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Chlorella vulgaris Has Preventive Effect on Cadmium Induced Liver Damage in Rats

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Abstract

We investigated if *Chlorella vulgaris* (CV) has protective effects on cadmium (Cd) induced liver damage in male Sprague-Dawley (SD) rats. Forty rats, aged 5 weeks old and weighed 90-110 g, were divided into a control (with Cd free water), 50 ppm of $CdCl_2$ in drinking water treated groups (Chlorella 0% diet group (Cd/CV0%), Chlorella 5% diet group (Cd/CV5%) or Chlorella 10% diet group (Cd/CV10%). All the rats had freely access to water and diet for 8 weeks. The results show that body weight gain and relative liver weight had significantly lower in Cd/CV0%-treated group than in Cd/CV-treated groups. Hepatic Cd contents showed significantly less by feeding CV ($P<0.05$). Cd/CV0%-treated rats had significantly ($P<0.05$) higher hepatic T-MTs, and Cd-MTs concentrations, compared to Cd/CV5% or Cd/CV10% treated rats. The MT I/II mRNA was expressed in the liver of all experimental rats. Its expression was more increased in Cd/CV5%- or Cd/CV10%-treated rats, compared to control and Cd-treated rats. Thus, this study suggested that CV would have a protective effect on Cd-treated liver injury by the reduction of Cd concentrations and stimulation of Cd-MT binds in the liver. However, more studies are needed to identify the proper mechanism of CV and liver toxicity.

Keywords: *Chlorella vulgaris*, Rats, Cadmium, Liver toxicity, Metallothionein

Cadmium (Cd) is one of heavy metals, which occurs naturally found everywhere in air, water, soils and foodstuff. It has many uses, including batteries, pigments, metal coatings, and plastics because it does not corrode easily. Otherwise, Cd is of public health significance since it is regarded as one of the main industrial and environmental pollutant[1]. Cd is can be easily ingested through contaminated food chain. Its biological half-life in the body estimates range from 20 years to 40 years[2]. Accordingly, exposure to Cd is associated with many chronic diseases such as liver damage[3], kidney tubular dysfunction[4], hypertension[5], osteoporosis[6], and cancer[7] since approximately 20-30 mg of cadmium can accumulate in the human body for their life-time.

Metallothioneins (MTs) are ubiquitous low molecular weight proteins (approxi. 6-7 kDa) and polypeptides of extremely high metal and sulfur content[8]. They are thought to play roles both in the intracellular fixation of the essential trace elements such as zinc and copper, in neutralizing the harmful influences of exposure to toxic elements such as Cd and mercury and in the protection from a variety of stress conditions such as alkylating agents, oxygen radicals, and ionizing radiations[9]. Especially, MTs play a key role in the biological detoxification for heavy metals such as cadmium by sequestration using cysteine residues[10-12]. The binding of cadmium to MTs prevents the free cadmium ions from exerting their toxic effects. Free cadmium ions in the cells as a result of the degradation of MTs initiate the synthesis of new MTs which then binds the cadmium thereby protecting the cell from the highly toxic free cadmium ions. Toxicity may be considered to occur when the binding capacity of MTs is surpassed. There is no evidence that the divalent cadmium cation undergoes biotransformation in man.

Chlorella among functional foods is drawing the public concerns and is widely sold as a heath food and health supplement in Korea, Japan, the U.S. and other countries. Chlorella is a single-celled freshwater alga that contains large amounts of chlorophyll along with protein with all essential amino acids, dietary fiber, and large amount of minerals and vitamins. Chlorella has demonstrated an ability to protect against gamma-radiation and toxic chemicals such as Cd, Hg and dioxins. It alleviated some of the side effects of 5-fluorouracil and helped prevent gastrointestinal absorption and promoted the excretion of dioxin already present in tissues in rats[13,14]. *Chlorella vulgaris*

Table 1. Body weight gain and liver weight of experimental rats[1].

Group[2]	Body weight gain (g)	Liver weight (g/100 g body weight)
Con	90.75±26.07[a]	2.71±0.43[a]
Cd/CV0%	43.79±12.55[b]	3.12±0.45[b]
Cd/CV5%	77.05±21.65[a]	2.75±0.59[a]
Cd/CV10%	77.09±33.57[a]	2.61±0.53[a]

[a-b]Values with different letters are significantly among groups at $P<0.05$ by Duncan's multiple range test.
[1]Values are mean±S.D.: n=10 rats per treatment group.
[2]Con: control group fed with Cd free water, Cd/CV0%: Cd treatment, chlorella free diet, Cd/CV5%: Cd treatment, chlorella supplementation (5% chlorella diet), Cd/CV10%: Cd treatment, chlorella supplementation (10% chlorella diet).

(CV) extracts by hot-water have been reported to show diverse antitumor activity[15], antioxidant activity[16], and antimicrobial activity[17]. Although the enormous amount of effort has been expanded to remove heavy metal by functional foods, almost all studies with CV functionality have been conducted with an extract form. Therefore, in order to investigate the effects of dried CV cells on the removal of Cd in the liver, we examined Cd content in liver, urine, and feces and changes in concentrations and gene expressions of MTs in the liver of male rats treated by 50 ppm Cd and Chlorella meal-based diet.

Body Weight Gain and Liver Weight

During the 8 weeks, body weight gain was shown in Table 1. Cd/CV0%-treated group had significantly ($P<0.05$) less body weight gain than Con, Cd/CV 5%, or Cd/CV10%-treated rats (Table 1). Cd/CV0%-treated rats had significantly ($P<0.05$) heavier liver weight than Con, Cd/CV5%, or Cd/CV10%-treated rats.

Cd Contents in Liver, Feces, and Urine

Cd contents in liver were significantly ($P<0.05$) different between Cd/CV0%-treated group and Cd/CV-treated groups as shown in Table 2. There were no significant differences among all the groups in urinary Cd content. On the other hand, there was significant difference in fecal Cd content by feeding CV ($P<0.05$).

Total MTs (T-MTs) and Cd-binding MTs (Cd-MTs) Concentrations in Liver

The concentration of T-MTs and Cd-MTs in Cd/CV0%-treated rats was 66.78 μg/g and 44.06 μg/g, respectively. It had significantly ($P<0.05$) higher than Cd/CV5%-(T-MTs: 21.84 μg/g, Cd-MTs: 14.87 μg/g), or Cd/CV10%-treated rats (T-MTs: 19.21 μg/g, Cd-MTs: 12.02 μg/g) (Figure 1). However, there were no ($P>0.05$) significant differences in T-MTs, and Cd-MTs concentrations between Cd/CV5% and Cd/CV10%-treated rats.

Table 2. Cd contents in feces and urine.

Group[1]	Cd (μg/g)	Fecal Cd (mg/kg)	Urinary Cd (mg/kg)
Con	0.28±0.24[b]	0.00±0.00[a]	0.02±0.02[a]
Cd/CV0%	66.78±35.38[a]	0.46±0.22[b]	0.02±0.01[a]
Cd/CV5%	21.84±15.90[b]	0.69±0.17[c]	0.02±0.00[a]
Cd/CV10%	19.21±13.76[b]	0.74±0.12[c]	0.22±0.12[b]

[1]Con: control group fed with Cd free water, Cd/CV0%: Cd treatment, chlorella free diet, Cd/CV5%: Cd treatment, chlorella supplementation (5% chlorella diet), Cd/CV10%: Cd treatment, chlorella supplementation (10% chlorella diet).

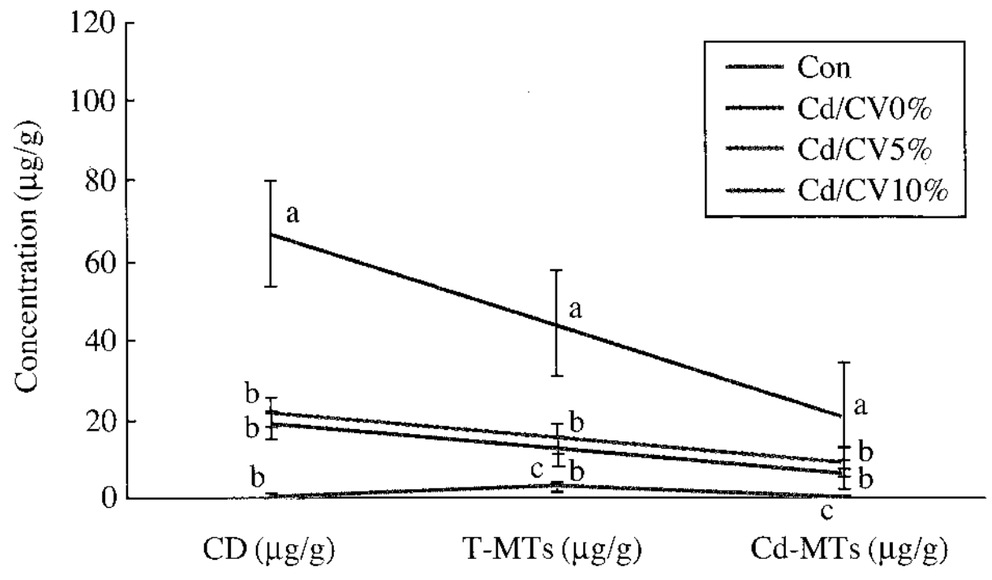

Figure 1. Concentrations of Cd, T-MTs and Cd-MTs in liver. Values are mean±S.D.: n=10 rats per treatment group. Con: control group fed with Cd free water, Cd/CV0%: Cd treatment, chlorella free diet, Cd/CV5%: Cd treatment, chlorella supplementation (5% chlorella diet), Cd/CV10%: Cd treatment, chlorella supplementation (10% chlorella diet).

The MT I/II mRNA Expression in Liver

The MT I/II mRNA expression in liver were shown in Figure 2. The MT I/II mRNA was expressed in liver of all experimental rats. Also, the expression was more increased in Cd/CV5% or Cd/CV10%-treated rats compared to Con and Cd/CV0%-treated rats. Based on this result, MTs can be abundantly present in the liver as the form of Cd-MTs by feeding CV.

Discussion

We examined this study to elucidate whether Cd toxicity in liver tissues is lowered and Cd is excreted by feeding CV or not. For this purpose, the rats treated with 50 ppm Cd in drinking water were fed with a 5% chlorella diet or a 10% chlorella diet for 8 weeks.

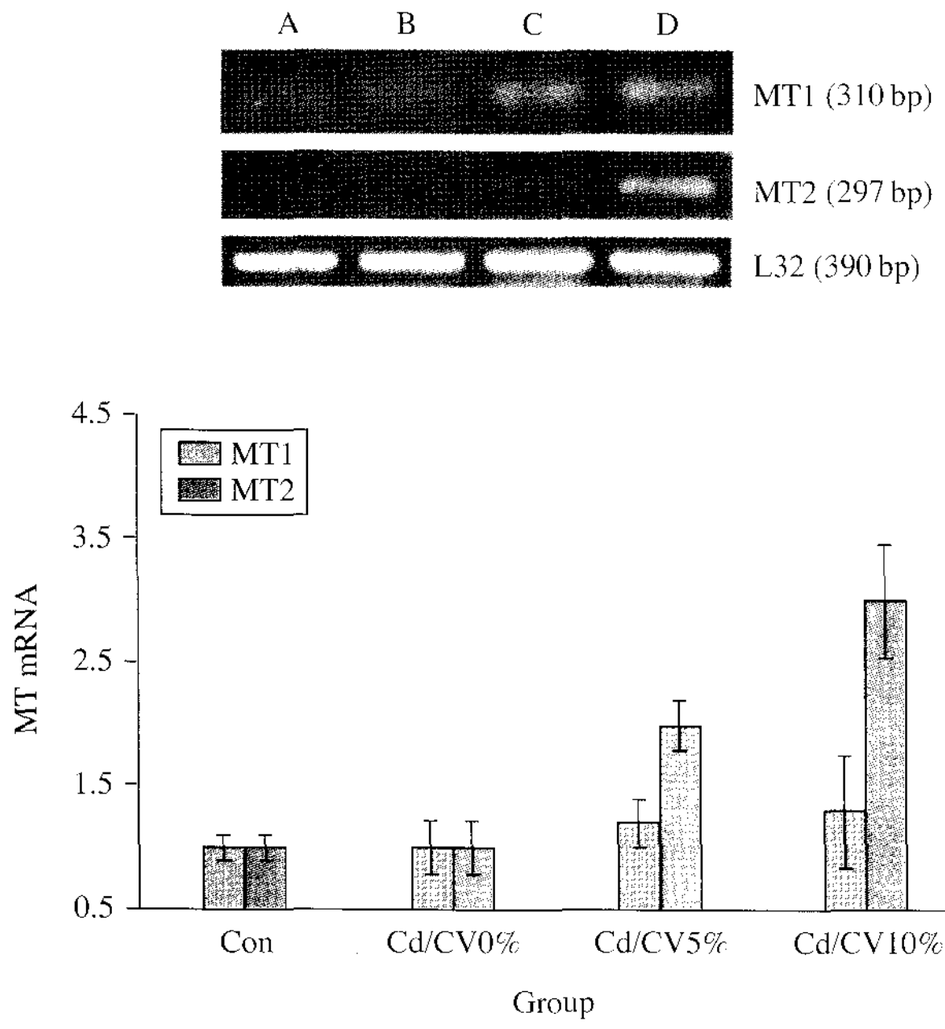

Figure 2. Expression of MT I/II mRNA in liver. A: control group fed with Cd free water, B: Cd treatment, chlorella free diet, C: Cd treatment, chlorella supplementation (5% chlorella diet), D: Cd treatment, chlorella supplementation (10% chlorella diet).

Body weight along with feed consumption is used as one of good indicators to determine if rats were intoxicated by heavy metals such as Cd[18]. In current study, rats treated with 50 ppm of Cd showed a significant ($P<0.05$) reduction in body weight compared to rats treated with CV. The data on body weight loss by Cd treatment was in accordance with that in Han *et al.*[19] study which reported the weight loss of rats administered Cd. However, adding 5% or 10% chlorella to regular feed diet brought increase in weight gain, about 11% over Cd/CV0%-treated group. Thus, body weight gain by CV treatment has been potentially attributed to stabilization of body metabolism. Liver weight was heavier in Cd/CV0%-treated rats than that in Cd/CV-treated rats. It showed that Cd absorbed via blood circulation system is firstly transported and accumulated into the liver although the Cd is transported and accumulated in the kidneys over the time. In order to identify this, Cd content in liver was determined. As expected, liver Cd contents were significantly ($P<0.05$) lower in Cd/CV5% and Cd/CV10%-treated rats compared to Cd-treated rats. Based on this result, Cd treatment has been associated with the hypertrophy of organ tissues such as liver and kidney[20,21]. However, Chlorella treatment may have the resistance to Cd-induced hepatotoxicity which is associated in part, with a lower accumulation of the metal in liver.

Cd induces synthesis of MTs and then binds to MTs, a metal-binding protein of low molecular weight. It was conceivable that the resistance to Cd-induced hepatotoxicity was due to a more efficient induction of MTs resulting in higher hepatic MTs levels. MTs can be biologically made in the liver in order to detoxify heavy metals, when heavy metals are uptake into body[22]. Cd toxicity depends on sequestration degree of Cd from cell or MTs concentrations[6,23]. The present study detected MTs levels in the liver tissue of 50 ppm Cd treated rats fed with varying doses (5 and 10%) of CV. After Cd treatment, an increase of MTs levels was observed in the Cd treated groups. Cd/CV5% or 10%-treated rats had significantly ($P<0.05$) lower T-MTs and Cd-MTs concentrations in the liver compared to Cd/CV0%-treated rats. It showed that the liver started to detoxify Cd absorbed where is main organ of detoxification. However, the ratio of Cd-MT/T-MT level in a single Cd/CV0%-treated group was lower than that in Cd/CV5% or 10%-treated groups. These results were in accordance with a study of Hwang YK *et al.*[24]. This increase in the concentration of Cd-MT by feeding Chlorella can be considered as induced synthesis of MT in the liver and then binding Cd-MT complexes for detoxification. Thus, feeding chlorella enriched in divalent minerals was found to stimulate MTs induction in liver with good efficacy. Cd binds to sulfhydryl groups on cysteine, which may compromise the function of enzymes and ion channels. Cd also interacts with DNA and RNA, resulting in reductions in protein synthesis.

MTs have a few of isoforms, MT I, II, III, IV but the two major forms, MT I/II are ubiquitous in most organs, particularly in liver and kidney. They have different isoelectric points but identical arrangements of cystinyl residues. After Cd-exposure, a clear induction of Cd-MT I and II was found in the liver and kidney. Specific motifs in the 3′-untranslated ends may result in different turnover rates for the mRNA isoforms in different tissues and the cell-type specific translation of the different MT isoforms. In the present study, only MT I and MT II have been examined. The MT I/II mRNA were expressed in the liver of all experimental rats. Also, they were more increased in Cd/CV5% or Cd/CV10%-treated rats than in Con and Cd/CV0%-treated rats. The increase in MT I expression was associated with the protective response of body against Cd, like a study of Alvarez *et al.*[25].

Cd is eliminated mainly in urine and feces. The amount excreted daily represents only about 0.005-0.010% of the total body burden 2. Excretion is proportional to the body burden[26,27] and increases up to 50-60 years of age[28]. Thus Cd contents in urine and

feces were determined in order to identify the effect of Chlorella on Cd excretion. As we expected, the present data showed that Cd excretion via feces was stimulated by feeding Chlorella. It is known that it is due to the properties of Chlorella where contains 55-67% protein, 1-4% chlorophyll, 9-18% dietary fiber and large amount of minerals and vitamins. It is possible that dietary fiber in Chlorella has binding characteristics to divalent metals such as Cd and can serve as a Cd chelate[29].

In conclusion, this study suggested that CV alleviate Cd intoxication due to the reduction of Cd concentrations and stimulation of Cd-MT binds in the liver. Accordingly, CV stimulates the excretion of Cd out of body via feces and urine. This study has further enhanced the current thinking on the health benefits of Chlorella. The further study is needed to identify the molecular mechanism by which Chlorella induces the synthesis of MTs and stimulates Cd detoxification in liver.

Methods

Experimental Animals and Diets

Five-week-old male Sprague-Dawley (SD) rats weighing 90-110 g were purchased from Orient Bio Inc. (Seoul, Korea) and allowed to acclimatize for one week prior to commencement of the test. The animals were housed in plastic cages in a room with controlled temperature (23±2°C), humidity (50-60%), and lighting (12 h light: 12 h darkness) with free access to water and lab chow. They were randomly divided into one control and three 50 ppm of $CdCl_2$ in drinking water (Cd)-treated groups. The Cd groups (n =10/group) included a Cd-treated group (Cd/CV0%-treated group), 5% Chlorella diet group (Cd/CV5%-treated group) or 10% Chlorella diet (Cd/CV10%-treated group). All the rats had freely access to water and diet for 8 weeks. CV was obtained from Daesang Wellife Co. (Seoul, Korea) and composition of normal and chlorella meal-based diet were made up 30% casein, 15% cornstarch, 50% sucrose, 5% cellulose, 5% coconut oil, 3.5% mineral mixture, 1% vitamin mixture. These diets had nearly the same composition except that chlorella meal-based diet contains 5% or 10% chlorella. All treatments and procedures were conducted in accordance with Hanyang University Lab Animal Care Committee (HALACC) animal use protocols.

Measurement of Cd Contents in Liver, Feces, and Urine

During the autopsy, liver was removed and preserved at −20°C before analysis. Before the scarification, 14-h urine was collected in individual plastic metabolic cages. The urine was centrifuged immediately after collection and volume of the 14-h urine and feces were recorded. The liver, feces and urine were wet-digested with 5 mL of HNO_3 and diluted up to 25 mL with water. The Cd contents were determined by Inductively Coupled Plasma Mass Spectrophotometry (ICP-MS, Model 3520, Perkin Elmer, Fremont, CA, USA).

Measurement of Total and Cd-binding Methallothionein Contents in Liver

About 1 g of liver was homogenized in 4 vol of 0.25 M sucrose and was centrifuged at 18,000 g for 20 min at 4°C. Aliquots 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 $CdCl_2$ solution (50 ppm Cd) and was incubated at room temperature for 5 min. The metal binding sites of metallothionein were saturated with Cd during this step. The complete saturation with Cd was ensured by using various aliquots of the sample. The excess of Cd was removed and precipitated by addition of rat RBC hemolysate (0.2 mL) and heat treatment in a water bath (about 100°C for 1 min). Rat hemoglobin has a high affinity for Cd and can remove Cd from all the bioligands except metallothionein. During the heat treatment Cd-bound hemoglobin was denatured and was removed by centrifugation at 1,000 g for 5 min at room temperature. The addition of the hemolysate and the heat treatment were repeated three times. The amount of Cd in the heated supernatant fraction is a measure of metallothionein bound Cd and was determined in an atomic absorption spectrophotometer (AAs, Cambridge, UK) by using an air-acetylene flame.

Measurement of mRNA Metallotheionein (MT) Expression by RT-PCR

Total RNA was extracted from the liver by homogenization TRIzol. Extracted total RNA was quantized by absorbance measurements at 260 and 280 nm and stored at −80°C. From each sample, 100 ng of RNA was reverse-transcribed (RT) using AMV reverse transcriptase, 1 mM dNTP, and oligo (dT12−18) 0.5 μg/mL. Then PCR analyses were performed on the aliquots of the cDNA preparations to detect MT I, MT II, and L32 (as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA, U.S.A.). The reactions were carried out in a volume of 20 μL containing (final concentration) 1 unit of Taq DNA polymerase, 0.2 mM dNTP, 10 μL reaction buffer, and 100 pmol of 5′ and

3′ primers. After initial denaturizing for 2 min at 95°C, thirty amplification cycles were performed for MT I (1 min of 94°C denaturizing, 1 min of 60°C annealing, and 1.5 min 72°C extension) MT II (1 min of 94°C denaturizing, 1 min of 60°C annealing, and 1.5 min 72°C extension) and L32 (1 min of 94°C denaturizing, 1 min of 55°C annealing, and 1 min 72°C extension). The PCR primers used in this study are listed below and were purchased from Bioneer Corp.: sense strand MT I, 5′-ACTGCCTTGTCGCTTA-3′; anti-sense strand MT I, 5′-TGGSGGTGTACGGCAAGACT-3′; sense strand MT II, 5′-CCAACTGCCGCCTCCATT-CG-3′; anti-sense strand MT II, 5′-GAAAAAAGTG-TGAGGAACCG-3′; sense strand L32, 5′-CGTGGG-CCGCCCTAGFCACCA-3′; anti-sense strand L32, 5′-TTGGCCTTAGGGTTCAGAGGGG-3′. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Statistical Analysis

All data were presented as means SD. Statistical analyses were performed the statistical package SPSS (Version 12.0, SPSS Inc., CA, U.S.A.) software, and significance of each group was verified with one-way analysis of variance followed by Duncan's multiple range test with significance set at $P<0.05$.

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