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Fucoidan Protects LLC-PK1 Cells against AAPH-induced Damage

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Abstract

This study was designed to investigate the protective effect of fucoidan against AAPH-induced oxidative stress in LLC-PK1 cells (porcine kidney epithelial cells). Oxidative stress was induced by exposing of LLC-PK1 cells to the 1 mM 2,2'-azobis(2-amidino propane) dihydrochloride (AAPH) for 24 hr. Exposure of LLC-PK1 cells to 1 mM AAPH for 24 hr resulted in a significant ($p<0.05$) decrease in cell viability, but fucoidan treatment protected LLC-PK1 cells from AAPH-induced cell damage in a dose dependant manner. To investigate the protective action of fucoidan against AAPH-induced damage of LLC-PK1 cells, we measured the effects of fucoidan on lipid peroxidation and antioxidant enzymes activities of AAPH treated cells as well as scavenging activities on superoxide anion radical and hydroxyl radical. Fucoidan had protective effect against the AAPH-induced LLC-PK1 cellular damage and decreased lipid peroxidation and increased activities of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-px). Furthermore, fucoidan showed strong scavenging activity against superoxide anion radical. The IC_{50} value of fucoidan was 48.37±1.54 μg/mL for superoxide anion radical scavenging activity. The fucoidan also had high hydroxyl radical scavenging activity (IC_{50}=32.03 μg/mL). These results indicate that fucoidan protects against AAPH-induced LLC-PK1 cell damage by inhibiting lipid peroxidation, increasing antioxidant enzyme activities and scavenging offree radicals.

Key words: fucoidan, AAPH, radical scavenging, antioxidant activity

INTRODUCTION

2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) generates free radicals at a constant and measurable rate by its thermal decomposition without biotransformation. The free radicals generated from AAPH react with oxygen molecules rapidly to yield peroxyl radicals. The lipid peroxyl radicals attack other lipid molecules to form lipid hydroperoxide and new lipid radicals. This reaction takes place repeatedly with resultant attacks upon various biological molecules, and it induces physiochemical alterations and cellular damage (1). Finally, AAPH causes a diverse array of pathological changes. Therefore, an AAPH-intoxication experiment may be a promising assay system for evaluating biological activities of antioxidants. In addition, LLC-PK1, a renal-tubular epithelial cell line, is susceptible to oxidative stress, resulting in cell death or injury. It was reported that AAPH led to the decreased viability of LLC-PK1 renal epithelial cells (2).

Oxidative stress is caused by an imbalance between antioxidant systems and the production of oxidants, including ROS. The reactive oxygen species (ROS) include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH); accumulation of these ROS can result in oxidative stress that has been related to human diseases such as cardiovascular diseases, cancers, aging and diabetes (3-5). Lipid peroxidation is also associated with an overproduction of ROS, including superoxide radical, hydroxyl radical and hydrogen peroxide. It is known that superoxide radical is associated with several pathophysiological processes, and it can not only directly initiate lipid peroxidation, but also be converted into more reactive radical species, such as hydroxyl radical that also initiate lipid peroxidation (6). Many natural products are known to produce significant amounts of antioxidants to control the oxidative stress caused by ROS (7,8).

Many studies have now confirmed that exogenic antioxidants are essential for counteracting oxidative stress. These antioxidants mainly come from plants in the form of phenolic compounds, ascorbic acid and carotenoids. The quest for natural antioxidants for dietary, cosmetic and pharmaceutical uses has become a major industrial and scientific research challenge over the last 20 years (9). Seaweeds have demonstrated free radical scavenging activities, and thus may help slow aging and prevent some chronic diseases. Almost all seaweed species have substantial ability to scavenge hydroxyl radicals (10) and are considered to be a rich source of antioxidants (11).

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Fucoidans, laminarans, and alginic acids are the most abundant polysaccharides of brown seaweeds. Especially fucoidans, the family of sulfated heteropolysaccharides, composed mainly of 1,3-linked fucose residues, are constituents of brown algae (12-14). Several biological activities have been attributed to the fucoidans, such as antioxidant, anticoagulant, antithrombotic, antiinflammatory, antitumoral and antiviral (15-22). However, no study has directly investigated the protective effects of fucoidanon oxidative damage.

On the basis of the above reports and our preliminary study, fucoidan is expected to be a potential therapeutic agent for free radical induced cell damage. The aim of this work was to investigate whether fucoidan can protect LLC-PK1 cells from AAPH toxicity. In addition, we evaluated the protective mechanism offucoidan, we examined the effects of fucoidan on lipid peroxidation of AAPH-treated cells and scavenging activity on free radicals.

MATERIALS AND METHODS

Materials and chemicals

Fucoidan purified from *F. vesiculosus*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,2′-azobis(2-amidinoproapne) dihydrochloride (AAPH) and bovine serum albumin were purchased from Sigma (St. Louis, MO). All other chemicals and solvents used were of analytical grade. The fucoidan was dissolved in phosphate buffered saline (PBS; Invitrogen, Carlsbad, CA) and used in concentrations of up to 100 μg/mL (25, 50, 100 μg/mL), but mainly 50 μg/mL in this study.

Cell culture

Commercially available porcine kidney epithelial cells (LLC-PK1 cells, passages 7～35) were maintained at 37°C in a humidified atmosphere of 5% CO_2 in culture plates with a 5% FBS-supplemented D-MEM/F-12 medium. After confluence had been reached, the cells were seeded into 24 well plates (4×10^5 cells/well) or 10-mm dishes (5×10^6 cells/dish). Two hours later, 1 mM AAPH was added to each of the wells, pre-incubated for 24 hr, and then incubated with fucoidan (25, 50, 100 μg/mL) for 24 hr (23). The proper concentration of AAPH and the incubation time were determined by the preliminary experiment.

Cell viability

The MTT assay of cell viability was performed following a well-described procedure with minor modifications (24). Cells were plated in 24-well cell culture plates at a density of 4×10^5 cells per 24 well. At the end of culture, 100 μL of MTT solution (5 mg/mL in PBS) was added to each well containing 1 mL medium. After 4 hr of incubation, the media were removed and formazan crystals were solubilized with 300 μL DMSO. The absorbance of each well was then read at 540 nm using a microplate reader.

Determination of lipid peroxidation

Lipid peroxidation was measured as thiobarbituric acid reactive substances (TBARS) production (25). Cells (4×10^4 cells/well) in 24-well plates were first incubated with 1 mM AAPH for 24 hr, and then incubated with fucoidan for 24 hr. 200 μL of each medium supernatant was mixed with 400 μL of TBARS solution then boiled at 95°C for 30 min. The absorbance at 532 nm was measured and TBARS concentrations were extrapolated from the 1,1,3,3-tetraethoxypropane serial dilution standard curve, TBARS values were then expressed as equivalent nmoles of malondialdehyde (MDA).

Superoxide anion radical scavenging activity

The scavenging potential for superoxide anion radical was analyzed via a hypoxanthine/xanthine oxidase generating system coupled with nitroblue tetrazolium (NBT) reduction following the method of Kirby and Schmidt (26). The reaction mixture contained 125 μL of buffer (50 mM K_2HPO_4/KOH, pH 7.4), 20 μL of 15 mM Na_2EDTA in buffer, 30 μL of 3 mM hypoxanthine in buffer, 50 μL of xanthine oxidase in buffer (1 unit per 10 mL buffer) and 25 μL of plant extract in buffer (a diluted sonicated solution of 10 μg per 250 μL buffer). The absorbance of the solution was measured at 540 nm. Superoxide scavenging activity was expressed as % inhibition compared to the blank.

Hydroxyl radical scavenging activity

The reaction mixture contained 0.45 mL of 0.2 M sodium phosphate buffer (pH 7.0), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$-EDTA, 0.15 mL of 10 mM H_2O_2, 0.525 mL of H_2O, and 0.075 mL of sample solution. The reaction was started by the addition of H_2O_2. After incubation at 37°C for 4 hr, the reaction was stopped by adding 0.75 mL of 2.8% trichloroacetic acid and 0.75 mL of 1.0% of 2-tribarbitric acid in 50 mM NaOH, the solution was boiled for 10 min, and then cooled in water. The absorbance of the solution was measured at 520 nm. Hydroxyl radical scavenging activity was evaluated as the inhibition rate of 2-deoxyribose oxidation by ·OH (27).

Antioxidant enzyme assays

Cells (5×10^6 cells/dish) in 10-mm dishes were pre-incubated for 2 hr. 1 mM of AAPH was added to all of the wells, pre-incubated for 24 hr, and then incubated with or without the indicated concentrations of fucoidan

for 24 hr. The medium was removed and the cells were washed twice with PBS. One mL of 50 mmol/L potassium phosphate buffer with 1 mmol/L EDTA (pH 7.0) was added and cells were scraped. Cell suspensions were sonicated on ice three times for 5 sec each time and then cell sonicates were centrifuged at 10,000×*g* for 20 min at 4°C. Cell supernatants were used for antioxidant enzyme activities. The protein concentration was measured by using the method of Bradford (28) with bovine serum albumin as the standard. SOD activity was determined by monitoring the auto-oxidation of pyrogallol (29). A unit of SOD activity was defined as the amount of enzyme that inhibited the rate of oxidation of pyrogallol by 50%. GSH-px activity was measured by using the method of Lawrence and Burk (30). One unit of GSH-px was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per minute.

Statistical analysis

The data are expressed as mean±SD. The statistical analysis was performed with SAS software. The values were evaluated by one-way analysis of variance (ANOVA) followed by post-hoc Duncan's multiple range tests.

RESULTS

Cell viability

LLC-PK1 cells were cultured with 0.01, 0.05, 0.1 and 1 mg/mL of fucoidan for 1 day and then cell viability was examined by the MTT assay (Fig. 1). Our results showed that fucoidan was not significantly cytotoxic to LLC-PK1 cells and the cytotoxicity of fucoidan itself was notobserved. Therefore, we evaluated the protective effect of fucoidan in AAPH-treated LLC-PK1 cells. The cells were pretreated with 1 mM of AAPH and pre-incubated for 24 hr, then added fucoidan of 25, 50, and 100 μg/mL. The cell survival was determined after 24 hr. As shown in Fig. 2, cell viability was notably decreased in cells treated with AAPH only. However, treatment with fucoidan resulted in increase of cell survival in a dose dependent manner. At a concentration of 100 μg/mL, fucoidan revised cell viability from 10.84% in cell treated with AAPH only to 80.23%.

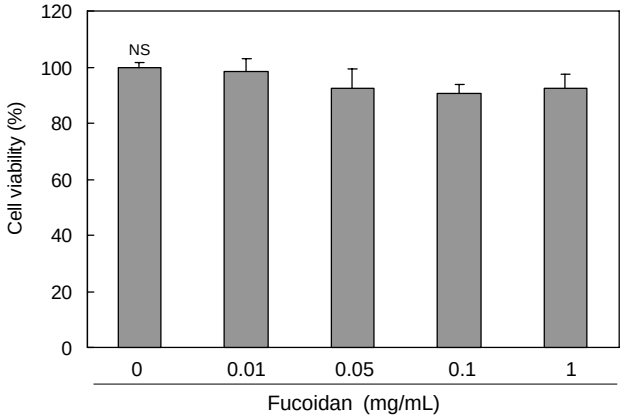

Fig. 1. Cytotoxicity of fucoidan on LLC-PK1. Cells were pretreated with various concentrations of fucoidan for 1 day and cell viability was determined by the MTT assay. Each value is expressed as mean±SD (n=3). NS: not significant.

Lipid peroxidation

To investigate the protective action of fucoidan against AAPH induced cytotoxicity, we examined the effects of fucoidan on lipid peroxidation and antioxidant enzyme activities of LLC-PK1 cells, and the scavenging activities of fucoidan towards reactive oxygen species, such as superoxide radical and hydroxyl radical. Fig. 3 showed the inhibitory effect of fucoidan on AAPH-induced lipid peroxidation in LLC-PK1 cells. The lipid peroxidation was markedly increased in the cells treated with AAPH, as shown by TBARS of 0.72±0.05 nmol MDA in the LLC-PK1 cells exposed to 1 mM AAPH compared with TBARS of 0.18±0.02 nmol MDA in untreated cells. However, treatment of fucoidan significantly inhibited the lipid peroxidation in a dose dependent manner ($p<0.05$). At a concentration of 25, 50, and 100 μg/mL, fucoidan showed significant decrease in MDA level ($p<0.05$).

Scavenging activity

Since lipid peroxidation is associated with overproduction of ROS, we examined the scavenging activity of fucoidan on ROS, such as superoxide anion radical

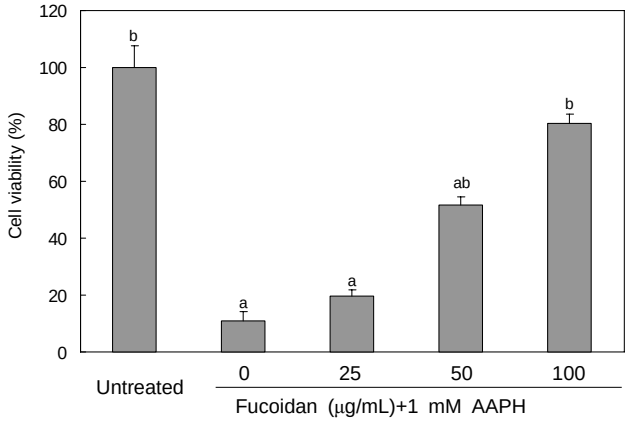

Fig. 2. Protective effects of fucoidan against AAPH-induced cell damage. The cells were seeded into 24 well plates (4×10^5 cells/well), two hours later, 1 mM of AAPH was added to all of the well and preincubated for 24 hr, then incubated with fucoidan (25, 50, 100 μg/mL) for 24 hr. Untreated is negative control without AAPH treatment. Each value is expressed as mean±SD (n=3). A value sharing the same superscript is not significantly different at $p<0.05$.

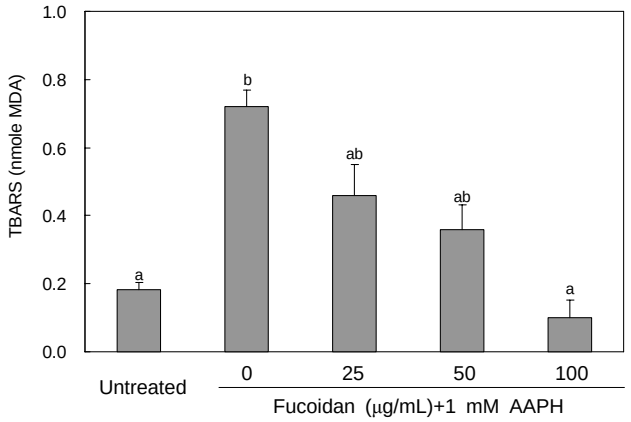

Fig. 3. Inhibitory effect of fucoidan on AAPH-induced lipid peroxidation of LLC-PK1 cells. The cells were seeded into 24 well plates (4×10^5 cells/well), two hours later, 1 mM of AAPH was added to all of the well and preincubated for 24 hr, then incubated with fucoidan (25, 50, 100 μg/mL) for 24 hr. Untreated is a negative control without AAPH treatment. Each value is expressed as mean±SD (n=3). A value sharing the same superscript is not significantly different at $p<0.05$.

and hydroxyl radical. Fig. 4 shows the percentage of superoxide anion radical scavenging activity by 25, 50 and 100 μg/mL of fucoidan. The superoxide anion radical scavenging activity was notably increased in the presence of 25, 50, 100 μg/mL of fucoidan. The percentage of superoxide anion radical scavenging activity with 50, μg/mL of fucoidan was 52.31%, with an IC_{50} value of 48.37±1.54 μg/mL. It is known that superoxide anion radical is associated with severe pathophysiological processes, and it can not only directly initiate lipid production, but also be converted into more reactive radical species, such as hydroxyl radical.

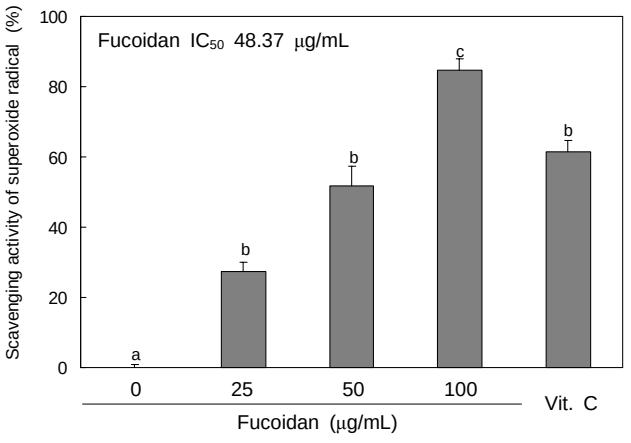

Fig. 4. Scavenging activity of fucoidan on the superoxide anion radical. IC_{50}: The concentration of sample required for 50% inhibition, Vit. C: vitamin C. The final concentration of vitamin C in the reaction mixture was 50 μg/mL. Each value is expressed as mean±SD (n=3). A values sharing the same superscript is not significantly different at $p<0.05$.

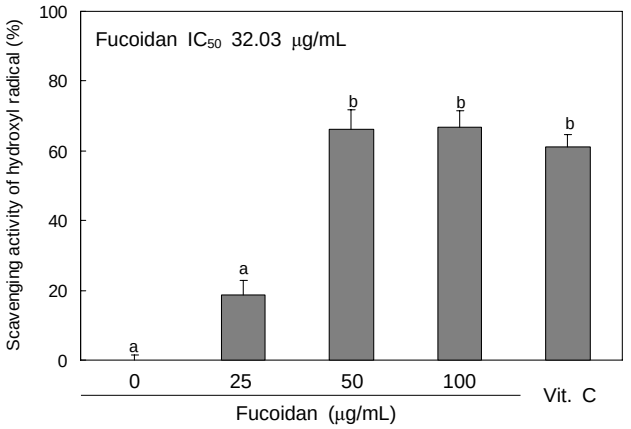

Fig. 5. Scavenging activity of fucoidan on hydroxyl radical. IC_{50}: The concentration of sample required for 50% inhibition, Vit. C: vitamin C. The final concentration of vitamin C in the reaction mixture was 50 μg/mL. Each value is expressed as mean±SD (n=3). A values sharing the same superscript is not significantly different at $p<0.05$.

The fucoidan also showed significant hydroxyl radical scavenging activity (Fig. 5). However, there was not a cumulative effect of fucoidan on hydroxyl radical scavenging activity at concentrations higher than 50 μg/mL. The scavenging activity of fucoidan against hydroxyl radical was higher than that of vit C, as 66.20% at concentration of 50 μg/mL. IC_{50} value of it was 32.03±2.34 μg/mL.

Antioxidant enzyme activities

Cells are protected from activated oxygen species by endogeneous antioxidant enzymes such as SOD and GSH-px. The effects of fucoidan on antioxidant enzyme activities in AAPH treated LLC-PK1 cells are shown in Table 1. Pretreatment with 1 mM AAPH for 24 hr significantly ($p<0.05$) decreased SOD activity of LLC-PK1 cells compared with untreated cells. Treatment of LLC-PK1 cells with fucoidan increased SOD activity of 1 mM AAPH pretreated cells. After the cells were treated with 25 and 50 μg/mL of fucoidan, SOD activities were not significantly increased. However, treatment with 100 μg/mL of fucoidan resulted in a significant ($p<0.05$) increase of SOD activity, as shown SOD activity of 63.44±3.26 unit/mg protein.

GSH-px activity in LLC-PK1 cells treated with 1 mM AAPH was significantly decreased in comparison to untreated cells ($p<0.05$). Treatment of 1mM AAPH pretreated cells with fucoidan resulted in an increase of GSH-px activity. GSH-px activities were also not significantly increased in the cells treated with 25 and 50 μg/mL of fucoidan. However, treatment with fucoidan of 100 μg/mL resulted in a significant ($p<0.05$) increase in GSH-px activity, as shown by the GSH-px activity

Table 1. Effects of fucoidan on antioxidant enzyme activities in AAPH treated LLC-PK1 cells

	Untreated	1 mM AAPH	Fucoidan (μg/mL)+1 mM AAPH		
			25	50	100
SOD (unit/mg protein)	62.19 ± 4.52^{b}	10.28 ± 2.19^{a}	13.10 ± 3.52^{a}	17.54 ± 0.32^{a}	63.44 ± 3.26^{b}
GSH-px (unit/mg protein)	5.70 ± 0.59^{b}	2.57 ± 0.25^{a}	2.11 ± 0.18^{a}	3.17 ± 0.14^{a}	5.86 ± 1.30^{b}

The cells were seeded into 10-mm dishes (5×10^6 cells/dish), two hours later, 1 mM of AAPH was added to all of the wells and preincubated for 24 hr, then incubated with fucoidan (25, 50, 100 μg/mL) for 24 hr. Untreated is negative control without AAPH treatment. Each value is expressed as mean ± SD (n=3). A value sharing the same superscript is not significantly different at $p < 0.05$. SOD: superoxide dismutase, GSH-px: glutathione peroxidase.

of 5.86 ± 1.30 unit/mg protein.

DISCUSSION

Fucoidan extracted from brown seaweed is a heteropolysaccharide, mainly composed of fucose, galactose and sulfate, with smaller amounts of mannose, glucuronic acid, glucose, rhamnose, arabinose and xylose. A previous study revealed that fucoidan and different fractions extracted from *L. japonica*, brown seaweed, were antioxidative, but their antioxidant abilities were quite different (31,32). In the present study, we investigated the protective effect of fucoidan on AAPH-induced LLC-PK1 cells damage. LLC-PK1 renal tubular epithelial cells are susceptible to free radicals (2) and an *in vitro* model of oxidative damage, in which LLC-PK1 cells are exposed to free radicals, would appear useful for searching for agents that can provide effective protection. Although various kinds of free radical initiators are known, AAPH has been used easily and successfully as a radical initiator. The free radicals generated from AAPH ultimately cause physicochemical alterations and cellular damage through the processes of oxidation reaction (33,34).

The exposure of LLC-PK1 cells to 1 mM AAPH for 24 hr resulted in a marked decrease in cell viability. However, fucoidan treatment exerted significant increase ($p < 0.05$) in cell viability in a dose dependent manner, suggesting that fucoidan protected LLC-PK1 cells from AAPH-induced cytotoxicity. To investigate the protective action of fucoidan against AAPH induced cytotoxicity, we measured the inhibitory effect of fucoidan on lipid peroxidation of AAPH treated cells. Lipid peroxidation in biological systems has long been thought to be a toxicological phenomenon, resulting in pathological consequences. Hence, measurement of lipid peroxidation end products such as TBARS provides a good index of cell destruction. Previous studies have shown that AAPH induced cytotoxicity is mediated, at least in part, through increases in the amount of lipid peroxidation of cell membranes (35,36). In this study, we demonstrated that lipid peroxidation was notably increased in LLC-PK1 cells pretreated with AAPH, but treatment of fucoidan inhibited lipid peroxidation in a dose dependent manner. Therefore, our results indicate that the inhibition of lipid peroxidtion may be one of the possible actions that are responsible for the protective effect of fucoidan against AAPH toxicity in LLC-PK1 cells.

Since lipid peroxidation is associated with overproduction of ROS and also regarded as one of the basic mechanisms of cell damage mediated by free radicals (37), we examined the scavenging activity of fucoidan on superoxide anion radical and hydroxyl radical. It is known that superoxide anion radical is associated with several pathophysiological processes, and it can not only directly initiate lipid peroxidation, but also be converted into more reactive radical species, such as hydroxyl radical, that also initiate lipid peroxidation (38). In this study, the superoxide anion radical scavenging activity was significantly increased in the presence of 25, 50, and 100 μg/mL of fucoidan. Previous studies have shown that superoxide radicals can be converted into hydrogen peroxide by SOD and the hydrogen peroxide can produce highly reactive hydroxyl radicals in the presence of metal ions (39). In our investigation, fucoidan over 50 μg/mL also showed significant hydroxyl radical scavenging activity ($p < 0.05$). Thus, the removal of hydroxyl radical as well as superoxide anion radical may play important roles in antioxidant defense within LLC-PK1 cells.

Our results also showed a significant decrease of antioxidant enzyme activities such as SOD and GSH-px in LLC-PK1 cells treated with AAPH compared with untreated cells. Fucoidan of 100 μg/mL increased the SOD activity significantly ($p < 0.05$). SOD, the endogenous scavenger, catalyses the dismutation of the highly reactive superoxide anion to H_2O_2 (40). The superoxide anion radical scavenging activity of SOD is effective only when it is followed by the actions of GSH-px, because the activity of SOD generates H_2O_2, which needs to be further scavenged by GSH-px. GSH-px catalyses the reduction of H_2O_2 at the expense of reduced GSH (i.e.

H_2O_2+2 GSH→2 H_2O+GSSG) (41). In the present study, GSH-px activity also significantly increased ($p < 0.05$) in the presence of fucoidan of 100 μg/mL.

In conclusion, we demonstrated that fucoidan can protect LLC-PK1 cells from AAPH-induced cellular damage, which may, in part, be linked to an inhibitiory effect of fucoidan on lipid peroxidation, ROS radical scavenging activity and increases in antioxidant enzyme activities by fucoidan.

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