Effects of 6-Hydroxydopamine Exposure on Motor Activity and Biochemical Expression in Zebrafish (*Danio Rerio*) Larvae

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Abstract

Parkinson's disease (PD) is a neurodegenerative disease that is characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra. However, current treatments for PD are mainly palliative. Recently, researchers discovered that neurotoxins can induce Parkinsonian-like symptoms in zebrafish. No study to date has investigated the characteristics of PD, such as neuroinflammation factors, oxidative stress, or ubiquitin dysfunction, in this model. Therefore, the current study was aimed at utilizing commonly used clinical drugs, minocycline, vitamin E, and Sinemet, to test the usefulness of this model. Previous studies had indicated that DA cell loss was greater with 6-hydroxydopamine (6-OHDA) than with other neurotoxins. Thus, we first challenged zebrafish with 6-OHDA immersion and found a significant reduction in zebrafish locomotor activity; we then reversed the locomotor disruptions by treatment with vitamin E, Sinemet, or minocycline. The present study also analyzed the mRNA expression of *parkin*, *pink1*, and *cd-11b*, because the expression of these molecular targets has been shown to result in attenuation in mammalian models of PD. Vitamin E, Sinemet, and minocycline significantly reversed 6-OHDA-induced changes of *parkin*, *pink1*, and *cd-11b* mRNA expression in zebrafish. Moreover, we assessed tyrosine hydroxylase (TH) expression to confirm the therapeutic effects of vitamin E tested on this PD model and established that vitamin E reversed the 6-OHDA-induced damage on TH expression. Our results provide some support for the validity of this *in vivo* Parkinson's model, and we hope that this model will be more widely used in the future.

Introduction

PARKINSON'S DISEASE (PD), a progressive neurodegenerative movement disorder, affects almost 0.2% of the world population and ~1% of the individuals older than 65 years of age.^{1–3} Previous studies have indicated that one of the main pathological features associated with PD is the selective death of dopamine neurons in the substantia nigra of the midbrain. Most patients with PD exhibit some clin-

ical symptoms, such as resting tremor, bradykinesia, and muscular rigidity,⁴ and these symptoms may progressively worsen with advancing stages. While much is known about the pathophysiology of this life-threatening disease, the cause of PD still remains unknown.⁵ Some clinical research groups have identified a subset of patients with PD who possess specific genes that are responsible for rare familial forms of the disease, and these findings have provided new insights into the molecular mechanisms underlying

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PD pathogenesis. Specifically, these familial genes, namely, *α-synuclein*, *parkin*, *uch-L1*, *dj-1*, and *pink1* (which encodes for PTEN-induced putative kinase 1), have been shown to be involved in dysfunction of the ubiquitin-proteasome system (UPS), oxidative stress, and Lewy body formation, all of which have been classified as players in idiopathic forms of PD. Other causes of PD, classified as sporadic forms of the disease, involve exposure to environmental toxins or other factors. In the clinic, 95% of cases are deemed sporadic; that is, these cases might be multifactorial, idiopathic disorders resulting from susceptibility to both environmental and genetic factors. The remaining 5% of clinical cases can be attributed to inherited forms of the disease. The two types of PD share clinical, pathological, and biochemical features, with natural aging and dysfunctioning of the mitochondria and associated molecular pathways representing a bridge between idiopathic and sporadic PD.

In addition, there is high co-morbidity of PD with other psychopathological syndromes, including affective disorders, cognitive deterioration, and perceptual and behavioral symptoms. Interestingly, anxiety is known to have a large impact on the prognosis of PD. For example, patients with PD who are also anxious score lower on scales assessing motor function and activities of daily living and exhibit more cognitive symptoms, which results in a lower quality of life.⁶ Therefore, the treatment of PD that includes the amelioration of cognitive symptoms may not only elevate quality of life, but also improve the effectiveness of treatment. An ideal scenario, then, would be if one could assess the therapeutic effects of candidate drugs on both PD and anxiety; however, no model that assesses these two diseases concurrently is currently available.

Tremendous emphasis has been placed on the investigation and discovery of new PD drugs. Although more and more pharmaceuticals aimed at treating PD are appearing in the market, most of them only relieve the clinical symptoms of the disease, and none of the agents has been shown to slow or halt the progression of PD. Furthermore, most of these drugs have severe adverse effects, such as psychiatric dyskinesia.⁷ In their efforts to discover new compounds that can improve the treatment of PD, many researchers have become committed to developing an animal model for this neurodegenerative motor disorder.^{8–10} A number of animal models of PD have already been established to further investigate the mechanisms of the disease or to screen for new therapeutic compounds.^{11–14} The first animal model of PD associated with the selective death of dopaminergic (DA) neurons was introduced more than 30 years ago through an intracerebroventricular injection of 6-hydroxydopamine (6-OHDA) in the rat.¹¹

The zebrafish is another candidate animal for a PD model. Although compared with zebrafish, rodents have a higher homology with humans, as an animal model, zebrafish has lower limitations than rodents in terms of breeding, cost, efficiency, and drug dosage. Some groups have been able to develop zebrafish as an animal model for PD.^{14,15} Some research groups using zebrafish have been able to model the two different forms of PD. One model was established using genetic defects, such as the loss function of *parkin*, *pink1*, and *lrrk2*.^{16–18} Another zebrafish model of PD was constructed by treating the fish with neurotoxins, such as 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).^{14,19,20} As mentioned earlier, only 5% of the cases of PD can be

attributed to genetic mutations.²¹ Hence, a model induced by neurotoxins is probably the closest in relation to the form of PD that is most commonly seen in the clinic. Parng *et al.*¹⁹ demonstrated that the administration of 6-OHDA caused DA neuron loss and neuronal oxidation in zebrafish larvae. However, except tyrosine hydroxylase (TH), no other molecular marker of PD has been investigated in this zebrafish model.

Some previous research groups have supported the hypothesis that the progression of PD includes four possible causes, such as ubiquitin system dysfunction, oxidative stress, inflammation, and mitochondrial dysfunction.²² Among these causes, ubiquitin system dysfunction and oxidative stress are considered early and late markers of PD progression, respectively. The UPS is responsible for the removal of short-lived regulatory proteins as well as for the degradation of damaged or misfolded proteins. The E3 ubiquitin-protein ligase, also called *parkin*, is the rate-limiting enzyme in UPS. Interestingly, some studies have demonstrated that mutations in *parkin* may lead to sporadic PD.^{23,24} On the other hand, some research has suggested that oxidative stress may play an important role in PD progression.^{25,26} Another potential marker of PD is *pink1* that encodes for a mitochondrial serine/threonine-protein kinase, which is considered as protecting cells from stress-induced mitochondrial dysfunction. Mutations in this gene cause one form of autosomal recessive early-onset PD. Sha *et al.* revealed that *pink1* is involved in anti-apoptosis and coordination of activity of E1 and E2 ligase.²⁷ Moreover, some research suggests these two molecules as genetic markers of PD.²⁸

Obviously, the underlying mechanisms, genes, and proteins involved in 6-OHDA-induced PD in zebrafish remain unknown. Moreover, no study has examined the effect of positive drugs on this model. Thus, our study intended to investigate zebrafish larvae behavior, including deficits in locomotor activity and anxiety-like behavior, and variations in the expression of some biomarkers of PD, including *pink1*, *parkin*, tumor necrosis factor- α (TNF- α), and *cd11b* in the hope of proving the feasibility of the 6-OHDA-induced zebrafish model of PD.

Materials and Methods

Fish maintenance

The AB strain of wild-type zebrafish was used for this study. Embryos were collected after natural spawning, staged according to standard criteria, and raised synchronously at 28.5°C in Hank's buffer (13.7 mM NaCl, 540 μ M KCl, 25 μ M Na₂HPO₄, 44 μ M KH₂PO₄, 300 μ M CaCl₂, 100 μ M MgSO₄, 420 μ M NaHCO₃, and pH 7.4). No additional maintenance was required, because the embryos receive nourishment from the attached yolk sac.

Locomotor behavioral test

Zebrafish larvae at 2 days post fertilization (dpf) were treated with 6-OHDA either in the absence or in the presence of tested drugs for 4 days in a 24-well plate. At 5 dpf, fish were transferred into 10-cm dishes (16 fish/dish), and swimming behavior was monitored by an animal behavior system with an automated video tracking (Singa Technology Co.; catalog no. TM-01).²⁹ Fish were transferred into a cuvette. The cuvette used for the present experiments was a

4.5-cm-high quartz cuvette, with a width and length of 1 cm. The cuvette was housed in a distinctive plastic box that was 16-cm long and 4.8-cm wide. The cuvettes were placed in front of a camera (Weichu Technology Co Ltd.; catalog no. IC-200) at a distance of 7.5 cm (Fig. 1). All instruments were adhered to a plastic plate that was 38- and 19-cm long and wide, respectively. During a test, four cuvettes were placed in a parallel arrangement. Individual zebrafish were gently put into the tank and tracked from the side of the tank so that it was possible to determine the swim height. Each animal was given a 2-min adaptation period, and then the swimming pattern of each fish was recorded for 10 min. The total distance moved was defined as the distance (in cm) that the fish moved during one 10 min session. In the cuvette diving test, a greater amount of time spent in the lowermost part of the tank indicated increased anxiety.³⁰

Whole-mount immunostaining

Zebrafish larvae were fixed in 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) for 5 h, rinsed, and stored at -20°C in EtOH. Whole-mount immunostaining was performed by standard methods. Briefly, fixed samples were blocked by 2% (v/v) lamb serum and 0.1% (w/v) bovine serum albumin in PBST for 1 h at room temperature. A mouse monoclonal anti-TH antibody (diluted 1:200 [v/v] in blocking buffer, MAB318; Millipore) was used as the primary antibody and incubated with the sample overnight at 4°C . A previous study has successfully used this antibody in zebrafish immunostaining.³¹ The next day, samples were washed six times with PBST (30 min each wash), followed by incubation with secondary antibody. After color development, the zebrafish were flat mounted with 3.5% methylcellulose and photographed.

FIG. 1. Diagram of animal behavior apparatus. The cuvette was a 4.5-cm-high quartz cuvette, with a width and length of 1 cm. They were housed in a distinctive plastic box that was 16-cm long and 4.8-cm wide. The cuvettes were placed in front of a camera at a distance of 7.5 cm. All instruments were adhered to a plastic plate that was 38- and 19-cm long and wide, respectively. During a test, four cuvettes were placed in a parallel arrangement.

Confocal microscopy

Imaging was acquired using a Leica TCS SP5 II confocal microscope (Leica Microsystems), and excitation was achieved with an argon laser at 488 nm. Images were captured using a $10\times$ objective, with 1024×1024 pixel resolution, and images were obtained by averaging 20 scans for each $100\text{-}\mu\text{m}$ section. Quantitation of the fluorescence was done using LCS Lite software (Leica Microsystems).

Total RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction

Zebrafish embryos at 9 hours post fertilization (hpf) were treated for 87 h with $10\text{ }\mu\text{M}$ minocycline, $100\text{ }\mu\text{M}$ vitamin E, or $25\text{ }\mu\text{g/mL}$ in the presence or absence of 6-OHDA. According to the manufacturer's instructions, total RNA was extracted from 20 zebrafish larvae of each treatment group using the TRIzol[®] Reagent (Invitrogen[™]). RNA was reverse transcribed to single-stranded cDNA using the iScript cDNA synthesis kit (Bio-Rad). The following reverse transcription-polymerase chain reaction (RT-PCR) using the gene expression assay primer for zebrafish *parkin* and *pink1* was used. Primers of *parkin*: forward: 5'-GCGAGTGTGTCTGA GCTGAA-3' reverse: 5'-CACACTGGAACACCAGCACT-3'. *pink1*: forward: 5'-GGCAATGAAGATGATGTGGAAC-3' reverse: 5'-GGTCGGCAGGACATCAGGA-3'. We then performed real-time PCR using the iQ[™] SYBR[®] Green (Bio-Rad) supermix for zebrafish *cd11b* in the Bio-Rad real-time PCR system (all materials were from Applied Biosystems). Primers of *cd11b* were forward: 5'-ACGTGACG CTGTTT GTCG-3' and reverse: 5'-GCCAGCAGCACAA GTCC-3'. The expression level of each gene was expressed as a relative fold change ($\log_2$ ratio) that was calculated using the comparative Ct method and using GAPDH as the internal reference.

Western blotting

Zebrafish embryos at 9 hpf were treated for 87 h with $10\text{ }\mu\text{M}$ minocycline, $100\text{ }\mu\text{M}$ vitamin E either in the presence or in the absence of 6-OHDA. Zebrafish larvae were then washed with ice-cold PBS, lysed in ice-cold lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, $100\text{ }\mu\text{g/mL}$ phenylmethylsulfonyl fluoride, and $1\text{ }\mu\text{g/mL}$ aprotinin), and then centrifuged at $20,000\text{ g}$ for 30 min at 4°C . The supernatant was decanted and reserved for western blotting, and protein concentrations were measured using the DC protein assay kit (Bio-Rad) that was modified from the method used by Lowry *et al.*³² Western blotting was then carried out as previously described.^{33,34} An equal volume of sample buffer (2% 2-mercaptoethanol, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue, 10% glycerol, and 50 mM Tris-HCl [pH 7.2]) was added to the sample, and protein lysates were loaded onto a 10% SDS-polyacrylamide gel. Electrophoresis was then carried out at 150 V for 90 min. After electrophoresis, the gels were transferred overnight at 4°C in transfer buffer (380 mM glycine, 50 mM Tris-HCl, 1% SDS, and 20% methanol) onto a polyvinylidene difluoride membrane (PVDF; Immobilon-P, Millipore Corp. [0.45- μm pore size]). The PVDF membrane was first blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween (20 mM Tris-HCl, 0.1% Tween 20,

and 137 mM NaCl [pH 7.4]) and then incubated overnight at 4°C with the anti-TH antibody (1:500 dilution; Millipore; MAB318), anti TNF- α (1:1000 dilution; AnaSpec; catalog no. 55383; polyclonal rabbit antibody). A horseradish peroxidase-conjugated secondary antibody was detected by chemiluminescence (Millipore Corp.). Images were obtained using the UVP BioChem Imaging System, and LabWorks 4.0 software (UVP) was used to quantify the relative densitometry. TH and TNF- α antibodies recognized bands at ~ 60 and ~ 40 kDa, respectively.

Statistical analysis

All experiments were repeated at least four times. All data are represented as the mean $\pm$ standard error of the mean. For immunoreactivity data, the intensity of each test band was expressed as the relative optical density (OD) that was calculated from the average control OD values obtained from

all controls. Wherever applicable, data were analyzed using one-way analysis of variance followed by Dunnett's test. A p value of less than 0.05 was considered statistically significant.

Results

6-OHDA treatment induced deficits in locomotor activity in both a dose- and time-dependent manner

The large amount of DA neuronal death is usually the cause of mobility deficits. Zebrafish were treated with 0, 10, 50, 100, and 250 μ M 6-OHDA (from 2 to 4 dpf). The effect of 6-OHDA on locomotor activity was then evaluated by examining typical swimming pattern (Fig. 2A), total swimming distance (Fig. 2B), and time spent in the bottom zone of the cuvette (Fig. 2C). The results of these evaluations clearly depicted a decrease in locomotor activity and an increase in the time spent in the bottom zone in 250 μ M

FIG. 2. Effect of different concentrations of 6-hydroxydopamine (6-OHDA) on zebrafish locomotor activity. (A) Typical swimming pattern at 5 days post fertilization (dpf) of zebrafish exposed to 0, 10, 50, 100, or 250 μ M 6-OHDA (from 2 to 4 dpf). (B) Quantitation of total distance of swimming for zebrafish treated with 6-OHDA at 5, 6, and 7 dpf. (C) Quantitation of time spent in the bottom zone of the cuvette at 5, 6, and 7 dpf for zebrafish treated with 6-OHDA at 2–5 dpf. The results indicate that treatment with 250 μ M 6-OHDA (from 2 to 4 dpf) significantly decreased the total swimming distance and increased the time that the fish spent in the bottom zone of the tank at 5, 6, and 7 dpf. Each value represents the mean of 16 fish, and error bars represent mean $\pm$ standard error of the mean (SEM). *Significantly different from the control group ($p < 0.05$).

6-OHDA-challenged zebrafish (Fig. 2). The total swimming distance in the 250 μ M 6-OHDA-treated group changed to 261.66 ± 59.64 mm from 795.84 ± 52.00 mm and accounted for almost 30–50% of the control group. Zebrafish were treated with 250 μ M 6-OHDA for 0, 2 (from 2 to 3 dpf), 3 (from 2 to 4 dpf), or 4 days (from 2 to 5 dpf). The effect of 6-OHDA on locomotor activity was also evaluated by visualizing typical swimming pattern (Fig. 3A), total swimming distance (Fig. 3B), and the time that the fish spent in the bottom zone of the tank (Fig. 3C). Our results clearly indicated a decrease in locomotor activity and an increase in the time spent in the bottom zone of the tank at 3 days of 6-OHDA treatment (from 2 to 4 dpf). Based on these data, we

decided to conduct further experiments using a concentration of 250 μ M 6-OHDA for a treatment period of 3 days.

Protective effect of the anti-oxidant vitamin E on 6-OHDA-treated zebrafish in locomotor activity

Oxidative stress is one of the most important factors in the etiology of PD; therefore, we used an anti-oxidant, vitamin E, in an effort to rescue the damage caused by 6-OHDA treatment. Zebrafish were treated with 100 μ M vitamin E (from 9 hpf to 4 dpf), and with 250 μ M 6-OHDA (from 2 to 4 dpf). We then assessed zebrafish swimming pattern (Fig. 4A), total swimming distance (Fig. 4B), and time spent in the bottom zone of the

FIG. 3. Effect of different 6-OHDA-treatment times on zebrafish locomotor activity. (A) Typical swimming pattern at 5 dpf after treatment with 250 μ M 6-OHDA for 0, 2 (from 2 to 3 dpf), 3 (from 2 to 4 dpf), or 4 days (from 2 to 5 dpf). (B) Quantitation of total swimming distance for zebrafish treated with 250 μ M 6-OHDA for 0, 2 (from 2 to 3 dpf), 3 (from 2 to 4 dpf), or 4 days (from 2 to 5 dpf) treatment. (C) Quantitation of time spent in the bottom zone of the tank for zebrafish treated with 250 μ M 6-OHDA for 0, 2 (from 2 to 3 dpf), 3 (from 2 to 4 dpf), or 4 days (from 2 to 5 dpf). We found that treatment with 250 μ M 6-OHDA for 3 days (from 2 to 4 dpf) significantly decreased the total swimming distance and increased the time spent in the bottom zone at 5, 6, and 7 dpf. Each value represents the mean for 16 fish, and error bars represent mean $\pm$ SEM. *Significantly different from the 0 day treatment group ($p < 0.05$).

cuvette (Fig. 4C) in order to determine whether the administration of vitamin E could rescue the locomotor deficits seen in 6-OHDA-treated fish. Our results demonstrated that vitamin E could apparently rescue the locomotor deficit caused by 6-OHDA with values changing from a total distance swam of 230.46 ± 122.13 mm pre-vitamin E to 514.83 ± 122.13 mm post-vitamin E. Moreover, the typical swimming pattern revealed a significant change, and treatment of 6-OHDA-challenged fish with vitamin E significantly reversed the increase in the time spent in the bottom zone of the cuvette. In previous

studies, adult zebrafish have shown this characteristic stress or anxiety-related behavior^{35–37} of spending more time at the bottom of the tank. To our knowledge, we are the first to reveal that zebrafish larvae also demonstrate the same behavior trend.

Vitamin E reversed 6-OHDA-induced decrease in TH expression in zebrafish

TH is the restricting enzyme for catecholamine synthesis, and it helps convert the amino acid tyrosine to a catecholamine

FIG. 4. Vitamin E protects against the 6-OHDA-induced locomotor activity deficiency. Zebrafish were treated with 100 μ M vitamin E (from 9 hours post fertilization (hpf) to 4 dpf) and with 250 μ M 6-OHDA (from 2 to 4 dpf). (A) Typical swimming pattern at 5 dpf for the control, 6-OHDA, 6-OHDA plus vitamin E, and vitamin E alone groups. (B) Quantitation of total swimming distance at 5, 6, and 7 dpf for the control, 6-OHDA, 6-OHDA plus vitamin E, and vitamin E alone groups. (C) Quantitation of time spent in the bottom zone of the tank at 5, 6, and 7 dpf for the control, 6-OHDA, 6-OHDA plus vitamin E, and vitamin E alone groups. Our results indicated that vitamin E significantly attenuated the 6-OHDA-induced deficiency in locomotor activity. Each value represents the mean for 12 fish, and error bars represent mean $\pm$ SEM. *Significantly different from the control group; #significantly different from the 6-OHDA group ($p < 0.05$).

called dopamine. In the current study, when zebrafish were treated with 250 μ M 6-OHDA (from 2 to 4 dpf), we observed an obvious decrease in TH expression in larvae head tissue at 5 dpf (Fig. 5); however, when fish were pre-treated with 100 μ M vitamin E (from 9 hpf to 4 dpf), we observed a marked attenuation of 6-OHDA-induced decrease in TH expression (Fig. 5).

Rescue of locomotor activity with minocycline administration to 6-OHDA-treated zebrafish

Neuroinflammation also plays an important role in PD. Therefore, we used a microglia inhibitor, minocycline, in an attempt to see whether we could produce any therapeutic effect

on this model. Zebrafish were treated with 10 μ M minocycline (from 9 hpf to 4 dpf) and 250 μ M 6-OHDA (from 2 to 4 dpf). The effect of minocycline on 6-OHDA-treated zebrafish locomotor activity was assessed by visualizing typical swimming pattern (Fig. 6A), total swim distance (Fig. 6B), and time spent in the bottom zone of the cuvette (Fig. 6C). Interestingly, our data demonstrated that minocycline significantly reversed the locomotor deficit caused by 6-OHDA in zebrafish larvae and reduced the time spent in the bottom zone that had initially increased with 6-OHDA. Before minocycline treatment, the total swimming distance of the control and 6-OHDA group decreased from 737.58 ± 40.9 mm to 102.84 ± 32.85 mm, respectively. However, the pre-treatment of minocycline rescued

FIG. 5. Vitamin E protects against 6-OHDA-induced decrease in tyrosine hydroxylase (TH) expression. Zebrafish were treated with 100 μ M vitamin E (from 9 hpf to 4 dpf) and with 250 μ M 6-OHDA (from 2 to 4 dpf). (A) Western blot analysis for TH expression at 5 dpf in the control, 6-OHDA, 6-OHDA plus vitamin E, and vitamin E alone groups. (B) Quantitative result of the bands of western blotting. Each bar was quantified by three samples, and each sample contained 20 zebrafish heads. (C) Whole-mount immunohistochemistry for TH expression at 5 dpf in the control, 6-OHDA, and 6-OHDA plus vitamin E groups. (D) Quantitative result for the control, 6-OHDA, and 6-OHDA plus vitamin E groups. Each bar indicates the value for five zebrafish larvae. Both analyses show that vitamin E significantly reversed the decrease in the expression of TH, which is a biomarker of dopaminergic neurons (white arrow). Each value represents the mean of three samples, and error bars represent mean $\pm$ SEM. *Significantly different from the control group; #significantly different from the 6-OHDA group ($p < 0.05$).

FIG. 6. Minocycline protects against 6-OHDA-induced locomotor activity deficiency. Zebrafish were treated with 10 μ M minocycline (from 9 hpf to 4 dpf) and with 250 μ M 6-OHDA (from 2 to 4 dpf). (A) Typical swimming pattern at 5 dpf for the control, 6-OHDA, 6-OHDA plus minocycline, and minocycline alone groups. (B) Total swimming distance at 5, 6, and 7 dpf for the control, 6-OHDA, and 6-OHDA with minocycline groups. (C) Quantitation of the time each fish spent in the bottom zone of the tank on 5, 6, and 7 dpf for the control, 6-OHDA, and 6-OHDA with minocycline groups. Our data showed that minocycline not only significantly reversed the 6-OHDA-induced deficiency of locomotor activity in zebrafish, but also attenuated the increase in the time spent in the bottom zone caused by 6-OHDA challenge. Each value represents the mean for 16 fish, and error bars represent mean $\pm$ SEM. *Significantly different from the control group; #significantly different from the 6-OHDA group ($p < 0.05$).

the total swimming distance from 102.84 ± 32.85 mm to 753.39 ± 63.12 mm. Importantly, minocycline alone did not have any effect on the total swimming distance.

Minocycline reversed 6-OHDA-induced expression of inflammatory genes and proteins in zebrafish

Inflammation plays a key role in 6-OHDA-induced DA neuron damage *in vivo*. We used quantitative real-time PCR (Fig. 7A) and western blotting (Fig. 7B) to measure inflammation-related gene and protein expression in 6-OHDA-treated zebrafish. As shown in Figure 7, 6-OHDA caused over-expression of TNF- α and *cd11b*, fivefold greater than that of

control fish. However, the pre-treatment with minocycline reversed the increased inflammatory gene and protein expression, indicating that the inflammatory response is probably involved in the zebrafish model of 6-OHDA-induced neuronal damage.

Rescue of locomotor activity with Sinemet in 6-OHDA-treated zebrafish

Sinemet is one of the most widely used clinical drugs on patients with PD. Therefore, we decided to test this drug on our zebrafish model in order to confirm the model's feasibility. Zebrafish were treated with 2.5 or 25 μ g/mL Sinemet (from 9 hpf to 4 dpf), and with 250 μ M 6-OHDA (from 2 to 4 dpf). The

FIG. 7. Minocycline attenuated the 6-OHDA-induced increase in pro-inflammatory cytokine expression. Zebrafish were treated with 10 μ M minocycline (from 9 hpf to 4 dpf) and with 250 μ M 6-OHDA (from 2 to 4 dpf). Our results indicated that the administration of minocycline significantly inhibited the increase in the expression of pro-inflammatory cytokines, measured as *cd-11b* in mRNA expression and tumor necrosis factor- α protein expression. Each bar represents the values for three samples. Each sample contained 20 zebrafish heads, and error bars represent mean $\pm$ SEM. *Significantly different from the control group; #significantly different from the 6-OHDA group ($p < 0.05$).

effect of Sinemet on locomotor activity of 6-OHDA-treated zebrafish was then assessed by visualizing typical swimming pattern (Fig. 8A), total swimming distance (Fig. 8B), and the time that the fish spent in the bottom zone of the cuvette (Fig. 8C). Our data demonstrated that Sinemet significantly rescued the locomotor deficit induced by 6-OHDA in zebrafish larvae (see Fig. 8A for examples of the typical swimming pattern of control fish, 6-OHDA-treated fish, and fish that had been pre-treated with Sinemet). In 6-OHDA-treated fish, we found that total swimming distance was decreased in comparison to controls from 582.54 ± 44.34 mm to 145.59 ± 53.07 mm. However, pre-treatment with Sinemet rescued the total swimming distance from 145.59 ± 53.07 mm without Sinemet to 523.32 ± 79.23 mm with Sinemet at 5 dpf. However, the pre-treatment of Sinemet could rescue the increase in time that the 6-OHDA-treated fish spent in the bottom zone of the tank only in 5 dpf.

Vitamin E and Sinemet reversed the 6-OHDA-induced decrease in *pink1* and *parkin* expression in zebrafish

Both *pink1* and *parkin* play an important role in the progression of PD. We, therefore, wanted to see whether the two

protective compounds that we used had any effect on the expression of these genes. 6-OHDA-treated zebrafish were treated with 100 μ M of vitamin E or 25 μ g/mL Sinemet (from 9 hpf to 4 dpf). We then used RT-PCR to measure PD-related gene expression of *pink1* (Fig. 9A) and *parkin* (Fig. 9B). The PCR product of *pink1* and *parkin* was 259 and 681 bp, respectively. As shown in Figure 9, 6-OHDA treatment decreased the expression of both *parkin* and *pink1*. However, co-treatment with either vitamin E or Sinemet was enough to reverse this decrease.

Discussion

Summary of our findings

Our current findings confirm zebrafish larva as a proper animal model for use in studies of PD. We first treated zebrafish with different doses of 6-OHDA and found a significant reduction in zebrafish locomotor activity using an optimal 250 μ M concentration of 6-OHDA (Figs. 2 and 3). We also found that three days of 6-OHDA treatment decreased the total swimming distance. We next tested a variety of clinical

FIG. 8. Sinemet protects against 6-OHDA-induced locomotor activity deficiency. Zebrafish were treated with 2.5 or 25 μg/mL Sinemet (from 9 hpf to 4 dpf) and with 250 μM 6-OHDA (from 2 to 4 dpf). **(A)** Typical swimming pattern at 5 dpf for the control, 6-OHDA, and 6-OHDA with Sinemet groups. **(B)** Quantitation of the total swimming distance at 5, 6, and 7 dpf for the control, 6-OHDA, and 6-OHDA with Sinemet groups. **(C)** Quantitation of time spent in the bottom zone of the tank at 5, 6, and 7 dpf for the control, 6-OHDA, and 6-OHDA with Sinemet groups. Our results indicated that Sinemet significantly reversed the 6-OHDA-induced deficiency of locomotor activity in zebrafish but rescued the increase in time that the 6-OHDA-treated fish spent in the bottom zone of the tank only in 5 dpf. Each value represents the mean for 12 fish, and error bars represent mean ± SEM. *Significantly different from the control group; #significantly different from the 6-OHDA group ($p < 0.05$).

drugs involved in targeting factors such as oxidative stress, inflammatory processes, mitochondrial dysfunction, and ubiquitin system dysfunction in order to confirm the zebrafish larva as an appropriate model of PD. Thus, we used vitamin E to confirm the oxidative stress process in this model. We did this in order to confirm that we could reverse the deleterious

FIG. 9. Vitamin E and Sinemet rescued the 6-OHDA-induced decrease in *parkin* and *pink1* mRNA expression. Zebrafish were treated with 100 μM of vitamin E or 25 μg/mL of Sinemet (from 9 hpf to 4 dpf) and with 250 μM 6-OHDA (from 2 to 4 dpf). The polymerase chain reaction product of *pink1* and *parkin* was 259 and 681 bp, respectively. **(A)** *pink1* messenger RNA expression at 3 dpf in the control (lane 1), 6-OHDA with vitamin E (lane 2), 6-OHDA with Sinemet (lane 3), and 6-OHDA groups (lane 4). **(B)** *Parkin* messenger RNA expression at 3 dpf in the control (lane 1), 6-OHDA with vitamin E (lane 2), 6-OHDA with Sinemet (lane 3), and 6-OHDA groups (lane 4). Our data demonstrate that vitamin E and Sinemet significantly inhibited the 6-OHDA-induced decrease in the expression of *parkin* and *pink1*. Each bar represents the values for three samples. Each sample contained 20 zebrafish heads, and error bars represent mean ± SEM.

effects of 6-OHDA treatment in this model by administering therapeutic agents, and we used this approach to verify that 6-OHDA damage included oxidative stress pathways. We found that vitamin E administration was able to rescue 6-OHDA-induced locomotor deficits (Fig. 4), and that vitamin E could reverse the 6-OHDA-induced decrease in the expression of TH (Fig. 5) and *parkin* and *pink1* (Fig. 9). With regard to the neuroinflammatory process, we found that minocycline rescued locomotor activity and attenuated the 6-OHDA-induced increase in TNF-α and *cd11b* expression (Fig. 7). In addition, in order to test this model's relation to the therapies used in clinical settings, we used Sinemet, the most widely used clinical drug in the market, and found that the administration of Sinemet could rescue the reduced locomotor activity in this model (Fig. 8).

Comparison between previous zebrafish PD models and our studies on behavior

In 2004, Bretau et al. first found that zebrafish could serve as an animal model of PD.¹⁴ This group found that by using neurotoxins such as MPTP, they could establish PD-like symptoms such as the reduction of locomotor activity in adult zebrafish and larvae. Bretau's group also demonstrated that paraquat and rotenone were neurotoxins that were not effective

in inducing PD-like phenotypes. Moreover, Anichtchik *et al.* also showed that intracerebral injections of 6-OHDA and MPTP could cause significant decreases in the locomotor activity of adult zebrafish.³⁸ Both of these previous studies monitored and recorded zebrafish horizontal swimming patterns in adult fish; however, the horizontal swimming pattern can only indicate exercise capacity. In contrast to these studies, we utilized an instrument that not only can record and measure the typical swimming pattern and exercise capacity but also can evaluate the time fish spent in each region.

Diving is a common reaction of prey fish in response to stress. Egan *et al.* first showed anxiety-related phenotypes in adult zebrafish after exposure to experimental stressors and pharmacological agents.³⁹ This group also took advantage of video-tracking tools and used them in their behavioral analysis to report that acute alarm pheromone exposure resulted in zebrafish which spent significantly less time in the upper portion of the tank. Egan *et al.*³⁹ confirmed zebrafish as a valid, reliable, and efficacious model for basic translational research of stress-related brain disorders. Consistent with these previous studies, in our study we were able to observe anxiogenic behavior in our 6-OHDA-treated zebrafish. The time that these fish spent in the bottom region of the cuvette significantly increased after the treatment of 6-OHDA. Moreover, we used zebrafish larvae in our experiment, as the use of larvae reduced the required dosage of drugs, providing a profit to the drug screening work, and because the high permeability of larvae and development of the blood-brain barrier at 3 dpf⁴⁰ could help the efficiency of drug treatment. In fact, we are the first group to investigate the administration of these clinical drugs to the zebrafish PD model.

Comparison between previous rat PD drug treatment and the zebrafish PD model

As in humans, Hughes and Collins demonstrated that chronic treatment of aging male and female rats with vitamin E could decrease anxiety.⁴¹ Our data have shown similar results in that the pre-treatment of vitamin E significantly reduced increases in the time which the zebrafish would spend in the bottom region of a cuvette. Further comparisons between rat and zebrafish models can be made in relation to the use of Sinemet, a combination of carbidopa and L-3,4-dihydroxyphenylalanine (L-DOPA). Several studies have reported the effect of L-DOPA on mood and anxiety. Specifically, it has been suggested that chronic L-DOPA therapy in severely DA-lesioned rats does not improve anxiogenic symptoms and may even impair non-DA processes.^{38,39} This implies that long-term L-DOPA therapy does not exert necessary neuroplastic changes needed for improving effect. Our data also show a similar trend when analyzing the time that our zebrafish model of PD spent in the bottom region of a testing tank. Specifically, the treatment with Sinemet did not reverse the increase in the time spent in the bottom zone of the cuvette that was induced by 6-OHDA.

Lastly, previous studies in rats have indicated that minocycline has an anti-Parkinsonian effect because of its anti-neuroinflammatory activity.^{42,43} Our results confirmed that minocycline could also exert a therapeutic effect over 6-OHDA-induced damage. Therefore, our studies are the first to reveal that these drugs demonstrate a therapeutic effect in the zebrafish model, which not only further verified this model's applicability but also improved its value.

Comparison between previous zebrafish models of PD and our results on the molecular mechanism of the disease

In the current study, the changes that we observed in motor behavior were also accompanied by changes in molecular targets. First, we showed decreased TH expression in the zebrafish larvae brain, which is consistent with PD clinical symptoms. In contrast, Anichtchik *et al.* showed no difference in TH in the adult zebrafish PD model.³⁸ Moreover, Zhang *et al.* revealed that the extract of *Fructus Alpinia oxyphylla* could rescue the 6-OHDA-induced increase in the expression of interleukin-1 β and TNF- α .⁴⁴ Along with TH, we examined other PD-related markers, such as *parkin* and *pink1*, that none of the previous studies had examined. Both *parkin* and *pink1* play an important role in human PD. *Parkin*, or E3 ubiquitin ligase, is a key enzyme of the ubiquitin system in the brain. Some clinical case reports have suggested that the mutation of E3 ubiquitin ligase results in autosomal recessive juvenile Parkinsonism.^{45,46} It also results in the aggregation of α -synuclein, which causes the death of dopamine neurons⁴⁷; a major pathologic hallmark of PD is the presence of cytoplasmic protein inclusions in the remaining surviving neurons.⁴⁸ Hence, the decreased expression of *parkin* protein in the brain of mammalian PD models is in agreement with our data. Specifically, we found that the expression of *parkin* significantly decreased after 6-OHDA treatment.

In addition to *parkin*, *pink1* gene expression was also decreased in our zebrafish PD model. Some studies have demonstrated that the overexpression of *pink1* helps protect against stress caused by mitochondrial defects and apoptotic processes.^{49,50} Exner *et al.* (2007) revealed that the loss of *pink1* function led to reductions in the functioning of complex I of the respiratory chain.⁵¹ Therefore, we evaluated *pink1* expression in order to validate the 6-OHDA treatment as a method for modeling PD in zebrafish.^{38,52,53} The mRNA expression of *parkin* and *pink1* in our data also showed a consistent trend to mammalian models of PD. After 6-OHDA treatment, mRNA expression of *parkin* and *pink1* decreased, and surprisingly, Sinemet was able to reverse this decrease. Sinemet is the combination of carbidopa and levodopa, both of which do not seem to aid in ubiquitin dysfunction or oxidative stress. However, Shin *et al.* showed that levodopa had a neuroprotective effect in MPTP-induced PD models,⁵⁴ suggesting that the drug could be responsible for activating the MAPK cascade. Importantly, activation of the MAPK is widely believed to participate in the survival pathway of dopamine neurons against oxidative stress.

Future directions and confirmation of behavior and molecular targets in the zebrafish model of PD

Until now, the phenotype and genotype of zebrafish models of PD have remained unclear. In order to increase the model's feasibility and relationship to PD, our current studies examined swimming behavior and pattern to confirm PD-like symptoms, and we also examined biomarkers related to PD progression, such as TH, TNF- α , *parkin*, *pink1*, and *cd11b*. Furthermore, the use of zebrafish as an animal model has some advantages compared with rat models or cell lines, such as the complete development of the zebrafish nervous system, low cost, and low dosages needed. Therefore, we hope that studies such as ours will increase the future application of the zebrafish PD

model in research and clinical practice in order to accelerate the development of therapeutic drugs.

Conclusions

Our present results showed that vitamin E, minocycline, and Sinemet can rescue the 6-OHDA-induced locomotor deficiency in zebrafish. In addition, we found that vitamin E and minocycline could reverse the 6-OHDA-induced increase in the time that these fish spent in the bottom region of the tank (an indication of anxiety). We also revealed that a decrease in the expression of *parkin* and *pink1* by 6-OHDA treatment could be reversed by vitamin E and Sinemet. Lastly, we showed that the 6-OHDA-induced increase in TNF- α and *cd-11b* expression could also be reversed by co-treatment with the microglia inhibitor minocycline.

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Disclosure Statement

No competing financial interests exist.

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