— **Regular Article** —

Protective Effect of *Chlorella vulgaris* against Lead-Induced Oxidative Stress in Rat Brains

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Lead (Pb) is a toxic heavy metal widely distributed in the environment. Recent studies suggest oxidative stress as one possible mechanism involved in Pb poisoning. The unicellular algae *Chlorella vulgaris* (CV) contains various bioactive substances with antioxidant for the prevention of oxidative stress by metals. We investigated the protective effects of CV on the oxidative system in five groups of male Sprague-Dawley rats fed American Institute of Nutrition (AIN)-76 diet, plus 2, 5 or 10% CV for 4 weeks. All animals were exposed to 200 mg/l lead acetate by drinking water except for the control (tap water). Body weight gains were significantly reduced in the Pb-exposed group (64%) relative to the control and CV groups. Brain weights were significantly increased in the Pb-exposed group (44%) relative to the others. In the experimental period, food intake, water intake and Pb intake were not different among the groups. The levels of Pb (87%) in brain obtained from the Pb-exposed group were significantly increased compared to the other groups. The levels of oxidative stress parameters in the brain such as superoxide dismutase (36%), glutathione peroxidase (63%), and glutathione reductase (30%) were decreased in the Pb-exposed group relative to the control but markedly increased in the CV groups. The CV also significantly increased glutathione levels by approximately 1.7-fold over the Pb-exposed group, while the malondialdehye concentration significantly decreased by approximately 47–71%. Based on these results, we found alterations in several indicators of oxidative stress of Pb intoxication, suggesting the antioxidant potential of CV. Therefore, CV may have protective effects on brain damage of low-level and short-term Pb exposure in the brains of rats.

Key words —— *Chlorella vulgaris*, lead toxicity, oxidative stress, antioxidant enzyme

INTRODUCTION

Lead (Pb) is an extensively used metal in applications such as batteries, paints and pigments in industries. The population may get exposed to lead by food and water contamination, and air pollution caused by industrial emission and gasoline containing lead compounds.[1)] Lead-exposure occurs through the respiratory and gastrointestinal systems, and lead which is ingested and absorbed is stored mainly in soft tissues and bone.[2)] Lead has deleterious effects on neurological, hematological, gastrointestinal, reproductive, circulatory, and immunological pathologies.[3)] One of the major symptoms in lead poisoning is generally known to be brain damage. Furthermore, an exposure to lower levels of lead is often related to behavioral abnormalities, learning disabilities, hearing dysfunctions and impaired cognitive functions in experimental animals and humans.[4–9)] Recent studies have reported that low-level lead exposure has a graded association with several diseases including components of oxidative stress. But the association between low level lead exposure and oxidative stress has not been explored systematically.[10)] One possible molecular mechanism involved in lead neurotoxicity is the disruption of the prooxidant/antioxidant balance,[11–13)] which can

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lead to brain injury via oxidative damage to critical biomolecules such as lipids, proteins and DNA.[14)]

Oxidative stress is one of the important mechanisms of the toxic effects of lead.[15–17)] Several studies reported alternations in antioxidant enzyme activities such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), and changes in the concentrations of some antioxidant molecules such as glutathione (GSH) in lead-exposed animals.[18, 19)] SOD activity is an important component of the cellular antioxidant system that protects cells from the harmful effects of O_2^- and its derivatives.[20)] Inhibition of SOD activity by Pb was also shown in an *in vitro* study where the authors indicated that this effect of Pb can lead to decreased scavenging of reactive oxygen species (ROS) and results in oxidative damage.[21)] Lead has a high affinity for sulfhydryl (SH) groups, mercaptides are formed with the SH group of cysteine, and less stable complexes with other amino acid side chains.[22)] GSH is a tripeptide containing cysteine and its active group is represented by the SH groups of cysteine residue in the antioxidant cellular defense.[23)] Lead binds exclusively to the SH groups, which decrease the GSH levels.[24)] Glutathione reductase (GR) reduces oxidized GSH (GSSG) back to GSH and supports the antioxidant defense system indirectly.[25)] GR possesses a disulfide at its active site, which was suggested as a target for lead, resulting in the inhibition of the enzyme.[12)] GPx activity represents the first protective response for small adjustments in H_2O_2 concentrations under normal physiological conditions.[26)] Malondialdehyde (MDA) is a marker for the determination of lipid peroxidation. The protective role of antioxidant such as α-tocopherol, ascorbic acid and methionine is reducing the lipid peroxidation as evidenced by a decreased MDA concentration in the brain because of Pb-induced oxidative stress.[27)] Taking these findings together, it is suggested that oxidative stress, which is induced by lead, is recovered by natural antioxidants, such as ascorbic acid,[28)] epigallocatechin-3-gallate[29)] and plant extracts *etc*.[30, 31)]

Marine algae have the potential to improve the biochemical imbalances induced by various toxins associated with free radicals. The unicellular green algae *Chlorella vulgaris* (CV) contains many bioactive substances with medicinal properties.[32)] Some studies have demonstrated the antioxidant properties of Chlorella against various diseases, heavy metals and UV-B exposure.[32–37)] Biochemically, Chlorella contains many dietary antioxidants such as lutein, α-carotene, β-carotene, ascorbic acid and α-tocopherol and these bioactive compounds have the ability to scavenge free radicals.[38–40)] In recent studies, we reported that supplementation of CV has a potential role in preventing toxin-caused oxidative stress and cadmium-induced hepatotoxicity.[41, 42)]

The present study was performed to examine the oxidative stress damage in lead-exposed rat brains and the protective effect of CV in recovering from lead-induced toxicity as a food-based natural antioxidant. We investigated the effect of lead and CV on GSH, several antioxidant enzymes and MDA in the brains of the rats which had been exposed 200 mg/l lead acetate and CV.

MATERIALS AND METHODS

Chemicals —— Lead acetate [$(C_2H_3O_2)_2Pb{\cdot}3H_2O$, Pb] was obtained from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). CV, a strain of unicellular green algae, was provided by Daesang Wellife (Seoul, Korea). All other chemicals and solvents used for this study were the highest grade available.

Animals and Treatment —— Male Sprague-Dawley rats (6 weeks old) weighing approximately 145–170 g were obtained from Shizuoka Laboratory Center Inc. (Hamamatsu, Japan) and acclimated for 1 week prior to experimental use. The animals were randomly assigned into five groups (ten animals each) and fed one of the following diets and drinking water: American Institute of Nutrition (AIN)-76 diet (control diet) and tap water (control group, Control), AIN-76 diet and 200 mg/l lead acetate water (Pb-exposed group, Pb), and AIN-76 modified diet plus 2, 5 or 10% CV (CV diet) and 200 mg/l lead acetate water (Pb+CV2, Pb+CV5, Pb+CV10). The dose level of Pb was designed as described by Jamieson *et al*.[43)] with modification. This concentration was chosen to produce a sub-clinical toxicity, as the dosage given for 10 weeks with a standard laboratory rat chow has shown not to produce significant alterations in hematopoiesis, histology and function.[44)] The compositions of the diets are provided in Table 1. After 4 weeks of treatment, animals were deprived of food for 12 hr and anesthetized by overexposure of CO_2. Brains were quickly removed, weighed, placed in ice-cold saline and stored at −20°C for further studies. Urine and feces samples were individually obtained in metabolic cages for 12 hr before sacrifice.

Table 1. Composition of Experimental Diet

(Unit: g/kg)

Ingredients	Control diet	CV diet 2%	CV diet 5%	CV diet 10%
Casein	200.0	184.5	160.9	125.8
DL-Methionine	3.0	3.0	3.0	3.0
Corn starch	150.0	150.0	150.0	150.0
Sucrose	500.0	496.0	489.9	479.8
Cellulose	50.0	49.7	49.2	48.4
Coconut oil	50.0	49.8	50.0	50.0
Mineral Mixture[a)]	35.0	35.0	35.0	35.0
Vitamin Mixture[b)]	10.0	10.0	10.0	10.0
Chlorine Bitartrate	2.0	2.0	2.0	2.0
Chlorella vulgaris (CV)	0.0	20.0	50.0	100.0
Total	1000.0	1000.0	1000.0	1000.0
Energy (kcal)	3862.0	3850.0	3834.0	3823.0

Mineral mixture (g/100 g): $CaHPO_4{\cdot}2H_2O$, 0.43; KH_2PO_4, 34.31; NaCl, 25.06; $Fe(C_6H_5O_7){\cdot}6H_2O$, 0.623; $MgSO_4{\cdot}7H_2O$, 9.98; $ZnCl_2$, 0.02; $MnSO_4{\cdot}4\text{-}5H_2O$, 0.121; $CuSO_4{\cdot}5H_2O$, 0.156; KI, 0.0005; $CaCO_3$, 29.29; $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, 0.0025. *b*) vitamin mixture (mg/100 g): vitamin A acetate, 93.2; vitamin D_3, 0.5825; α-tocopherol-acetate, 1200; vitamin K_3, 6.0; vitamin B_1 hydrochloride, 59.0; vitamin B_2, 59.0; vitamin B_6 hydrochloride, 29.0; vitamin B_{12}, 0.2; vitamin C, 588.0; D-biotin, 1.0; foric acid, 2.0; pantothenic acid, 235.0; nicotinic acid, 294.0; inositol, 1176.0; lactose, 96257.0.

The animals were housed in plastic cages and provided with free access to their allocated diet and drinking water. They were kept in a temperature-controlled environment animal facility at $22 \pm 2°C$ with a 12 hr light/dark cycle. For chelation of divalent ions, all of the used experimental appliances were prepared with ethylenediaminetetraacetic acid (EDTA) treated solution before usage. All animals were maintained and managed in accordance with Hanyang University Lab Animal Care Committee (HULACC) animal use protocols.

Lead Estimation —— For Pb determination, wet weights were recorded. Samples were digested with 25 ml of concentrated nitric acid, and diluted up to 25 ml with distilled water. Pb concentration was determined in the samples by a Shimadzu inductively coupled plasma mass spectrometer (model ICPM-8500; Shimadzu, Tokyo, Japan).

Preparation of Brain Extracts —— For measurement of antioxidant enzyme activities, GSH and lipid peroxidation, brain extracts were prepared. Briefly, a portion of brain tissue was homogenized in a sucrose buffer (0.25 M sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.4) and the homogenate was centrifuged at $10000 \times g$ for 60 min at 4°C for the assay of SOD activity. The supernatants were collected and stored at −80°C for use. The rest of the brain tissue was homogenized in cold phosphate buffered saline (PBS) with 1 mM EDTA, and the homogenate was centrifuged at $12000 \times g$ for 15 min at 4°C. The supernatants were analyzed for GR activity, GPx activity, GSH and MDA concentration.

Measurement of SOD, GR and GPx Activities —— To measure the level of oxidative stress in the brains of the animals after treatment with lead, CV, or both, biomarkers related to oxidative stress were measured. SOD, GR and GPx activities were determined with commercial assay kits and procedures were performed according to the instructions of the manufacturer.

SOD activity was determined by the modified method of NADPH-phenazinemethosulphate-nitroblue tetrazolium formazan inhibition reaction spectrophotometrically using a commercial kit (SOD assay kit-WST, Dojindo Laboratories, Inc., Kumamoto, Japan). The activity of GR in brain homogenate was assayed using a commercially available GR Activity Assay Kit (NWK-GR01, Northwest Life Science Specialties, LLC., Vancouver, WA, U.S.A.). GPx activities were measured by the modified method according to Paglia and Valentine (1967) using a commercial kit (NWK-GPX01, Northwest Life Science Specialties, LLC.).[45)]

Estimation of GSH —— GSH concentration of the brain homogenate was determined, as described previously with some modification,[46)] by commercial available Assay Kits (NWK-GSH01, Northwest Life Science Specialties, LLC.), according to the manufacturer's instructions.

Lipid Peroxidation —— The concentration of MDA in brain homogenate was measured, as described previously with some modification,[47)] using commercial available Assay Kits (NWK-MDA01, Northwest Life Science Specialties, LLC.), according to the manufacturer's protocol.

Total Protein Determination —— The protein content of the supernatant was determined using a commercial protein assay kit (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Inc., Richmond, CA, U.S.A.). Crystalline bovine serum albumin (BSA) was used for the standard curve. The concentration was measured using a spectrophotometer at 595 nm.

Statistical Analysis —— Data were expressed as means ± S.E. The results were analyzed for statistically significant experimental differences using one-way analysis of variance (ANOVA) and post-hoc Duncan's multiple range tests. Differences were considered statistically significant at p-value < 0.05.

Table 2. Body Weight Gain and Brain Weight in Rats for 4 Weeks

Groups	Body weight Gain (g)	Brain weight	
		Absolute (g)	Relative (g)*
Control	122.88 ± 2.92	1.73 ± 0.04$^{a,b)}$	0.50 ± 0.01
Pb	44.80 ± 3.32**	1.67 ± 0.07$^{a)}$	0.72 ± 0.38**
Pb + CV2	121.44 ± 3.54	1.78 ± 0.05$^{a,b)}$	0.48 ± 0.15
Pb + CV5	125.14 ± 3.45	1.91 ± 0.04$^{b)}$	0.50 ± 0.08
Pb + CV10	126.50 ± 4.65	1.82 ± 0.04$^{a,b)}$	0.48 ± 0.15

All values are means ± S.E. (n = 10). Control (Pb free drinking water + control diet); Pb (drinking water containing 200 mg/l Pb + control diet); Pb + CV2 (drinking water containing 200 mg/l Pb + 2% chlorella diet); Pb + CV5 (drinking water containing 200 mg/l Pb + 5% chlorella diet); Pb + CV10 (drinking water containing 200 mg/l Pb + 10% chlorella diet). * Brain weight/100 g body weight. ** Values are significantly different among columns at p-values < 0.05 by Duncan's multiple range tests. $a), b)$ Values with different letters are significantly different among columns at p-values < 0.05 by Duncan's multiple range tests.

Table 3. Food, Water and Lead Intake in Rats for 4 Weeks

Groups	Food intake (g/day)	Water intake (g/day)	Lead intake (mg/day)
Control	21.72 ± 1.34^{ns}	23.33 ± 0.11^{ns}	—
Pb	19.78 ± 1.42	23.23 ± 0.12	4.64 ± 0.03^{ns}
Pb + CV2	21.18 ± 0.83	23.38 ± 0.78	4.68 ± 0.17
Pb + CV5	20.86 ± 0.70	23.32 ± 0.82	4.66 ± 0.15
Pb + CV10	20.92 ± 0.44	23.00 ± 0.71	4.58 ± 0.13

All values are means ± S.E. (n = 10). Control (Pb free drinking water + control diet); Pb (drinking water containing 200 mg/l Pb + control diet); Pb + CV2 (drinking water containing 200 mg/l Pb + 2% chlorella diet); Pb + CV5 (drinking water containing 200 mg/l Pb + 5% chlorella diet); Pb + CV10 (drinking water containing 200 mg/l Pb + 10% chlorella diet). ^{ns}Non-significant.

RESULTS

Changes in Body Weight Gain, Brain Weight, Food, Water and Lead Intake

Body weight gains and the brain weights of the rats are presented in Table 2. A significant decrease was observed for the Pb-exposed group (44.80 ± 3.32 g) when compared to the control (122.88 ± 2.92 g), Pb+CV2 (121.44 ± 3.54 g), Pb+CV5 (125.14 ± 3.45 g) and Pb+CV10 (126.50 ± 4.65 g) groups. The absolute brain weight of the Pb-exposed group (1.67 ± 0.07 g) was significantly lower than the Pb+CV5 (1.91 ± 0.04 g) group. However, the relative brain weight of the Pb-exposed group was significantly higher than the control (0.50 ± 0.01 g), Pb+CV2 (0.48 ± 0.15 g), Pb+CV5 (0.50 ± 0.08 g) and Pb+CV10 (0.48 ± 0.15 g) groups. As shown in Table 3, the food, water and lead intakes for 4 weeks were not significantly different among the groups.

Concentrations of Lead in Tissues, Urine and Feces

The lead concentrations in brain, urine and feces of rats are listed Table 4. The brain lead level in the Pb-exposed group (92.46 ± 8.02 μg/g wet weight) was significantly higher than in the control (49.14 ± 2.53 μg/g wet weight). In contrast, the brain lead levels were significantly reduced in the Pb+CV2 (47.81 ± 3.31 μg/g wet weight), Pb+CV5 (47.00 ± 4.70 μg/g wet weight) and Pb+CV10 (51.60 ± 5.85 μg/g wet weight) groups compared with the Pb-exposed group. Urinary lead levels had no significant differences in between Pb and Pb+CV groups. The lead concentrations of liver and kidney in the Pb-exposed group were shown significant increases compared to the control. On the other hand, the liver lead concentrations were significantly decreased in the Pb+CV5 and Pb+CV10 supplemented groups compared with the Pb-exposed groups. The lead levels in kidney were also shown significant decreases in the Pb+CV5 and Pb+CV10 supplemented groups compared to the Pb-exposed groups. There were significant differences in the

Table 4. Lead Concentration in Tissues, Urine and Feces

Groups	Tissue (μg/g wet weight)			Urine	Feces
	Brain	Liver	Kidney	(mg/ml urine)	(mg/g feces)
Control	$49.14 \pm 2.53^{b)}$	$5.05 \pm 0.02^{c)}$	$31.27 \pm 2.97^{c)}$	$0.07 \pm 0.01^{b)}$	$0.45 \pm 0.13^{c)}$
Pb	$92.46 \pm 8.02^{a)}$	$50.12 \pm 7.54^{a)}$	$175.53 \pm 8.45^{a)}$	$0.70 \pm 0.31^{a)}$	$13.70 \pm 0.84^{b)}$
Pb + CV2	$47.81 \pm 3.31^{b)}$	$48.11 \pm 6.47^{a)}$	$169.26 \pm 8.74^{a)}$	$0.90 \pm 0.59^{a)}$	$21.04 \pm 0.52^{a)}$
Pb + CV5	$47.00 \pm 4.70^{b)}$	$31.42 \pm 5.36^{b)}$	$115.95 \pm 9.63^{b)}$	$0.49 \pm 0.35^{a)}$	$14.93 \pm 2.30^{b)}$
Pb + CV10	$51.60 \pm 5.85^{b)}$	$25.81 \pm 4.87^{b)}$	$134.57 \pm 7.81^{b)}$	$0.68 \pm 0.44^{a)}$	$12.60 \pm 1.17^{b)}$

All values are means ± S.E. (n = 10). Control (Pb free drinking water + control diet); Pb (drinking water containing 200 mg/l Pb + control diet); Pb + CV2 (drinking water containing 200 mg/l Pb + 2% chlorella diet); Pb + CV5 (drinking water containing 200 mg/l Pb + 5% chlorella diet); Pb + CV10 (drinking water containing 200 mg/l Pb + 10% chlorella diet). a, b, c) Values with different letters are significantly different among columns at p-values < 0.05 by Duncan's multiple range tests.

Table 5. Antioxidant Enzyme Activities in the Brains of Rats

Groups	SOD	GPx	GR
	(U/mg protein)	(mU/100 mg protein)	(mU/mg protein)
Control	$17.71 \pm 2.68^{b)}$	$2.21 \pm 0.86^{b)}$	$33.04 \pm 5.40^{c)}$
Pb	$11.74 \pm 3.14^{b)}$	$0.81 \pm 0.24^{a)}$	$23.52 \pm 5.42^{d)}$
Pb + CV2	$21.28 \pm 3.81^{a)}$	$2.04 \pm 0.42^{b)}$	$46.38 \pm 9.10^{b)}$
Pb + CV5	$21.99 \pm 3.18^{a)}$	$3.76 \pm 0.88^{c)}$	$99.74 \pm 3.52^{a)}$
Pb + CV10	$26.77 \pm 3.94^{a)}$	$4.21 \pm 0.47^{c)}$	$66.36 \pm 7.14^{b)}$

All values are means ± S.E. (n = 10). Control (Pb free drinking water + control diet); Pb (drinking water containing 200 mg/l Pb + control diet); Pb + CV2 (drinking water containing 200 mg/l Pb + 2% chlorella diet); Pb + CV5 (drinking water containing 200 mg/l Pb + 5% chlorella diet); Pb + CV10 (drinking water containing 200 mg/l Pb + 10% chlorella diet). a, b, c) Values with different letters are significantly different among columns at p-values < 0.05 by Duncan's multiple range tests.

fecal lead levels between the control and Pb-exposed groups (control: 0.45 ± 0.13 mg/g feces, Pb-exposed group: 13.70 ± 0.84 mg/g feces). Fecal lead levels in the Pb+CV5 and Pb+CV10 groups were not increased compared with the Pb-exposed group, while that in the Pb+CV2 (21.04 ± 0.52 mg/g feces) group was significantly increased relative to the Pb-exposed group.

Antioxidant Enzyme Activities in Brain

Table 5 listed the selected parameters (SOD, GPx and GR) related to oxidative stress in the brain. SOD activity of the Pb-exposed group was slightly decreased compared to the control, whereas that of the CV-treated animals was markedly increased when compared with the Pb-exposed group. GPx activity in the Pb-exposed group was markedly decreased to about 50% that of the control group. In contrast, these parameters were highly increased in the Pb+CV groups compared with Pb-exposed group. GR activities were significantly decreased in the Pb-exposed group in comparison with the control while its activities in the CV-treated groups were significantly elevated compared with the control and Pb-exposed group.

GSH Level in Brain

As shown in Fig. 1, a significant decrease of the GSH level was observed in the Pb-exposed group (2.80 ± 0.42 μM/mg protein) compared to the control (5.24 ± 0.67 μM/mg protein). In contrast, the level was significantly higher in the Pb+CV groups (Pb+CV2: 4.82 ± 0.45 μM/mg protein, Pb+CV5: 5.40 ± 0.37 μM/mg protein, Pb+CV10: 5.62 ± 0.73 μM/mg protein) than in the Pb-exposed group.

Lipid Peroxide (LPO) Level in Brain

The MDA level in brain, an end product indicative of the extent of lipid peroxidation, is represented in Fig. 2. The MDA concentration levels in the brains were significantly increased in the Pb-exposed group (3.75 ± 0.16 μM/mg protein) when compared with the control (2.46 ± 0.50 μM/mg protein). On the other hand, following the supplementation of CV2 and CV5 in rats, the MDA levels showed a reversal to near to the control level, and this parameter in the Pb+CV10 group

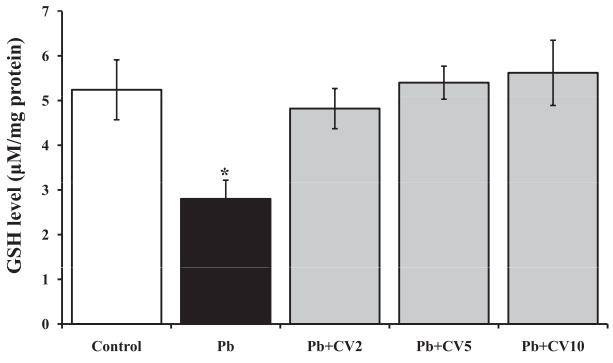

Fig. 1. GSH Level in Brains of Rats

All values are means ± S.E. ($n = 10$). The used animals in this study were randomly assigned into five groups (ten animals each). Control (Pb free drinking water + control diet); Pb (drinking water containing 200 mg/l Pb + control diet); Pb + CV2 (drinking water containing 200 mg/l Pb + 2% chlorella diet); Pb + CV5 (drinking water containing 200 mg/l Pb + 5% chlorella diet); Pb + CV10 (drinking water containing 200 mg/l Pb + 10% chlorella diet). *Values with different letters are significantly different among the groups at p-values < 0.05 by Duncan's multiple range tests.

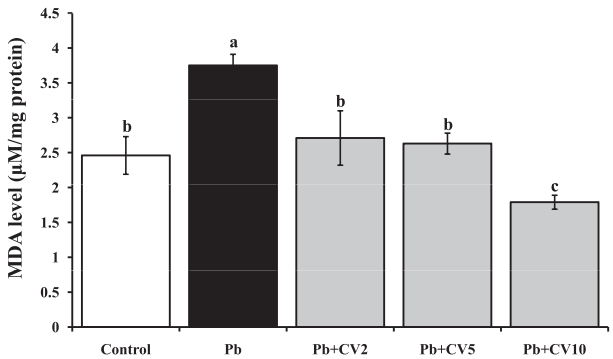

Fig. 2. Lipid Peroxide Level in Brains of Rats

All values are means ± S.E. ($n = 10$). The used animals in this study were randomly assigned into five groups (ten animals each). Control (Pb free drinking water + control diet); Pb (drinking water containing 200 mg/l Pb + control diet); Pb + CV2 (drinking water containing 200 mg/l Pb + 2% chlorella diet); Pb + CV5 (drinking water containing 200 mg/l Pb + 5% chlorella diet); Pb + CV10 (drinking water containing 200 mg/l Pb + 10% chlorella diet). ^{a,b,c}Values with different letters are significantly different among the groups at p-values < 0.05 by Duncan's multiple range tests.

(1.79 ± 0.20 μM/mg protein) was significantly reduced compared with the control.

DISCUSSION

Lead is a one of the major heavy metals known to be toxic to mammals. Lead poisoning is associated with physiological problems such as mental retardation, learning disabilities, low birth weight and behavioral problems.[48] In particular, lead can cause some serious damage in the brain and oxidative stress has been proposed as a potential mechanism in the pathogenesis of lead toxicity.[49]

The present study investigated the lead-induced oxidative stress and antioxidant ability of CV in the brains of rats which were given a low-level exposure to lead acetate for 4 weeks. A significant decrease in body weight gain was observed in the Pb-exposed group compared with the control. Our results were in agreement with measurements described by Jamieson *et al.*, who stated that the weights of the rats which received treatments with 200 mg/l lead for 3 weeks were significantly decreased.[50] However, rats that were fed with CV recovered their body weights to that of the control within 4 weeks, although food, water consumption and lead exposure levels were not different among the groups. The lead concentration in the tissues (brain, liver and kidney) of the Pb-exposed group was significantly higher than of the control group. However, a decrease of lead concentration in tissues (brain, liver and kidney) was shown in the CV-fed groups. An increase in the fecal lead concentration was also observed in the CV-fed groups relative to the Pb-exposed group. The data from our studies indicated CV supplementation caused the recovery of body weight gains, inhibited bioaccumulation in tissue and prompted lead excretion into the feces. There are several lead-nutrient interactions that have been studied and supported by experimental evidence, in which the nutritional factors have been demonstrated to have an impact on health outcomes following lead exposure.[51,52] CV is known to contain various nutritional ingredients such as 13.7% carbohydrate, 61.6% proteins, 12.5% fat, 9–18% dietary fiber, trace elements (selenium, zinc, calcium and magnesium) and vitamins (thiamine, B1, B2, ascorbic acid, D, E and K).[37,53]

Lead-induced disruption of the prooxidant/antioxidant balance in the brain could induce injury via oxidative damage to critical biological macromolecules. Oxidative damage is considered a major cause of lead-induced brain damage, because the brain is believed to be particularly vulnerable to oxidative stress due to the high rate of oxygen free radical generation without commensurate levels of antioxidant defenses.[54,55] Generation of highly ROS, such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH) and LPO, in the result of heavy metal ions are known to damage various cellular components including proteins, membrane lipids and nucleic acids.[56] *In*

vitro and *in vivo* studies pointed that lead exposure might cause the generation of ROS and changes in the antioxidant defense systems in animals.[57–60)]

Antioxidant enzymes such as SOD, GPx, and GR are supposed to be the primary defenses that prevent biological macromolecules from oxidative damage. SOD is the most important line of the antioxidant enzyme defense system against ROS and, in particular, superoxide anion radicals. Moreira *et al*. (2001) demonstrated that brain antioxidant defenses (SOD and GPx) from individuals exposed to Pb decreased significantly.[14)] In our study, SOD activity tends to be decreased in the brain after Pb exposure, while in CV-treated groups it had increased. GPx needs GSH to decompose H_2O_2 or other peroxides with the simultaneous oxidation of GSH into GSSG. GPx is a potential target for lead toxicity because a lead-associated reduction in the selenium uptake may increase the susceptibility of cells to oxidative stress. An antagonistic effect between selenium and Pb was found to affect GPx activity that requires selenium as a cofactor.[61)] Wang *et al*. (2006) described that the GPx activity of mouse brains was decreased in the Pb-exposure group compared with the control group.[19)] Similar results were found in our study; GPx activities in the Pb-exposed group were decreased relative to the groups fed 5 and 10% CV. GR, another component of the antioxidant defense system, reduces GSSG back to GSH and thereby supports the antioxidant defense system indirectly. GR possesses a disulfide at its active site that was suggested as a target for lead, resulting in the inhibition of the enzyme.[62)] The inhibition results in a decrease in the GSH/GSSG ratio that renders cells more susceptible to oxidative damage. Moreira *et al*. (2001) have reported that the effect of lead exposure induced a decrease in the activity of GR.[14)] Our study has shown that GR activity in the brain of the Pb-exposed group was lower than in the control, while GR activity increased in the 5 and 10% CV groups in comparison with the Pb-exposed group.

GSH is synthesized in the cytoplasm of the liver cells and then distributed through the circulatory/transport system into different organs.[63)] GSH plays a crucial role in both scavenging ROS and in the detoxification of chemical compounds. Therefore, perturbation in the redox status of GSH cannot only impair cell defenses against toxic compounds, but also result in enhanced oxidative stress and tissue injury.[64)] Lead has a very high affinity for SH groups and therefore results in a lead-exposed decrease in GSH levels. We also observed that a significant decrease (87.1%) of the GSH level was observed in the Pb-exposed group relative to the control, and an increase in the GSH level was shown in the administration of CV. Similarly, Aykin-Burns *et al*. (2003) described that GSH levels in the brains of Pb-treated groups were found to be lower than in the control.[65)] However, the concurrent treatment of Chlorella prevented GSH depletion, indicating the activity of a protective antioxidant.

A number of *in vitro* and *in vivo* studies have shown that a significant increase of the MDA level is correlated with an increase in the lead exposure.[66, 67)] Pande *et al*. (2001) demonstrated that, by means of a certain antioxidant supply, the production of MDA was reduced by 30–40%.[68)] In agreement with these research results, we observed that lipid peroxidation is amplified (52.0%) in the brain after exposure to lead. However, the MDA level of rats that were fed the CV mixed diet was interestingly significant decreased compared to the Pb-exposure group.

In conclusion, our study showed that the administration of CV is capable of reducing free radical damage by directly acting as a free radical scavenger and by indirectly stimulating antioxidant enzyme activities when animals were given a subchronic low-level exposure to lead. Based on the results obtained from this study, an increase of antioxidant abilities is expected when animals are given CV simultaneously to lead exposure. The protective effects of CV against lead-induced toxicity may be due to various bioactive ingredients in CV, which react with various ROS as well as inhibits oxidation processes in lipids and in the cellular compartment. Further studies are required to confirm and to better understand the underlying molecular mechanisms for the protective effect of CV.

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REFERENCES

1) Pande, M. and Flora, S. J. S. (2002) Lead induced oxidative damage and its response to combined administration of α-lipoic acid and succimers in rats. *Toxicology*, **177**, 187–196.
2) Mudipalli, A. (2007) Lead hepatotoxicity & potential health effects. *Indian J. Med. Res.*, **126**, 518–

527.
3) Patrick, L. (2006) Lead Toxicity Part II: the role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. *Altern. Med. Rev.*, **11**, 114–127.
4) Goyer, R. A. (1996) Results of lead research: prenatal exposure and neurological consequences. *Environ. Health Perspect.*, **104**, 1050–1054.
5) Liuji, C., Xianqiang, Y., Hongli, J. and Baolu, Z. (2002) Tea catechins protect against lead-induced cytotoxicity, lipid peroxidation, and membrane fluidity in HepG2 cells. *Toxicol. Sci.*, **69**, 149–156.
6) Lockitch, G. (1993) Perspectives on lead toxicity. *Clin. Biochem.*, **26**, 371–381.
7) Meadows, R. (1996) Growing pains. *Environ. Health Perspect.*, **104**, 146–149.
8) Ruff, H. A., Markowitz, M. E., Bijur, P. E. and Rosen, J. F. (1996) Relationships among blood lead levels, iron deficiency, and cognitive development in two-year-old children. *Environ. Health Perspect.*, **104**, 180–185.
9) West, W. L., Knight, E. M., Edwards, C. H., Manning, M., Spurlock, B., James, H., Johnson, A. A., Oyemade, U. J., Cole, O. J., Westney, O. E., Laryea, H., Jones, S. and Westney, L. S. (1994) Maternal low level lead and pregnancy outcomes. *J. Nutr.*, **124**, 981S–986S.
10) Ahamed, M. and Siddiqui, M. K. J. (2007) Low level lead exposure and oxidative stress: Current opinions. *Clin. Chim. Acta*, **383**, 57–64.
11) Hermes-Lima, M., Pereira, B. and Bechara, E. J. (1991) Are free radicals involved in lead poisoning? *Xenobiotica*, **21**, 1085–1090.
12) Sandhir, R., Julka, D. and Gill, K. D. (1994) Lipoperoxidative damage on lead exposure in rat brain and its implications on membrane bound enzymes. *Pharmacol. Toxicol.*, **74**, 66–71.
13) Adonaylo, V. N. and Oteiza, P. I. (1999) Lead intoxication: antioxidant defenses and oxidative damage in rat brain. *Toxicology*, **135**, 77–85.
14) Moreira, E. G., Rosa, G. J., Barros, S. B., Vassilieff, V. S. and Vassillieff, I. (2001) Antioxidant defense in rat brain regions after developmental lead exposure. *Toxicology*, **169**, 145–151.
15) Ercal, N., Treeratphan, P., Hammond, T. C., Matthews, R. H., Grannemann, N. H. and Spitz, D. R. (1996) In vivo indices of oxidative stress in lead-exposed C57BL/6 mice are reduced by treatment with meso-2,3-dimercaptosuccinic acid or N-acetylcysteine. *Free Radic. Biol. Med.*, **21**, 157–161.
16) Gürer, H., Özgünes, H., Neal, R., Spitz, D. R. and Ercal, N. (1998) Antioxidant effects of N-acetylcysteine and succimer in red blood cells from lead-exposed rats. *Toxicology*, **128**, 181–189.
17) Neal, R., Cooper, K., Gürer, H. and Ercal, N. (1998) Effects of N-acetylcysteine and 2,3-dimercaptosuccinic acid on lead induced oxidative stress in rat lenses. *Toxicology*, **130**, 167–174.
18) McGowan, C. and Donaldson, W. E. (1986) Changes in organ nonprotein sulfhydryl and glutathione concentrations during acute and chronic administration of inorganic lead to chicks. *Biol. Trace Elem. Res.*, **10**, 37–46.
19) Wang, J., Wu, J. and Zhang, Z. (2006) Oxidative stress in mouse brain exposed to lead. *Ann. Occup. Hyg.*, **50**, 405–409.
20) Afonso, V., Champy, R., Mitrovic, D., Collin, P. and Lomri, A. (2007) Reactive oxygen species and superoxide dismutases: role in joint diseases. *Joint Bone Spine*, **74**, 324–329.
21) Adler, A. J., Barth, R. H. and Berlyne, G. M. (1993) Effect of lead on oxygen free radical metabolism: inhibition of superoxide dismutase activity. *Trace Elem. Med.*, **10**, 93–96.
22) Vallee, B. L. and Ulmer, D. D. (1972) Biochemical effects of mercury, cadmium and lead. *Annu. Rev. Biochem.*, **41**, 91–128.
23) Pastore, A., Federici, G., Bertini, E. and Piemonte, F. (2003) Analysis of glutathione: Implication in redox and detoxification. *Clin. Chim. Acta*, **333**, 19–39.
24) Korsrud, G. O. and Meldrum, J. B. (1988) Effect of diet on the response in rats to lead acetate given orally or in the drinking water. *Biol. Trace Elem. Res.*, **17**, 167–173.
25) Gürer, H. and Ercal, N. (2000) Can antioxidnat be beneficial in the treatment of lead poisoning? *Free Radic. Biol. Med.*, **29**, 927–945.
26) Drevet, J. R. (2006) The antioxidant glutathione peroxidase family and spermatozoa: A complex story. *Mol. Cell. Endocrinol.*, **250**, 70–79.
27) Patra, R. C., Swarup, D. and Dwivedi, S. K. (2001) Antioxidant effects of α-tocopherol, ascorbic acid and L-methionine on lead induced oxidative stress to the liver, kidney and brain in rats. *Toxicology*, **162**, 81–88.
28) Iwata, N., Okazaki, M., Kamuichi, S. and Hibino, Y. (2010) Protective effects of oral administrated ascorbic acid against oxidative stress and neuronal damage after cerebral ischemia/reperfusion in diabetic rats. *J. Health Sci.*, **56**, 20–30.
29) Yin, S. T., Tang, M. L., Su, L., Chen, L., Hu, P., Wang, H. L., Wang, M. and Ruan, D. Y. (2008) Effects of epigallocatechin-3-gallate on lead-induced oxidative damage. *Toxicology*, **249**, 45–54.
30) Kharoubi, O., Slimani, M., Krouf, D., Seddik, L.

and Aoues, A. (2008) Role of wormwood (*Artemisia absinthium*) extract on oxidative stress in ameliorating lead induced haematotoxicity. *Afr. J. Tradit. Complement. Altern. Med.*, **5**, 263–270.
31) Lee, M. K., Cho, S. Y., Kim, D. J., Jang, J. Y., Shin, K. H., Park, S. A., Park, E. M., Lee, J. S., Choi, M. S., Lee, J. S. and Kim, M. J. (2005) Du-zhong (*Eucommia ulmoides* Oliv.) cortex water extract alters heme biosynthesis and erythrocyte antioxidant defense system in lead-administered rats. *J. Med. Food*, **8**, 86–92.
32) Vijayavel, K., Anbuselvam, C. and Balasubramanian, M. P. (2007) Antioxidant effect of the marine algae *Chlorella vulgaris* against naphthalene-induced oxidative stress in the albino rats. *Mol. Cell. Biochem.*, **303**, 39–44.
33) Konishi, F., Tanaka, K., Himeno, K., Taniguchi, K. and Nomoto, K. (1985) Antitumor effect induced by a hot water extract of *Chlorella vulgaris* (CE): resistance to meth-A tumor growth mediated by CE-induced polymorphonuclear leukocytes. *Cancer Immunol. Immunother.*, **19**, 73–78.
34) Morimoto, T., Nagatsu, A., Murakami, N., Sakakibara, J., Tokuda, H., Nishino, H. and Iwashima, A. (1995) Anti-tumor-promoting glyceroglycolipids from the green algae, *Chlorella vulgaris*. *Phytochemistry*, **40**, 1433–1437.
35) Malanga, G. and Puntarulo, S. (1995) Oxidative stress and antioxidant content in *Chlorella vulgaris* after exposure to ultraviolet-B radiation. *Physiol. Plant.*, **94**, 672–679.
36) Lee, H. S., Choi, C. Y., Cho, C. and Song, Y. (2003) Attenuating effect of chlorella supplementation on oxidative stress and NFkappaB activation in peritoneal macrophages and liver of C57BL/6 mice fed on an atherogenic diet. *Biosci. Biotechnol. Biochem.*, **67**, 2083–2090.
37) Blas-Valdivia, V., Ortiz-Butrón, R., Pineda-Reynoso, M., Hernández-Garcia, A. and Cano-Europa, E. (2010) *Chlorella vulgaris* administration prevents HgCl2-caused oxidative stress and cellular damage in the kidney. *J. Appl. Phycol.*, **23**, 53–58.
38) Hashimoto, S., Setoyama, T., Yokokura, T. and Mutai, M. (1982) Effects of soybean phosphilid, chlorella phospholipid and clofibrate on collagen and elastin synthesis in the aorta and on the serum and liver lipid contents in rats. *Exp. Mol. Pathol.*, **36**, 99–106.
39) Shibata, S., Oda, K., Onodera-Masuoka, N., Matsubara, S., Kikuchi-Hayakawa, H., Ishikawa, F., Iwabuchi, A. and Sansawa, H. (2001) Hypocholesterolemic effects of indigestible fraction of Chlorella regularis in cholesterol-fed rats. *J. Nutr. Sci. Vitaminol.* (Tokyo), **47**, 373–377.
40) Shibata, S., Natori, Y., Nishhara, T., Tomisaka, K., Matsumoto, K., Sansawa, H. and Nguyen, V. C. (2003) Antioxidant and anti-cataract effects of Chlorella on rats with streptozocin-induced diabetes. *J. Nutri. Sci. Vitaminol.* (Tokyo), **49**, 334–339.
41) Shim, J. Y. and Om, A. S. (2008) *Chlorella vulgaris* has preventive effect on cadmium induced liver damage in rats. *Molecular & Cellular Toxicology*, **4**, 138–143.
42) Shim, J. Y., Shin, H. S., Han, J. G., Park, H. S., Lim, B. L., Chung, K. W. and Om, A. S. (2008) Protective effects of *Chlorella vulgaris* on liver toxicity in cadmium-administered rats. *J. Med. Food*, **11**, 479–485.
43) Jamieson, J. A., Stringer, D. M., Zahradka, P. Z. and Taylor, C. G. (2008) Dietary zinc attenuates renal lead deposition but metallothionein is not directly involved. *Biometals*, **21**, 29–40.
44) Six, K. M. and Goyer, R. A. (1970) Experimental enhancement of lead toxicity by low dietary calcium. *J. Pediatr.*, **135**, 108–110.
45) Paglia, D. E. and Valentine, W. N. (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.*, **70**, 158–169.
46) Teitze, F. (1969) Enzymatic method for the quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.*, **27**, 502–522.
47) Buege, J. A. and Aust, S. D. (1978) Microsomal lipid peroxidation. *Methods Enzymol.*, **52**, 302–310.
48) Sanders, T., Liu, Y., Buchner, V. and Tchounwou, P. B. (2009) Neurotoxic effects and biomarkers of lead exposure: a review. *Rev. Environ. Health*, **24**, 15–45.
49) Verstraeten, S. V., Aimo, L. and Oteiza, P. I. (2008) Aluminium and lead: molecular mechanisms of brain toxicity. *Arch. Toxicol.*, **82**, 789–802.
50) Jamieson, J. A., Taylor, C. G. and Weiler, H. A. (2006) Marginal zinc deficiency exacerbates bone lead accumulation and high dietary zinc attenuates lead accumulation at the expense of bone density in growing rats. *Toxicol. Sci.*, **92**, 286–294.
51) Hsu, P. C. and Guo, Y. L. (2002) Antioxidant nutrients and lead toxicity. *Toxicology*, **180**, 33–44.
52) Prasanthi, R. P. J., Reddy, G. H. and Reddy, G. R. (2006) Calcium or zinc supplementation reduces lead toxicity: assessment of behavioral dysfunction in young and adult mice. *Nutr. Res.*, **26**, 537–545.
53) Morita, K., Matsueda, T., Iida, T. and Hasegawa, T. (1999) Chlorella accelerates dioxin excretion in rats.

J. Nutr., **129**, 1731–1736.
54) Adhami, V. M., Husain, R., Agarwal, A. K. and Seth, P. K. (2000) Intrahippocampal cholinergic-rich transplants restore lead-induced deficits: a preliminary study in rats. *Neurotoxicol. Teratol.*, **22**, 41–53.
55) Villeda-Hernandez, J., Barroso-Mouguel, R., Mendez-Armenta, M., Nava-Ruiz, C., Huerta-Romero, R. and Rios, C. (2001) Enhanced brain regional lipid peroxidation in developing rats exposed to low level lead acetate. *Brain Res. Bull.*, **55**, 247–251.
56) Halliwell, B. and Gutteridge, J. M. C. (1989) Protection against oxidants in biological systems: The superoxide theory of oxygen toxicity. In: *Free Radical in Biology and Medicine*, Clarendon Press, Oxford, pp. 86–123.
57) Ribarov, S. R. and Bochev, P. G. (1982) Lead-hemoglobin interaction as a possible source of reactive oxygen species—a chemiluminescent study. *Arch. Biochem. Biophys.*, **213**, 288–292.
58) Lawton, L. J. and Donaldson, W. E. (1991) Lead-induced tissue fatty acid alterations and lipid peroxidation. *Biol. Trace Elem. Res.*, **28**, 83–97.
59) Monterio, H. P., Bechara, E. J. and Abdalla, D. S. (1991) Free radical involvement in neurological porphyries and lead poisoning. *Mol. Cell. Biochem.*, **103**, 73–83.
60) Hsu, P. C., Liu, M. Y., Hsu, C. C., Chen, L. Y. and Leen Guo, Y. (1997) Lead exposure causes generation of reactive oxygen species and functional impairment in rat sperm. *Toxicology*, **122**, 133–143.
61) Schrauzer, G. N. (1987) Effects of selenium antagonists on cancer susceptibility: new aspects of chronic heavy metal toxicity. *J. UOEH*, **9**, 208–215.
62) Sandhir, R. and Gill, K. D. (1995) Effect of lead on lipid peroxidation in liver of rats. *Biol. Trace Elem. Res.*, **48**, 91–97.
63) Meister, A. and Anderson, M. E. (1983) Glutathione. *Annu. Rev. Biochem.*, **52**, 711–760.
64) Meister, A. (1995) Glutathione metabolism. *Methods Enzymol.*, **251**, 3–7.
65) Aykin-Burns, N., Laegeler, A., Kellogg, G. and Ercal, N. (2003) Oxidative effects of lead in young and adult fisher 344 Rats. *Arch. Environ. Contam. Toxicol.*, **44**, 417–420.
66) Yiin, S. J. and Lin, T. H. (1995) Lead-catalyzed peroxidation of essential unsaturated fatty acid. *Biol. Trace Elem. Res.*, **50**, 167–172.
67) Rehman, S., Rehman, S., Chandra, O. and Abdulla, M. (1995) Evaluation of malondialdehyde as an index of lead damage in rat brain homogenates. *Biometals*, **8**, 275–279.
68) Pande, M., Mehta, A., Pant, B. P. and Flora, S. J. S. (2001) Combined administration of a chelating agent and an antioxidant in the prevention and treatment of acute lead intoxication in rats. *Environ. Toxicol. Pharmacol.*, **9**, 173–184.