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Protective Effects of the Alga Fucoidan Against Amyloid- β -Induced Neurotoxicity in SH-SY5Y Cells

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Dementia is expected to affect an increasing number of patients with global aging populations. About 70% of all dementia is related to Alzheimer's disease (AD). Overaccumulation of amyloid- β protein (A β) in the brain forms senile plaques, one of the main features of neurodegeneration in AD. However, there are few drugs available to specifically inhibit senile plaque formation. Fucoidan, a sulfated polysaccharide derived from brown algae, has various bioactivities, such as anti-tumoral and anti-obesity effects. This study aimed to clarify the mechanism underlying the protective effect of fucoidan against A β -induced neurotoxicity in human neuroblastoma SH-SY5Y cells. Cell viability and A β -induced cytotoxicity were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, calcein AM, and ethidium homodimer-1. A β -induced oxidative stress was evaluated through reactive oxygen species (ROS), cell membrane phospholipid peroxidation, mitochondrial ROS, and Mn-SOD, a mitochondrial radical scavenger. In addition, mitochondrial membrane permeability transition, and ATP concentration were evaluated. Fucoidan significantly improved A β -reduced cell viability. With respect to oxidative stress, A β exposure increased ROS, lipid peroxidation, and mitochondrial ROS, while fucoidan significantly suppressed these changes. Fucoidan also suppressed the decline in mitochondrial permeability transition and ATP caused by A β . Therefore, through its numerous antioxidant activities, fucoidan might have a neuroprotective role in preventing A β -induced neurotoxicity.

Key words Alzheimer's disease, amyloid β -protein, fucoidan, oxidative stress, neurotoxicity

INTRODUCTION

Alzheimer's disease (AD) is an age-related progressive neurodegenerative disease characterized by an excess of senile plaques and neurofibrillary changes. These cellular abnormalities lead to neuronal loss in the hippocampus and cerebral cortex, resulting in memory and cognitive impairments.¹⁾ The amyloid cascade hypothesis is the most plausible mechanism of AD, where accumulation of the amyloid- β protein (A β) in the brain and phosphorylation of the tau protein cause neurofibrillary changes, leading to neuronal cell death and brain atrophy. Recently, the United States Food and Drug Administration granted fast-track approval to aducanumab, a monoclonal antibody that selectively reduces A β aggregates in the brain, as a new treatment for AD; however, this approval is controversial, and its clinical usefulness uncertain. Other approved therapies for AD do not treat the disease but only delay symptom progression; therefore, new therapies for AD are urgently required. Furthermore, since AD is an irreversible disease that takes about 20 years to progress from the beginning of A β accumulation to AD onset, it is necessary to treat AD with drugs that inhibit A β aggregation and reduce its toxicity from the asymptomatic stage.^{2,3)}

The use of long-term supplements and nutrients to maintain and restore physical function has lately gained attention for preventing AD onset. Various nutrients and supplements, including polyphenols such as curcumin and epigallocatechin gallate, with properties that improve cognitive function have been reported.⁴⁻⁸⁾ Although polyphenols have a strong antioxidant effect, they have low permeability across the blood-brain barrier (BBB) and are required in large doses. Recently, polysaccharides derived from seaweed have been reported to be able to cross the BBB⁹⁾ and have neuroprotective effects.¹⁰⁾ Accordingly, their potential for long-term prevention of dementia has also been investigated.¹¹⁾ Fucoidan, an intercellular mucosal polysaccharide, is a slippery component found in brown algae such as kelp, wakame seaweed, and mozuku seaweed.¹²⁾ It reaches the different organs via carrier-mediated transport after absorption¹³⁾ and has various biological effects, such as improvement of blood circulation,¹⁴⁾ antitumoral and antimetastatic effects,^{15,16)} improvement of the immune system function,¹⁷⁾ and anti-obesity effects.¹⁸⁾ Recently, fucoidan has been reported to suppress the increase in reactive oxygen species (ROS) production in AD-transgenic *C. elegans*,¹⁹⁾ and after A β -induced neurotoxicity in primary rat neuron cultures.²⁰⁾ However, the mechanism through which fucoidan reduces ROS production has not been yet elucidated.

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This study aimed to clarify the mechanism underlying the neuroprotective effect of fucoidan using an A β -treated neurodegeneration model *in vitro*.

MATERIALS AND METHODS

Drugs and Reagents Highly purified A β -protein (Human, 1-42) was purchased from the Peptide Institute Inc. (Osaka, Japan). A β ₁₋₄₂ was dissolved in dimethyl sulfoxide (DMSO) and incubated at 37°C for 24 h to allow for self-aggregation and oligomerization before being prepared in medium, without fetal bovine serum (FBS), to 5 μ M. The final DMSO concentration was set to 0.2%. DMEM/Ham's F-12 medium and all-trans retinoic acid (ATRA) were procured from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). FBS was purchased from Sigma-Aldrich Company (MO, USA). The fucoidan from *Laminaria japonica* (scientific name: *Saccharina japonica*) with \geq 85% purity was purchased from Cayman Chemical Company (MI, USA). In this study, all other compounds were used at the highest purity commercially available.

Cell Culture SH-SY5Y cells (human neuroblastoma, EC-94030304) were purchased from the European Collection of Authenticated Cell Cultures (London, UK). SH-SY5Y cells were cultured in D-MEM/Ham's F-12 containing 10% FBS at 37°C under 5% CO₂/95% air atmosphere. SH-SY5Y cells are morphologically and neurochemically comparable to neurons, and widely used to assess neuronal damage and cytotoxicity in neurodegenerative diseases, cerebral ischemia-reperfusion, and epilepsy.⁴⁰⁾ To examine the effect of fucoidan, SH-SY5Y cells were treated with 10 μ M ATRA for 5 d and differentiated. Differentiated SH-SY5Y cells were then exposed to 5 μ M A β with or without fucoidan 1 or 10 μ g/mL in DMEM/Ham's F-12. Cells cultured in medium alone, without FBS, were used as control.

3-(4,5-Dimethylthiazoyl-2-yl)-2,5-Diphenyltetrazolium Bromide Viability Cell Assay We measured cell viability using Cell Proliferation Kit I (Roche Diagnostics K.K., Mannheim, Germany). The assay is based on the formation of blue formazan from 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondria, a dehydrogenase active only in living cells. SH-SY5Y cells suspensions of 1.0 x 10⁶ cells/mL were seeded into 96-well plates, incubated at 37°C for 24 h, exposed to A β , and treated with fucoidan and A β +fucoidan for 3 or 24 h. The formazan was detected at 540 nm using a microplate reader (Spectra Max i3; Molecular Devices, CA, USA), and viability showed as a percentage of non-treated cells.

Calcein and Ethidium Homodimer-1 (live/dead) Cell Assay SH-SY5Y cells suspensions of 1.0 x 10⁶ cells/mL were seeded into 96-well plates, incubated at 37°C for 24 h, and exposed to A β and treated with fucoidan for 3 h. Then, treated-cells were stained with 2 μ M calcein AM and 10 μ M ethidium homodimer-1 (Molecular Probes, OR, USA). The green, fluorescent calcein hydrolyzed by intracellular some esterases depends on the number of surviving cells, and ethidium homodimer-1 enters only cells with damaged membranes where it binds to nucleic acids, emitting bright red fluorescence proportional to the number of dead cells. The green fluorescence intensity was measured at Ex: 495 nm and Em: 530 nm and the red fluorescence intensity was measured at Ex: 495 nm and Em: 645 nm using Spectra Max i3 (Molecular Devices). The morphology of individual cells was also assessed after

imaging with a fluorescence microscope (BZX800; Keyence, Osaka, Japan).

Detection of Reactive Oxygen Species To investigate the effects of A β exposure on ROS production, the chloromethyl derivative 2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; Thermo Fisher Scientific, MA, USA), a useful indicator for ROS detection, was used. SH-SY5Y cells were incubated at 37°C for 24 h, after which these cells were exposed to A β and treated with fucoidan for 30 min. ROS-mediated fluorescence was measured at Ex: 488 nm and Em: 525 nm using Spectra Max i3 (Molecular Devices).

Assay of Mitochondrial ROS SH-SY5Y cells at 1.0 x 10⁶ cells/mL were plated in 96-well plates, incubated at 37°C for 24 h, exposed to A β and treated with fucoidan for 30 min. Mitochondrial ROS was detected using the Mitochondrial ROS Detection Kit (Cayman Chemical Company, MI, USA). The fluorescence intensity was measured at Ex: 500 nm and Em: 580 nm using Spectra Max i3 (Molecular Devices).

Assay of Phospholipid Peroxidation in Membranes To measure phospholipid peroxidation in the membranes of A β -exposed cells, SH-SY5Y cells were treated with 5 μ M of diphenyl-1-pyrenylphosphine (DPPP; Thermo Fisher Scientific, MA, USA). DPPP reacts with hydroperoxides to produce fluorescent DPPP oxide.⁴¹⁾ SH-SY5Y cells at 1.0x10⁶ cells/mL plated in 96-well plates were incubated with DPPP at 37°C for 10 min, then exposed to A β , and treated with fucoidan for 30 min. The fluorescence intensity of DPPP oxide was measured at Ex: 351 nm and Em: 380 nm using Spectra Max i3 (Molecular Devices).

Assay of Manganese-Superoxide Dismutase SH-SY5Y cells were incubated at 37°C for 24 h, after which cells were exposed to A β and treated with fucoidan for 24 h. The mitochondrial SOD isozyme content in cell lysates was determined and measured by ELISA using a monoclonal antibody (Human SOD2 ELISA Kit; Abcam, Cambridge, UK). These ELISA systems did not show any cross-reactivity with other SOD isozymes. SOD protein level was determined using the OxiSelect™ Superoxide Dismutase Activity Assay (Cell Biolabs, Inc., CA, USA), and measured at 450 nm using Spectra Max i3 (Molecular Devices).

Observation of Mitochondrial Membrane Permeability Transition SH-SY5Y cells were incubated at 37°C for 24 h, after which these cells were exposed to A β , and treated with fucoidan for 3 h. Changes in mitochondrial membrane permeability transition (MPT) were measured using the Mito Capture Apoptosis Detection Kit (Bio Vision Inc., CA, America). Fluorescence intensities were measured at Ex:488 nm, Em:590 nm (Lm1:red), and Em:530 nm (Lm2:green) using Spectra Max i3 (Molecular Devices), and the Lm1/Lm2 ratio was determined.

ATP Assay ATP levels in cells were measured by the firefly luciferase luminescence method using an intracellular ATP assay kit (TOYO B-NET Co. Ltd, Tokyo, Japan). SH-SY5Y cells were incubated at 37°C for 24 h, after which cells were exposed to A β and treated with fucoidan for 3 h. After incubation, the ATP extracted from SH-SY5Y cells was measured for luminescence with Spectra Max i3 (Molecular Devices).

Statistical Analysis All results of the cell experiments are shown as mean \pm S.E.M. values. Each measurement was repeated three times. Comparisons with untreated SH-SY5Y cells were performed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. P-values < 0.05 were considered statistically significant.

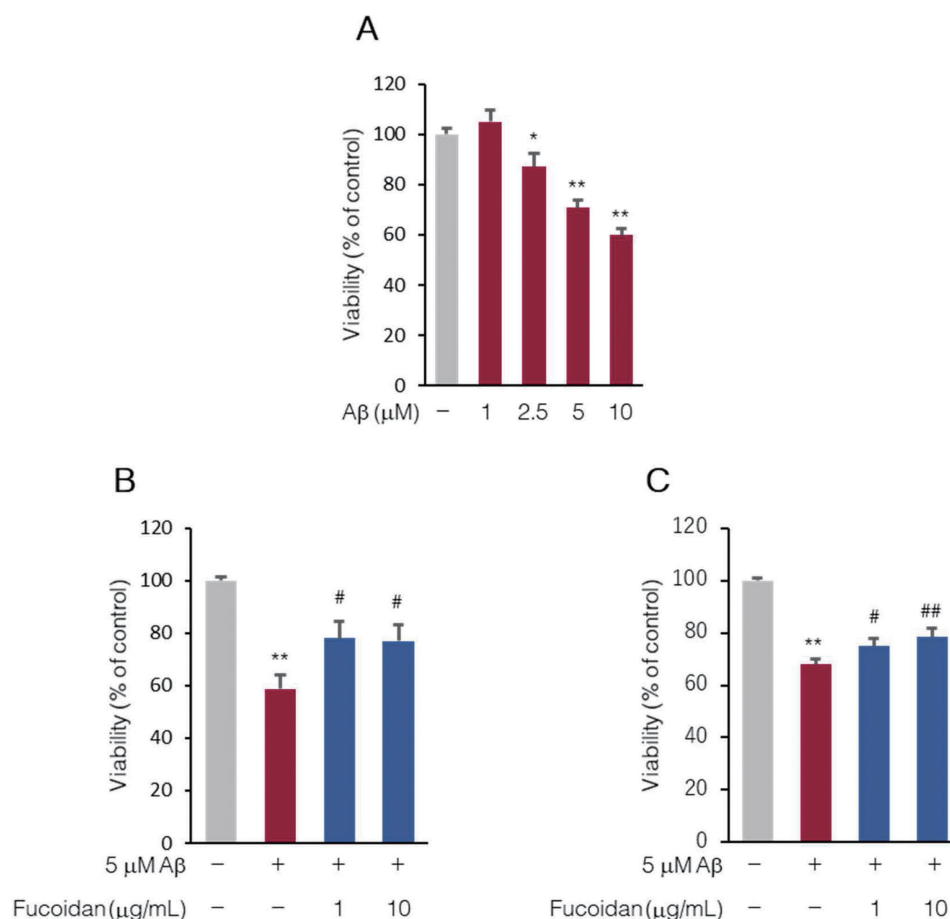


Fig. 1. Effect of Fucoidan on SH-SY5Y Cell Viability

Cell viability of SH-SY5Y cells assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **(A)** Viability (% of control) of SH-SY5Y cells treated with 1, 2.5, 5, and 10 μM Aβ for 3 h. **p < 0.01 vs. control cells. *p < 0.05 vs. control cells. **(B)** Viability (% of control) of SH-SY5Y cells treated with 5 μM Aβ and fucoidan (1 and 10 μg/mL) for 3 h. **p < 0.01 vs. control cells. #p < 0.05 vs. Aβ exposed cells. **(C)** Viability (% of control) of SH-SY5Y cells treated with 5 μM Aβ and fucoidan (1 and 10 μg/mL) for 24 h. **p < 0.01 vs. control cells. #p < 0.05 vs. Aβ exposed cells. ##p < 0.01 vs. Aβ exposed cells. Values in all graphs (A–C) are expressed as the mean of six independently treated culture wells plus S. E. M. Each measurement was repeated three times with similar results.

RESULTS

Effect of Fucoidan on Aβ-Induced Neuronal Injury To determine the Aβ concentration that induces neuronal toxicity, SH-SY5Y cells were incubated with 1–10 μM Aβ. The changes in cell viability were assessed using the MTT assay. No significant change in cell viability was observed at 1 μM Aβ, but at ≥ 2.5 μM, Aβ significantly reduced cell viability in a concentration-dependent manner (Fig. 1A). Since exposure to 5 μM Aβ decreased cell viability by ~30%, we used this concentration in subsequent experiments. Exposure of SH-SY5Y cells to Aβ (5 μM) for 3 and 24 h resulted in a marked decrease in cell viability compared to control cells without Aβ exposure. Although there was no significant difference in viability depending on incubation time, treatment with fucoidan at 1 and 10 μg/mL significantly reversed the Aβ-induced decrease in cell viability (Fig. 1B and C).

To further investigate Aβ cytotoxicity, SH-SY5Y cells were stained with calcein AM and ethidium homodimer-1 (Fig. 2A–F). Calcein AM emits green fluorescence in the cytoplasm of viable cells via esterase activity. When the cell membrane is damaged, ethidium homodimer-1 enters the cell, binds to DNA and RNA, and emits bright red fluorescence. There-

fore, double staining with calcein AM and ethidium homodimer-1 allowed the simultaneous analysis of the viability and cytotoxicity of Aβ treated cells and control cells. Fluorescence imaging of control cells only showed green fluorescence, with a few cells having red-stained nuclei (Fig. 2D). Aβ treatment was associated with a change in cell morphology, i.e., shortening of the protrusion length (Fig. 2B), and an increase in the number of red-stained nuclei (Fig. 2E). Co-treatment of SH-SY5Y cells with Aβ and fucoidan (10 μg/mL) decreased the changes to cell morphology (Fig. 2C) and the number of red-stained nuclei (Fig. 2F). SH-SY5Y cells incubated with Aβ for 3 h showed significantly reduced viability (Fig. 2G) and increased cytotoxicity compared to control cells (Fig. 2H). Fucoidan (1 and 10 μg/mL) significantly improved cell viability in Aβ-treated cells (Fig. 2G). Additionally, fucoidan (10 μg/mL) significantly reduced Aβ-induced cytotoxicity (Fig. 2H).

Oxidative Stress ROS production was determined by chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA) staining using fluorescence microscopy. Since ROS are produced from an early stage, experiments were conducted after a 30 min incubation. ROS production due to Aβ exposure was significantly reduced in cells treat-

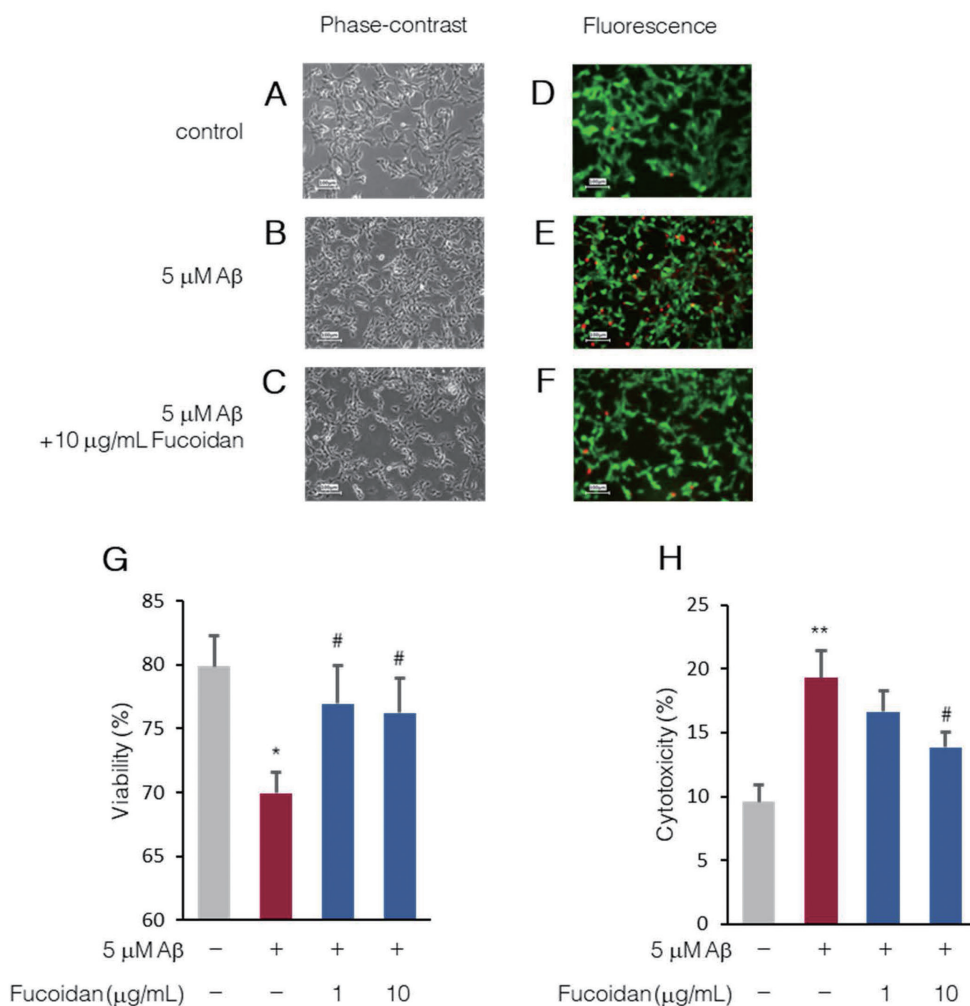


Fig. 2. The Viability and Cytotoxicity of SH-SY5Y Cells were Evaluated using the Calcein and Ethidium Homodimer-1 (live/dead) Cell Assay

(A–F) SH-SY5Y cells stained with calcein AM and ethidium homodimer-1 observed using phase-contrast (A–C) and fluorescence microscopy (D–F). The scale bar represents 100 μ m. (G) Viability (%) of SH-SY5Y cells treated with 5 μ M A β and fucoidan (1 and 10 μ g/mL). * p < 0.05, vs. control cells. # p < 0.05 vs. A β exposed cells. (H) Cytotoxicity (%) of SH-SY5Y cells treated with 5 μ M A β and fucoidan (1 and 10 μ g/mL). ** p < 0.01 vs. control cells. # p < 0.05 vs. A β exposed cells. Values in graphs (G) and (H) are expressed as the mean of six independently treated culture wells plus S. E. M. Each measurement was repeated three times with similar results.

ed with fucoidan for 30 min. Microscopy images are shown in Fig. 3A–F. Green dichlorofluorescein fluorescence increased after A β exposure (Fig. 3E), but it was attenuated by 10 μ g/mL fucoidan treatment (Fig. 3F). ROS production was significantly higher in SH-SY5Y cells exposed to A β for 30 min than in control cells (Fig. 3G).

The production of mitochondrial ROS, which consists mainly of superoxide anions, significantly increased in SH-SY5Y cells treated with A β for 30 min (Fig. 4). Fucoidan (1 μ g/mL) suppressed A β -induced mitochondrial ROS production. No significant difference in mitochondrial ROS was observed at the concentration of 10 μ g/mL, and no significant difference was observed between the two concentrations of 1 and 10 μ g/mL. Therefore, the mitochondrial ROS at both concentrations of 1 and 10 μ g/mL maybe the same.

Phospholipid peroxidation was then measured in the cell membrane using diphenyl-1-pyrenylphosphine (DPPP), which reacts stoichiometrically with lipid hydroperoxides to yield fluorescent DPPP oxide. Lipid peroxidation was significantly higher in SH-SY5Y cells treated with 5 μ M A β than in control cells (Fig. 5). However, 10 μ g/mL fucoidan significantly sup-

pressed A β -induced lipid peroxidation.

Effects of Fucoidan on A β -Induced Mitochondrial Dysfunction A β -induced cell death is associated with mitochondrial membrane injury. Mitochondrial membrane damage reduces MPT and decreases ATP and scavenger production. MPT and ATP were analysed after incubation for 3 h according to a previous study.²¹⁾

SH-SY5Y cells treated with A β for 24 h showed a decrease of ~50% in manganese superoxide dismutase (Mn-SOD) levels compared to control cells. Fucoidan significantly increased Mn-SOD levels in a concentration-dependent manner (Fig. 6). Incubation time was set to 24 h to allow Mn-SOD protein expression.

MPT significantly decreased in SH-SY5Y cells treated with A β for 3 h to about one-third of that in control cells. Fucoidan significantly ameliorated the A β -mediated decrease in MPT (Fig. 7).

A decrease in ATP levels leads to the uncoupling of oxidative phosphorylation, further resulting in ATP depletion. Therefore, we investigated whether ATP production was altered by A β treatment and whether fucoidan treatment

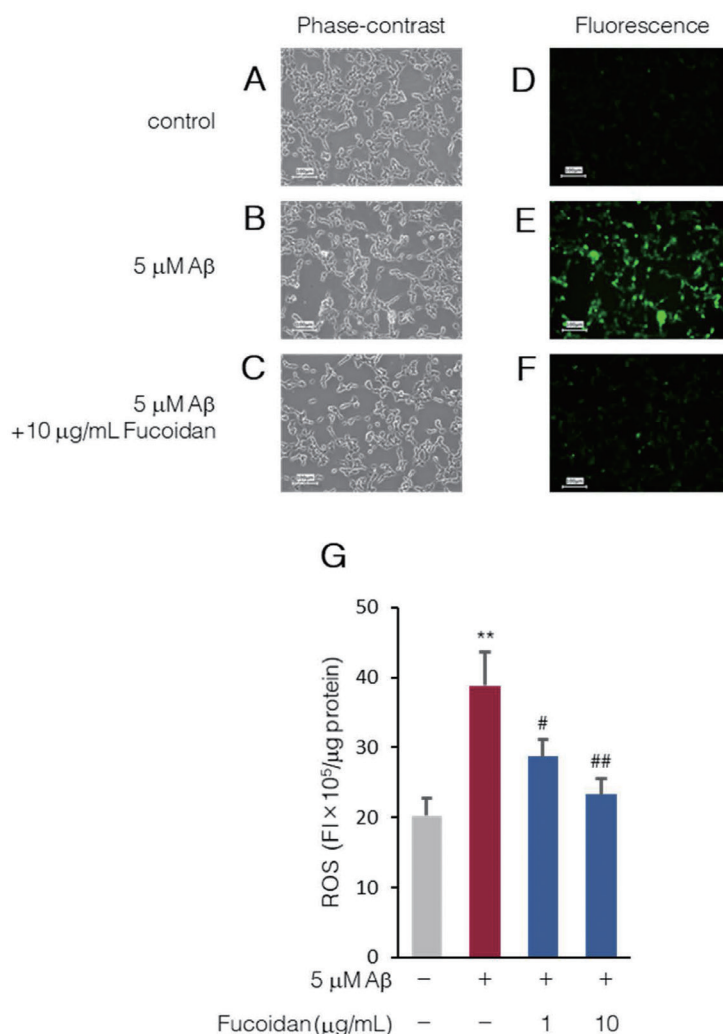


Fig. 3. Determination of Reactive Oxygen Species (ROS) Generation

ROS generation in SH-SY5Y cells was evaluated using chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate. (A–F) SH-SY5Y cells observed using phase-contrast (A–C) and fluorescence microscopy (D–F). The scale bar represents 100 μ m. (G) ROS generation (fluorescence intensity [FI] $\times 10^5/\mu$ g protein) in SH-SY5Y cells treated with 5 μ M A β and fucoidan (1 and 10 μ g/mL). **p < 0.01 vs. control cells. #p < 0.05 vs. A β exposed cells. ##p < 0.01 vs. A β exposed cells. Values in (G) are expressed as the mean of six independently treated culture wells plus S. E. M. Each measurement was repeated three times with similar results.

reversed this effect in the current experimental model. The results showed that ATP levels after A β treatment were nearly 80% of those in control cells. Fucoidan (10 μ g/mL) significantly recovered the A β -induced ATP decrease (Fig. 8).

DISCUSSION

This study demonstrated the protective effect of fucoidan against A β -induced neurotoxicity, especially oxidative stress in cell membranes and mitochondrial damage, in SH-SY5Y cells. Fucoidan reduced A β -induced cell membrane damage by ameliorating phospholipid peroxidation, maintaining cell membrane integrity, and increasing cell viability. Additionally, fucoidan reduced mitochondrial damage by alleviating the A β -mediated decrease in MPT, decreasing mitochondrial ROS production, and increasing ATP levels. Although the protective effects of fucoidan have been previously reported, the mechanism underlying these effects has not been fully elucidated. Fucoidan has been reported to exhibit various biological activities, such as anti-dyslipidemia, anti-arteriosclerosis,²²⁾ and

anti-cancer effects,²³⁾ which are related to its anti-inflammatory and anti-oxidative effects. This study showed that fucoidan could have a preventive role against neurodegenerative diseases as well.

A single oral fucoidan administration to both male and female mice reported an lethal dose 50 (LD₅₀) and approximate lethal dose (LD) value of 2000 mg / kg, indicating that the concentration of fucoidan used in this study is safe.²⁴⁾ Here, SH-SY5Y cells were treated with 1 and 10 μ g/mL of fucoidan, far lower concentrations than those used in previous reports (50–800 μ g/mL fucoidan). Those reports showed that fucoidan treatment ameliorated 1-methyl-4-phenylpyridinium ion-induced neurotoxicity in SH-SY5Y cells.^{25,26)} The human plasma concentration of fucoidan was approximately 13 μ g/mL after oral administration of 2.25 g of fucoidan for 12 d²⁷⁾; therefore, the fucoidan concentrations used in this study were within the appropriate range.

Furthermore, fucoidan penetrated the BBB at a rate of 10.4×10^{-6} cm/s *in vitro*, where the BBB was constructed with astrocytes, vascular endothelial cells, and pericytes.⁹⁾ Howev-

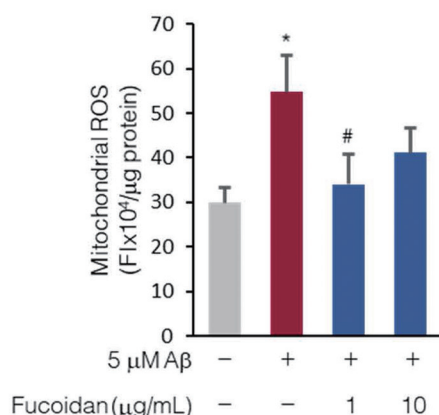


Fig. 4. Determination of Oxidative Stress in Mitochondria

The generation of oxidative stress from mitochondrial ROS in SH-SY5Y cells was evaluated using a mitochondrial ROS detection kit. The graph shows mitochondrial ROS levels (fluorescence intensity [FI] $\times 10^4/\mu$ g protein) in SH-SY5Y cells treated with 5 μ M A β and fucoidan (1 and 10 μ g/mL). * $p < 0.05$ vs. control cells. # $p < 0.05$ vs. A β exposed cells. Values are expressed as the mean of six independently treated culture wells plus S. E. M. Each measurement was repeated three times with similar results.

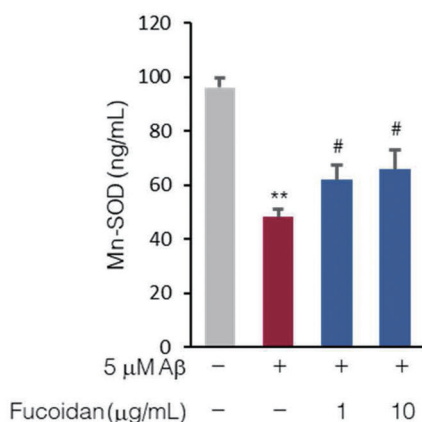


Fig. 6. Determination of Manganese Superoxide Dismutase (Mn-SOD) Levels

Mn-SOD concentration in SH-SY5Y cells was evaluated using the Human SOD2 ELISA Kit. The graph shows Mn-SOD levels (ng/mL) in SH-SY5Y cells treated with 5 μ M A β and fucoidan (1 and 10 μ g/mL). ** $p < 0.01$ vs. control cells. # $p < 0.05$ vs. A β exposed cells. Values are expressed as the mean of six independently treated culture wells plus S. E. M. Each measurement was repeated three times with similar results.

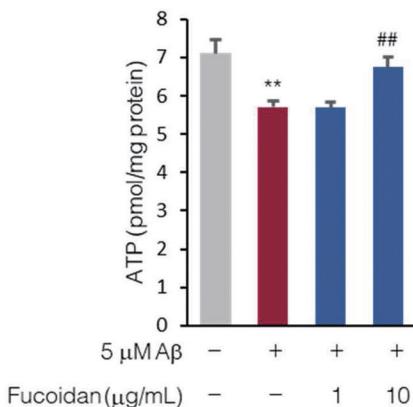


Fig. 8. ATP Determination.

ATP levels were measured using luminescence. The graph shows ATP levels (pmol/mg protein) in SH-SY5Y cells treated with 5 μ M A β and fucoidan (1 and 10 μ g/mL). ** $p < 0.01$ vs. control cells. ## $p < 0.01$ vs. A β exposed cells. Values are expressed as the mean of six independently treated culture wells plus S. E. M. Each measurement was repeated three times with similar results.

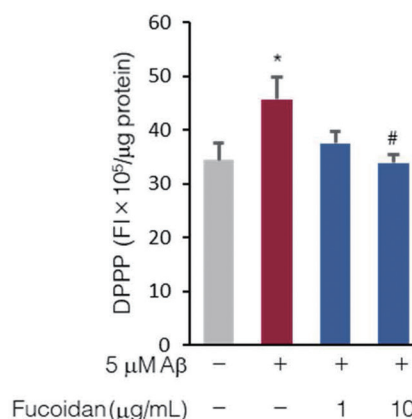


Fig. 5. Determination of Membrane Lipid Peroxidation

Membrane lipid peroxidation in SH-SY5Y cells was evaluated by a lipid peroxidation assay using diphenyl-1-pyrenylphosphine (DPPP). The graph shows DPPP levels (fluorescence intensity [FI] $\times 10^5/\mu$ g protein) in SH-SY5Y cells treated with 5 μ M A β and fucoidan (1 and 10 μ g/mL). * $p < 0.05$ vs. control cells. # $p < 0.05$ vs. A β exposed cells. Values are expressed as the mean of six independently treated culture wells plus S. E. M. Each measurement was repeated three times with similar results.

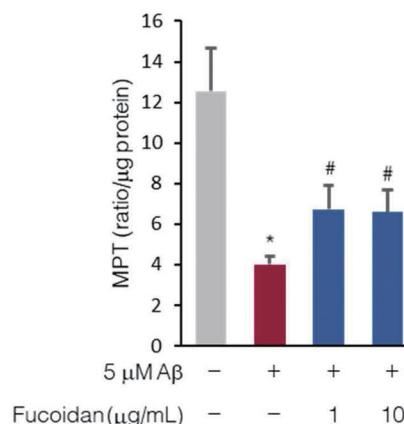


Fig. 7. Determination of Mitochondrial Membrane Permeability Transition (MPT)

The graph shows mitochondrial MPT (ratio/ μ g protein) in SH-SY5Y cells treated with 5 μ M A β and fucoidan (1 and 10 μ g/mL). The change in membrane potential was measured by comparing the ratio of emission at 585 nm/514 nm. * $p < 0.05$ vs. control cells. # $p < 0.05$ vs. A β exposed cells. Values are expressed as the mean of six independently treated culture wells plus S. E. M. Each measurement was repeated three times with similar results.

er, it is unclear whether fucoidan passes through the BBB or transitions into the brain *in vivo*, thus, further research is needed in this area. This study confirmed that A β increases mitochondrial ROS, particularly mitochondrial superoxide anions, decreases Mn-SOD, and induces oxidative stress. Oxidative stress is related with health issues²⁸⁾ and results in excessive ROS production, which can be mitigated by cellular antioxidant activity. Additionally, free radicals affect the structure and function of neurons and contribute to neurodegenerative diseases.²⁹⁾ There is increasing evidence that oxidative stress plays a central role in the pathophysiology of AD.³⁰⁾ Therefore, the development of antioxidative medications that decrease oxidative stress and promote neuronal survival will help in the prevention and early treatment of AD. Seaweed compounds, with their antioxidant effects via ROS scavenging,³¹⁾ are one such promising medication. In this study, fucoidan reduced A β -induced ROS production and oxidative stress.

A β increases phospholipid peroxidation in the cell membrane. Considering the brain is one of the most lipid-rich organs, brain phospholipid changes can lead to various pathogenic processes.³²⁾ A β is the main component of senile plaques, a major pathological feature of AD. It is produced by cleavage of the amyloid precursor protein (APP) by β -site amyloid precursor protein cleaving enzyme-1, an endogenous membrane protein in the brain, and γ -secretase. The APP metabolism is closely associated with membrane phospholipids. Furthermore, changes in phospholipid composition have been reported in patients with AD, including a sphingomyelin decrease³³⁾ and high oxidative stress-related changes in erythrocyte phospholipids.³⁴⁾ The inhibitory effect of fucoidan on A β -induced cell membrane damage can be useful in maintaining membrane integrity.

Considering the ameliorating effect of fucoidan on oxidative stress, we investigated mitochondrial function, where ROS are generated. Fucoidan showed a protective effect against the suppression of mitochondrial function by A β . A β increased production of mitochondrial ROS and reduced Mn-SOD, which removes superoxide produced by mitochondrial electron transport chain complexes. Fucoidan alleviated this A β -induced effect by reducing mitochondrial ROS production and restoring MPT and ATP levels.

Fucoidan, a cell wall component of brown algae, is a sulfated polysaccharide whose main component is fucose. Fucoidan can capture radicals. The antioxidation of sulfated polysaccharides is decided by their structural characteristics, such as sulfation degree, molecular weight, major sugar types, glycoside branching,^{35,36)} and sulfate content. The reducing power of fucoidan increases with increasing sulfate content and molecular weight.^{37,38)} Fucoidan from *Porphyra haitanensis* had a high

proportion of sulfate, and the derived sulfated polysaccharide fraction showed enhanced SOD, glutathione peroxidase, and antioxidant activity in various tissues of aged mice.³⁹⁾

A β also reduced MPT and ATP production. MPT is affected by mitochondrial Ca²⁺, ATP, and ROS, which decreases with opening of MPT pores. Fucoidan suppressed mitochondrial ROS production and increased ATP production. Additionally, it increased the potential difference reduced by mitochondrial membrane damage. This indicates that fucoidan minimizes A β -induced mitochondrial damage.

In summary, fucoidan protects against A β -induced neuronal toxicity by directly inhibiting A β -induced oxidative stress in the cell membranes and mitochondria (Fig. 9). Therefore, fucoidan is a valuable nutritional supplement for long-term consumption with the potential to slow down the progression or even prevent the onset of AD.

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Conflict of interest The authors declare no conflict of interest.

REFERENCES

- 1) Moya-Alvarado G, Gershoni-Emek N, Perlson E, Bronfman FC. Neurodegeneration and Alzheimer's disease (AD). What Can Proteomics Tell Us About the Alzheimer's Brain? *Mol. Cell. Proteomics*, **15**, 409–425 (2016).
- 2) Roher AE, Kokjohn TA, Clarke SG, Sierks MR, Maarouf CL, Serrano GE, Sabbagh MS, Beach TG. APP/A β structural diversity and Alz-

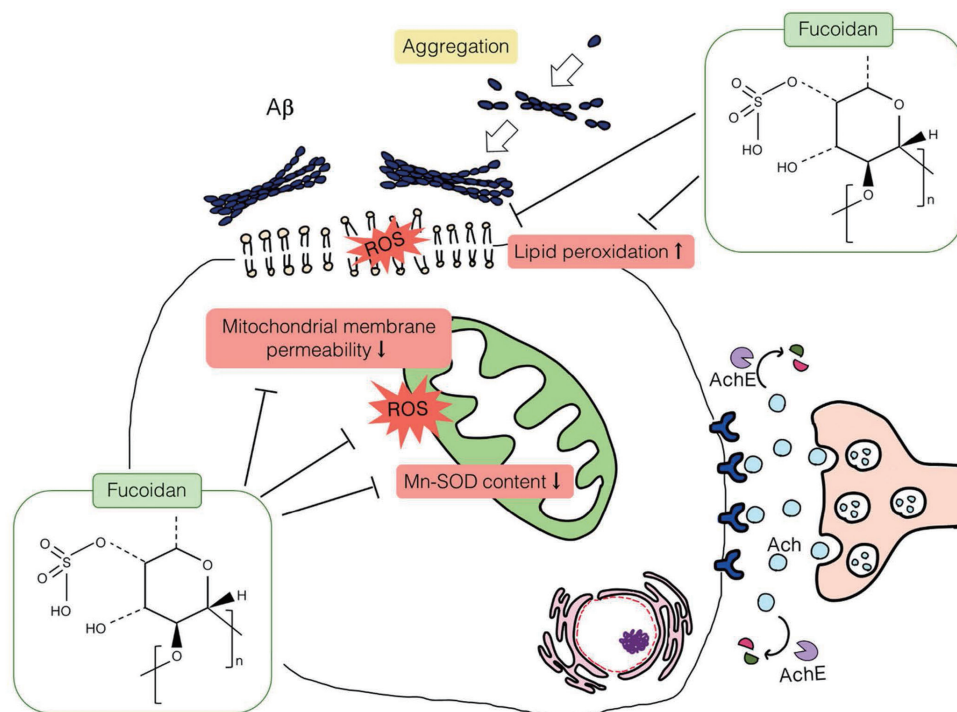


Fig. 9. Schematic Diagram of the Mechanism of A β -Induced Neurotoxicity and its Inhibition by Fucoidan

Aggregated A β is associated with the pathogenesis of AD. A β damages the neuronal membranes and mitochondria through multiple mechanisms, and fucoidan significantly inhibits the A β -induced damage.

- heimer's disease pathogenesis. *Neurochem. Int.*, **110**, 1–13 (2017).
- 3) Sadigh-Eteghad S, Sabermarouf B, Majdi A, Talebi M, Farhoudi M, Mahmoudi J. Amyloid-beta: a crucial factor in Alzheimer's disease. *Med. Princ. Pract.*, **24**, 1–10 (2015).
 - 4) Botchway BOA, Moore MK, Akinleye FO, Iyer IC, Fang M. Nutrition: Review on the Possible Treatment for Alzheimer's Disease. *J. Alzheimers Dis.*, **61**, 867–883 (2018).
 - 5) Patrick RP. Role of phosphatidylcholine-DHA in preventing APOE4-associated Alzheimer's disease. *FASEB J.*, **33**, 1554–1564 (2019).
 - 6) Reddy PH, Manczak M, Yin X, Grady MC, Mitchell A, Tonk S, Kuruva CS, Bhatti JS, Kandimalla R, Vijayan M, Kumar S, Wang R, Pradeepkiran JA, Ogunmokun G, Thamarai K, Quesada K, Boles A, Reddy AP. Protective Effects of Indian Spice Curcumin Against Amyloid-beta in Alzheimer's Disease. *J. Alzheimers Dis.*, **61**, 843–866 (2018).
 - 7) Ono K, Yoshiike Y, Takashima A, Hasegawa K, Naiki H, Yamada M. Potent anti-amyloidogenic and fibril-destabilizing effects of polyphenols *in vitro*: implications for the prevention and therapeutics of Alzheimer's disease. *J. Neurochem.*, **87**, 172–181 (2003).
 - 8) Singh NA, Mandal AK, Khan ZA. Potential neuroprotective properties of epigallocatechin-3-gallate (EGCG). *Nutr. J.*, **15**, 60 (2016).
 - 9) Subaraja M, Anantha Krishnan D, Edwin Hillary V, William Raja TR, Mathew P, Ravikumar S, Gabriel Paulraj M, Ignacimuthu S. Fucoidan serves a neuroprotective effect in an Alzheimer's disease model. *Front. Biosci. (Elite Ed.)*, **12**, 1–34 (2020).
 - 10) Hannan MA, Dash R, Haque MN, Mohibbullah M, Sohag AAM, Rahman MA, Uddin MJ, Alam M, Moon IS. Neuroprotective Potentials of Marine Algae and Their Bioactive Metabolites: Pharmacological Insights and Therapeutic Advances. *Mar. Drugs*, **18**, (2020).
 - 11) Wei H, Gao Z, Zheng L, Zhang C, Liu Z, Yang Y, Teng H, Hou L, Yin Y, Zou X. Protective Effects of Fucoidan on Abeta25-35 and d-Gal-Induced Neurotoxicity in PC12 Cells and d-Gal-Induced Cognitive Dysfunction in Mice. *Mar. Drugs*, **15**, (2017).
 - 12) Zhao Y, Zheng Y, Wang J, Ma S, Yu Y, White WL, Yang S, Yang F, Lu J. Fucoidan Extracted from *Undaria pinnatifida*: Source for Nutraceuticals/Functional Foods. *Mar. Drugs*, **16**, (2018).
 - 13) Bai X, Zhang E, Hu B, Liang H, Song S, Ji A. Study on Absorption Mechanism and Tissue Distribution of Fucoidan. *Molecules*, **25**, (2020).
 - 14) Kim H, Ahn JH, Song M, Kim DW, Lee TK, Lee JC, Kim YM, Kim JD, Cho JH, Hwang IK, Yan BC, Won MH, Park JH. Pretreated fucoidan confers neuroprotection against transient global cerebral ischemic injury in the gerbil hippocampal CA1 area via reducing of glial cell activation and oxidative stress. *Biomed. Pharmacother.*, **109**, 1718–1727 (2019).
 - 15) Xu L, Liu F, Li C, Li S, Wu H, Guo B, Gu J, Wang L. Fucoidan suppresses the gastric cancer cell malignant phenotype and production of TGF-beta1 via CLEC-2. *Glycobiology*, **30**, 301–311 (2020).
 - 16) Kawamoto H, Miki Y, Kimura T, Tanaka K, Nakagawa T, Kawamukai M, Matsuda H. Effects of Fucoidan from Mozuku on Human Stomach Cell Lines. *Food Sci. Technol. Res.*, **12**, 218–222 (2006).
 - 17) Apostolova E, Lukova P, Baldzhieva A, Katsarov P, Nikolova M, Iliev I, Peychev L, Trica B, Oancea F, Delattre C, Kokova V. Immunomodulatory and Anti-Inflammatory Effects of Fucoidan: A Review. *Polymers (Basel)*, **12** (2020).
 - 18) Miyashita K, Beppu F, Hosokawa M, Liu X, Wang S. Nutritional characteristics of the brown seaweed carotenoid fucoxanthin. *Arch. Biochem. Biophys.*, **686**, 108364 (2020).
 - 19) Wang X, Yi K, Zhao Y. Fucoidan inhibits amyloid-beta-induced toxicity in transgenic *Caenorhabditis elegans* by reducing the accumulation of amyloid-beta and decreasing the production of reactive oxygen species. *Food Funct.*, **9**, 552–560 (2018).
 - 20) Jhamandas JH, Wie MB, Harris K, MacTavish D, Kar S. Fucoidan inhibits cellular and neurotoxic effects of beta-amyloid (A beta) in rat cholinergic basal forebrain neurons. *Eur. J. Neurosci.*, **21**, 2649–2659 (2005).
 - 21) Kimura AM, Tsuji M, Yasumoto T, Mori Y, Oguchi T, Tsuji Y, Umino M, Umino A, Nishikawa T, Nakamura S, Inoue T, Kiuchi Y, Yamada M, Teplow DB, Ono K. Myricetin prevents high molecular weight Abeta1-42 oligomer-induced neurotoxicity through antioxidant effects in cell membranes and mitochondria. *Free Radic. Biol. Med.*, **171**, 232–244 (2021).
 - 22) Yokota T, Nomura K, Nagashima M, Kamimura N. Fucoidan alleviates high-fat diet-induced dyslipidemia and atherosclerosis in ApoE(shl) mice deficient in apolipoprotein E expression. *J. Nutr. Biochem.*, **32**, 46–54 (2016).
 - 23) Atya ME, El-Hawiet A, Alyeldeen MA, Ghareeb DA, Abdel-Daim MM, El-Sadek MM. *In vitro* biological activities and *in vivo* hepatoprotective role of brown algae-isolated fucoidans. *Environ. Sci. Pollut. Res. Int.*, **28**, 19664–19676 (2021).
 - 24) Jung YM, Yoo KM, Park DC, Kim TK, Lee HS, Ku SK. Oral Single Dose Toxicity Study of Low Molecular Fucoidan in Mice. *Toxicol. Res.*, **24**, 79–86 (2008).
 - 25) Han YS, Lee JH, Lee SH. Fucoidan Suppresses Mitochondrial Dysfunction and Cell Death against 1-Methyl-4-Phenylpyridinium-Induced Neuronal Cytotoxicity via Regulation of PGC-1alpha Expression. *Mar. Drugs*, **17**, (2019).
 - 26) Liu H, Wang J, Zhang Q, Geng L, Yang Y, Wu N. Protective Effect of Fucoidan against MPP(+)-Induced SH-SY5Y Cells Apoptosis by Affecting the PI3K/Akt Pathway. *Mar. Drugs*, **18**, (2020).
 - 27) Irhimeh MR, Fitton JH, Lowenthal RM, Kongtawelert P. A quantitative method to detect fucoidan in human plasma using a novel antibody. *Methods Find. Exp. Clin. Pharmacol.*, **27**, 705–710 (2005).
 - 28) Vatner SF, Zhang J, Oydanich M, Berkman T, Naftalovich R, Vatner DE. Healthful aging mediated by inhibition of oxidative stress. *Ageing Res. Rev.*, **64**, 101194 (2020).
 - 29) Jiang T, Sun Q, Chen S. Oxidative stress: A major pathogenesis and potential therapeutic target of antioxidative agents in Parkinson's disease and Alzheimer's disease. *Prog. Neurobiol.*, **147**, 1–19 (2016).
 - 30) Chen Z, Zhong C. Oxidative stress in Alzheimer's disease. *Neurosci. Bull.*, **30**, 271–281 (2014).
 - 31) Omidbakhshfar MA, Sujeeth N, Gupta S, Omranian N, Guinan KJ, Brotman Y, Nikoloski Z, Fernie AR, Mueller-Roeber B, Gechev TS. A Biostimulant Obtained from the Seaweed *Ascophyllum nodosum* Protects *Arabidopsis thaliana* from Severe Oxidative Stress. *Int. J. Mol. Sci.*, **21** (2020).
 - 32) Ledesma MD, Martin MG, Dotti CG. Lipid changes in the aged brain: effect on synaptic function and neuronal survival. *Prog. Lipid Res.*, **51**, 23–35 (2012).
 - 33) Kosicek M, Hecimovic S. Phospholipids and Alzheimer's disease: alterations, mechanisms and potential biomarkers. *Int. J. Mol. Sci.*, **14**, 1310–1322 (2013).
 - 34) Leskova GF, Kryzhanovsky GN. Changes in phospholipid composition of cardiomyocyte plasma membranes during hemorrhagic shock. *Bull. Exp. Biol. Med.*, **151**, 284–287 (2011).
 - 35) Pangestuti R, Kim SK. Neuroprotective effects of marine algae. *Mar. Drugs*, **9**, 803–818 (2011).
 - 36) Pozharitskaya ON, Obluchinskaya ED, Shikov AN. Mechanisms of Bioactivities of Fucoidan from the Brown Seaweed *Fucus vesiculosus* L. of the Barents Sea. *Mar. Drugs*, **18** (2020).
 - 37) Yuan Y, Macquarrie D. Microwave assisted extraction of sulfated polysaccharides (fucoidan) from *Ascophyllum nodosum* and its antioxidant activity. *Carbohydr. Polym.*, **129**, 101–107 (2015).
 - 38) Qi H, Zhang Q, Zhao T, Chen R, Zhang H, Niu X, Li Z. Antioxidant activity of different sulfate derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta) *in vitro*. *Int. J. Biol. Macromol.*, **37**, 195–199 (2005).
 - 39) Zhang Q, Li N, Zhou G, Lu X, Xu Z, Li Z. *In vivo* antioxidant activity of polysaccharide fraction from *Porphyra haitanensis* (Rhodophyta) in aging mice. *Pharmacol. Res.*, **48**, 151–155 (2003).
 - 40) Krishtal J, Bragina O, Metsla K, Palumaa P, Tougou V. *In situ* fibrillizing amyloid-beta 1-42 induces neurite degeneration and apoptosis of differentiated SH-SY5Y cells. *PLoS One*, **12**, e0186636 (2017).
 - 41) Okimotoa Y, Watanabea A, Nikia E, Yamashitab T, Noguchia N. A novel fluorescent probe diphenyl-1-pyrenylphosphine to follow lipid peroxidation in cell membranes. *FEBS Lett.*, **474**, 137–140 (2000).