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Effect of *Chlorella vulgaris* Intake on Antioxidative Capacity in Rats Oxidatively Stressed with Dietary Cadmium

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Abstract This study was conducted to investigate whether dietary chlorella intake could have an effect on antioxidative capacity in rats oxidatively stressed with cadmium (Cd). Sprague-Dawley rats fed dietary chlorella (0, 5, and 10%) for 4 weeks after induction of oxidative stress by exposing to Cd (200 ppm) for 8 weeks. After the oxidative stress applied, plasma and liver malondialdehyde concentrations and xanthine oxidase activities were decreased in 5% chlorella fed group compared to chlorella free group. Although liver heme oxygenase-1 protein expression was not affected by chlorella, the enzyme activity was improved in 5% chlorella fed group. Erythrocyte superoxide dismutase activity and hepatic metallothionein concentration were increased in 5% chlorella fed group. However, 10% chlorella intake had no effect on the improvement of oxidative stress-related enzymes and proteins. These findings suggest that, after induction of oxidative stress with Cd, 5% chlorella intake might improve antioxidative capacity against oxidative stress.

Keywords: *Chlorella vulgaris*, cadmium, antioxidative capacity, heme oxygenase-1, oxidative stress

Introduction

Organisms are able to adapt to fluctuating stresses and protect themselves against damage by reactive oxygen species (ROS) and other radicals through repair processes, compartmentalization of free radical production, defense enzymes, and endogenous and exogenous antioxidants (free radical scavengers) (1). However, in the case of disturbed balance between the formation of free radicals and antioxidant defense, ROS are accumulated in various tissues, yield cell damage, and oxidative stress occurs. Oxidative stress that damages cellular lipids, proteins, and DNA is thought to be implicated in a wide variety of several diseases or conditions, including some cancers, cardiovascular diseases, neurodegenerative disorders, age-related disorders, and perhaps even factors underlying the aging process itself (1).

Cadmium (Cd) is a highly toxic environmental heavy metal. The detailed mechanisms for the toxic effects of cadmium are still largely unknown. Actually, it has been thought that Cd may replace redox active metals such as iron and copper, which in turn cause free radical-induced damage via indirect generation of various radicals involving the superoxide radical, hydroxyl radical, and nitricoxide through Fenton reactions (2,3). Besides, an increase in unbound free/chelated copper and iron ions could participate in oxidative stress via same reaction (4). Exposure of cultured hepatocytes to Cd causes lipid peroxidation, based on the formation of thiobarbiturate-reactive substances (TBARS) (5). When animal was exposed to high doses of Cd, the activities of antioxidant enzymes were altered (6,7). In this study, Cd was used as an oxidative stress-causing material based on several studies (8-10).

Chlorella vulgaris (chlorella) are freshwater unicellular algae. Chlorella contains many dietary antioxidants such as lutein, α-carotene, β-carotene, ascorbic acid, and α-tocopherol (11,12). Particularly, chlorophyll and phenolic compounds have been suggested as the active components with antioxidative activity (13-15). These bioactive components have the capacity to scavenge the free radicals.

In previous studies in this lab by Son *et al.* (16), when rats were fed chlorella (3 or 5% of kg diet, w/w) and Cd 160 ppm simultaneously, chlorella intake might reduce oxidative stress through inhibition of radical production. Therefore, the overall goal of this follow-up study was to investigate whether chlorella could have some effects on betterment of antioxidant capacity on rats after induction of distinct oxidative stress.

Materials and Methods

Chlorella powder sample Chlorella sample used in this study was obtained from Daesang Co. (Seoul, Korea). The composition of chlorella has the same specifications as shown in Son *et al.* (16).

Experimental animals and diets Fifty 14-week-old male Sprague Dawley (SD) rats purchased from Jung-Ang Lab. Animal, Inc. (Seoul, Korea) were placed in individual stainless steel wire mesh cages in a climate-controlled room. The room had a 12:12 hr light-dark cycle, a temperature of 22-24°C and a relative humidity of 45±5%. The rats were fed a pellet diet (Samyang Co., Seoul, Korea) for the first 7 days (adaptation period). To induce oxidative stress, the rats were randomly divided into Cd free group (n=10) and Cd group (n=40), and fed the modified American Institute of Nutrition (AIN)-93G diet with or without Cd 200 ppm ($CdCl_2$ 406 ppm, 0.04% of kg diet, Sigma-Aldrich, St. Louis, MO, USA) for 8 weeks. In previous study (16), dietary Cd level (160 ppm) showed no

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Table 1. Specification of chlorella powder

Ingredients	Content (g/100 g powder)	Amino acid composition	Content (g/100 g powder)
Protein	60.6 g	Alanine	4.38
Carbohydrate	3.7 g	Arginine	3.51
Fat	12.8 g	Aspartic acid	4.86
Dietary fiber	13.0 g	Cystine	0.71
Ash	4.5 g	Glutamic acid	6.60
Moisture	5.4 g	Glycine	3.19
Chlorophyll	2,400 mg	Histidine*[1)]	1.16
Carotene	106 mg	Isoleucine*	2.04
Vitamin A potency	58,900 IU	Leucine*	4.52
Vitamin C	74 mg	Lysine*	4.88
Vitamin E	22.8 mg	Methionine*	1.20
Calcium	5.1 mg	Phenylalanine*	2.48
Copper	0.14 mg	Proline	2.54
Zinc	3.37 mg	Serine	2.19
Iron	22.4 mg	Threonine*	2.38
Magnesium	310 mg	Tryptophan*	1.01
Manganese	3.84 mg	Tyrosine	1.64
Phosphorus	1,060 mg	Valine*	3.14
Calorie		372 kcal	

[1)]Essential amino acids.

apparent induction of oxidative stress and dosage of chlorella (0, 3, and 5% of kg diet, w/w) showed no observable antioxidant capacity. According to several studies (17-21), dietary cadmium supply was chosen that did not trigger intoxication, but could induce oxidative stress. For comparing oxidative stress-induced group with control group, all rats of the Cd free group and 10 rats of the Cd group were sacrificed.

Remaining 30 rats that already fed dietary Cd were then stratified according to body weight and randomly blocked into 3 treatment groups according to the chlorella on a different level (0, 5, and 10% of kg diet, w/w) for the experimental period, which would be lasted for 4 weeks. Dose of chlorella was chosen in accordance with several studies. (16,22-24) The specifications of chlorella are shown in Table 1. The measurement was made in accordance with the methods recorded in the Food Code (25) and the Health Functional Food Code (26) at the Korea Health Supplement Institute. If chlorella substituted for 1 macronutrient (carbohydrates, protein, or lipids), there were imbalance of constituents of food intake due to extreme change of energy ratio and total calorie. Chlorella was replaced from all nutrients proportionally. And then, there was no imbalance of constituents of food intake or nutritional dilution of diet. The composition of the experimental diets is shown in Table 2. During the experimental period, the rats were allowed *ad libitum* access to the experimental diets and distilled water. Body weight was recorded twice a week and food intake was recorded 3 times a week. For preventing contamination of other minerals, all instruments were treated 0.4% ethylenediamine tetraacetic acid (EDTA) solution and washed with distilled deionized water.

Tissue preparation At the end of the experimental period, animals were deprived of food for 12 hr and sacrificed after anesthetization with diethyl ether. After blood samples were collected directly from the heart using syringes treated with sodium citrate or non-treated syringes. Blood samples collected with sodium citrate treated syringes were transferred to polypropylene tubes containing EDTA. All blood samples were centrifuged at 1,000×g for 30 min at 4°C (Union 55R Centrifuge; Hanil, Seoul, Korea), plasma and serum were obtained, and they were frozen at −80°C. To extract erythrocytes, the remaining pellets were washed with ice-cold saline (1:1, v/v) and rewashed 3 times by centrifugation at 1,000×g for 10 min at 4°C. Erythrocytes were suspended in ice-cold saline (1:1, v/v dilution) and stored at −80°C. The liver was perfused with saline, blotted with tissue paper, cut into small pieces, and stored at −80°C.

Analytical methods Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and total protein and albumin concentrations were measured using a kit (Asan Pharmaceutical, Seoul, Korea). Malondialdehyde (MDA) concentration was determined using a lipid peroxidation assay kit (Oxford Biochemical Research, Inc., Oxford, MI, USA).

Xanthine oxidase (XO) activity was determined using the method of Yoon (27). Liver (1 g) was homogenized in 10 vol. of 0.25 M ice-cold sucrose and centrifuged at 600×g (Union 55R; Hanil) for 10 min at 4°C. The supernatant was centrifuged at 12,000×g (Ultra 80; Sorvall, Waltham, MA, USA) for 30 min at 4°C as described by Mayanil *et al.* (28) and Roy *et al.* (29). The supernatant was then recentrifuged at 105,000×g (Ultra 80; Sorvall) for 60 min. Plasma or tissue homogenate was diluted with deionized water and 0.3 M Tris buffer (pH 8.0). A 0.45 mM xanthine solution was added to the mixture and incubated at 30°C for 20 min. A blank reaction of each sample was also conducted

Table 2. Composition of experimental diets

(g/kg diet)

Ingredients \ Groups	Cd +	Cd -		
	Chlorella 0%	Chlorella 0%	Chlorella 5%	Chlorella 10%
Corn starch	396.78	400.2	380.19	360.18
Dextrinized cornstarch	132	132	125.4	118.8
Sucrose	100	100	95	90
Casein (>85% protein)	200	200	190	180
Soybean oil	70	70	66.5	63
Fiber	50	50	47.5	45
Mineral mix[1)]	35	35	33.25	31.5
Vitamin mix[2)]	10	10	9.5	9
Choline bitartrate	2.5	2.5	2.375	2.25
tert-Butyl hydroquinone	0.014	0.014	0.0133	0.0126
Chlorella powder	0	0	50	100
Cadmium chloride	0.406	0	0	0
Total amount	1,000	1,000	1,000	1,000

[1)]AIN-93G-MIX (Dyets Inc., Bethlehem, PA, USA) (g/kg mixture): anhydrous calcium carbonate, 357; monobasic potassium phosphate, 196; sodium chloride, 74; potassium sulfate, 46.6; tripotassium citrate monohydrate, 70.78; magnesium oxide, 24; ferric citrate, 6.06; zinc carbonate, 1.65; manganous carbonate, 0.63; cupric carbonate, 0.3; potassium iodate, 0.01; anhydrous sodium selenate, 0.01025; ammoniumparamolybdate 4-hydrate, 0.00795; sodium metasilicate 9-hydrate, 1.45; chromium potassium sulfate 12-hydrate, 0.275; boric acid, 0.0815; sodium fluoride, 0.0635; nickel carbonate, 0.0318; lithium chloride, 0.0174; ammonium vanadate, 0.0066; powered sucrose 221.026.

[2)]AIN-93-VX (Dyets Inc.) (g/kg mixture): niacin, 3; calcium pantothenate, 1.60; pyridoxine HCl, 0.70; thiamine HCl, 0.60; riboflavin, 0.60; folic acid, 0.20; biotin, 0.02; vitamin E acetate (500 IU/g), 15; vitamin B_{12} (0.1%), 2.50; vitamin A palmitate (500,000 IU/g) 0.80, vitamin D_3 (400,000 IU/g) 0.25, vitamin K_1/dextrose mix (10 mg/g),7.50; sucrose 967.23.

without incubation. The reaction was terminated by the addition of 0.9 N H_2SO_4. After the addition of Na_2WO_4 (2%), the solution was incubated for 5 min at room temperature and centrifuged at 3,000×g (Micro 17R; Hanil) for 15 min. Phosphotungstic acid and alkali solution were added to supernatant. After 20 min, absorbance at 710 nm was measured using a spectrophotometer (Genesys 10 UV; Thermo Electron Co., Waltham, MA, USA). Xanthine oxidase activity was expressed as μmol or nmol of uric acid produced from the reaction of 1 L plasma or 1 mg liver protein/min with the substrate. The Lowry method was used for determination of protein content (30).

Superoxide dismutase (SOD) activity was determined using the method of Flohe *et al.* (31). One unit of SOD is defined as that amount of enzyme which inhibits the rate of cytochrome *c* reduction, under the conditions specified, by 50% and referred as McCord and Fridovich unit (U/min/mg protein) (32). Liver (1 g) was homogenized in 10 vol.(w/v) of 50 mM phosphate-0.25 M sucrose-0.5 mM EDTA buffer (pH 7.4) and the homogenate was centrifuged at 10,000×g (High Speed Union 55R; Hanil) for 10 min at 4°C. After ultrasonication, the supernatant, which extracted to 0.4 vol.(w/v) of chloroform:ethanol solution (5:3, v/v) and strongly mixed for 2 min, was centrifuged at 12,000×g (Ultra 80; Sorvall) for 30 min at 4°C. An aliquot of the supernatant was used for the assay of liver SOD activity. For the assay of erythrocyte SOD activity, the erythrocyte suspension was resuspended in 10 vol. of 10 mM Tris-1 mM EDTA buffer (pH 7.4) and hemolyzed. Resuspended erythrocyte was added to 0.4 vol. of chloroform:ethanol (5:3) solution. Erythrocyte or tissue homogenate was added to solution A and solution B. Solution A was made up of 5 μM xanthine in 0.001 N NaOH and 2 μM cytochrome *c* are admixed with 50 mM phosphate buffer (pH 7.8) containing 0.1 M EDTA. Solution B is freshly prepared solution of 0.2 U/mL xanthine oxidase in 0.1 mM EDTA. Absorbance at 550 nm was measured for a period of 3 min using a spectrophotometer (Genesys 10UV; Thermo Electron Co.). The Lowry method was used to determine the protein contents of samples (30).

Heme oxygenase-1 (HO-1) activity was measured using the heme oxygenase-1 enzyme immunoassay (EIA) kit (Takara Bio Inc., Otsu, Shiga, Japan).

The procedure of Western blot analysis of HO-1 protein was shown below. Liver (50 mg) was homogenized in 10 vol. of PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Inc., Seongnam, Korea), sonicated for 20 min, incubated for 30 min on ice, and centrifuged at 14,000×g for 10 min at 4°C. Protein concentration was determined by using the Bradford assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as standard; equal amount of protein was discontinuously subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophorosis (SDS-PAGE, Bio-Rad), and the separated polypeptides were then transferred electrophoretically from the gel to a nitrocellulose membrane (Whatman, Dassel, Germany) using a transfer buffer. For blockade of nonspecific binding sites, the nitrocellulose blot was incubated at room temperature for 1 hr with Tris-buffered saline plus 0.1% Tween-20 (TBS-T) containing 5%(w/v) nonfat dry milk. And membrane was incubated at 4°C with goat polyclonal anti-HO-1 (1:5,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as primary antibodies (1:1,000 dilution in TBS-T with 5% milk) for overnight, which specifically recognizes the 32-kd HO-1 proteins, respectively. Blot was subsequently washed in TBS-T and incubated with horseradish peroxidase-conjugated rabbit anti-goat IgG antibody (1:1,000 dilution

Table 3. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in rats fed diets with different levels of dietary cadmium

Group[1)]	AST (U/L)	ALT (U/L)	Total protein (g/dL)	Albumin (g/dL)
Cd-	58.26±3.31[NS2)]	22.03±3.31[NS]	7.66±0.12[a]	3.66±0.09[NS]
Cd+	54.22±4.83	20.77±1.01	6.53±0.12[b]	3.90±0.09

[1)]Cd-, no cadmium with 0%(w/w) chlorella powder; Cd+, cadmium (200 ppm) with 0%(w/w) chlorella powder.
[2)]Mean±SE; Values within a column with different letters are significantly different at α=0.05 level by unpaired *t*-test; [NS]Not significant.

in TBS-T with 5% milk, Santa Cruz Biotechnology, Inc.) for 1 hr as secondary antibody. Actin signal was served as equal loading controls (a mouse monoclonal anti-actin as primary antibody, 1:1,000, and goat anti-mouse antibody as secondary antibody, 1:4,000). Protein expression was visualized by means of WEST-one™ Western blot detection system (iNtRON Biotechnology, Inc.). The band intensity was determined with LAS-3000 imaging system (LAS-3000; Fujifilm, Tokyo, Japan) and calculated using the image analysis program (MultiGauge V3.0; Fujifilm).

Determination of metallothionein (MT) was measured by the methods of cadmium/hemoglobin affinity assay (33,34). One g of liver was homogenized in 1 vol. of 10 mM Tris-HCl buffer (pH 7.4, 4°C) and centrifuged at 10,000×g for 10 min at 4°C (Micro 17R; Micro Refrigerated Centrifuge, Hanil). The supernatant fraction was heated for 2 min in a boiling water bath. The heated samples were then centrifuged at 10,000×g for 2 min to remove precipitated proteins. The sample was mixed with 1 mL of 0.26 mM $CdCl_2$ solution and was incubated at room temperature for 15 min. The metal binding sites of MT were saturated with Cd during this incubation step. For removal of excessive Cd, it was precipitated by addition of rat red blood cell (RBC) hymolysate which was prepared following the method of Onosaka and Cherian (35), and heat treatment in a boiling water bath for 2 min. The heat treated Cd-bound hemoglobin was removed by centrifugation at 10,000×g for 2 min at 4°C. The amount of Cd in the heated supernatant fraction is a measure of MT-bound Cd and was determined in using an atomic absorption spectrophotometer (AAS, Model 6701F; Shimadzu Co., Kyoto, Japan). The analysis was performed at the 228.8 nm resonance line. The concentration of MT in each tissue was calculated by assuming that 7 g atom of Cd is bound to each mole of thionein which has a molecular weight of 6,050 by amino acid analysis.

Statistical analysis All statistical analyses were performed by the SAS program package version 9.1. All results are expressed as the mean±standard error (SE). The data between Cd fed and Cd non-fed group were analyzed by unpaired *t*-test. The data between chlorella fed and chlorella non-fed group were analyzed by one-way analysis of variance (ANOVA) and differences among experimental groups were evaluated using Duncan's multiple range tests at the $p<0.05$ significant level. All analyses were performed in duplicate or triplicate.

Results and Discussion

Induction of oxidative stress by exposure to Cd There were no significant differences between the rats with Cd-containing diet and the rats with Cd-free diet in serum AST and ALT activities, and albumin concentration, except total protein concentrations (Table 3). All those indicators were within the normal reference range of SD rats.

Table 4. Concentrations of malondialdehyde (MDA) and metallothionein (MT) in rats fed diets with different levels of dietary cadmium

Group[1)]	Plasma MDA (nmol/dL)	Liver MDA (nmol/g wet liver)	Liver MT (μg/g wet wt)
Cd-	58.00±3.29[a2)]	11.39±0.62[NS]	2.28±0.72[b]
Cd+	118.11±3.73[b]	15.79±2.09	13.13±1.32[a]

[1)]Cd-, no cadmium with 0%(w/w) chlorella powder; Cd+, cadmium (200 ppm) with 0%(w/w) chlorella powder.
[2)]Mean±SE; Values within a column with different letters are significantly different at α=0.05 level by unpaired *t*-test; [NS]Not significant.

Significant increase in plasma MDA concentration indicated that oxidative stress was induced through Cd treatment (Table 4). Plasma and liver XO activities were significantly increased and erythrocyte and liver SOD activities were reduced by dietary Cd intake (Table 5). This result is consistent with several studies. Halliwell (36) and Kim *et al.* (17) reported that XO activities were increased by Cd intake. Erdogan *et al.* (37) reported that cadmium increased the plasma MDA concentration and reduced blood SOD activity. In previous study (16), plasma and liver XO activities, and plasma MDA concentration were significantly increased and liver SOD activity was significantly reduced by exposure to Cd. Park *et al.* (18) reported that erythrocyte and liver SOD activities were significantly decreased by Cd administration.

There was no significant difference in liver MDA concentration between Cd fed group and Cd free group (Table 4). No significant change in Cd-induced lipid peroxidation was reported by several studies (10,38,39). It has been proposed that the enhancement of lipid peroxidation by Cd in rats is a consequence of a decrease in SOD activity (9).

Although antioxidant enzyme activity is reduced, the insignificant change in MDA concentration was probably caused by the activation of other defensive factors. The MT, protective agent, was considered to play an important role in detoxification processes (40,41). An induced MT synthesis has also been observed in various rat tissues after exposure to Cd (42). In this study, hepatic MT concentration was significantly increased with dietary Cd intake (Table 4). This finding was in accordance with other studies that hepatic MT concentration was significantly increased in rats exposed Cd (19,43).

The protein expression and the enzymatic activity of liver

Table 5. Xanthine oxidase (XO), hemeoxygenase (HO)-1, and superoxide dismutase (SOD) activities in rats fed diets with different levels of dietary cadmium

Group[1)]	Plasma XO (μmol/min/L)	Liver XO (nmol/min/mg Protein)	Liver HO-1 (ng/mg protein)	Erythrocyte SOD (U[2)]/min/mg protein)	Liver SOD (U/min/mg protein)
Cd-	8.84±1.35[b3)]	0.21±0.03[b]	195.83±33.90[b]	153.49±18.92[a]	34.04±4.63[a]
Cd+	15.50±0.61[a]	0.37±0.05[a]	873.40±65.30[a]	107.93±10.04[b]	17.69±2.32[b]

[1)]Cd-, no cadmium with 0%(w/w) chlorella powder; Cd+, cadmium (200 ppm) with 0%(w/w) chlorella powder.
[2)]1 unit (U) inhibits cytochrome C reduction rate by 50% at pH 7.8 and 25°C.
[3)]Mean±SE; Values within a column with different letters are significantly different at α=0.05 level by unpaired *t*-test.

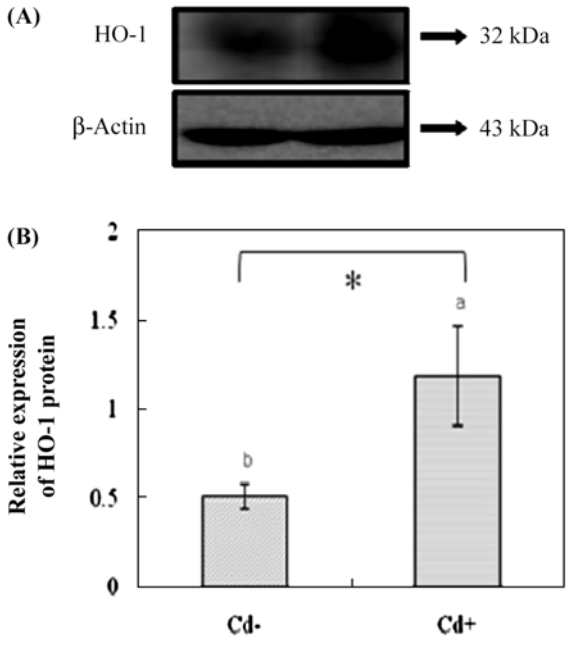

Fig. 1. Liver hemeoxygenase (HO)-1 protein expression in rats fed diets with different levels of cadmium. (A) Western blot analysis. Each lane contained 50 μg of total protein. (B) Relative expression of HO-1 protein was normalized by β-actin (*p<0.001).

HO-1 were significantly increased by Cd intake (Table 5, Fig. 1). It was reported that cadmium ion is a powerful inducer of HO and expression of its protein was increased in a dose- and time-dependent manner (10,44,45). Ossola and Tomaro (46) showed increased liver HO activity in albino wistar rats after Cd administration. Increased HO-1 enzyme activity and its protein expression by exposure to Cd seemed to minimize the Cd-derived oxidative damage.

In this study, cadmium (200 ppm) exposure to 14-week-old male SD rats influenced the decrease in SOD activities and the increase in XO activities in liver and blood; it also affected increased plasma MDA concentrations and liver HO-1 activity and its protein expression. Cadmium dose and feeding period used in this work seemed to be sufficient for induction of oxidative stress. Oxidative stress rather than cadmium-induced protein malnutrition was then induced in 14-week-old male SD rats exposed to 200 ppm Cd (0.04% of total kg diet) for 8 weeks.

Effect of chlorella on antioxidative capacity After induction of oxidative stress, there were no significant differences in serum AST and ALT activities, and total protein and albumin concentrations among all chlorella fed groups (CV-0, CV-5, and CV-10) (Table 6). Serum AST and ALT activities, and total protein and albumin concentrations were not influenced by dietary chlorella intake.

Plasma and liver MDA concentrations in CV-5 group were significantly lower than those in other groups (CV-0 and CV-10) (Table 7). Plasma and liver XO activities were significantly reduced and erythrocyte SOD activity was significantly induced in CV-5 group compared to CV-0 and CV-10 group (Table 8). Although there was no significant difference in liver SOD activity between CV-0 and CV-5 group, liver SOD activity in CV-5 group was more increased than that in CV-10 group.

There was no significant difference in liver HO-1 protein expression among all 3 experimental groups (Fig. 2). However, liver HO-1 enzyme activity in CV-5 group was significantly lower than that in CV-0 and CV-10 group (Table 8). There was no significant difference in liver HO-1 activity between CV-0 and CV-10 group. It could be mentioned that liver HO-1 activity was improved by dietary chlorella intake, although liver HO-1 protein expression did not affect. It is thought that existing protein which its function was maximized has done its job without eliciting newly synthesized protein.

Oxidative stress was attenuated by chlorella intake which might suppress production of Cd-induced ROS, inferring by decreased radical generating plasma and liver XO activities and increased superoxide anion radical eliminating erythrocyte SOD activity (Table 7). In addition, reduced ROS would lower ROS-dependent HO-1 activity. This radical scavenging effect of chlorella supplementation

Table 6. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, total protein, and albumin concentrations in rats fed diets with different levels of chlorella powder

Group[1)]	AST (U/L)	ALT (U/L)	Total protein (g/dL)	Albumin (g/dL)
CV-0	46.21±3.422[NS2)]	19.55±4.79[NS]	7.37±0.06[NS]	3.95±0.11[NS]
CV-5	49.14±2.82	18.15±2.46	7.27±0.08	3.86±0.10
CV-10	54.85±4.41	18.88±2.56	7.24±0.09	3.77±0.11

[1)]CV-0, CV-5, and CV-10 : no cadmium with 0, 5, and 10%(w/w) chlorella powder, respectively.
[2)]Mean±SE; [NS]Values within a column are not significant at α=0.05 level by Duncan's multiple range test.

Table 7. Concentrations of malondialdehyde (MDA) and metallothionein (MT) in rats fed diets with different levels of chlorella powder

Group[1)]	Plasma MDA (nmol/dL)	Liver MDA (nmol/g wet liver)	Hepatic MT (μg/g wet wt)
CV-0	98.47±6.97[a2)]	15.91±2.02[a]	13.68±1.73[b]
CV-5	76.88±4.41[b]	10.63±0.18[b]	20.71±1.60[a]
CV-10	85.20±3.55[ab]	13.43±0.70[ab]	18.11±0.97[ab]

[1)]CV-0, CV-5, and CV-10: no cadmium with 0, 5, and 10%(w/w) chlorella powder, respectively.
[2)]Mean±SE; Different letters within a column are not significant at α=0.05 level by Duncan's multiple range test.

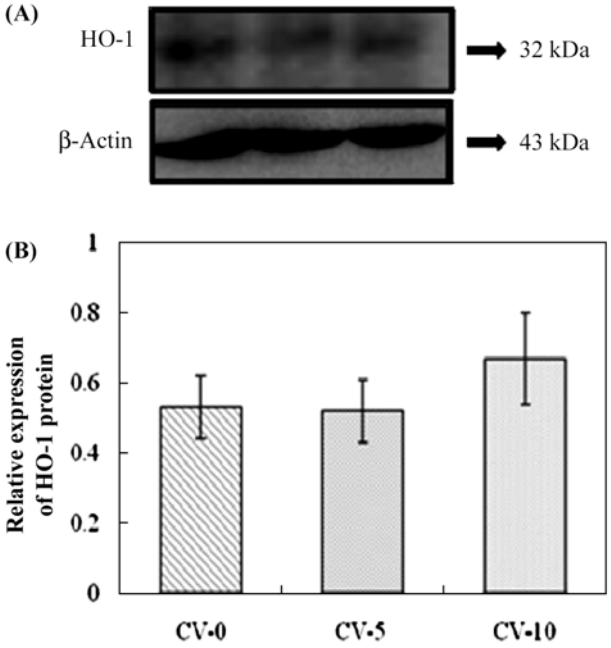

Fig. 2. Liver hemeoxygenase (HO)-1 protein expression in rats fed diets with different levels of chlorella powder. (A) Western blot analysis. Each lane contained 50 μg of total protein. (B) Relative expression of HO-1 protein was normalized by β-actin.

might lead to reduced MDA concentrations, because attenuated radical has a lower chance of increasing lipid peroxidation level. According to Shibata *et al.* (12), chlorella-containing diets showed significantly lower serum MDA concentration. The results in Vijayavel *et al.* (47) was reported that alcoholic extract of chlorella reduced lipid membrane peroxidation and increased serum and hepatic SOD activities by inhibiting the production of ROS with its free radical scavenging effect or by improving the antioxidant capacity of the cells. And it was also mentioned that the alcoholic extract of chlorella is responsible for reducing the oxidative stress by removing the toxic metabolites from the target organ and/or by directly scavenging ROS by its sulfhydryl groups (47).

In the light of decreased MDA concentrations and improved enzymes activities, antioxidants in chlorella such as chlorophyll, polyphenol, vitamins, and sulfur-containing compounds may exert their radical scavenging effect (48). Also, the finding that the ingestion of chlorella tablet increased serum carotene and lutein concentrations in human was observed (12). Moreover, several lines of evidence suggested the free radical scavenging activities of chlorella. Miranda *et al.* (13) found antioxidative capacity of chlorella in phenol compounds of the methanolic extract and Guzman *et al.* (49) confirmed antioxidative effects of hydrophilic compounds presented in the aqueous extract of chlorella. It was reported that radical scavenging effects may have been due to the presence of chlorophyll (50). It was also known that chlorophyllin, a soluble form of chlorophyll, had potent antioxidative ability involving scavenging various physiologically important ROS (51).

This result can be supported by hepatic MT concentration, as well. Hepatic MT level in CV-5 group was significantly higher than CV-0 group. (Table 7) It is considered that 5% chlorella intake stimulated hepatic MT *de novo* synthesis and led increased MT to scavenge Cd-induced ROS. MT was suggested to have the activity of a reactive oxygen scavenger against oxidative stress (52,53). In *in vivo* studies, high protein containing diet showed increasing MT synthesis in rat organs (20,21). Since chlorella contains 55-67% protein and an amount of minerals (16), MT *de novo* synthesis was promoted during chlorella intake after oxidative stress applied.

The effect of dietary chlorella intake on antioxidative capacity was not dose-dependent manner. There were no any differences in MDA concentrations, XO and SOD activities, and liver HO-1 activity and its protein expression between CV-0 and CV-10 groups. Also, 10% chlorella diet did not stimulate hepatic MT, as well. Johnson and Loo (54) reported that high concentrations of antioxidants induced oxidative damage to cellular DNA and acted as pro-oxidant. It is likely that, in the case of the diet containing 10% chlorella, excess nutrients such as minerals and antioxidants might interfere with the absorption of each other. In accordance with Sareen *et al.* (55), vitamin C can reduce transition metals and aggravate oxidative damage rather than act as antioxidant. Divalent metal ions share their transporters and excessively ingested divalent

Table 8. Xanthine oxidase (XO), hemeoxygenase (HO)-1, and superoxide dismutase (SOD) activities in rats fed diets with different levels of chlorella powder

Group[1)]	Plasma XO (μmol/min/L)	Liver XO (nmol/min/mg protein)	Liver HO-1 (ng/mg protein)	Erythrocyte SOD (U[2)]/min/mg protein)	Liver SOD (U/min/mg protein)
CV-0	13.29±0.68[a3)]	0.29±0.02[a]	531.29±68.92[a]	98.62±5.81[b]	20.34±1.97[ab]
CV-5	11.21±0.65[b]	0.18±0.04[b]	270.57±48.20[b]	155.36±8.93[a]	25.33±1.66[a]
CV-10	13.61±0.56[a]	0.32±0.04[a]	582.43±38.17[a]	114.66±11.49[b]	16.87±2.43[b]

[1)]CV-0, CV-5, and CV-10 : no cadmium with 0, 5, and 10%(w/w) chlorella powder, respectively.
[2)]1 unit (U) inhibits cytochrome C reduction rate by 50% at pH 7.8 and 25°C.
[3)]Mean±SE; Values within a column with different letters are significantly different at α=0.05 level by unpaired *t*-test.

cations such as Cu, Fe, and Zn impede their absorption with each other. Excessive chlorella intake seemed to aggravate oxidative damage and inhibit MT synthesis due to interrupting nutrient absorption.

In conclusion, chlorella supplementation might improve antioxidative capacity against oxidative stress. It can be suggested that, without the change in the protein expression of ROS-dependent antioxidant, chlorella intake seemed to maximize radical scavenging capacity by reducing the production of radicals and promoting the capture and elimination of radicals. It is considered that antioxidants in chlorella such as chlorophyll, polyphenol, vitamins, and sulfur-containing compounds may exert their radical scavenging effect.

In previous study (16), it was hard to mention that which amount to consume for antioxidative capacity works better, 3 or 5%(w/w) chlorella of kg diet. In this study, however, 5% chlorella intake had more antioxidative effect than 10% chlorella intake did, showing higher radical scavenging capacity of oxidative stress-related enzymes and protein. Since chlorella had an improving effect on antioxidative capacity, it could be considered the helpful food source to prevent oxidation-linked diseases/disorders induced or deteriorated by oxidative stress.

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