Fucoidan protects against dopaminergic neuron death in vivo and in vitro

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ARTICLE INFO
Article history:
Received 30 November 2008
Received in revised form 22 May 2009
Accepted 3 June 2009
Available online 21 June 2009

Keywords:
Fucoidan
Neuroprotection
Antioxidant activity
Oxidative stress
1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
Parkinson's disease

ABSTRACT
Parkinson's disease is a neurodegenerative disorder of uncertain pathogenesis characterized by a loss of dopaminergic neurons in substantia nigra pars compacta, and can be modeled by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Oxidative stress may contribute to MPTP- and Parkinson's disease-related neurodegeneration. Fucoidan is a sulfated polysaccharide extracted from brown seaweeds which possesses a wide variety of biological activities including potent antioxidant effects. Here we investigated the effect of fucoidan treatment on locomotor activities of animals, striatal dopamine and its metabolites and survival of nigral dopaminergic neurons in MPTP-induced animal model of Parkinsonism in C57/BL mice in vivo and on the neuronal damage induced by 1-methyl-4-phenylpyridinium (MPP+•) in vitro, and to study the possible mechanisms. When administered prior to MPTP, fucoidan reduced behavioral deficits, increased striatal dopamine and its metabolites levels, reduced cell death, and led to a marked increase in tyrosine hydroxylase expression relative to mice treated with MPTP alone. Furthermore, we found that fucoidan inhibited MPTP-induced lipid peroxidation and reduction of antioxidant enzyme activity. In addition, pre-treatment with fucoidan significantly protected against MPP+•-induced damage in MN9D cells. Taken together, these findings suggest that fucoidan has protective effect in MPTP-induced neurotoxicity in this model of Parkinson's disease via its antioxidative activity.© 2009 Elsevier B.V. All rights reserved.

1. Introduction
Parkinson's disease is a progressive neurological disorder characterized by the loss of dopamine in the striatum, which occurs mainly due to the death of the dopaminergic neurons in the substantia nigra compacta pars. A meperidine analog, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), causes selective nigral dopaminergic lesions, resulting in Parkinsonian syndromes in humans, primates and mice (Bloem et al., 1990). Numerous studies have subsequently demonstrated that the toxicity of MPTP depends on its conversion to its active metabolite, 1-methyl-4-phenylpyridinium (MPP+) (Tipton and Singer, 1993). MPP+• rapidly accumulates in dopaminergic neurons via the plasma membrane dopamine transporter. Once inside cells, MPP+• accumulates in mitochondria where it inhibits complex I of the mitochondrial electron transport chain. This leads to impairment of energy production and increased free radical generation, and eventually causes dopaminergic neuron death (Alcaraz-Zubeldia et al., 2001; Thomas et al., 2000). It was reported that MPTP might preferentially target dopaminergic neurons rather than other neurons in the same region. Such toxicity on the dopaminergic nigrostriatal system seems reasonable since the substantia nigra is rich in dopamine, which can undergo both enzymatic and non-enzymatic oxidation to produce free radicals (Fahn and Cohen, 1992). However, the antioxidant system in the nigrostriatal tract is severely attenuated in drug-induced Parkinsonism or in Parkinson's disease. For example, MPTP depletes striatal glutathione (GSH) in mice, and the said effects may make dopaminergic neurons more susceptible to oxidative stress (Aoyama et al., 2008). Agents with antioxidant capabilities could theoretically prevent, at least in part, the progression of Parkinson's disease. There are several lines of evidence from animal models which imply that a variety of antioxidative strategies, such as overexpression of Cu, Zn superoxide dismutase, Mn superoxide dismutase, or treatment with copper sulfate provide resistance to MPTP (Alcaraz-Zubeldia et al., 2001; Klivenyi et al., 1998; Przedborski et al., 1992).

In our recent efforts to explore new drugs for Parkinson's disease treatments, fucoidan from Laminaria japonica has drawn our attention. Fucoidan (sulfated fucans) is the collective name for algal sulfated polysaccharides extracted from the edible brown seaweeds and their structure consists chiefly of polymers formed by branched polysaccharide sulfate esters with L-fucose building blocks (Davis et al., 2003). Fucoidan exhibits various biological activities, acting as an anti-inflammatory in the brain (Del Bigio et al., 1999), as an antioxidant (Ruperez et al., 2002) and as a neuroprotectant...
2.2. Animals and treatments

Its average molecular weight was determined to be 7000 by high analysis of fucoidan showed that fucose was the main component. In our research has shown significant antioxidant activity in vitro and a protective effect against chronic renal failure of rats (Zhang et al., 2003). So, it is interesting to know whether fucoidan has a neuroprotective effect against dopaminergic neurodegeneration.

2.3. Behavioral analysis and evaluation of locomotor activity

Locomotor activity was assessed in automated activity chambers connected to a digiscan analyzer that transmitted the number of beam crossings per movement (cm) across the 60-min recording period. Recorded as total distance traveled (cm), mean velocity (cm/s) and mean movement rate was set at 1.2 ml/min. Striata from five to nine animals in each treatment group were used.

2.4. Quantiﬁcation of dopamine and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)

For these studies, striatum processed and stored at −80 °C was used. The contents of dopamine and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were determined using an HPLC apparatus with an electrochemical detector (Model 5600A CoulArray Detector System ESA, Brighton, MA, U.S.A.). Briefly, tissues were homogenized in 0.2 M ice-cold perchloric acid. The homogenate was placed in an ice bath for 60 min. Subsequently, the sample was centrifuged at 15,000 g for 20 min at 4 °C and the supernatant was transferred to a clean tube and measured for volume.

2.5. Immunohistochemistry and quantification of dopaminergic neuronal survival profiles

Tissues for histological analysis were ﬁxed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) by immersion for 48 h at 4 °C, and then cryoprotected in 30% sucrose overnight at 4 °C. Brains were sectioned at 30 μm thickness on a sliding microtome for free-floating tissue sections. Every sixth section from a given area was stained with polyclonal antibodies against neuronal TH (1:2000 Sigma-Aldrich, St. Louis, MO, U.S.A.). Sections were incubated with primary antibodies for 24 h at 4 °C. Inclusion of diluted nonimmune goat serum instead of the primary antibodies served as negative controls. Sections were incubated in biotinylated goat anti-mouse antibody and then in the avidin–biotin–peroxidase complex for 30 min at 37 °C. The bound complex was visualized by incubating sections in a solution containing 0.1% 3,3′-diaminobenzidine (Sigma, St. Louis, MO, U.S.A.), 1% H2O2, and 8% ammonium nickel sulfate (Fluka Chemie GmbH, Switzerland). Sections were then mounted on gelatinized slides, left to dry overnight, dehydrated in ascending alcohol concentrations, and mounted on Permount (Fisher Scientific, Fair Lawn, NJ). TH positive neurons with distinct nuclei were counted in six sections throughout the entire rostrocaudal extent of the substantia nigra. All sections were coded and examined blind.

2.6. Western blot analysis

Cellular proteins were extracted from the striatal samples with an extract buffer containing 0.5% Triton X-100 and protease-inhibitor cocktail (1:1000, Sigma-Aldrich, St. Louis, MO). The tissues were homogenized in this buffer with the Fisher model 100 sonic dismembranator and put on ice for 1 h. The soluble extracts were separated by centrifugation at 11,500 rpm for 5 min at 4 °C. Equal amounts of protein samples (20 μg) were mixed with the loading buffer (0.06 M Tris-HCl, 2% SDS, and 2% β-mercaptoethanol, pH 7.2), boiled for 5 min, resolved by SDS-polyacrylamide gels, and transferred to a nitrocellulose filter (Millipore, Bedford, MA) using a semidry blotting apparatus (Bio-Rad Laboratories, Hercules, CA). After blocking with a solution containing 5% nonfat milk, the filters were incubated with TH (1:1000 Boehringer-Mannheim, Indianapolis, IN) or GAPDH (Sigma, St. Louis, MO) antibodies for detection of the level of dopaminergic neuronal terminals, and for normalization of the loading protein. Then the filters were incubated with IRDye 800-labeled secondary antibody (1:10,000, Rockland Immunocchemicals, U.S.A.). The signal was visualized by Odyssey infrared imaging system according to the instructions of the manufacturer (LI-COR instrument, U.S.A.). The fluorometric intensity measurement of each band was performed with corresponding image software.

2.7. Assay of MPP+ levels

In order to assess MPP+ level in striatum, additional groups of five mice which had received fucoidan (25 mg/kg) were sacrificed 2 h...
after the last MPTP injection. Striatal tissue was prepared as described above and analysed for MPP⁺ level using HPLC and UV detection at 295 nm. In brief, the mobile phase (0.14 M sodium dihydrogen phosphate, acetonitrile (30%) adjusted to pH 2.5 with H₃PO₄) was delivered at a rate of 0.5 ml/min onto the reversed-phase column (125 mm × 3 mm with precolumn 5 mm × 3 mm, both filled with

Fig. 1. Effects of fucoidan on the locomotor activity in MPTP mice. MPTP-HCl (15 mg/kg, 4 times, at 2 h interval, i.p.) was injected on the 11th day of 18-day duration of fucoidan treatment (12.5 mg/kg or 25 mg/kg). Locomotor activity in 60 min was detected and analysed by Truscan 2.0 system on the 7th day after MPTP injections. Data of total movement distance (A), mean velocity (B) and mean distance per movement (C) were shown as mean ± S.E.M., n = 5–8. *P < 0.05, **P < 0.01, ***P < 0.001 vs. MPTP group. (D) shows the effects of fucoidan on the 30 min-track-plot-pictures of MPTP mice. a, control (without MPTP and fucoidan); b, MPTP; c, fucoidan (12.5 mg/kg) + MPTP; d, fucoidan 25 mg/kg + MPTP.

Fig. 2. Effects of fucoidan on the contents of dopamine, DOPAC, HVA and (DOPAC + HVA)/dopamine in the striatum of MPTP mice. MPTP-HCl (15 mg/kg, 4 times, at 2 h interval, i.p.) was injected on the 11th day of 18-day duration of fucoidan treatment (12.5 mg/kg or 25 mg/kg). The contents of dopamine, DOPAC and HVA were detected by HPLC-ECD on the 7th day after MPTP injections and the ratio of dopamine to (DOPAC + HVA) was calculated. Data of dopamine (A), DOPAC (B), HVA (C) and (DOPAC + HVA)/dopamine (D) were shown as mean ± S.E.M. n = 4–6. *P < 0.05, **P < 0.01, ***P < 0.001 vs. MPTP group.
Nucleosil 120-C18, Macherey-Nagel). Aliquots (20 μl) were analysed using an autosampler with a cooling module set at 4 °C. Data were calculated by an external standard calibration.

2.8. MDA, GSH, SOD, GSH-Px, catalase and total antioxidant capability assay

The substantia nigra was removed, blotted and weighed, and then homogenized in a 0.1 M phosphate buffer solution (pH 7.4). The samples were centrifuged at 3000 × g and 4 °C for 30 min, and the supernatants were then subjected to the measurement of malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase and total antioxidant capability by spectrophotometric methods according to the procedures provided by the assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, PR China). MDA level was analyzed by 2-thiobarbituric acid (Placer et al., 1966), GSH content was determined by spectrophotometry using 5, 5′-dithiobis (2-nitrobenzoic acid) (Davidson and Hird, 1964); SOD activity was analyzed by monitoring the inhibition of the reduction of nitro blue tetrazolium by the sample at 560 nm (Winterbourn et al., 1975). GSH-Px activity was detected with 5-5′-dithiobis-p-nitrobenzoic acid (Hafemen, 1974); Catalase activity was taken as the rate of decrease in absorbance of H2O2 at 240 nm/min/mg protein in the presence of catalase (Aebi, 1984); Total antioxidant capability was measured by the method of ferric reducing/antioxidant power assay (Benzie and Stain, 1996).

2.9. In vitro observation of neuroprotective effects of fucoidan

We used a dopaminergic cell line, MN9D, to establish an in vitro model for MPP+ toxicity study. MN9D cells were kindly provided by Dr. Bastian Hengerer (Novartis Institute for BioMedical Research, Basel, Switzerland) and maintained in Dulbecco’s modified Eagle medium/F12 supplemented with 10% newborn calf serum (PAA Company, Pasching, AUT) in an incubator with an atmosphere of 5% CO2 at 37 °C. We aimed to determine whether fucoidan can protect dopaminergic cells. MN9D cells were plated at a density of 6 × 10³ cells/well in a 96-well plate 24 h before the experiment. Next, MN9D cells were incubated in the serum-free medium containing 0.01, 0.1, 1.0 mg/ml fucoidan for 30 min except for the control cells receiving saline instead. Then, MN9D cells were exposed to the neurotoxin MPP+ at the final concentration of 50 μM for further 36 h. The supernatants in each well were separately harvested and used to determine lactate dehydrogenase (LDH) activity with the spectrophotometrical method by LDH analysis kits (Biovision Incorporation, California, U.S.A.). The cells were washed twice with phosphate buffer solution (pH 7.4) and the mitochondrial activity was quantitatively assessed using the MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl tetrazolium bromide] assay. Formazan dissolved in DMSO was detected by an automatic plate reader (BioRad Company, USA) at 570 nm wavelength. Data were expressed as percentage of normal control value. Unless stated otherwise, all other chemicals were purchased from Sigma-Aldrich.

2.10. Statistical analysis

The data were expressed as mean ± S.E.M.. Statistical significance of difference between groups were determined by one-way analysis of variance (ANOVA) followed by Dunnett’s t-test or Bonferroni’s test as appropriate.
3. Results

3.1. Fucoidan attenuates MPTP-induced locomotor activity deficits

Fig. 1 shows the results for the locomotion activity. MPTP administration result in a significant reduction in the total movement distance, mean velocity and mean distance per movement in mice compared to the saline-treated control animals ($P < 0.01$, $P < 0.01$, $P < 0.001$, respectively). This finding points the correlate of loss of nigral dopaminergic neurons due to MPTP neurotoxicity. However, these reductions were partially rescued in fucoidan (25 mg/kg) treatment mice ($P < 0.05$), while fucoidan (25 mg/kg) alone didn’t affect the locomotor activity in mice compared to the saline-treated control animals. Fig. 1D shows the effects of fucoidan on the 30 min-track-plot-pictures of MPTP mice.

3.2. Fucoidan prevents against MPTP-induced striatal depletion of dopamine and DOPAC

The neurotoxicity of MPTP was confirmed by a decrease of striatal content of dopamine and its metabolites as measured by HPLC-ECD (Fig. 2). In accordance with previous reports, MPTP administration led to a marked reduction in striatal dopamine ($1677.55 \pm 167.04$ ng/g tissue), DOPAC ($199.57 \pm 21.75$ ng/g tissue) and HVA levels ($1181.56 \pm 211.81$ ng/g tissue) compared to the saline-treated control mice (dopamine, $21925.89 \pm 750.14$ ng/g tissue, DOPAC, $1110.36 \pm 53.84$ ng/g tissue, HVA, $2463.79 \pm 140.90$ ng/g tissue) ($P < 0.001$). Treatment with fucoidan (25 mg/kg) markedly prevented the decrease of the content of dopamine ($5407.55 \pm 554.94$ ng/g tissue) and DOPAC ($422.30 \pm 39.23$ ng/g tissue) in the striatum of MPTP mice ($P < 0.01$ and $P < 0.05$, respectively). Fucoidan (25 mg/kg) treatment effectively reversed the abnormally increased ratio of (DOPAC + HVA) to dopamine induced by MPTP ($P < 0.001$), suggesting that fucoidan inhibited the turnover of dopamine in the striatum of MPTP mice. Fucoidan at lower dose (12.5 mg/kg) showed slight preventative effect on the MPTP-induced reduction of striatal dopamine and DOPAC levels, which indicated that the effect of fucoidan is dose dependent. Fucoidan (25 mg/kg) treatment alone didn’t affect the content of striatal dopamine and its metabolites compared to control mice ($P > 0.05$).

3.3. Fucoidan prevents MPTP-induced loss of TH-positive neurons

Fig. 3 revealed that MPTP exposure leads to a marked loss of TH positive neurons in the substantia nigra pars compacta compared to the...
Saline-treated control mice (37.60±3.96 vs 75.00±4.24, *P*<0.001). Fucoidan (25 mg/kg) treatment significantly recovered this reduction of nigral TH immunoreactivity (56.60±4.18, *P*<0.05). TH protein expression was also analyzed by immunoblotting and quantified using GAPDH as an internal protein level control. MPTP treatment markedly reduced the levels of nigral TH protein compared to the saline-treated control mice (*P*<0.001). The normalized fluorimetric results revealed that fucoidan treatment restored the TH levels in MPTP mice (*P*<0.05). Fucoidan (25 mg/kg) treatment alone showed no significant changes compared to the saline-treated control mice (*P*<0.05).

### 3.4. Fucoidan protects against MPTP-induced lipid peroxidation, GSH, SOD, GSH-Px, catalase activity and total antioxidant capacity

Fig. 4 showed that MPTP exposure significantly increased the level of lipid peroxidation (MDA) and decreased antioxidative activities in the substantia nigra of mice as compared with the saline control group. However, the treatment with fucoidan significantly decreased MDA content, and increased GSH, SOD, GSH-Px, catalase activity and total antioxidant capacity in the substantia nigra of mice in comparison with the MPTP mice (*P*<0.05 or 0.01).

### 3.5. Fucoidan didn’t affect the pharmacokinetics of MPTP

Potential interference of fucoidan and MPTP pharmacokinetics is a critical aspect in determining the relevance of the above results. We thus tested if the resistance to toxicity was due to a lower delivery of MPTP to the brain following intraperitoneal injection, a reduced brain biotransformation of MPTP to MPP⁺ or to diminished striatal mitochondrial monoamine oxidase B activity. We did not observe any significant differences in striatal levels of MPP⁺ between MPTP mice pretreated with saline (6.60±0.83 ng/mg of tissue) and those pretreated with fucoidan (25 mg/kg, i.p.) for 11 days (6.46±0.90 ng/mg of tissue). Thus, fucoidan didn’t modify MPTP brain availability, and its protection is not the consequence of an interaction with the neurotoxin.

### 3.6. Fucoidan protects against MPP⁺-induced damage in MN9D cells

Fig. 5 summarized the effect of fucoidan on the neuronal injury induced by MPP⁺ in vitro. In the experiment, exposure of the MN9D cells to MPP⁺ significantly reduced the mitochondrial activity and increased release of intracellular LDH to the medium, whereas fucoidan at the concentrations used reversed the decreased mitochondrial activity and the increased LDH release induced by MPP⁺ (*P*<0.01 or 0.001). The in vitro results provide further evidence that fucoidan also exerts a direct protection against the neuronal injury caused by MPP⁺. Fig. 5C showed that fucoidan prevents the MPP⁺-induced morphological changes on MN9D cells.

### 4. Discussion

Neuroprotection with fucoidan has been demonstrated in models of cerebral ischemia and Alzheimer’s disease (Uhm et al., 2003; Alarcon et al., 2005; Jhamandas et al., 2005). In the present study, fucoidan treatment significantly improve the locomotor activity, protects against depletion of both striatal dopamine and TH positive neurons in the substantia nigra pars compacta in MPTP animal model of Parkinson’s disease. Fucoidan pretreatment also directly protect dopaminergic neurons from the damage of neurotoxins, MPP⁺, in an *in vitro* model system of Parkinson’s disease in dopaminergic MN9D cells. To our knowledge, this is the first study to demonstrate that fucoidan possesses potent neuroprotective effects on dopaminergic neurons in *vivo* and *in vitro*. Furthermore, administration of fucoidan (25 mg/kg) effectively limited lipid peroxidation and increased the level/activities of tissue non-enzymic (GSH) and enzymic (SOD, GSH-Px, catalase) antioxidants of substantia nigra in MPTP mice. Thus, the neuroprotective effect may partly be mediated through the antioxidative activity of fucoidan. However, the precise mechanism involved in this effect remains to be delineated.

The MPTP model in mice is widely used to study neuroprotection because it replicates the key pathobiochemical features of Parkinson’s disease such as oxidative stress, mitochondrial dysfunction, excitotoxicity, inflammatory processes and apoptosis (Beal, 2001; Schmidt and Ferger, 2001). Drugs that are able to reduce the neurotoxicity may prove to be neuroprotective. However, interference with the biotransformation of MPTP by inhibiting its uptake into the brain, or by blocking its conversion via monoamine oxidase B to MPP⁺, will also result in reduced toxicity by decreasing the concentration of the ultimate

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**Fig. 5.** Effects of fucoidan on MPP⁺-induced damage in MN9D cells. MN9D cells were pretreated with different concentration of fucoidan for 1 h followed by co-treatment with 50 μM MPP⁺ for 36 h. (A) Cell vitality was detected by MTT assay. (B) Culture supernatants were collected and assayed for LDH with commercial LDH kit. (C) Phase contrast microscopy of MN9D cells after treatment with MPP⁺ (50 μM) and different concentrations of fucoidan for 36 h. a, control (without MPP⁺ and fucoidan); b, MPP⁺ only; c, fucoidan (0.1 mg/ml) + MPP⁺; d, fucoidan (10 mg/ml) + MPP⁺. Scale bar, 20 μm. The results are mean±S.E.M., n=5. **P*<0.01, ***P*<0.001 vs. MPTP group.
Thus, the dopaminergic terminals may be more difficult to rescue than the dopaminergic cell bodies and subsequently the dopaminergic terminals may be more difficult to protect in a model including dopaminergic cell death.

In conclusion, fucoidan treatment provides protection against the neurotoxic effects of MPTP in rodents and the mechanisms of protection induced by this agent may be partly related to its antioxidative activity. It must be noted that the possible mechanisms of protection for fucoidan are rather speculative at this stage and needs further studies.

Acknowledgements

This study is supported by the National Basic Research Program of China (2006CB500700), NSF fund (30571704), and NSF fund (30430280). We thank Dr. Suzhen Chen from Harvard Medical School for her critical reading of the manuscript.

References


