<Original Article>

**Fucoidan attenuates 6-hydroxydopamine-induced neurotoxicity by exerting anti-oxidative and anti-apoptotic actions in SH-SY5Y cells**

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**Abstract:** Parkinson’s disease (PD) is an irreversible neurological disorder with related locomotor dysfunction and is characterized by the selective loss of nigral neurons. PD can be experimentally induced by 6-hydroxydopamine (6-OHDA). It has been reported that reactive oxygen species, which deplete endogenous glutathione (GSH) levels, may play important roles in the dopaminergic cell death characteristic of PD. Fucoidan, a sulfated algal polysaccharide, exhibits anti-inflammatory and anti-oxidant actions. In this study, we investigated whether fucoidan can protect against 6-OHDA-mediated cytotoxicity in SH-SY5Y cells. Cytotoxicity was evaluated by using MTT and LDH assays. Fucoidan alleviated cell damage evoked by 6-OHDA dose-dependently. Fucoidan reduced the number of apoptotic nuclei and the extent of annexin-V-associated apoptosis, as revealed by DAPI staining and flow cytometry. Elevation of lipid peroxidation and caspase-3/7 activities induced by 6-OHDA was attenuated by fucoidan, which also protected against cytotoxicity evoked by buthionine-sulfoximine-mediated GSH depletion. Reduction in the glutathione/glutathione disulfide ratio induced by 6-OHDA was reversed by fucoidan, which also inhibited 6-OHDA-induced disruption of mitochondrial membrane potential. The results indicate that fucoidan may have protective action against 6-OHDA-mediated neurotoxicity by modulating oxidative injury and apoptosis through GSH depletion.

**Keywords:** apoptosis, caspase-3/7, fucoidan, glutathione/glutathione disulfide ratio, 6-hydroxydopamine

**Introduction**

PD is known as one of chronic neurological disorders associated with oxidative damage to dopamine-producing neurons in human midbrain [17]. The neuronal death of this area is connected with various motor dysfunction, such as stiffness or slowness [19]. Although the precise etiology of neuronal injury remains unknown, accumulating evidence suggests that impairment in the ability of mitochondria to regulate the levels of lipid peroxides made by oxidative stress and inflammatory processes may be critical [6, 14]. Glutathione (GSH) is an endogenous antioxidant that protects against various oxidative stressors by regulating reactive oxygen species (ROS) levels in PD patients [17]. GSH concentration is decreased in the nigral regions of PD patients [12, 20]. The antioxidant and neuroprotective characteristics of GSH have been amply demonstrated in various models of oxidative injury, including models in which GSH is depleted by the addition of L-buthionine-(S,R)-sulfoximine (BSO) [10, 20].

Fucoidan is a fucose-rich, sulfated polysaccharide derived from brown seaweeds and has been used as a component of foods, cosmetics, and dietary supplements for many centuries in Asian countries. The pharmacological actions of fucoidan include anti-inflammatory, anti-oxidant, and anti-thrombotic effects [9, 21]. However, fucoidan has not yet been shown to be neuroprotective.

6-hydroxydopamine (6-OHDA) acts as a dopamine depleting agent which can occur toxicity to dopaminergic neurons. Thus, it can be applicable to make an experimental form of PD [1, 16]. Recently, fucoidan has been shown to inhibit the toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by exerting anti-oxidant activities [11]. However, no report has yet proposed the neuroprotective effect of fucoidan on 6-OHDA-mediated toxicity in dopaminergic SH-SY5Y neuroblastoma cells. Previously, we found that fucoidan protected against β-amyloid-mediated neuronal damage in rat neuronal cultures; fucoidan exerted potent anti-oxidant effects and inhibitory action of apoptosis [9]. In this study, we explored whether
fucoidan protected against various oxidative injuries (including GSH deprivation) attributable to BSO, and mitochondrial membrane dysfunction evoked by 6-OHDA.

**Materials and Methods**

**Chemicals**

Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (USA). Penicillin-streptomycin mixture antibiotic was obtained from Lonza (USA). Fucoidan (from *Fucus vesiculosus*), 6-OHDA, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), NADH, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (USA).

**Cell culture**

The human dopaminergic SH-SY5Y cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 µg/mL of streptomycin, and 100 U/mL of penicillin. Cells were maintained at 37°C under an atmosphere of 95% air and 5% CO₂. All experiments used cell monolayers at 70% confluence.

**MTT assay**

Cells were plated into 96-well culture plates (20,000 cells/well) at 37°C and grown in an incubator under 5% CO₂. After 24 h, cells were treated with 60 µM 6-OHDA in a volume of 200 µL; incubation continued for 15 to 17 h. Fucoidan was pretreated 2 h prior to the exposure of 6-OHDA. The cells were incubated with MTT solution (final concentration, 5 mg/mL) for 3 h. Then, the media were removed, 200 µL of DMSO was added, and incubation continued for extra 30 min. The absorbance was measured at 570 nm (Molecular Devices, USA).

**Lactate dehydrogenase (LDH) measurement**

Cells were plated (2 × 10⁴ cells/well) into 24-well plates, and treated with 60 µM 6-OHDA either alone or in combination with fucoidan for 24 h. The cytotoxicity was analyzed by assessing the level of LDH released from destructed membrane into the surrounding media 20-24 h after exposure to 6-OHDA or fucoidan. LDH activities were measured using an absorbance at 405 nm using a GSH assay kit (Enzo Life Sciences) and expressed as GSH/GSSG (picomolar) ratios.

**Thiobarbituric acid-reactive substances (TBARS) assay**

Cellular levels of lipid peroxidation were analyzed using OXlek TBARS Assay Kits (Enzo Life Sciences, USA). Briefly, cultured cells were lysed with 2% (w/v) sodium dodecyl sulfate. Harvested cells were sonicated in PBS for 5 sec at 40 V in an ice bath, and mixed with 100 µL of sodium dodecyl sulfate solution and 2.5 mL of TBS/Buffer reagent. The sample mixtures were incubated at 95°C for 60 min, and cooled in an ice bath. The supernatants were obtained by centrifugation (1,500 × g) for 30 min. The levels of TBARS were measured at an absorbance of 532 nm. The results were calculated by standard curve which made according to difference of malondialdehyde (MDA) concentration.

**Glutathione (GSH) assay**

GSH was analyzed according to the method of Chang et al. [3]. Cultured cells were detached using trypsin and harvested with a cell scraper into 15-mL conical tubes, centrifuged briefly. Cell washing with cold PBS was repeated twice. The cells were suspended in 5% (w/v) metaphosphoric acid and sonicated in an ice bath. The cells were next centrifuged at 12,000 × g for 5 min at 4°C. Total GSH and glutathione disulfide (GSSG) levels were estimated by absorbance at 405 nm using a GSH assay kit (Enzo Life Sciences) and expressed as GSH/GSSG (picomolar) ratios.

**DAPI staining**

Cells were seeded on coverglasses into bottom-culture dishes. After incubation for 24 h, the cells were treated with 6-OHDA or fucoidan, fixed in 70% (v/v) ethanol after washing with PBS. Cells were stained with DAPI (1 µg/mL). Morphological changes in DNA were observed under an inverted fluorescence microscope (Carl Zeiss, Germany).

**Caspase-3/7 assay**

To assess caspase-3/7 activities, cells were plated at a density of 0.5 × 10⁴ cells/well. After treatment of cells with 60 µM 6-OHDA, either alone or with fucoidan for 12 h, caspase-3/7 activity was evaluated by commercial kit (Millipore, USA). The caspase-3/7 reagent was mixed with cell suspension. After incubation, 7-amino-actinomycin D (7-AAD) reagent was added, and data were collected by Muse Cell Analyzer (Millipore).

**Apoptosis assay**

Flow cytometric assay on apoptosis quantitatively measured using Annexin-V and 7-AAD kits (Millipore). Annexin V binds phosphatidylserine which translocated to the outer cell membrane during apoptotic process. 7-AAD is known as a fluorescent, DNA-intercalating agent. All assays were carried out conformity with the assay protocol. Briefly, after exposure to 6-OHDA alone or 6-OHDA with fucoidan for 24 h, the cells were collected and resuspended in assay buffer. Stained cells were evaluated by the Muse Cell Analyzer.

**Assay of mitochondrial membrane potential (MMP)**

The diminution of cellular MMP is known as a disruption of energy production and sign of early apoptosis. After treatment with 60 µM 6-OHDA, either alone or with fucoidan for 12 h, MMP was analyzed using the Muse MitoPotential Kit (Millipore). Briefly, harvested cell suspension was mixed with the Muse MitoPotential reagent followed by incubation at 37°C for 20 min. Finally, the 7-AAD reagent was added, and data were collected using the Muse Cell Analyzer.
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Statistics
Results were assessed by one-way ANOVA with Dunnett’s test. A value of $p < 0.05$ or $p < 0.01$ was considered statistically significant. All data were expressed as means ± SEM.

Results
Fucoidan protects SH-SY5Y cells against 6-OHDA-mediated neurotoxicity
Treatment of cells with 6-OHDA (60 µM) triggered 50 to 60% neurotoxicity, as revealed by the MTT assay. Thus, we used 60 µM 6-OHDA as the control level in all experiments. Fucoidan exerted significant protective effects at concentrations of 4, 20, 40, and 60 µg/mL (Fig. 1).

Phase contrast photomicrography and DAPI staining
The protective effects of fucoidan were reflected morphologically, as shown in Figure 2A–C. In the control (vehicle-treated) group, most neurites and cell bodies were intact (Fig. 2A). However, the majority of cell bodies and neurites were damaged after exposure to 6-OHDA for 24 h (Fig. 2B). Fucoidan mitigated 6-OHDA-induced neurotoxicity (Fig. 2C). DAPI staining showed that 6-OHDA induced nuclear breakdown and fragmentation (Fig. 2E), to a greater extent than occurred in control samples (Fig. 2D). Fucoidan prevented such cellular injuries (Fig. 2F).

Effects of fucoidan on TBARS levels, the glutathione redox system, and neurotoxicity mediated by glutathione deprivation
We used the TBARS assay to explore the effects of fucoidan on lipid peroxide formation. Likewise in Figure 3A, treatment of cells with 6-OHDA increased the TBARS levels by about two-fold (compared to control values). Fucoidan pretreatment significantly inhibited lipid peroxidation. We evaluated the action of fucoidan on GSH redox reaction, and markers of oxidative stress, by determining GSH/GSSG ratios. Treatment of cells with 6-OHDA markedly diminished in GSH/GSSG ratio (Fig. 3B). Under such circumstances, fucoidan significantly inhibited the decline of the GSH/GSSG ratio induced by 6-OHDA, such that the values remained normal. Prolonged exposure (48 h) of cells to a high concentration (3 mM) of L-buthionine-(S,R)-sulfoximine (BSO; an inhibitor

Fig. 1. Protective effects of fucoidan on 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in SH-SY5Y cells. Cultures were pretreated with 2–60 µg/mL fucoidan for 2 h prior to exposure to 60 µM 6-OHDA for 24 h. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Optical density was measured at 570 nm. Values are means ± SEM of three independent experiments, each performed in triplicate. *$p < 0.05$, **$p < 0.01$ compared to control, and $p < 0.05$ compared to vehicle.

Fig. 2. Morphological evidence of protection afforded by fucoidan to cells exposed to 6-OHDA-induced neurotoxicity. (A-C) Phase-contrast photomicrographs of cultured SH-SY5Y cells exposed to vehicle (A), 60 µM 6-OHDA (the control agent) (B), and 40 µg/mL fucoidan and 6-OHDA (C). (D-F). Each group contains examples of cells exposed to vehicle (D), 60 µM 6-OHDA (E), and 60 µM 6-OHDA+40 µg/mL fucoidan (F). Red arrows indicate apoptotic cells (E). DAPI stain (D-F). 200× (A-C), 400× (D-F).
Fig. 3. Fucoidan inhibits 6-OHDA-induced reactive oxygen species (ROS) production in, and L-buthionine-(S,R)-sulfoximine (BSO)-induced neuronal injury to, SH-SY5Y cells. Fucoidan significantly inhibited the extent of lipid peroxidation, as measured by the thiobarbituric acid-reactive substances (TBARS) assay (A), and accelerated recovery as assessed by evaluation of the glutathione/glutathione disulfide (GSH/GSSG) ratio (B). Fucoidan significantly attenuated neuronal injury induced by glutathione deprivation of SH-SY5Y cells (C). All values are means ± SEM of three independent experiments, each performed in triplicate. *p < 0.05, **p < 0.01 compared to control, and #p < 0.05 compared to vehicle.

Fig. 4. Changes in cumulative apoptotic cell ratio (live, early apoptotic, apoptotic/dead, and dead) as determined by assay of apoptotic status based on caspase-3/7 activation of SH-SY5Y cells exposed to various concentrations of fucoidan and 6-OHDA, measured by flow cytometry (A). Representative flow cytometric data are shown in (B). Apoptotic cell ratio by caspase-3/7 activation was observed 24 h after exposure to 6-OHDA. All values are means ± SEM of three independent experiments, each performed in triplicate. *p < 0.05, **p < 0.01 compared to control, and #p < 0.05 compared to vehicle.
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Effects of fucoidan on caspase-3/7 activities

We evaluated the anti-apoptotic activities of fucoidan by measuring caspase-3/7 activities using flow cytometry. Treatment of cells with 6-OHDA elevated apoptotic status (apoptotic/dead and dead cells) based on caspase-3/7 activation. Among cumulative cell counts, the percentage of apoptotic/dead and dead cells increased two- and five-folds compared to vehicle group, respectively. Fucoidan pretreatment significantly inhibited such elevations in both apoptotic/dead and dead cells induced by 6-OHDA (Fig. 4A). Representative flow cytometric profiles on apoptotic cell counts are shown in Figure 4B.

Effects of fucoidan on apoptosis

Treatment of cells with 6-OHDA increased the percentage of early and late apoptosis by 13% and 12%, respectively. Fucoidan pretreatment (40 µg/mL) significantly decreased by 5 and 2% (Fig. 5A). Representative apoptotic flow cytometric profiles on apoptotic cell counts are shown in Figure 5B.

Effects of fucoidan on MMP

The 6-OHDA reduced the proportion of SH-SY5Y cells exhibiting a decrease in mitochondrial depolarization. The average percentage of depolarized dead cells in 6-OHDA-treated group increased from 9.6% to 38%. Pretreatment with fucoidan (40 µg/mL) significantly inhibited by 16.7% compared to 6-OHDA (control) group (Fig. 6A). Representative flow cytometric profiles of cumulative cell counts (Depolarized/live and depolarized/dead cells except live and dead cells were shown) are shown in Figure 6B.

Discussion

Although many researchers have sought to define the pathogenesis of PD, the mechanism underlying neurodegeneration in the substantia nigra remains unclear. However, oxidative stress, including elevation of iron concentrations and reduced GSH levels, may be key in this context [15]. In accordance with these ideas, the elevation of TBARS and reduction of GSH/GSSG ratio evoked by 6-OHDA were well observed in same cell line [3]. Especially, reduction of glutathione level in nigral regions of brain is considered as a critical factor in PD pathogenesis and secondary to complex I inhibition [12]. In in vivo studies, fucoidan treatment has
showed a distinct amelioration of neurodegenerative symptoms in experimental PD models induced by MPTP and 6-OHDA, respectively [11, 22]. Hence, our results suggest that fucoidan may be one of the potential neuroprotective candidates derived from various natural antioxidants. Recently, fucoidan has been reported to suppress the microglial activation via increase in ROS-generating enzyme, NADPH oxidase-1 (Nox 1) expression in dopaminergic neurons of rat substantia nigra region after 6-OHDA injection [22]. These results mean that fucoidan could be contributable to neuronal survival through modulatory action of nitric oxide, ROS and a key proinflammatory cytokine, such as tumor necrosis factor-α [4, 5]. Here, Cui et al. [5] have demonstrated that fucoidan remarkably inhibited an elevation of inducible nitric oxide synthase protein and microglial activation induced by lipopolysaccharide at similar concentrations like us. Mitochondrial dysfunction caused by inhibition of complex I activity may correlate with the extent of injury to dopaminergic neurons in PD patients. Unfortunately, there have been few pharmacological reports on the ability of fucoidan to prevent PD. Previously, we screened some natural medicines using an in vitro PD model (human dopaminergic SH-SY5Y neuroblastoma cells) [3].

In the present study, we found that fucoidan inhibited both lipid peroxidation, and the disturbance of GSH metabolism, induced by 6-OHDA in SH-SY5Y cells. These results suggest that fucoidan may serve as a useful, natural GSH-sparing agent that partially alleviates PD symptoms. Fucoidan inhibits the neuronal damage triggered by deprivation of endogenous GSH. Although source of fucoidan is somewhat different, the neuroprotective basis seems to be mainly attributable to the anti-oxidant and anti-inflammatory action [4, 11, 13, 21, 22]. Previously, we showed that fucoidan protected neuronal cells by inhibiting oxidative effects, as well as the stimulation of protein kinase C and caspase 3/9, which is required for β-amyloid-induced neurotoxicity [9]. Similarly, we now show that fucoidan inhibits the elevation of caspase 3/7 activity evoked by 6-OHDA and consequent apoptotic death. An earlier report showed that fucoidan inhibited the elevation of caspase-3 activity in a model of H2O2-induced neurotoxicity [7], and protected against MPTP-induced lipid peroxidation of, and 1-methyl-4-phenylpyridinium (MPP+)-induced neuronal injury to, MN9D cells [11]. Thus, fucoidan acts a neuroprotectant in experimental models of PD, such as MPTP or 6-OHDA. However, fucoidan has not yet been shown the capability to permeate the blood-brain barrier. The MMP decreases when the mitochondrial membrane is damaged or collapses after exposure to a 6-OHDA-induced auto-oxidation cascade [2, 8]. Fucoidan increased the number of cells in which depolarized mitochondria was apparent after 6-OHDA treatment. Recently, fucoidan has been shown to alleviate the mitochondrial dysfunction that develops after traumatic injury [18]. However, the exact pharmacological properties of fucoidan are difficult to conclude easily because sulfated polysaccharide is complex and possess broad spectrum therapeutic properties. Overall, our results suggest that fucoidan pretreatment improves neuronal survival by exerting anti-oxidative and anti-apoptotic effects when 6-OHDA induces neuronal damage to SH-SY5Y cells.

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