Therapeutic Effects of Fucoidan in 6-Hydroxydopamine-Lesioned Rat Model of Parkinson's disease: Role of NADPH oxidase-1

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SUMMARY

Aims: To explore the effect of fucoidan treatment on oxidative stress-mediated dopaminergic neuronal damage and its potential mechanisms. **Methods:** The effect of fucoidan was investigated in a 6-hydroxydopamine (6-OHDA) rat model of PD, an animal model considered appropriate for preclinical studies of PD therapy. The effects of fucoidan treatment on animal behavior and the survival ratio of dopaminergic neurons were investigated. We further observed the effect of fucoidan on microglia and the NADPH oxidases-1 (Nox1), a family of enzymes generating reactive oxygen species (ROS). **Results:** We found that chronic fucoidan administration mitigated the motor dysfunction induced by 6-OHDA. Similarly, fucoidan reduced the loss of DA neurons in the SNc and DA fibers in the striatum in 6-OHDA-lesioned rats. Moreover, we found that fucoidan inhibited the 6-OHDA-stimulating expression of Nox1 in both tyrosine hydroxylase (TH)-positive neurons and non-TH-positive neurons, prevented Nox1-sensitive oxidative stress and cell damage in SNc neurons. Fucoidan also effectively inhibited nigral microglial activation. **Conclusion:** These results support the beneficial effect of fucoidan in 6-OHDA-lesioned rat model of PD. Fucoidan may suppress the Nox1-triggered oxidative stress in the SNc to protect DA neurons from 6-OHDA-induced toxicity and achieve its beneficial effect.

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder resulting from progressive death of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNc). This disease usually shows severe clinical symptoms, mainly including resting tremor, rigidity, and bradykinesia [1–3]. The mechanism underlying DA neuronal degeneration remains elusive, paralleling with the lack of effective therapy for PD at present [4]. As such, there is an urgent need to exploit neuroprotective agents that can prevent DA neurons from degeneration in the progression of PD.

While the mechanism for the loss of DA neurons is unclear, oxidative stress is believed to play a role [5,6]. DA neurons are characteristically sensitive to oxidative stress owing to their reduced antioxidant capacity and high content of oxidation-prone dopamine, lipids, and iron [7,8]. In addition, DA neurons are enriched with nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox), a family of enzymes generating reactive oxygen species (ROS) and thus promoting oxidative stress [9]. The first Nox enzyme was discovered in neutrophils [10–12]. Seven homologues of Nox (Nox1–5 and Duox1/2) have since been identified

in various tissues [13–15]. Recently, expression of Nox1 and Nox1-derived ROS in SNc DA neurons was found to be increased in PD animals in response to environmental toxins, such as paraquat, 6-hydroxydopamine (6-OHDA), and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [16–18]. Thus, Nox1 and Nox1-directed oxidative stress are thought to contribute to the death of DA neurons and the pathogenesis of PD. Further evidence for this notion is that oxidative stress facilitated activation of microglia [19,20], a critical step leading to degeneration of DA neurons [21–23]. Due to these recent progresses, pharmaceutical targeting of oxidative devices has become a valuable approach for PD therapy.

Fucoidan (sulfated fucans) is a sulfated fucosylated polymer from brown algae, which has been documented in traditional Chinese medicines for more than 1000 years and is a common food worldwide today. Noticeably, fucoidan exhibited the protective effect on DA neurons in MPTP-treated mice and lipopolysaccharide-treated rats according to our previous studies [24,25]. This indicates that fucoidan has a potential neuroprotective effect in PD model animal, although its beneficial properties have not been fully characterized in a PD model and particularly underlying

mechanisms for its effect remain to be elucidated. We thus conducted this study to characterize the effect of fucoidan on a neurochemical loss of nigral DA neurons and behavioral deficits in a rat model of PD induced by 6-OHDA. Moreover, we carried out a series of mechanistic studies to evaluate the role of Nox1 and oxidative stress in mediating the effect of fucoidan.

Materials and Methods

Source of Fucoidan

Fucoidan was isolated from *Laminaria japonica* commercially cultured in Qingdao, China, as described previously [26]. Its chemical composition was analyzed as follows: 48% total sugar content, 28% fucose content, and 29% sulfate content. Neutral monosaccharide analysis of fucoidan showed that fucose was the main component with its molar ratio to galactose at 1:0.24. Its average molecular weight was 7000 Da as determined by high-performance steric exclusion chromatography analysis. Fucoidan was dissolved in 0.9% saline for animal treatment.

Animals

Male Sprague–Dawley rats, weighing 250–300 g, were supplied by the Vital River Laboratory Animal Technology Co. Ltd (Beijing, China) and housed with a standard 12-h on/off light cycle with food and water *ad libitum* in their home cages. All animal procedures were performed by following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Use and Care Committee of Capital Medical University. The number of animals used was the minimal animals required for statistical analysis, and all procedures were taken to minimize animal suffering.

Unilateral 6-OHDA Lesions

Rats were anesthetized with chloral hydrate (350 mg/kg) and positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). 6-OHDA (Sigma, St. Louis, MO, USA) was dissolved to 6 µg/µL in 0.9% saline (ascorbic acid, 0.2%). A total volume of 3.0 µL was injected into the left striatum at three adjacent points using coordinates relative to the bregma and dura (antero-posterior: +1.0 mm, medio-lateral: +2.0 mm, and dorso-ventral: −4.5/−5.0/−5.5 mm). The same volume of saline was injected into the left striatum at the three points as sham controls. Injections were carefully controlled at a constant rate of 1 µL/min. After each injection, the needle was held for an additional 5 min to avoid back reflux along the injection track.

Behavior Test and Fucoidan Administration

To examine rotational behavior induced by apomorphine (APO) purchased from Sigma, rats were placed into a cylinder rotameter (Columbus Instruments, Columbus, OH, USA). After rats were adapted to the testing environment, they were subcutaneously (s.c.) injected with APO at 0.5 mg/kg. Measurements of rotational activity began 5 min after APO injection and lasted for 30 min. The rotameter recorded the number of full clockwise and counter-

clockwise turns the animals performed during the testing period. Clockwise turns (contralateral to 6-OHDA injection) were counted as positive value, and counterclockwise turns (ipsilateral to 6-OHDA injection) were counted as negative value. The rats with ≥60 contralateral rotations per 30 min were used as valid PD rats. These PD rats were randomly divided into three groups: (i) 6-OHDA rats treated with intraperitoneal (i.p.) injection of saline once daily for 3 weeks, (ii) 6-OHDA rats treated with fucoidan (10 mg/kg, i.p.) once daily for 3 weeks, and (iii) 6-OHDA rats treated with fucoidan (20 mg/kg, i.p.) once daily for 3 weeks. The rats in the sham group were treated with saline (i.p.) once daily for 3 weeks. Locomotor activity was assessed in Truscan-automated activity chambers (Columbus Instruments, Columbus, OH, USA) connected to a digiscan analyzer, which transmitted the number of beam breaks to a computer.

Immunohistochemistry

After anesthesia with chloral hydrate, rats were perfused with 0.9% saline followed by 4% cold paraformaldehyde. Brains were removed and postfixed overnight in sucrose/PBS at 4 °C. Coronal sections (30 µm) were cut by a Leica freezing microtome (Solms, Germany). Sections through the striatum (0.26–1.6 mm posterior to bregma) and substantia nigra (SN) (4.80–6.30 mm posterior to bregma) were collected. These sections were incubated overnight with a mouse antibody against tyrosine hydroxylase (TH) (1:2000; Sigma, MO, USA) or a rabbit antibody against Iba1 (1:300, WAKO, Osaka, Japan). TH-immunoreactive (ir) neurons in the SNc were examined as described previously [27]. Briefly, cells were counted using a 10 × objective lens. Cells were counted only when they exhibited at least one neurite or had a visible nucleus. The density of TH-ir fibers in the striatum was measured by quantifying optical segmentation of acquired 10 × images. The average optic density value in the SN from each Iba1-stained section was determined using an image analysis system. All sections were coded and examined blindly.

Double-Fluorescent Immunostaining

Coronal sections were incubated in 0.2% Triton X-100 (30 min) and blocked in 3% normal goat serum (30 min). The sections were then incubated overnight with mixed primary antibodies in 2% normal goat serum/PBS at 4 °C. The antibodies used include a mouse antibody against TH (1:2000), NeuN (1:500; Chemicon, Temecula, CA, USA), glial fibrillary acidic protein (GFAP, 1:500; Abcam, MA, USA), or CD11B (1:300; Abcam) and a rabbit antibody against Nox1 (1:200; Sigma). These sections were incubated in AlexaFluor-conjugated goat anti-rabbit (594, ZSGB-BIO, Beijing, China) and AlexaFluor-conjugated goat anti-mouse (488, ZSGB-BIO) antibodies for 1 h at room temperature, washed with PBS, and then mounted with DAPI in antifade solution for 5 min. Fluorescent images were acquired by a fluorescence microscope (Olympus, Tokyo, Japan).

Western Blot Analysis

Proteins were extracted using the RIPA buffer (Beyotime, Beijing, China) containing a protease inhibitor cocktail (Roche,

Mannheim, Germany). Protein concentrations were determined using a BCA kit (Thermo Fisher Scientific, Rockford, IL, USA). Protein samples were separated on 10% SDS–polyacrylamide gels and then transferred to PVDF membranes (Millipore, MA, USA) using a semidry blotting apparatus (Bio-Rad Laboratories, Hercules, CA). After blocking with 5% nonfat milk, membranes were probed overnight at 4°C with a mouse antibody against TH (1:5000, Sigma) or β -actin (1:5000, Sigma), or a rabbit antibody against Nox1 (1:500, Sigma). Membranes were washed with 0.5% Tween-20/PBS, incubated with an IRDye 800-labeled secondary antibody (1:10 000, Rockland Immunochemicals, Gilbertsville, PA, USA), and scanned with an Odyssey infrared imaging system (LI-COR instrument, Lincoln, NE, USA). TH and Nox1 signals were normalized to β -actin levels.

Oxyblot Analysis

Protein oxidation levels were detected using an Oxyblot™ protein oxidation detection kit (Chemicon, Temecula, CA) according to

the manufacturer's protocol. In brief, 5 μ L (20 μ g) of protein extract, 5 μ L of 12% SDS, and 10 μ L of dinitrophenylhydrazine derivatizing agent were mixed and incubated at room temperature for 15 min. The reaction was terminated by adding 7.5 μ L of neutralization buffer. Carbonyl-derivatized samples were loaded on 12% SDS–polyacrylamide gels and then transferred onto a PVDF membrane (Millipore). Transferred membranes were blocked in a solution containing 5% nonfat milk for 1 h at room temperature and probed with a primary antibody (1:150) provided by the kit at 4°C overnight followed by incubation of an IRDye 700-labelled secondary antibody (1:5000, Rockland) for 1 h at room temperature. Positive bands of 2,4-dinitrophenyl (DNP)-modified proteins were scanned and measured using software from LI-COR Biosciences (Lincoln, NE, USA).

Statistical Analysis

Data are presented as the mean $\pm$ SEM. Statistical analysis was performed using analysis of variance (ANOVA) followed by the

Figure 1 Fucoidan attenuates 6-OHDA-induced motor dysfunction in PD model rats. **(A)** At 5 weeks after the 6-OHDA lesion, rats were tested for their rotational responses to APO. The rats that exhibited ≥ 60 contralateral rotations during a 30-min measurement period were considered as the PD model rats for further study. Rotational activities measured at 5 weeks in PD model rats that were randomly divided into 3 groups: 6-OHDA alone, 6-OHDA + 10 mg/kg fucoidan (Fu), and 6-OHDA + 20 mg/kg fucoidan. Effects of fucoidan on APO-induced rotational behavior in PD model rats. Rotational tests were performed 2 and 3 weeks after fucoidan treatments, that is, 7 and 8 weeks after the 6-OHDA lesion. **(B)** Effects of fucoidan on spontaneous locomotor activity measured after 3-week treatments with fucoidan. Data are expressed as means $\pm$ SEM ($n = 8$ per group). **(A)** Two-way repeated-measures ANOVA followed by Tukey's multiple comparisons test. **(B)** One-way ANOVA followed by Newman–Keuls test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus 6-OHDA alone.

Tukey's multiple comparisons test or Newman–Keuls test. Differences at the $P < 0.05$ level were considered statistically significant.

Results

Fucoidan Attenuates Motor Dysfunction in PD Model Rats

We first carried out a series of behavioral experiments to test the effect of fucoidan on motor dysfunction in a rat PD model. To this end, we subjected rats to the unilateral 6-OHDA lesion. We then administered fucoidan (10 or 20 mg/kg, i.p., once daily) starting at 5 weeks after the 6-OHDA lesion for 3 weeks. Rotational behavior in response to a single dose of APO (0.5 mg/kg, s.c.) was measured before, during, and after fucoidan treatment. Before fucoidan treatment (baseline), no significant difference in rotational responses to APO was seen among three groups: (i) 6-OHDA, (ii) 6-OHDA + 10 mg/kg fucoidan, and (iii) 6-OHDA + 20 mg/kg fucoidan (Figure 1A). At 2 weeks after fucoidan treatment (7 weeks after the 6-OHDA lesion), a declining tendency of rotational behavior was shown in rats treated with 10 mg/kg fucoidan, although it did not reach a statistically significant level as compared to 6-OHDA alone ($P > 0.05$) (Figure 1A). At a dose of 20 mg/kg, fucoidan significantly reduced rotational activities ($P < 0.01$). Following a longer period (3 weeks) of treatment with fucoidan (8 weeks after the 6-OHDA lesion), fucoidan at both doses showed a significant effect (10 mg/kg, $P < 0.05$ and 20 mg/kg, $P < 0.001$). In addition to rotational behavior in response to APO, we monitored spontaneous locomotor activity following 3-week treatment with fucoidan. While 6-OHDA lesions decreased locomotor activity ($P < 0.001$), a significantly less decrease was seen in rats treated with fucoidan at the two

doses ($P < 0.05$) (Figure 1B). These behavioral data demonstrate that fucoidan possesses the ability to alleviate motor deficits in PD model rats.

Fucoidan Partially Attenuates the Loss of TH Immunoreactivity in PD Model Rats

We next performed histological experiments to evaluate the effect of fucoidan on changes in the dopaminergic system induced by 6-OHDA. Rats were sacrificed after 3-week fucoidan treatments (8 weeks after 6-OHDA administration) for histological staining of TH-ir fibers in the striatum and TH-ir neurons in the SNc. As expected, there was a significant loss of TH-ir fibers in the striatum ipsilateral to 6-OHDA injection relative to the side contralateral to 6-OHDA injection based on imaging analysis (Figure 2A) and quantification of optical density of TH-ir fibers (Figure 2C). Fucoidan at 10 mg/kg had a minimal effect on the loss of striatal TH-ir fibers ($P > 0.05$). Remarkably, fucoidan at 20 mg/kg markedly prevented the loss ($P < 0.01$). Similar results were observed in the SNc in terms of TH-ir neurons (Figure 2B and D). Fucoidan at a higher dose (20 mg/kg) increased the number of TH-ir neurons in the SNc as compared to 6-OHDA-treated rats ($P < 0.001$). These results indicate that fucoidan can prevent the damage of dopaminergic system in the striatum and SNc derived from 6-OHDA insults.

Fucoidan Inhibits Microglial Activation in the SN

Iba-1 immunostaining was performed to detect microglial activation in relation to neurodegenerative inflammation. As shown in Figure 3A, cells in the SN of a rat from the sham group represented the resting microglia, which usually has two or three fine processes. After 6-OHDA injection, microglial cells were activated.

Figure 2 Fucoidan partially attenuates the loss of striatal TH-ir fibers and SNc TH-ir neurons in PD model rats. (A and B) Representative photomicrographs illustrating the effect of fucoidan on the 6-OHDA-induced loss of striatal TH-ir fibers (A) and SNc TH-ir neurons (B). Scale bar = 1 mm (A) and 200 μ m (B). (C) Quantification of TH-ir fibers in the striatum. Optical density (OD) of TH-ir fibers was measured and then calculated as a ratio of the ipsilateral (left) to contralateral (right) striatum. (D) Quantification of TH-ir neurons in the SNc. The number of TH-ir neurons was counted and then calculated as a ratio of the ipsilateral (left) to contralateral (right) SNc. Note that fucoidan at a higher dose (20 mg/kg) substantially prevented the loss of striatal TH-ir fibers and SNc TH-ir neurons. Data are expressed as means $\pm$ SEM ($n = 4$ per group). One-way ANOVA followed by Newman–Keuls test. ** $P < 0.01$ and *** $P < 0.001$ versus 6-OHDA alone.

Figure 3 Fucoidan inhibits microglial activation in the SN of PD model rats. **(A)** Representative photomicrographs of Iba-1 immunostaining illustrating microglial activation in the SN following the 6-OHDA lesion with or without fucoidan treatment. Scale bar = 200 μ m (left) and scale bar = 100 μ m (right). **(B)** Quantitative analysis of the effect of fucoidan on Iba-1 expression in the SN. Optical density (OD) of immunoreactive Iba1 was measured and calculated as a ratio of the ipsilateral (left) to contralateral (right) SN. Data are expressed as means $\pm$ SEM ($n = 4$ per group). One-way ANOVA followed by Newman–Keuls test. * $P < 0.05$ versus sham group, # $P < 0.05$ versus 6-OHDA group.

Figure 4 Fucoidan attenuates protein carbonylation in the SN of PD model rats. **(A)** A representative Oxyblot illustrating the effect of fucoidan on protein carbonylation in the ipsilateral SN of PD model rats. Note that carbonylation signals were reduced in rats treated with fucoidan and 6-OHDA as compared to rats treated with 6-OHDA alone. **(B)** Quantitative analysis of protein carbonyl levels in the ipsilateral SN of four different groups. Data are shown as means $\pm$ SEM ($n = 3$ per group). One-way ANOVA followed by Newman–Keuls test. *** $P < 0.001$ versus sham group, * $P < 0.05$ versus 6-OHDA group.

These activated cells were readily identified throughout the SN by their thicker processes and more rounded cell bodies. Fucoidan (10 or 20 mg/kg, once daily for 3 weeks) significantly inhibited activation of microglia as more microglial cells exhibited the rami-

fied morphology similar to the sham group. Quantification of expression levels of Iba-1 by measuring optical density of Iba-1 staining confirmed the imaging impression. A consistent increase in nigral Iba-1 expression was seen after the 6-OHDA lesion

Figure 5 Fucoidan reduces the increase in Nox1 expression in the SN of PD model rats. **(A)** Immunofluorescent images visualizing expression of TH (green), Nox1 (red), and DAPI (blue) in the SN. TH-positive neurons showed Nox1 colabeling signals (magenta arrows), and adjacent non-TH-positive cells displayed intense Nox1 staining (white arrows). Scale bars = 200 μm. **(B)** Representative Western blots illustrating the effect of fucoidan on 6-OHDA-stimulated Nox1 expression in the SN. **(C)** and **(D)** Quantification of Western blot results illustrating the effect of fucoidan on 6-OHDA-induced changes in Nox1 **(C)** and TH **(D)** expressions in the SN. Data are expressed as means ± SEM ($n = 3$ per group). One-way ANOVA followed by Newman–Keuls test. * $P < 0.05$ versus the 6-OHDA group, *** $P < 0.001$ versus 6-OHDA group.

($P < 0.05$) (Figure 3B). This increase was significantly reduced by 20 mg/kg fucoidan ($P < 0.05$). These results reveal an inhibitory effect of fucoidan on the 6-OHDA-induced microglial activation in the SN.

Fucoidan Reduces Oxidative Stress in the SN

The 6-OHDA insult facilitates protein carbonylation, a type of protein oxidation that often leads to irreversible protein and thus cell damage. To determine the effect of fucoidan on the 6-OHDA-induced oxidative stress in the local SN, we assayed changes in the level of carbonylated proteins in ipsilateral SN lysates using an Oxy-blot kit. A representative blot is shown in Figure 4A, and results from quantification of all blots are shown in Figure 4B. A higher level of carbonylated proteins in the SN was seen in 6-OHDA-lesioned rats compared with sham rats, establishing that 6-OHDA promotes local protein carbonylation ($P < 0.001$). This enhanced protein carbonylation was reduced following fucoidan administration at either 10 or 20 mg/kg ($P < 0.05$). Thus, fucoidan is able to suppress the nigral oxidative stress in response to 6-OHDA.

Fucoidan Limits the Increase in Nox1 Protein Expression in the SN

The above observation that fucoidan inhibited protein carbonylation responses to 6-OHDA indicates the role of oxidative stress in mediating 6-OHDA toxicity. Nox is a primary enzyme for producing ROS, especially superoxide, which leads to oxidative stress in a variety of cell types, including glial cells and neurons [11]. Thus, in this study, we investigated responses of Nox1 to the 6-OHDA lesion in the presence or absence of fucoidan. From immunofluorescent labeling, 6-OHDA seemed to enhance SN Nox1 staining in 6-OHDA-treated rats compared with sham rats (Figure 5A). Noticeably, in 6-OHDA-lesioned rats, a few TH-positive neurons showed Nox1 colabeling signals (magenta arrows), while a greater number of adjacent non-TH-positive cells displayed intense Nox1 staining (white arrows). Fucoidan at both doses induced overall reduction in Nox1 immunoreactive signals in the SN. The results from Western blot assays verified the immunofluorescent impression. As shown in Figure 5B and C, 6-OHDA increased Nox1 protein levels in the ipsilateral SN ($P < 0.001$). However, this increase

Figure 6 Expression of Nox1 in NeuN-positive neurons in the SN. **(A)** Immunofluorescent photomicrographs illustrating expression of Nox1 (red), NeuN (green), and DAPI (blue) in the SN. A same set of multiple labeling is shown for each of four groups of rats. Scale bar = 200 μ m. **(B)** Immunofluorescent photomicrographs illustrating expression of Nox1 in astrocytes and microglia in the SN. Nox1 (red) was not colabeled with the astrocytic marker GFAP (green) or microglial marker CD11B (green). Scale bar = 30 μ m.

was not seen in rats treated with fucoidan (10 mg/kg and 20 mg/kg, $P < 0.05$). Corresponding with altered Nox1 expression, TH protein levels in the SN were reduced by 6-OHDA (Figure 5D). Fucoidan at 20 mg/kg partially prevented this reduction ($P < 0.001$). These data suggest that nigral Nox1 expression is sensitive to dopamine inputs. 6-OHDA upregulates its expression, while fucoidan blocks its responses to 6-OHDA.

Fucoidan Affects Nox1 Expression Mainly in Neuronal Cells

To identify the cell type that expresses Nox1, we performed multiple labeling with a mouse antibody against a neuronal or glial marker together with a rabbit antibody against Nox1. We found that SN cells immunoreactive to Nox1 coexpressed the neuronal marker NeuN (Figure 6A). In contrast, no Nox1-positive cells contained the astrocytic marker (GFAP) or microglial marker (CD11B) (Figure 6B). Thus, Nox1 is mainly expressed in neuronal rather than glial cells. In addition, Nox1 expression was not confined to the SNc (in circle). Abundant Nox1 staining was seen in the reticular part of SN. Of note, fluorescent signals of NeuN and

Nox1 in Figure 6A were altered in an opposite direction by 6-OHDA or 6-OHDA + fucoidan. While NeuN signals were reduced by 6-OHDA, Nox1 signals were elevated. These opposite changes were partially inhibited by fucoidan.

Discussion

PD is a severe movement disorder due to progressive degeneration of the nigrostriatal dopaminergic pathway [28]. Despite decades of studies, underlying mechanisms for DA degeneration remain poorly understood [29,30]. Recent attention has suggested the important role of oxidative stress in the DA degeneration, although the clinical trial of antioxidants has failed in PD therapy [31–33]. The fucoidan has been reported to show the therapeutic potential for the brain disease in animal models, including Alzheimer's disease [34], ischemia–reperfusion injury [35], and depression disorder [36], although how it passes blood–brain barrier and acts on brain remains unknown. We previously found that fucoidan had therapeutic potential for mouse MPTP PD and rat lipopolysaccharide PD [24,25]. Its therapeutic effect might result from its antioxidative effect action [24]. In this

study, we expanded the study of fucoidan to a rat 6-OHDA model which has been used extensively as a preclinical model of PD to assess the antiparkinsonian effects and neuroprotection of new pharmacological therapies [37–39]. We found that the 20 mg/kg fucoidan significantly mitigated the motor dysfunction responses to 6-OHDA and attenuated the damage of dopaminergic system in the striatum and SNc. While fucoidan at 10 mg/kg had no dramatic effects on microglial activation, the loss of striatal TH-ir fibers and TH-ir neurons in SNc exhibited a statistically significant effect on behavioral dysfunction, protein carbonylation, and Nox1 expression. It suggests that fucoidan at 10 mg/kg may be a marginal dose for this PD model rat. Furthermore, a Nox1-directed oxidative stress in the SN may be a target of fucoidan. Those beneficial effects are at least partially due to fucoidan against this stress.

It has been well documented that the 6-OHDA lesion induces rapid activation of microglia and subsequent release of neurotoxic proinflammatory factors such as ROS in the SNc [40,41]. This excessive microglial activation is believed to play a role in the development and progression of PD [42,43]. Consistent with this, we observed robust activation of microglia in the SN following the 6-OHDA lesion. More interestingly, fucoidan suppressed the 6-OHDA-induced microglia activation. Thus, fucoidan is reasoned to exert its protective effect through a mechanism involving the inhibition of microglial responses to the neurotoxicant.

Increasing evidence supports a contributing role of the ROS-generating enzyme Nox to PD. Nox is expressed in neutrophils as well as other cell types including neurons [11]. Among those, Nox1 expression levels were recently found to be upregulated in the SNc of PD animals in response to paraquat, 6-OHDA, or MPTP as well as in the postmortem brain tissue of patients with PD [16–18]. The upregulated Nox1 expression is believed to promote local oxidative stress and facilitate cellular degeneration. As such, apocynin, a putative Nox inhibitor, or adeno-associated virus-mediated Nox1 knockdown reduced DNA oxidative damage and DA neuronal degeneration in paraquat-insulted animals [17]. Of note, the increase in Nox1 expression in the rat SNc DA neurons occurred 3 days after 6-OHDA injection [18], while the death of DA neurons has not been seen yet at this time point. Thus, the upregulation of Nox1 expression is considered to be a preceding event that leads to subsequent ROS production and oxidative stress. As oxidative stress is one of the important mediators for

activation of microglia [19,20,44], Nox1 may act as a major regular for persistent activation of microglia in PD model rats. Our studies here provided evidence supporting the role of Nox1: (i) 6-OHDA lesions induced an increase in Nox1 expression in the SN and (ii) 6-OHDA also induced a parallel increase in protein oxidation (i.e., an increased protein carbonylation) in the SN. Remarkably, all major Nox1-related steps (upregulated Nox1 expression, increased protein oxidation, and microglial activation) were sensitive to fucoidan. Chronic fucoidan administration significantly inhibited the changes in these steps induced by 6-OHDA. Based on these results, we proposed a signaling mechanism that may primarily underlie the beneficial effect of fucoidan. That is, fucoidan may exert its effect via suppressing the response of the Nox1-oxidation pathway to the 6-OHDA lesion.

We for the first time discovered that Nox1 is highly expressed in non-TH-positive neurons in the SNc, in addition to some survived TH-containing neurons, but not in glial cells. Based on this finding and others [16,18], we consider that Nox1 expressed in TH neurons is directly involved in the death of DA neurons usually in the early onset of PD model rats induced by 6-OHDA, while Nox1 enriched in non-TH-positive neurons may contribute to sustaining the activation of microglia and exacerbating the degenerative process of DA neurons. While this assumption is attractive, further studies are needed in the future to prove and characterize this model. Nevertheless, our data pave a new avenue to explore the molecular mechanism for the fucoidan effect and to develop much needed therapeutic agents for PD by targeting the Nox1 system.

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Conflict of Interest

The authors declare no conflict of interest.

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