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### **Protective effects of *Chlorella vulgaris* on liver toxicity in cadmium-administered rats**

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**ABSTRACT** The biochemical mechanisms of *Chlorella vulgaris* protection against cadmium (Cd)-induced liver toxicity were investigated in male Sprague-Dawley rats (5 weeks of age, weighing 90-110 g). Forty rats were randomly divided into one control and three groups treated with 10 ppm Cd: one Cd without *Chlorella* (Cd-0C), one Cd with 5% *Chlorella* (Cd-5C), and one Cd with 10% *Chlorella* (Cd-10C) groups. The rats had free access to water and diet for 8 weeks. Body weight gain and relative liver weight were significantly lower in the Cd-0C group than in Cd-5C and Cd-10C groups. Rats in the Cd-0C group had significantly higher hepatic concentrations of Cd and metallothioneins (MTs) than in the Cd-5C or Cd-10C group. The hepatic MT I/II mRNA was expressed in all experimental rats. MT II was more expressed in the Cd-5C and Cd-10C groups than in the Cd-0C group. Morphologically, a higher level of congestion and vacuolation was observed in the livers of the Cd-0C group compared to those of the Cd-5C and Cd-10C groups. Therefore, this study suggests that *C. vulgaris* has a protective effect against Cd-induced liver damage by reducing Cd accumulation and stimulating the expression of MT II in liver. However, the details of the mechanism of *C. vulgaris* on liver toxicity remains to be clarified by further studies.

**KEY WORDS:** \* cadmium \* *Chlorella vulgaris* \* liver \* rats

#### INTRODUCTION

CADMIUM (CD), A HEAVY METAL, is of public health significance because it is regarded as one of the major industrial and environmental pollutants. Cd is mostly dispersed in nature in soil, water, and air and can be easily ingested through contaminated plants and crops or inhaled via cigarette smoke or contaminated air. Because approximately 20-30 mg of Cd can accumulate in the human body over a lifetime, the toxicity of Cd can cause human diseases including lung fibrosis, (1) kidney tubular dysfunction, (2) hypertension, (3) osteoporosis, (4) cancer, (5) and hepatic damage. (6)

The majority of Cd absorbed eventually complexes with metallothioneins (MTs). MTs are produced by several tissues such as liver and kidney and are largely intracellular but readily detectable at low levels in the circulation. MTs, which are widely present in eukaryotic and prokaryotic organisms, are a group of cysteine-rich heavy metal-binding proteins with low molecular masses (approximately 6-7 kDa). (7) MTs can protect cells against damage caused by alkylating agents, oxygen free radicals, and ionizing radiations. (8) Also, MTs are involved in intracellular metal regulation. In particular, MTs play a key role in the biological detoxification of heavy metals such as Cd by sequestration using cysteine residues. (9,10)

There has been increasing interest in finding natural food(s) or biomaterial(s) that can alleviate the intoxication of toxic metals in the body. Accordingly, studies have demonstrated that a calcium-rich diet, (11) a protein-rich diet, (12) a diet containing alginate, (13) a green tea containing catechin, (14) and an extract of Omija (15) can stimulate the excretion of heavy metals. In addition, the optimum amount of dietary copper and iron with increased dietary calcium and protein (16) and vitamin C (17,18) can be involved in the mechanism of metal detoxification. Recently, a supplementation of *Chlorella vulgaris* was also shown to alleviate the Cd toxicity in rats. (19)

*Chlorella*, a unicellular green microalgae, is considered to be an important functional food in many developed countries because it contains 55-67% protein with all the essential amino acids, 1-4% chlorophyll, 9-18% dietary fiber, and a large amount of minerals and vitamins, and dietary fiber. (20) Furthermore, *C. vulgaris* hot water extracts have been reported to show diverse antitumor activity, (21-23) antioxidant activity, (24) and antimicrobial activity. (25-28) However, almost all studies of *C. vulgaris* functionality have been conducted with extracts. Therefore, we investigated whether dried *C. vulgaris* powder may assist the removal of Cd-MT complexes in the liver, examining the changes in hepatic concentrations and gene expressions of MTs of male rats administered Cd.

#### MATERIALS AND METHODS

##### Chemicals

*C. vulgaris* for the *Chlorella* meal-based diet was obtained from Daesang Wellife Co. (Seoul, Republic of Korea). Unless noted, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). RNazol (Difco Laboratories, Sparks, MD), the avian myeloblastosis virus kit (Roche Co., Meylan, France), and AccuPower[™] polymerase chain reaction (PCR) PreMix (Bioneer Co., Seoul) were used

for reverse transcription (RT)-PCR.

#### Animals and housing conditions

Five-week-old male Sprague-Dawley rats weighing 90-110 g were allowed to acclimatize for 1 week. The rats were housed in plastic cages under controlled temperature (23 [+ or -] 2[degrees]C), humidity (50-60%), and photoperiod (12-hour light:12-hour dark) with free access to water and diet for 8 weeks. All treatments and procedures were conducted in accordance with Hanyang University (Seoul) Lab Animal Care Committee animal use protocols.

#### Experimental design

Each experimental group consisted of 10 rats. All the rats were randomly divided into control group, one Chlorella-free diet group (Cd-0C), one 5% Chlorella diet (Cd-5C) group, and one 10% Chlorella diet (Cd-10C) group. Rats in the Cd groups were provided drinking water with 10 ppm of Cd (Cd[Cl.sub.2]x[H.sub.2]O). Diet compositions are shown in Table 1.

#### Measurement of Cd contents in experimental diet and liver

Accurately measured samples of experimental diets and livers (about 1 g) were digested in concentrated nitric acid (3.0 mL) using a heating block. The digested samples made up to 5.0 mL with double glass distilled water and read for Cd (228.8 nm) on an inductively coupled plasma-mass spectrometer (model 3520, Perkin Elmer, Fremont, CA) against suitable standards for each metal in linear a range of 0.5-5.0 [micro]g/mL. The standards were processed identically. (29)

#### Measurement of MT contents in liver

About 1 g of liver was homogenized in 4 volumes of 0.25 M sucrose and then centrifuged at 18,000 g for 20 minutes at 4[degrees]C. Aliquots of 0.1, 0.2, or 0.5 mL of the cytosol were first adjusted to a sample volume of 2.4 mL with 0.03 M Tris-HCl buffer, pH 8. The sample was mixed with 1 mL of Cd[Cl.sub.2] solution (10 ppm Cd) and then incubated at room temperature for 5 minutes. The metal binding sites of MT were saturated with Cd during this step. The complete saturation with Cd was ensured by using various aliquots of the sample. The excess Cd was removed and precipitated by addition of rat red blood cell hemolysate (0.2 mL) and heat treatment in a water bath (about 100[degrees]C for 1 minute). Rat hemoglobin has a high affinity for Cd and can remove Cd from all the bioligands except MT. During the heat treatment Cd-bound hemoglobin was denatured and then removed by centrifugation at 1,000 g for 5 minutes at room temperature. The addition of the hemolysate and the heat treatment were repeated three times. The accurately measured samples (about 1 mL) were digested in concentrated nitric acid (3.0 mL) using a heating block. The digested samples were made up to 5.0 mL with double glass distilled water and read for Cd (228.8 nm) on an inductively coupled plasma-mass spectrometer (model 3520, Perkin Elmer) against suitable standards for each metal in a linear range of 0.5-5.0 [micro]g/mL. The standards were processed identically.

The rat red blood cell hemolysate for Cd-heme determination was prepared as follows: control rats (not previously exposed to Cd) were bled from the abdominal aorta under phenobarbital anesthesia into heparinized tubes. Twenty milliliters of 1.15% KCl was added to 10 mL of blood, mixed well, and centrifuged at 3,000 g for 5 minutes at 10[degrees]C. The pellet containing the red blood cells was suspended in 30 mL of 1.15% KCl and centrifuged. The washing and centrifugation steps were done with 20 mL of 30 mM Tris-HCl buffer (pH 8.0), and the resultant solution kept at room temperature for 10 minutes for hemolysis. The membrane fraction was removed by centrifugation at 9,000 g for 20[degrees]C. The supernatant fraction was collected and used for the hemolysate for the Cd-heme method. The hemolysate samples can be stored at 4[degrees]C for 2-3 weeks (until they turned dark). If a precipitate formed, the hemolysate was stirred before use. (30)

#### Measurement of mRNA MT expression by RT-PCR

Total RNA was extracted from the liver by homogenization in TRIzol. Extracted total RNA was quantified by absorbance measurements at 260 and 280 nm and stored at -80[degrees]C. From each sample, 1.5 [micro]g of RNA was reverse-transcribed using avian myeloblastosis virus reverse transcriptase, 1 mM deoxynucleotide triphosphates, and 0.5 mg/mL oligo(dT12-18). PCR analysis was then performed on the aliquots of the cDNA preparations to detect MT I, MT II, and glyceraldehyde 3-phosphate dehydrogenase (as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA). The reactions were carried out in a volume of 20 [micro]L containing (final concentration) 1 unit of Taq DNA polymerase, 0.2 mM deoxynucleotide triphosphates, 10 [micro]L of 10x reaction buffer, and 100 pmol of 5' and 3' primers. After initial denaturation for 2 minutes at 95[degrees]C, 30 amplification cycles were performed for MT I (1 minute of 95[degrees]C denaturation, 1 minute of 60[degrees]C annealing, and 1.5 minutes of 72[degrees]C extension), MT II (1 minute of 94[degrees]C denaturation, 1 minute of 60[degrees]C annealing, and 1 minute of 72[degrees]C extension), and L32 (1 minute of 95[degrees]C denaturation, 1 minute of 55[degrees]C annealing, and 1 minute of 72[degrees]C extension). The PCR primers used in this study are listed in Table 2. After amplification, portions of the PCR procedures were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and ultraviolet irradiation.

#### Histology

From each treatment group, paraffin-embedded liver slices from two to four animals were used for histological examination. Formalin-fixed hepatic tissues were sectioned and processed sequentially in ethanol, xylene, and paraffin using a Thermo Electron (Waltham, MA) Excelsior. Tissues were then embedded in paraffin using a Miles (Elkhart, IN) Tissue Tek II embedding center, after which paraffin blocks were sectioned at 5 [micro]m with a rotary microtome. Sections were placed on glass microscope slides, dried, and stained with hematoxylin and eosin. All histological processing was performed at the Kyung-Hee University Histology Laboratory (Seoul).

## Statistical analysis

Means and standard deviations (SD) of all variables were computed for each of the groups. Analysis of variance was first performed on the means to determine whether there were significant differences ( $P < .05$ ). When analysis of variance indicated statistical significance, Duncan's multiple range test was used to determine which means were significantly different. SPSS (Chicago, IL) software was used for all statistical analyses.

## RESULTS

### Body weight gain, Cd consumption, and relative liver weight

Changes in body weight of all experimental rats are shown in Table 3. Rats in the Cd-0C group (196.25 [+ or -] 45.18 g/8 weeks) had a significantly ( $P < .05$ ) lower body weight gain than those in the control (273.33 [+ or -] 22.12 g/8 weeks), Cd-5C (235.00 [+ or -] 35.86 g/8 weeks), and Cd-10C (239.17 [+ or -] 44.78 g/8 weeks) groups. Cd consumption through drinking water did not differ among the Cd-treated groups (Table 3). Rats in the Cd-0C group (2.96 [+ or -] 0.35 g/8 weeks) had significantly ( $P < .05$ ) heavier relative liver weight than those in the control (2.37 [+ or -] 0.08 g/100 g of body weight), Cd-5C (2.70 [+ or -] 0.34 g/100 g of body weight), and Cd-10C (2.51 [+ or -] 0.34 g/100 g of body weight) groups (Table 3).

### Cd concentrations in experimental diet/liver and MTs concentrations in liver

Cd was not detected in the experimental diets (normal, 5% Chlorella, and 10% Chlorella diet). Rats in the Cd-0C group (Cd, 51.49 [+ or -] 1.39 [micro]g/g; MTs, 34.74 [+ or -] 3.11 [micro]g/g) had significantly ( $P < .05$ ) higher concentrations of hepatic Cd and MTs compared to the Cd-5C (Cd, 45.98 [+ or -] 1.97 [micro]g/g; MTs, 15.08 [+ or -] 2.60 [micro]g/g) or Cd-10C (Cd, 45.98 [+ or -] 2.87 [micro]g/g; MTs, 12.50 [+ or -] 2.57 [micro]g/g) group (Table 4). However, there were no ( $P > .05$ ) significant differences in Cd and MTs concentrations between the Cd-5C and Cd-10C groups.

### MT I/II mRNA expression in liver

Hepatic MT I and II mRNA expressions are shown in Figure 1. The MT I and II mRNAs were expressed in liver of all experimental rats. Also, MT II mRNA expression was greater in Cd-5C (1.22 [+ or -] 0.30)- or Cd-10C (1.89 [+ or -] 0.29)-administered rats compared to control and Cd-0C (1.09 [+ or -] 0.10)-administered rats.

### Histology

Congestion of blood vessels in liver occurred in all Cd-administered groups, especially within the portal veins (Fig. 2). A definitely higher level of congestion was observed in the Cd-0C group compared to the Cd-5C or Cd-10C group. Vacuolation of hepatocytes was observed in Cd-administered groups (Fig. 2). A dramatic decrease in these vacuolated cells was, however, observed in the Cd-5C or Cd-10C group compared to the Cd-0C group.

## DISCUSSION

The present study was undertaken to examine the effect of *C. vulgaris* on liver toxicity of Cd-administered rats. For this purpose, body and liver weights were measured as an indicator of Cd toxicity. The body weight gain in Cd-administered rats (Cd-0C) was significantly ( $P < .05$ ) less than that in controls. There are a few reports on body weight loss in Cd-administered rats with similar results to this current study. An example is one study in which rats intoxicated with Cd exhibited reduced body weight gain along with a reduction in feed consumption. (31) The loss of body weight apparently resulted from an absorption and metabolism disorder caused by Cd administration. However, body weight gains in the Cd-5C and Cd-10C rats were similar to that for control group rats. The Chlorella doses of Cd-5C and Cd-10C are equivalent to a dose of 0.1 g/kg of body weight/day and 0.2 g/kg of body weight/day, respectively, for humans.

[FIGURE 1 OMITTED]

The liver is well known as the primary target organ of toxicity when a high dose of Cd [Cl.sub.2] is administered. (32-35) Most of the Cd absorbed during chronic exposure is initially trapped in liver and gradually redistributed to other organs, mainly to kidney. (36) In order to elucidate whether Cd accumulated in liver tissue may be lowered by administering Chlorella, we determined Cd concentrations in liver. As expected, the Cd concentrations in liver were significantly lower in Chlorella-administered rats. Thus, it appears that the degree of Cd-induced hepatotoxicity is associated in part, with less accumulation of the metal in liver by administering Chlorella.

[FIGURE 2 OMITTED]

The properties of *C. vulgaris* (24) are potentially attributed to stabilization of body metabolism since it contains phytochemicals, chlorophyll, diverse minerals, and vitamins related to antioxidants as well as protein. Ca, Zn, Mg, and protein in Chlorella may inhibit absorption of Cd in the intestine and thereby prevent cellular toxicity. (19) Chlorophyll is known to reduce the absorption of carcinogens such as heterocyclic amines, polycyclic hydrocarbons, and aflatoxin. It is also postulated that Chlorella, because of its high chlorophyll content, is able to assist in the detoxification of heavy metals and pesticides. (37)

Production of MTs increased in all Cd-treated groups compared to the control group. MTs were produced more in the Cd-0C group than

in the Cd-5C and Cd-10C groups, demonstrating that MTs were not simply produced in a dose-dependent manner. This finding contradicts a previous study (38) reporting that exposure of Cd to rats led to high MT production by feeding *Chlorella* diet. MT I is involved in the metabolism or detoxification of toxic metals such as Cd, and MT II is responsible for the homeostasis of essential metals such as Ca, Fe, and Cu. This present study showed that MT I was expressed in all groups and MT II was increased by administering *Chlorella*. Consequently, we can affirm that the elevated concentration of total MT is due to an increased expression of MT I. In the study of Alvarez et al., (39) MT I mRNA expression was increased in testis exposed to Cd. They reported that this fact was associated with the response of body protection against Cd. We consider that *Chlorella* may inhibit Cd absorption in small intestine, preventing it from reaching the liver. In another study, the contents of Cd in the small intestine showed an inverse relationship with the contents of dietary calcium, chlorophyll, etc. (40) This study reported that Ca binding protein is increasingly produced in the small intestine and thus Cd is highly absorbed. Therefore, high contents of chlorophyll inhibit production of Cd binding protein in the border of the intestine. Therefore, ingested Cd may not be absorbed into the intestine, and it may be excreted directly through feces or urine. The Cd content in feces and urine was increased by administering *Chlorella* (data not shown).

Cd-induced liver toxicity involves two distinct pathways: the first is caused by the direct toxic effects of the metal and/or ischemia due to endothelial cell injury, and the second process is one in which Kupffer cell activation and neutrophil infiltration play a major role by triggering a complex cascade of inflammatory mediators. Accordingly, the liver was analyzed using hematoxylin and eosin staining. The Cd-0C group revealed enlarged sinusoids and a tendency for hepatocytes to form irregular patches of cells, some in the process of necrosis and congestion of vessels. However, histological evaluation revealed less damage in Cd-5C or Cd-10C rats compared to Cd-0C rats. Unlike production of MTs, histological damage was prevented by feeding *Chlorella* in a dose-dependent manner. It is considered that MTs are the body's primary protection against toxic metals such as Cd, Hg, Pb, etc., and exposures to heavy metals normally lead to their adaptive up-regulation.

This showed that *Chlorella* may help the body trap and excrete Cd either in the form of MTs or by direct excretion before its intestinal absorption. Accordingly, *C. vulgaris* plays a role in the alleviation of Cd intoxication. In conclusion, some substances in *Chlorella*, such as chlorophyll and dietary fiber, may bind Cd and help eliminate it from the body before its intestinal absorption, which may lead to less Cd accumulation in the liver. Further study will be required to identify the mechanism by which *Chlorella* alleviates heavy metal intoxication.

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#### AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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TABLE 1. COMPOSITION OF THE DIETS (g/kg) Normal diet Chlorella Chlorella Ingredient 5% diet 10% diet Casein 200 160.9 125.8 DL-Methionine 3 3 3 Cornstarch 150 150 150 Sucrose 500 490.4 479.8 Cellulose 50 49.2 48.4 Coconut oil 50 49.5 49.0 Mineral mixture (a) 35 35 35 Vitamin mixture (b) 10 10 10 Choline bitartrate 2 2 2 Chlorella 0 50 100 Total 1,000 1,000 1,004 Energy 3,862 3,834.3 3,822.6 (a) Mineral mixture (in g/100 g): CaHP[0.sub.4] x 2[H.sub.2]0,0.43; K[H.sub.2]P[O.sub.4], 34.31; NaCl, 25.06; Fe([C.sub.6][H.sub.5] [O.sub.7]) 6[H.sub.2]0, 0.623; MgS[O.sub.4] x 7[H.sub.2]0, 9.98; Zn[Cl.sub.2], 0.02; MnS[O.sub.4] x 4-5[H.sub.2]0, 0.121; CUS[O.sub.4] x 5[H.sub.2]0, 0.156; KI, 0.0005; CaC[O.sub.3], 29.29; (N[H.sub.4]) 6[Mo.sub.7]7[O.sub.2] - 4[H.sub.2]0, 0.0025. (b) Vitamin mixture (in mg/100 g): vitamin A acetate, 93.2; vitamin [D.sub.3], 0.5825; [alpha]-tocopherol acetate, 1,200; vitamin [K.sub.3], 6.0; vitamin [B.sub.1] hydrochloride 59.0; vitamin [B.sub.2], 59.0; vitamin [B.sub.6] hydrochloride, 29.0; vitamin [B.sub.12], 0.2; vitamin C, 588; D-biotin, 1.0; folic acid, 2; pantothenic acid, 235; nicotinic acid, 294; inositol, 1,176; lactose, 96.257. TABLE 2. SEQUENCE OF THE PRIMERS USED TO AMPLIFY DIFFERENT GENES BY RT-PCR AND SIZES OF THE FRAGMENTS GENERATED cDNA Forward Reverse Size (bp) MT-I ACTGCCTTGTCGCTTA TGGSGGTGTACGGCAAGACT 312 MT-II CCAACTGCCGCCTCCATTCG GAAAAAAGTGTGAGGAACCG 297 L32 CGTGGCCCGCCCTAGFCACCA TTGGCCTTAGGGTTCAGAGGGG 243 TABLE 3. BODY WEIGHT GAIN, CD CONSUMPTION, AND LIVER WEIGHT Body weight gain Cd consumption Group (g/8 weeks) ([micro]g/24 hours/rat) Control 273.33 [+ or -] 22.12 (b) -- Cd-OC 196.25 [+ or -] 45.18 (a) 340.0 [+ or -] 9.5 (NS) Cd-5C 235.00 [+ or -] 35.86 (b) 346.0 [+ or -] 7.5 Cd-10C 239.17 [+ or -] 44.78 (b) 350.0 [+ or -] 10.5 Liver weight Group (g/100g body weight) Control 2.37 [+ or -] 0.08 (a) Cd-OC 2.96 [+ or -] 0.35 (b) Cd-5C 2.70 [+ or -] 0.34 (ab) Cd-10C 2.51 [+ or -] 0.34 (ab) Data are mean [+ or -] SD values (n = 10 rats per treatment group). Control, control group fed without 10 ppm Cd in drinking water; Cd-OC, Cd treatment, Chlorella-free diet; Cd-5C, Cd treatment, Chlorella supplementation (5% Chlorella diet); Cd-10C, Cd treatment, Chlorella supplementation (10% Chlorella diet). (ab) Values with different letters are significantly among groups at P < .05 by Duncan's multiple range test. NS, not significant. TABLE 4. CONCENTRATIONS OF CD AND NITS IN LIVER Group Cd ([micro]g/g) MTS ([micro]g/g) Control 0.01 [+ or -] 0.01 (c) 2.93 [+ or -] 1.43 (a) Cd-OC 51.49 [+ or -] 1.39 (a) 34.74 [+ or -] 3.1 (c) Cd-5C 45.98 [+ or -] 1.97 (b) 15.18 [+ or -] 2.60 (b) Cd-10C 45.98 [+ or -] 2.87 (b) 12.51 [+ or -] 2.57 (b) Data are mean [+ or -] SE values (n = 10 rats per treatment group). Control, control group fed without 10 ppm Cd in drinking water; Cd-OC, Cd treatment, Chlorella-free diet; Cd-5C, Cd treatment, Chlorella supplementation (5% Chlorella diet); Cd-10C, Cd treatment, Chlorella supplementation (10% Chlorella diet). (a-c) Values with different letters are significantly among groups at P < .05 by Duncan's multiple range test.

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