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RESEARCH ARTICLE

Antioxidant Effect of *Phaeodactylum tricornutum* in Mice Fed High-fat Diet

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Abstract High-fat diet (HFD)-induced obesity is associated with oxidative stress. The purpose of this study was to examine the antioxidant effect of *Phaeodactylum tricornutum* extract in mice with diet-induced obesity. Four-week-old C57BL/6J mice were fed a normal diet or HFD with and without 0.7% *P. tricornutum* lipid extract corresponding to 0.2% fucoxanthin for 8 weeks. *P. tricornutum* significantly decreased body weight and epididymal white adipose tissue in mice fed the HFD. Serum triglyceride, glucose, insulin, and leptin levels, as well as homeostasis model assessment for insulin resistance (HOMA-IR) values, were significantly lower in the *P. tricornutum* group than in the HFD group. *P. tricornutum* significantly decreased thiobarbituric acid reactive substances (TBARS) and increased glutathione and the activities of superoxide dismutase, catalase, and glutathione peroxidase in the liver compared with the HFD group. Thus, *P. tricornutum* could exert anti-obesity and antioxidant effects in mice fed a HFD.

Keywords: *Phaeodactylum tricornutum,* fucoxanthin, obesity, antioxidant, glucose

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Introduction

Obesity, which is defined as the excessive accumulation of body fat that may impair health, has become an epidemic worldwide health problem. Obesity represents a strong risk for the development of type 2 diabetes, hyperlipidemia, cardiovascular diseases (CVDs), and some cancers (1). The consumption of a high-fat diet (HFD) has been demonstrated to induce obesity, insulin resistance, and hyperlipidemia in experimental animals (2-4). Mounting evidence indicates that obesity induces oxidative stress (5,6). Reactive oxygen species (ROS) are overproduced and the antioxidant defense system, including antioxidants and antioxidant enzymes, is inhibited in obese animals and humans. Such oxidative stress could underlie the pathogenesis of obesity-related chronic diseases, such as CVDs and diabetes (7).

Recently, fucoxanthin has received much attention as a compound having an anti-obesity effect. Fucoxanthin is a carotenoid that is abundant in marine environments, especially in macroalgae and microalgae (8). The fucoxanthin-rich fraction or fucoxanthin prepared from macroalgae such as *Undaria pinnatifida* has been shown to exert anti-obesity and hypolipidemic effects in diet-induced and genetically obese animals (3,4,9-11). Recently, the marine diatom *Phaeodactylum tricornutum* has been reported to be a good source of fucoxanthin (12). *P. tricornutum* is a microalga that contains fucoxanthin as a major pigment, which plays a crucial role in the light-harvesting complexes of photosystems (13). Because fucoxanthin is at least 10 times more abundant in *P. tricornutum* than in macroalgae (12), *P. tricornutum* is expected to be beneficial in the management of obesity. However, the anti-obesity effect of *P. tricornutum* has not been studied *in vivo.*

Fucoxanthin has demonstrated antioxidant activity *in vitro*. It has singlet oxygen-quenching and radical scavenging activities (14-16) and has been shown to inhibit the

production of intracellular ROS and cell damage induced by hydrogen peroxide (17). Thus, fucoxanthin could be effective in alleviating oxidative stress associated with obesity. However, most investigations of the antioxidant effects of fucoxanthin have focused on *in vitro* studies, whereas *in vivo* studies have been limited. Therefore, in the current study, the effects of fucoxanthin-rich extract prepared from *P. tricornutum* on obesity and antioxidant status in HFD-induced obese mice were evaluated.

Materials and Methods

Reagents Casein, D,L-methionine, a mineral mixture, and a vitamin mixture were purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA, USA) and *tert*-butyl hydroquinone from Fluka Co. (Milwaukee, WI, USA). Cornstarch was acquired from Daesang Co. (Seoul, Korea), and sucrose and soybean oil were purchased from Cheiljedang Co. (Seoul, Korea). Lard was obtained from Lotte Samgang Co. (Seoul, Korea). Assay kits for triglycerides, cholesterol, and glucose were obtained from Asan Co. (Seoul, Korea). The insulin assay kit was purchased from Linco Co. (St. Charles, MO, USA) and the leptin assay kit was from BioVendor Laboratorní Medicina a.s. (Modrice, Czech Republic). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Cellulose, choline bitartrate, and all other reagent-grade chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of fucoxanthin-rich lipid extract of *P. tricornutum* *P. tricornutum* was obtained from the Korea Marine Microalgae Culture Center (Seoul, Korea). The alga was cultivated in a 30-L plastic cylinder at 20°C, where air was continuously supplied at 5 L/min by air lift. The light intensity provided by 60 W fluorescent lamps was maintained at 2,500 lx. The alga was cultured in Conway medium, which was prepared with filter-sterilized seawater, for 5 days. The cells were precipitated by adding 200 ppm $Al_2(SO_4)_3$ (v/v), collected by centrifugation at 10,000×*g* for 10 min using a basket centrifuge (Hansung, Seoul, Korea), and freeze dried. The sample was pulverized into powder by grinding in a mortar and stored at −80°C prior to extraction.

P. tricornutum was extracted with 4 volumes of acetone at 45°C for 2 h and centrifuged at 10,000×*g* for 10 min. After the supernatant was filtered with Whatman no. 2 filter paper, it was dried using a rotary evaporator under vacuum at 40°C. The dried supernatant (60 mg) was dissolved in methanol (100 mL) and water (10 mL) and then hexane (100 mL) was added for partitioning in methanol/water/hexane (10:1:10, v/v/v). The lower phase (methanol/water phase) was washed several times with hexane until colorless and then concentrated using a rotary evaporator under vacuum. The concentrated methanol/water phase (2 mL) was loaded onto a silica gel column (2.0×30.0 cm), and then eluted with 100 mL hexane/acetone (6:3, v/v). The eluent was concentrated using a rotary evaporator.

The concentration of fucoxanthin in the extract was analyzed by HPLC (Trilution LC 2.1; Gilson Inc., Middleton, WI, USA) using an ODS-80Ts column (4.6×150 mm, 5.0 μm; Tosoh Corporation, Tokyo, Japan) according to the method of Liu *et al*. (18). The mobile phase consisted of methanol/acetonitrile (70:30, v/v) and the flow rate was 0.8 mL/min. For the detection of fucoxanthin, the absorbance was monitored at 450 nm using a UV-Vis detector. The *P. tricornutum* extract was determined to contain 31.9% fucoxanthin.

Animals and experimental protocol Four-week-old male C57BL/6J mice (n=21) obtained from Bio Genomics, Inc. (Seoul, Korea) were housed individually in plastic cages under standard laboratory conditions at a temperature of 24±5°C and a relative humidity of 55±5% with a regular light cycle (06:00-18:00 light, 18:00-06:00 dark). All mice were divided randomly into 3 groups after 1 week of adaptation, during which time they had free access to commercial chow. Normal diet (ND), HFD, and HFD with *P. tricornutum* (HFD+PT) groups were fed a basal diet based on the standard AIN-76 diet composition containing 5% corn oil, a HFD containing 3% corn oil and 17% lard, and a HFD containing 0.7% *P. tricornutum* extract, respectively, *ad libitum* for 8 weeks (Table 1). The compositions of the ND and HFD were selected based on a study by Kim *et al.* (2), in which the fat-calorie percentages were 11.5 and 39.0%, respectively. Body weight and food intake were measured once and 3 times a week, respectively. All procedures for this study were approved by the Animal Resource Center at Inje University.

Analytical sample preparation At the end of the experiment, the mice were sacrificed by heart puncture following an overnight fast. Blood and liver samples were collected immediately and blood samples were centrifuged at 1,500×*g* for 15 min to acquire serum. Serum and liver samples were stored at −70°C for further analysis. Epididymal white adipose tissue (WAT) samples were collected and weighed.

Biochemical analyses of serum Serum triglyceride, total cholesterol, and glucose levels were measured by enzymatic methods using commercial kits. Serum leptin and insulin levels were determined by enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay, respectively, using

Table 1. Composition of experimental diets (%)

Ingredient	Group[1)]		
	ND	HFD	HFD+PT
Casein	20.00	20.00	20.00
Corn starch	15.00	12.10	12.10
Sucrose	50.00	37.00	37.00
α-Cellulose	5.00	5.00	5.00
Corn oil	5.00	3.00	2.30
Lard	-	17.00	17.00
Vitamin mixture[2)]	1.00	1.20	1.20
Mineral mixture[2)]	3.50	4.20	4.20
D,L-Methionine	0.30	0.30	0.30
Choline bitratrate	0.20	0.20	0.20
tert-Butyl hydroquinone[3)]	0.001	0.004	0.004
P. tricornutum extract	-	-	0.70

[1)]NC, normal diet; HFD, high-fat diet; HFD+PT, high-fat diet containing 0.7% *P. tricornutum* extract
[2)]AIN-76 Vitamin mixture, AIN-76 Mineral mixture, ICN Pharmaceuticals, Costa Mesa, CA, USA
[3)]Antioxidative agent, 0.01 g/50 g of lipids

commercial kits. Homeostasis model assessment of insulin resistance (HOMA-IR) values was estimated by dividing the product of fasting insulin (μU/mL) and glucose (mmol/L) levels by 22.5 (19).

Measurement of lipid peroxide and glutathione concentrations in liver Hepatic lipid peroxidation was evaluated as thiobarbituric acid reactive substances (TBARS) using the method of Ohkawa *et al.* (20). Liver tissue was homogenized in 5 volumes of 10 mM sodium phosphate buffer (pH 7.4). The homogenate (0.5 mL) and the solution composed of 15% trichloroacetic acid (TCA), 0.4% thiobarbituric acid (TBA), and 2.5% HCl (1 mL) were combined and placed at 100°C for 45 min. After cooling, the reaction mixture was centrifuged at 1,500×*g* for 10 min. The absorbance of adduct of malondialdehyde (MDA) and TBA formed was measured at 534 nm. The protein content was measured using the Bradford method (21) with bovine serum albumin as the standard. The level of lipid peroxides was expressed as nmol MDA/mg protein. The glutathione (GSH) level in the liver was determined using the method of Ellman (22). Each liver sample was homogenized in 9 volumes of 0.1 mM phosphate buffer (pH 7.4). After centrifugation (10,000×*g* at 4°C for 30 min), the supernatant (0.5 mL) was mixed with 4.5 mL 5,5-dithiobis-2-nitrobenzonic acid (DTNB) working solution containing 10 mM DTNB and 0.1 M phosphate buffer (pH 8.0; 1:90, v/v). The reaction mixture was incubated at room temperature for 15 min and then the absorbance was measured at 534 nm.

Measurement of antioxidant enzyme activities in the liver To measure the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), liver tissue samples were prepared. Each liver sample was homogenized in 10 volumes of 50 mM phosphate buffer (pH 7.4) and centrifuged at 1,000×*g* at 4°C for 10 min. The supernatant was used for the measurement of CAT and GSH-Px activities according to the Abei method (23) and the method developed by Paglia and Valentine (24), respectively. One unit of CAT activity was defined as μmol of hydrogen peroxide reduced/min. One unit of GSH-Px activity was defined as nmol of NADPH substrate converted to $NADP^+$/min. To measure SOD activity, the supernatant was further centrifuged at 10,000×*g* for 20 min and activity was measured in the final supernatant according to the method developed by Marklund and Marklund (25). One unit of SOD activity was defined as the amount of enzyme that reduced the rate of pyrogallol autoxidation by 50%. All assays were carried out in triplicate using a spectrophotometer (U-2000; Hitachi Ltd., Tokyo, Japan). The enzyme activities were expressed as specific activity (U/mg protein).

Statistical analyses All data are expressed as the mean± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) with a *post hoc* Tukey's test was used to evaluate significant differences among groups ($p<0.05$).

Results and Discussion

Body weight, epididymal fat weight, and food intake The body weights, weight gains, weights of epididymal WAT, food intakes, and feed efficiency ratios (FERs) of the animals are listed in Table 2. The final body weight, weight gain, and epididymal fat pad weight (mg/100 g BW) were significantly higher in the HFD group than in the ND group. However, the consumption of fucoxanthin-rich lipid extract of *P. tricornutum* at 0.7% of the diet significantly reduced these levels by 8, 29, and 24%, respectively, compared with the HFD group ($p<0.05$, $p<0.01$, and $p<0.01$, respectively). These values of the HFD+PT group did not differ significantly from those of the ND group. Although food intake did not differ significantly among the 3 groups, the FER was highest in the HFD group, lowest in the ND group, and intermediate in the HFD+PT group ($p<0.05$).

Since Maeda *et al*. (9) first reported that lipids from *U. pinnatifida* offered at 2% of diet (equivalent to 0.2% fucoxanthin) decreased abdominal WAT weights in rats and *KK-Ay* mice, many studies have shown anti-obesity

Table 2. Body weight, food intake, and feed efficiency ratio of C57BL/6J mice fed the experimental diets

Group[1)]	ND	HFD	HFD+PT
Initial body weight (g)	20.7±0.2[ns2)]	20.5±0.2	20.8±0.3
Final body weight (g)	25.4±0.4[a]*	29.7±0.9[b]	27.3±0.5[a]
Weight gain (g/day)	0.085±0.006[a]**	0.163±0.018[b]	0.115±0.011[a]
Epididymal fat pad weight (mg/g BW)	20.0±1.2[a]**	28.2±1.7[b]	21.5±1.2[a]
Food intake (g/day)	3.55±0.15[ns]	3.31±0.14	3.21±0.14
FER[3)] (%)	2.43±0.21[a]*	4.87±0.40[c]	3.58±0.32[b]

[1)]NC, normal diet; HFD, high-fat diet; HFD+PT, high-fat diet containing 0.7% *P. tricornutum* extract
[2)]Values are presented as mean±SEM (n=7); Means in the same row not sharing a common alphabet are significantly different at *p<0.05 and **p<0.01; ns, not significant
[3)]Feed efficiency ratio (FER, %)=[body weight gain (g/day)/food intake (g/day)]×100

Table 3. Serum biochemisty and HOMA-IR in C57BL/6J mice fed the experimental diets

Group[1)]	ND	HFD	HFD+PT
Triglycerdie (mg/dL)	77.4±5.2[a]*[2)]	99.7±6.1[b]	82.4±4.0[a]
Total cholesterol (mg/dL)	98.4±5.2[ns]	116.4±6.8	96.6±5.8
Glucose (mg/dL)	102.5±4.3[a]*	135.2±6.2[b]	113.1±4.7[a]
Insulin (μU/mL)	26.7±1.8[a]**	45.5±2.0[b]	32.5±1.7[a]
Leptin (ng/mL)	5.9±0.3[a]**	9.9±0.4[b]	6.4±0.3[a]
HOMA-IR[3)]	6.8±0.6[a]**	15.3±1.2[b]	9.1±0.8[a]

[1)]NC, normal diet; HFD, high-fat diet; HFD+PT, high-fat diet containing 0.7% *P. tricornutum* extract
[2)]Values are presented as mean±SEM (n=7); Means in the same row not sharing a common alphabet are significantly different at *p<0.05 and **p<0.01; ns, not significant
[3)]Homeostasis model assessment for insulin resistance (HOMA-IR)=[insulin (μU/mL)×glucose (mmol/L)]/22.5

effects of fucoxanthin-rich lipid extract or pure fucoxanthin obtained from macroalgae in genetically and diet-induced obese rodents (3,4,9-11,26-28). In this study, *P. tricornutum* extract containing 31.9% fucoxanthin was offered to mice at 0.7% of HFD to investigate anti-obesity effect. The 0.7% *P. tricornutum* extract, which contains the equivalent of 0.2% fucoxanthin, effectively alleviated HFD-induced obesity. Fucoxanthin has shown anti-obesity effects by up-regulating the expression of uncoupling protein-1 (UCP-1) in WAT, which could play an important role in energy metabolism to avoid the excessive accumulation of body fat (9,26). Fucoxanthin has also been reported to inhibit pancreatic lipase activities *in vitro* and suppress triglyceride absorption *in vivo* (29). *P. tricornutum* extract could reduce weight gain and WAT accumulation by increasing UCP-1 expression in WAT and inhibiting the absorption of dietary fat by fucoxanthin.

Hypolipidemic and hypoglycemic effects of *P. tricornutum*

Serum triglyceride levels were significantly higher in the HFD group (99.7±6.1 mg/dL) than in the ND group (77.4 ±5.2 mg/dL; p<0.05; Table 3). However, the consumption of *P. tricornutum* significantly lowered serum triglyceride levels (82.4±4.0 mg/dL; p<0.05) by 17% compared with those in the HFD group. Serum total cholesterol levels did not differ significantly among the 3 groups. Fucoxanthin has been reported to reduce serum triglyceride levels in mice fed a HFD (27,28). The consumption of fucoxanthin effectively reduced the activities of fatty acid synthase, malic enzyme, and glucose-6-phosphate dehydrogenase in the liver (4,27) and WAT (28). In addition, supplementation of fucoxanthin resulted in increased excretion of fecal lipids (4). Thus, the hypotriglycemic effect of *P. tricornutum* could be due to reduced activities of lipogenic enzymes and increased excretion of dietary fat by fucoxanthin.

In the HFD group, serum glucose levels (135.2±6.2 mg/dL) were significantly higher compared with the ND group (102.5±4.4 mg/dL; p<0.05); the HFD+PT group showed a 16% reduction in glucose levels (113.1±4.7 mg/dL; p< 0.05) compared with the ND group. Serum insulin and leptin levels in the HFD group (45.5±2.0 μU/mL and 9.9± 0.4 ng/mL, respectively) were elevated compared with the ND group (26.7±1.8 μU/mL and 5.9±0.3 ng/mL, respectively; p<0.01), whereas these levels were significantly reduced in the HFD+PT group by both 29% (32.5±1.7 μU/mL and 6.4±0.3 ng/mL, respectively; p<0.01) compared with the HFD group. Serum glucose, insulin, and leptin levels of the HFD+PT and ND groups did not differ significantly. HOMA-IR values were significantly lower in the ND and HFD+PT groups (6.8±0.6 and 9.1±0.8, respectively) than in the HFD group (15.3±1.2; p<0.01).

A HFD promotes hyperleptinemia, hyperinsulinemia, and moderate hyperglycemia along with insulin resistance (3,11,26). In this study, mice fed a HFD showed higher

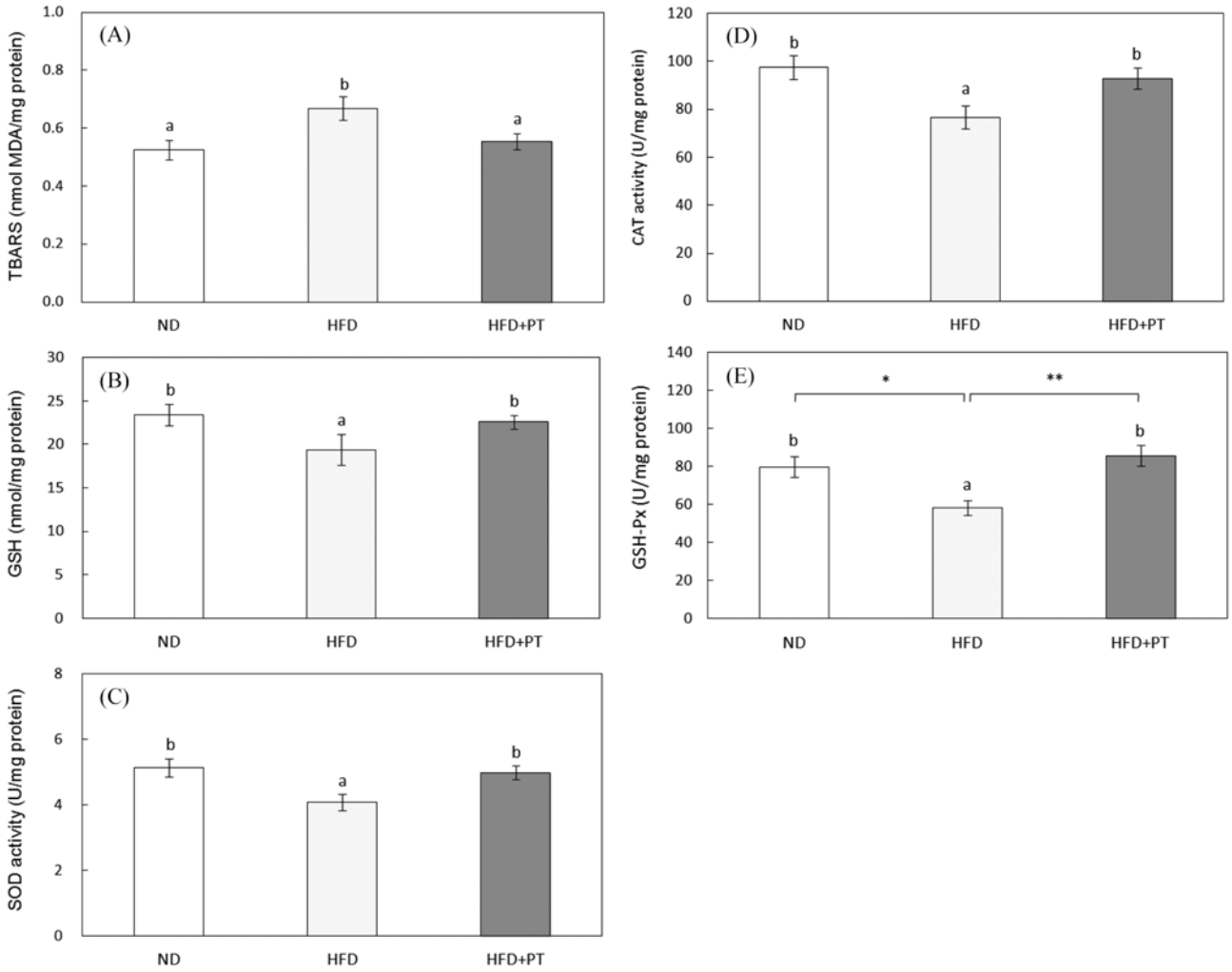

Fig. 1. Lipid peroxide and glutathione levels and activities of antioixdative enzymes of the liver in C57BL/6J mice fed the experimental diets. A, TBARS; B, GSH; C, SOD activity; D, CAT activity; and E, GSH-Px activity. NC, normal diet; HFD, high-fat diet; HFD+PT, high-fat diet containing 0.7% *P. tricornutum* extract. Values are presented as mean±SEM (n=7); Each bar with different letters is significantly different at $p<0.05$ (A, B, C, and D) and *$p<0.05$ and **$p<0.01$ (E).

serum insulin, leptin, and glucose levels and HOMA-IR, an index of insulin resistance, relative to the ND-fed mice. These elevated parameters were reduced by consumption of *P. tricornutum* to become comparable with those of the ND group. Leptin is secreted from adipocytes and controls body weight and fat accumulation by regulating energy expenditure (30). Obesity is related to leptin resistance and hyperleptinemia (31). Previous studies have reported that fucoxanthin decreases plasma leptin levels (27,28) and mRNA expression of leptin in the WAT of obese mice (26), which have been associated with the reduction of body fat accumulation. Visceral adipose tissue in obesity is strongly associated with insulin resistance (32). The reduction of body weight and epididymal WAT by *P. tricornutum* could contribute to the improvement of insulin resistance and, thereby, the suppression of hyperglycemia induced by a HFD. Nishikawa *et al.* (33) reported that fucoxanthin alleviated hyperglycemia and hyperinsulinemia by promoting glucose transporter 4 (GLUT4) translocation and expression in diabetic/obese *KK-Ay* mice. Further, fucoxanthin increased activities of glycolytic enzyme such as hepatic glucokinase and decreased activities of gluconeogenic enzymes such as glucose-6-phosphase and phosphoenolpyruvate carboxykinase in HFD-fed mice (27). Therefore, the hypoglycemic effect of *P. tricornutum* could be mediated partly by activation of the insulin signaling pathway and modification of the activities of glucose-regulating enzymes by fucoxanthin.

Antioxidant effect of *P. tricornutum* The effects of *P. tricornutum* on lipid peroxide and GSH concentrations and the activities of antioxidant enzymes in the liver are shown in Fig. 1. Feeding with a HFD increased hepatic TBARS (0.669±0.041 nmol MDA/mg protein) and decreased GSH levels (19.4±0.8 nmol/mg protein) compared with the ND

group (0.525±0.033 nmol MDA/mg protein and 23.4±1.2 nmol/mg protein, respectively; $p<0.05$). The activities of hepatic SOD, CAT, and GSH-Px were significantly lower in the HFD group (4.08±0.24, 76.7±4.7, and 58.3±3.9 U/mg protein, respectively) than in the ND group (5.14±0.28, 97.4±5.0, and 79.8±5.5 U/mg protein; $p<0.05$). These results are consistent with those of previous studies, which demonstrated that high-fat feeding increased lipid peroxides and reduced GSH content and the activities of antioxidative enzymes of the liver in rodents (34). However, the consumption of *P. tricornutum* reduced TBARS and elevated GSH levels of mice fed a HFD (0.555±0.027 nmol MDA/mg protein and 22.6±0.8 nmol/mg protein, respectively; $p<0.05$), to values comparable with those of the ND group. In addition, supplementation of *P. tricornutum* significantly induced SOD, CAT, and GSH-Px activities (4.98±0.21, 92.9±4.5, and 85.6±5.4 U/mg protein, respectively; $p<0.05$, $p<0.05$, and $p<0.01$, respectively) compared with the HFD group.

HFD-induced oxidative stress has been reported to trigger insulin resistance (34). In addition, oxidative stress is strongly related to the development of CVDs and type 2 diabetes in obesity (7). In this study, *P. tricornutum* alleviated the oxidative stress in HFD-fed mice by increasing hepatic GSH levels and potentiating the antioxidative enzyme system. GSH is a potent antioxidant that is involved in scavenging ROS and removing lipid peroxides (35). SOD, CAT, and GSH-Px also play a crucial role in the antioxidant defense mechanism (36). SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide, which can then be degraded into water by CAT or be involved in the oxidization of GSH to glutathione disulfide (GSSG) by GSH-Px. Therefore, the alleviation of oxidative stress by *P. tricornutum* could contribute to the improvement of insulin sensitivity and reduction of risks for CVDs and type 2 diabetes in diet-induced obese mice. Marxen *et al.* (37) reported that *P. tricornutum* extract exerted DPPH radical scavenging activity *in vitro* and suggested that carotenoids could be responsible for the antioxidant activity. Fucoxanthin showed antioxidant activity *in vitro* (14-17) and was reported to be the major antioxidant in *P. tricornutum* (15) and *Hijikia fusiformis,* a brown algae (16). Thus, the antioxidant activity of *P. tricornutum* in HFD-fed mice could be due to fucoxanthin.

In conclusion, *P. tricornutum* effectively reduced body weight and epididymal WAT and alleviated hyperglycemia, hyperinsulinemia, and hypertriglyceridemia in mice fed a HFD. It also decreased hepatic lipid peroxides and increased GSH and antioxidant enzyme activities. Therefore, *P. tricornutum* may be useful in preventing obesity and insulin resistance induced by high-fat feeding in mice.

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