicating that OE exerts protection against DAergic neuronal degeneration induced by rotenone, which is similar to the function of SE.

**DISCUSSION**

**Rotenone-Induced Cell and Animal Models of PD.**

Rotenone is a major widely used ingredient in pesticides. Because rotenone inhibits complex I activity in the mitochondrial respiratory chain, induces oxidative stress and degeneration of DAergic neurons, and reproduces behavioral features reminiscent of human PD, it is therefore implicated as an environmental toxin in PD pathogenesis and is used widely in the establishment of PD cell and animal models.24,25

SH-SYSY human neuroblastoma cells are DAergic neurons. These cells express tyrosine hydroxylase and dopamine-β-hydroxylase, and they possess the ability to synthesize dopamine and norepinephrine. Moreover, these cells also express the dopamine transporter, a protein expressed only in DAergic neurons within the central nervous system.24 In this study, we used rotenone to induce neurotoxicity in SH-SYSY cells and evaluated the effect of OE on rotenone-induced toxicity in this PD cell model.

Intravenous, intraperitoneal, subcutaneous, and intragastric administration or stereotoxic injection of rotenone to the brain has been applied in rats or mice to establish PD animal models.24,25 Most recently, based on systematic comparison of the dosage and treatment time of rotenone, Inden and co-workers reported that oral administration of rotenone at 30 mg/(kg·d) for 56 days to C57BL/6 mice was appropriate to establish PD mouse model, as demonstrated by decreased lifespan, impaired motor function, decreased TH-positive neurons in the SNpc of the midbrain, and increased α-synuclein expression in the sustained TH-positive neurons.27 In the present study, we used the same PD mouse model and evaluated the effect of OE on this model.

**Mechanism for the Neuroprotective Effects of OE on PD Cell and Animal Models.** Endogenous neurotransmitter dopamine is present in high concentration in axon terminals of DAergic neurons. Dopamine is metabolized by various enzymes, including mitochondrial monoamine oxidase (MAO), and may also readily undergo auto-oxidation due to its quinone structure, and both pathways were shown to generate ROS, for example, superoxide and hydrogen peroxide. The oxidation products of dopamine may exert toxic effects on various cellular components and induce lipid peroxidation. It was suggested that imbalance in dopamine metabolism may lead to excess production of ROS or a decrease in the capacity of the natural protective mechanisms within the substantia nigra, contributing to the pathogenesis of Parkinson’s disease.28 Oxidative stress is also implicated in the pathogenesis of rotenone-induced PD models. In the present PD cell model, ROS levels were significantly elevated after rotenone treatment for 24 h. Oxidative stress was also observed in the present rotenone-treated mouse model, as reflected by decreased activity levels of SOD, a major antioxidant defense enzyme, in the striatum and blood plasma and by increased levels of MDA, an end-product of lipid peroxidation, in the midbrain, striatum, and blood plasma. OE reduced ROS levels in the PD cell model, OE at high concentrations increased SOD activity and decreased the MDA contents in the plasma, midbrain, and striatum of PD mice (Table 1), and our previous experiment demonstrated that OE possessed potent radical-scavenging activity,27 therefore, the underlying mechanism of the neuroprotective effects of OE was majorly due to its radical-scavenging and antioxidant activity. Through alleviation of oxidative stress, OE protected and preserved more DAergic neurons from rotenone-induced damage and maintained production of DA.

Moreover, our PD cell and animal models both demonstrated that the neuroprotective function of OE might be associated with its inhibition of sustained ERK1/2 activation. ERK1/2 (also known as p42/p44 MAPK) are two isoforms of extracellular signal-regulated kinase (ERK) that belong to the MAPK (mitogen-activated protein kinase) family. The RAS–RAF–MEK1/2→ERK1/2 signaling pathway amplifies and transduces the signal from the cell membrane to the nucleus through a sequential phosphorylation cascade. Upon receptor activation by an extracellular signal, membrane-bound UDP-loaded RAS recruits RAF kinase into a complex where it is activated. Then, RAF phosphorylates two serine residues on MEK1/2 (mitogen-activated protein kinase kinase 1/2), which in turn phosphorylates ERK1/2 on threonine and tyrosine residues on the dual-specific motif. Phosphorylated ERK1/2 modulates cytoplasmic and nuclear targets that play important roles in executing normal biological functions.29 ERK activation has dual functions in neuronal cells. On one hand, ERK activation often contributes to neuronal cell survival and proliferation. On the other hand, ERK activation can also promote neuronal cell death and is involved in the pathogenesis of neurodegeneration. As reported, phospho-ERK was found in aggregates in the substantia nigra of patients with PD but not in normal subjects.30 Neuron toxins, including glutamate, 6-hydroxy-dopamine, and rotenone, were reported to induce ERK activation and lead to neuronal death, whereas specific MEK1/2 inhibitors, such as 98095, can reduce ERK activation and alleviate neuronal damage induced by these toxins.31−35 The factor that decides the function of the ERK signaling pathway is not clear; some evidence suggests that kinetics and the duration of ERK activation play important roles in the function of ERK. The prompt activation of ERK primarily promotes cell survival. In contrast, the chronic and sustained activation of ERK triggers neurodegeneration and accelerates neuronal death.36 In our experiment, we found that rotenone
induced sustained ERK activation in SH-SY5Y cells more significantly than the control at 24 h (Figure 5). In agreement with this finding in the PD cell model, elevated ERK phosphorylation was also observed in the striatum and midbrain of mice after chronic administration of rotenone (Figure 8). OE inhibited the sustained ERK phosphorylation not only in the rotenone-poisoned PD cell model but also in the midbrain and striatum of the PD mouse model.

It was reported that ROS are an important factor that regulates the ERK signaling pathway. Under normal conditions, phosphorylation of ERK is maintained in a dynamic balance. Phosphatase exerts negative feedback through dephosphorylation of activated ERK. RAS and RAF, two upstream kinases of ERK, can be activated by ROS partially by direct oxidation, and ROS have been shown to inhibit ERK-direct phosphatases by oxidation of their catalytic cysteine residues, thereby leading to sustained activation of ERK.29 From the above analysis, it can be inferred that inhibition of persistent ERK activation by OE may partially account for its scavenging activity against ROS.

Depending on the cell type and the nature of the injury, persistent activation of ERK is associated with the intrinsic apoptotic pathway (mitochondria-dependent), characterized by the release of cytochrome C and activation of the apoptosis executor caspase-9, or with the extrinsic apoptotic pathway, characterized by the activation of the initiator caspase-8.29 Phosphorylated ERK enters the nucleus and induces apoptosis through phosphorylation of transcription factors involved in apoptosis; other phosphorylated ERKs stay in the cytoplasm to phosphorylate the downstream apoptosis-related proteins. ERK activity has been shown to directly affect mitochondrial function by decreasing mitochondrial respiration and mitochondrial membrane potential, leading to mitochondrial membrane disruption and cytochrome C release. ERK activity could also promote cytochrome C release by modulating Bcl-2 family protein expression by up-regulating pro-apoptotic proteins such as Bax, and by down-regulating antiapoptotic proteins, such as Bcl-2.29 The combined actions of Bax and Bcl-2 control the permeability of the mitochondrial membrane. Bax promotes mitochondrial permeability, whereas Bcl-2 counteracts the effect of Bax. Once Bax expression is above the threshold, a large transition pole on the mitochondrial membrane can be formed.27 Mitochondria are damaged and membrane potential is decreased, leading to the release of cytochrome C. Furthermore, cytochrome C leakage supports the formation of an apoptosome complex, which activates caspase 9 and, in turn, activates caspase-3. Caspase-3 cleaves the inhibitor of caspase-activated DNase, leading to DNA degradation or fragmentation. Moreover, activation of caspase-8 leads to the activation of other caspases, including apoptosis executor caspase-3, which ultimately leads to cell apoptosis.29 Consistent with the above reports, we found that rotenone treatment for 24 h elevated the expression of Bax, cytochrome C, and cleaved caspase-3 in SH-SY5Y cells. Down-regulation of Bax, cytochrome C, and cleaved caspase-3 by OE is possibly associated with its evident inhibition of sustained activation of ERK1/2. However, although significant ERK activation was observed in the PD animal model, Bax and Bcl-2 expression in the midbrain and striatum was not affected. This divergence between the PD cell and animal model may be due to the complexity of the cell types in the animal brain.

Taken together, rotenone increased ROS levels in SH-SY5Y cells, leading to persistent phosphorylation of ERK and up-regulation of Bax expression, which in turn damaged the mitochondrial membrane, accelerated cytochrome C release and caspase-3 activation, and led to cell damage. The animal experiments also indicated that rotenone increased oxidative stress and ERK1/2 phosphorylation in the brain, induced DAergic neuron degeneration, and caused dysfunction in motor ability. Our results demonstrated that OE offered protection against rotenone toxicity in both cell and mouse models of PD through scavenging ROS, reducing oxidative stress, inhibiting ERK phosphorylation, alleviating neuronal damage or inhibiting neuronal apoptosis. Tetrahydroxyquinoline OE is concluded to be an important agent responsible for the anti-PD function of the traditional Chinese medicine P. oleracea; OE may also serve as a neuroprotective candidate for the prevention and treatment of PD.

### Insufficiency of the Present Study

In addition to the above discovery, some differences between the results of our experiment and literature reports and some limitations of the present study still need to be addressed.

1. As reported in the literature,27 oral administration of rotenone (30 mg/(kg·d), 56 d) decreased the life-span of C57BL/6 mice to approximately 30% within 1 week, and from then on to the 56th day, the survival rate did not change. In our experiment, except for one mouse in the Con+OE(H) group that died without a clear reason, no death occurred in the groups. Moreover, the literature reported that rotenone treatment (30 mg/(kg·d), 56 d) did not affect the body weight of C57BL/6 mice.27 However, significant weight loss occurred in rotenone-treated C57BL/6 mice in our present study. These divergences may be related to the difference in mice age, feeding environment, or personal manipulation.

2. SE is a monoamine oxidase B (MAO-B) inhibitor. Through inhibiting dopamine oxidation, it increases dopamine concentration in the brain. In addition, SE has potent antioxidant and neuroprotective functions.38 In view of structure similarity between antioxidants OE and SE, a phenethylamine derivative drug used in clinic for the treatment of PD, SE was used as the positive control in the present study. SE (10 mg/(kg·d), ip) was reported to alleviate asymmetric rotation in 6-hydroxy-dopamine intoxicated C57BL/6J mice, increase the moving time on the rota-rod, and inhibit lipid peroxidation in the midbrain.39 In our experiment, SE (10 mg/(kg·d), intragastrically) was found to improve motor coordination ability in rotenone-induced PD mice, reduce oxidative stress in the brain and plasma, and increase the density of TH-positive fibers. SE had no effect on ERK1/2 phosphorylation in the midbrain and striatum of rotenone-treated mice. Therefore, the molecular mechanisms for the neuroprotective effects of SE and OE are perhaps different in some aspects. In addition, OE is a catecholic tetrahydroxyquinoline possessing dopamine moiety, and whether OE can influence MAO-B, or dopamine receptors, or endogenous dopamine concentrations in the brain of toxin-induced PD mice needs further intensive study.

3. In addition to the ERK signaling pathway, whether OE influences P38 and JNK, two other branches of the MAPK signaling pathway, still needs to be studied.
**METHODS**

**Cell Experiments.** Cell Culture. Human neuroblastoma SH-SYSY cells (Cell Bank of Chinese Academy of Science, Shanghai, China) were cultured in 25 cm² flask supplemented with DMEM-Ham’s F12 medium [M&C Gene Technology (Beijing) Ltd., China] containing 10% FBS (Tianjin Hao Yang Biological Manufacture Co. Ltd., China) and 100 U/mL penicillin/streptomycin (HyClone Biological Company, USA) and then maintained at 37 °C in the incubator (Thermo Company, USA) containing 95% air and 5% CO₂.

**Compound Treatment.** Racemic OE was synthesized by our laboratory and identified by H NMR and 13C NMR, with a purity of 99.89% as detected by HPLC. When the cell density in a 25 cm² flask reached 70–80% confluence, the cells were subcultured and seeded in 24-well-plates (for LDH assay and TUNEL staining) with a density of 5 × 10⁴ cells/well or in 10 cm dishes (for the Western blot assay) with a density of 1 × 10⁶ cells/dish. Then, 24 h after plating, cells were starved by changing 10% FBS to 1% FBS. After the cells were starved for 12 h, the cells were treated for either (1) LDH assay or (2) Western blot assay. (1) For the LDH assay, cells in a 24-well-plate were treated with 0.1, 1, 10, 20, or 50 μM OE for 24 h, and 1 μL of DMSO was added as the vehicle control; alternatively, the cells on a 24-well-plate were pretreated with 0.1, 1, or 10 μM OE or DMSO for 2 h and then cotreated with 5 μM rotenone (Sigma-Aldrich Corp., USA) or DMSO for 24 h. (2) For the Western blot assay, cells on a 10 cm dish were pretreated with 10 μM OE or DMSO for 2 h and then cotreated with 5 μM rotenone or DMSO for 1, 12, or 24 h.

**LDH Assay.** Briefly, 120 μL of culture medium in each well of a 24-well-plate was transferred to a 96-well plate. LDH working solution was freshly prepared according to the guidelines provided by the manufacturers of the LDH Kit (Beyotime Institute of Biotechnology, China). Then, the 120 μL of culture medium was mixed with 60 μL of LDH working solution and incubated in the dark on a shaker for 30 min at room temperature. Absorbance was read at 490 nm with a M680 plate reader (Bio-Rad Company, USA). Cells in the 24-well-plate were then incubated with 10 μL of 10% Triton X-100 for 10 min at 37 °C to induce cell lysis. Then, 120 μL of medium was removed to a 96-well-plate for total LDH determination as described above. The LDH value of the medium from the control well (without cells) was used as the background. The LDH release rate (%) = (LDH released into the medium − background)/(total LDH − background). All the experiments were repeated three times.

**Apoptosis Assay.** After rotenone treatment for 24 h, the culture medium was removed, and the cells on coverslip in the 24-well-plate were washed twice gently with PBS (10 mM, pH 7.2). Cells were fixed with 4% paraformaldehyde (PFA) for 1 h at room temperature and then rinsed with PBS (3 × 5 min). The plate was then put on ice, and the cells were treated with 0.1% Triton X-100 resolved in 0.1% citrate sodium for 2 min and then rinsed with PBS (2 × 5 min). TUNEL reaction solutions were prepared according to the protocol of the TUNEL apoptosis detection kit (Roche, Switzerland). To each well, 50 μL of the TUNEL reaction solution was added, and the plate was incubated at 37 °C for 1 h. After the cells were rinsed with PBS (3 × 5 min), the cells were stained with 50 μL of DAPI (Sigma-Aldrich Corp., USA) for 60 s then rinsed with PBS (3 × 5 min). Cells were photographed using an Olympus BX63 fluorescence microscope. The numbers of blue- and red-stained cells in 12 individual fields of vision (at 40X magnification) for each sample were counted, and the ratio of the number of red-stained cells to the number of blue-stained cells was calculated as the apoptosis rate.

**ROS Determination.** After rotenone treatment, cells on a 24-well-plate were treated with 1 μL of 10 mM DCFH-DA (Sigma-Aldrich Corp., USA) solution and maintained at 37 °C for 30 min. The plate was rotated every 5 min. For the flow cytometry assay, the culture medium was removed, and then the cells were washed with PBS (10 mM, pH 7.2). Cells were trypsinized for 90 s, centrifuged at 1000 r/min for 3 min, and then washed with PBS. The centrifugation and washing steps were repeated three times to sufficiently remove the remaining DCFH-DA. The fluorescence intensity was determined by flow cytometry (BD Company, USA), with the excitation wavelength at 485 nm and the emission wavelength at 525 nm. For each group, the experiment was repeated three times, and the results were analyzed by FlowJo software. For fluorescence imaging, after the cells were incubated with DCFH-DA and washed with PBS three times, the cells were directly imaged with a fluorescence microscope.

**Western Blot Assay.** Cells in a 10 cm dish were washed twice with PBS after removal of the culture medium, and then 800 μL of RIPA lysis buffer (Beyotime Institute of Biotechnology) was added for 15 min. The lysis buffer was mixed with 100 μL PMSF (Sangon Biotech Shanghai Ltd., Co., China) in isopropanol at a ratio of 99:1 before use. The cells were collected, broken ultrasonically (5 × 2 s), and then centrifuged at 12000 rpm for 20 min at 4 °C. The protein concentration of the supernatant was determined by BCA protein assay kit (Beyotime Biotechnology Company, China). The proteins in the lysate (30 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% gel for β-actin, ERK1/2, p-ERK1/2, and p-MEK1/2; 12% gel for Bax and Bcl-2; and 15% gel for cytochrome C and cleaved caspase-3), transferred to a nitrocellulose membrane (Pall Corporation, USA), and then probed with antibodies against the following proteins: ERK (44/42 kDa), p-ERK1/2 (Thr202/Tyr204, 44/42 kDa), p-MEK1/2 (Ser177/211, 45 kDa) (Cell Signaling Technology Company, USA), Bax (21 kDa), Bcl-2 (26 kDa), cytochrome C (12 kDa), β-actin (42 kDa) (Proteintech Group Company, USA), and cleaved caspase-3 (17 kDa) (Abcam company, USA). The blots were developed by ECL solution.

**Animal Experiments.** **Animal Grouping and Treatment.** One hundred 8-week-old C57BL/6j male mice [SCXK (Jing) 2009-0007], weighing 22–25 g and purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China), were bred at 22–25 °C, with 50 ± 10% RH, a 12 h light/dark circle, and free access to food and water. Mice were randomly divided into 5 groups with 20 in each group: a vehicle control group (Con), rotenone model group (Rot), rotenone model group treated with the positive control selegiline hydrochloride (Rot + SE), rotenone model group treated with a low dose of oleracein E (Rot +OE(L)), and a rotenone model group treated with a high dose of oleracein E (Rot +OE(H)). The Con group treated with the positive control selegiline hydrochloride (Rot + SE), rotenone model group treated with a low dose of oleracein E (Rot +OE(L)), and a rotenone model group treated with a high dose of oleracein E (Rot +OE(H)) were intragastrically administered 3 mg/mL rotenone (Sigma Co.) suspended in 0.5% CMC solution at a dosage of 30 mg/kg-d for 56 days. The Con group was administered an equal volume of 0.5% CMC solution. After rotenone treatment for 1 h, the Rot +OE(L) and Rot +OE(H) groups were intragastrically administered 0.3 and 1.5 mg/mL OE suspended in 0.5% CMC solution at a dosage of 3 mg/kg-d and 15 mg/kg-d, respectively, for 56 days. The Rot +SE group was intragastrically administered 1 mg/mL SE suspended in 0.5% CMC solution at a dosage of 10 mg/kg-d. The Con and Rot groups were treated with an equal volume of 0.5% CMC. All experimental procedures were approved by the Shandong University Animal Care and Use Committee.

**Spontaneous Activity Test.** The mouse was put into a JLBehv-LAM-4 open field apparatus (Shanghai Jiliang Software Technology Co., Ltd.) that included four sound-insulating boxes, each with a 25 cm × 25 cm cage. The moving distance and moving trial within 30 min were recorded and analyzed by image analysis software.

**Rota-rod Test.** The apparatus was set up by our group, with one rotary machine, one wood rod (15 cm length × 3 cm diameter), and two round baﬄe plates (25 cm diameter) with a distance of 9 cm. The distance between the rod and the table level was 40 cm. The mouse was placed on the rod between the two baﬄe plates, and the machine was turned on. The rotary speed was maintained at 24 r/min. The sustained time on the rod was recorded, that is, the time from the beginning of rotation to the point that the mouse fell from the rod. If the falling time was less than 4 min, the real time was recorded as the sustained time, whereas if the falling time was more than 4 min, 4 min was recorded as the sustained time. Each mouse was tested on the apparatus 3 times, and the average time was used for statistical analysis.

**Biochemical Assay.** After behavioral tests, the animals were fasted overnight. Blood was collected from the eye and put into an Eppendorf tube prerinsed with 2500 U/mL heparin. After blood samples were centrifuged at 3000 r/min for 10 min at 4 °C, the plasma...
was collected and preserved at −80 °C until analysis. After blood collection, the brain was immediately excised, and the midbrain and striatum were dissected on ice. Tissues were washed with cold saline, dried with filter paper, and weighed. Cold saline was added to the midbrain or striatum (9:1), and then the samples were homogenized (12500 r/min, 4 times, 10 s/time) over an ice bath with an IKA R104 homogenizer (Beijing Dequan Xinye Co. Ltd., China). The obtained 10% (m/v) brain homogenates were centrifuged at 3000 r/min for 10 min at 4 °C. The supernatants were stored at −80 °C before analysis. The protein content of the brain homogenate was determined according to the Coomassie blue method. 

The SOD activity and glucose-6-phosphate dehydrogenase (G6PDH) in the brain homogenates were measured by the G6PDH and SOD assay kits (Jiancheng Bioengineering Institute, China). The obtained 10% (m/v) brain homogenates were mixed with 100 mM PMSF at the ratio of 99:1 before use, and homogenized (12500 r/min, 4 times, 10 s/time) over an ice bath with an IKA R104 homogenizer (Beijing Dequan Xinye Co. Ltd., China). The obtained 10% (m/v) brain homogenates were centrifuged at 3000 r/min for 10 min at 4 °C. The supernatants were stored at −80 °C before analysis.

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**ASSOCIATED CONTENT**

Supporting Information

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Effect of OE on body weight in rotenone-induced PD mice and effect of OE on apoptosis-related protein expression in the midbrain and striatum in rotenone-induced PD mice (PDF)

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**Author Contributions**

H.S. and X.H. are co-first authors. H.S. and X.H. collaborated to conduct the PD cell and animal experiments; C.L. synthesized racemic oleacine E, L.L. and R.Z. helped to perform behavioral tests and some biochemical and Western blot assays. T.J., S.Y., D.F., J.G., J.S., and J.J. helped to collect blood and tissues and prepare brain homogenates and plasma samples. L.X., as the corresponding author, guided and organized all experiments and wrote this manuscript.

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS**

Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein; caspase-3, cysteinyllaspartate specific proteinase-3; CMC, sodium carboxymethyl cellulose; Con, vehicle control; DAB, diaminobenzidine; DAPI, dopaminergic; DAPI, 4′,6-diamidino-2-phenylindole; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DEMEM, Dulbecco’s modified Eagle medium; DMSO, dimethyl sulfoxide; DNase, deoxyribonuclease; DPPH, 1,1-diphenyl-2-picyrylhydrazyl radical; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; H2O2, hydrogen peroxide; HPLC, high performance liquid chromatography; LDH, lactic acid dehydrogenase; MAO-B, monoamine oxidase-B; MAPK, mitogen activated protein kinase; MDA, malonaldehyde; MEK1/2, mitogen-activated protein kinase 1 and 2; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; O6, oleacine E; PBS, phosphate buffered saline; PD, Parkinson’s disease; p-ERK1/2, phosphorylated ERK1/2; p-MEK1/2, phosphorylated MEK1/2; PMSF, phenylmethylsulfonyl fluoride; PFA, paraformaldehyde; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SE, seleagine hydrochloride; SNPc, substantia nigra pars compacta; SOD, superoxide dismutase; TETQ, tetrahydroisoquinoline; Rot, rotenone; t-ERK1/2, total ERK1/2; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; UTF, uridine-triphosphatase.

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