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Letter

The enhanced elimination of tissue methylmercury in *Parachlorella beijerinckii*-fed mice

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ABSTRACT — To investigate the influence of *Chlorella* (*Parachlorella beijerinckii*) on the excretion and tissue accumulation of methylmercury (MeHg), we orally administered 5 mg/kg of MeHg chloride (4 mg Hg/kg) to female C57BL/6N mice (aged 10 weeks). The mice were housed in metabolism cages to collect urine and feces for 3 weeks with diets containing 0%, 5%, or 10% *P. beijerinckii* powder (BP) in a basal diet (CE-2). The lowered blood Hg levels in the 5% and 10% BP groups became significant compared to those of the control group (0% BP) as early as day 7. During the 21 days of testing, significant increases in the cumulative Hg eliminations into urine (5% BP) and feces (5% and 10% BP) were found in the BP groups. Twenty-one days after administration, the organ Hg levels in both BP groups tended to decrease compared to that of the control group. The reduction of Hg levels in the kidney and brain were significant, whereas that in the liver was not. Although tissue Hg levels are known to be closely related to glutathione (GSH) metabolism, no difference was found in GSH levels in the blood or organs between the control group and the 10% BP group. These results suggest that continuous BP intake accelerates the excretion of MeHg and subsequently decreases tissue Hg levels in mice, with no alteration of GSH metabolism. We should conduct further research to elucidate details regarding the mechanism of BP-induced enhancement of MeHg excretion.

Key words: Methylmercury, *Chlorella*, *Parachlorella beijerinckii*, Excretion, Detoxification

INTRODUCTION

Methylmercury (MeHg) is a neurotoxic metal compound that is widely distributed in the natural environment. Since environmental MeHg is accumulated in seafood through the food web, consumption of fish and shellfish is the major source of MeHg exposure for humans. In recent years, pregnant women have increasingly been cautioned against consuming seafood in several countries (FDA, 2001; European Commission, 2004).

Chlorella (Parachlorella beijerinckii CK-5) is a unicellular green algae approximately 3-8 μm in diameter. It was originally identified as *Chlorella vulgaris* based on its morphological characteristics, according to the description of Fott and Nováková (1969), and was re-identified as *P. beijerinckii* based on both the sequence of 18S rDNA gene and its morphological characteristics (Krienitz *et al.,* 2004). *Chlorella* has been eaten as a nutritional food for many years because it contains abundant nutritional components such as proteins, vitamins, minerals and dietary fibers. *Chlorella* has previously been reported to be useful in detoxifying dioxins, cadmium (Cd), and lead in animal experiments (Morita *et al.*, 2001; Nagano *et al.*, 1983; Uchikawa *et al.*, 2009).

We have previously reported that BP intake induces an increase in Hg excretion into feces and urine in mice during the 24-hr period after MeHg administration (Uchikawa *et al.*, 2010). However, this 24-hr period was found to be too short to observe significant decreases in blood and organ Hg levels of BP-fed mice. Therefore, to confirm that continuous BP intake could decrease the tissue Hg accumulation, we examined in the present

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study Hg accumulation in tissue as well as Hg excretion in MeHg-administered mice in a 3-week experiment.

MATERIALS AND METHODS

The preparation of *P. beijerinckii* powder (BP) and BP diet

P. beijerinckii CK-5, a unicellular green alga, was used in this study. The algal cells were cultured in an outdoor pool, harvested, and washed with water using a centrifuge separator (5,000 × g). The obtained algal slurry was heated at 118°C for 1 min with a heat-exchanger (Morinaga Engineering Co., Ltd., Tokyo, Japan) and was powdered with a spray-drier under a blower temperature of 170°C. The basal diet (pelleted rodent diet, CE-2) and BP diets, which contained 5% or 10% BP in the basal diet, were obtained from CLEA Japan, Inc. (Tokyo, Japan).

Animals and chemicals

MeHg chloride and all chemicals were obtained from Wako Pure Chemicals Industries (Osaka, Japan) and dissolved in distilled water (200 µg/ml) for administration to mice. Female C57BL/6N mice (aged 10 weeks) were purchased from Charles River Japan Co., Ltd. (Kanagawa, Japan) and randomly divided into three groups (control group, 5% BP group and 10% BP group) consisting of six animals each. After oral administration of MeHg chloride (5 mg/kg), animals were housed in metabolism cages (one mouse per cage) with a 12-hr light cycle (6:00 to 18:00) at 23 ± 0.5°C and 55 ± 5% relative humidity for 3 weeks and allowed free access to feed and water. After MeHg administration, blood (10 µl) was collected from the tail vein at 1, 7, and 14 days. Twenty-one days after administration, 0.5 ml of the blood was collected from the inferior vena cava under pentobarbital anesthesia, the animals were perfused with phosphate-buffered saline (pH 7.3), and the liver, kidneys, and brain were removed for Hg analysis. The animals were cared for according to the NIH published guidelines.

Analysis of total Hg

All samples were degraded by the wet-ashing method (Ministry of Environment of Japanese Government, 2004), and total Hg levels were determined by the reducing-vaporization method using a Mercury Analyzer RA-3320 (Nippon Instruments Co., Tokyo, Japan).

Glutathione analysis

In the control and 10% BP groups, the glutathione (GSH) contents of the blood, liver and kidney were determined according to the method of Tietze (1969). Blood was immediately diluted with a matching volume of ice-cold 4% perchloric acid (1 mM EDTA), and liver and kidney tissues were immediately homogenized in ice-cold 4% perchloric acid (1 mM EDTA). The samples thus obtained were kept at -80°C until analysis.

Statistical analysis

The significance of difference was calculated according to the Student's *t*-test using Microsoft Office Excel 2007 for Windows (Microsoft Co. Ltd., Tokyo, Japan). Each value of $p < 0.05$* or $p < 0.01$** was considered statistically significant.

RESULTS AND DISCUSSION

To examine the effects of BP on MeHg metabolism, we administered two kinds of BP diet containing either 5% or 10% BP in the basal diet. The diet compositions are shown in Table 1. Since *Chlorella* contains abundant proteins and dietary fibers, the crude protein and crude fiber contents of the BP diets were slightly higher than those of the basal diet, although the physiological fuel values were mostly the same among the three diets. There were no differences in the amounts of diet intake or the body weights during the 3 weeks among the three groups (data not shown). The amount of water intake for the 3 weeks showed a slight increase, 73.0 ± 5.1 ml in the control group to 81.7 ± 10.1 ml in the 10% BP group, but the difference was not significant.

We have previously found that BP intake causes an increase in Hg excretion into both feces and urine in mice 24 hr after MeHg administration (Uchikawa *et al.*, 2010). In the present study, after MeHg administration (4 mg Hg/kg), feces and urine were collected for 3 weeks. The cumulative Hg excretions into feces and urine of the control mice were 54.7 ± 2.7% and 8.2 ± 1.1% of dose,

Table 1. The composition of each diet (100 g)

	Basal diet (CE-2)	5% BP diet	10% BP diet
Moisture (%)	9.3	9.1	8.9
Crude protein (%)	25.1	26.9	28.8
Crude fat (%)	4.8	5.1	5.3
Crude fiber (%)	4.2	4.9	5.5
Crude ash (%)	6.7	6.7	6.7
NFE[a] (%)	50.0	47.4	44.8
Physiological fuel value (kcal)	343.1	342.7	342.3

[a] : Nitrogen-free extracts.

respectively. The Hg amounts of fecal excretion in the 5% and 10% BP groups increased by 12.2% and 10.2% compared to the control group, respectively (Fig. 1A). The total amount of feces in the control group during the 3 weeks was 20.2 ± 2.9 g, and BP caused increases of 14% (5% BP group) and 5% (10% BP group), though the increases were not statistically significant (data not shown). The BP-induced increases in the fecal Hg excretion could be accounted for, at least in part, by the fecal amounts. *Chlorella* has also been reported to contribute to increased fecal amounts in human ingested *Chlorella vulgaris* tablets (Fujiwara *et al.*, 1998).

The urinary Hg excretion in the 5% and 10% BP groups increased by 22.7% and 15.2% compared to the control group, respectively, though the increase in the latter group was not significant (Fig. 1B). In addition, a significant increase in the urine volume was found from the control (22.1 ± 2.3 ml) to the 10% BP group (29.9 ± 5.8 ml). The slightly higher excretion in the 5% BP group than the 10% BP group might suggest some inhibition of Hg urinary excretion at a higher BP content in the diet. The increased portions of the total excreted Hg into feces and urine during 3 weeks were 8.5% and 6.8% of dose in the 5% and 10% BP groups, respectively.

The BP-induced stimulation of Hg excretion caused alterations in the tissue Hg levels. The time-dependent changes in blood Hg levels are shown in Fig. 2. Although no difference was found on day 1, as reported previously (Uchikawa *et al.*, 2010), the blood Hg levels in both BP groups became significantly lower than that in the control group on day 7, at which time the Hg levels in the 5% and 10% BP groups were 25% and 41% lower than those of the control group, respectively. The lowered blood Hg levels in the BP groups were maintained thereafter, except for that of the 5% BP group on day 14. However, the blood Hg concentrations in the 10% BP group were approaching the 5% BP group levels on the final day. These results suggest that the inhibitory effect of high-dose BP on the Hg excretion mentioned above might be present in the later phase. The lack of BP-induced differences in blood Hg levels on day 1 suggests that MeHg absorption at the gastrointestinal tract would have occurred mostly at equal rates among the groups.

Three weeks after MeHg administration, BP-induced decreases in tissue Hg accumulations were evident in the brain and kidney. The Hg levels in brains of the 10% BP group were significantly lower than those of the control group (Fig. 3A). However, the alteration in the 5% BP group was not significant, though the amount of Hg accumulation was slightly lower than that of the control group. The renal Hg levels of both BP groups were 27% to 30% lower than those of the control group (Fig. 3B). Although BP caused a significant reduction in the renal Hg, a dose-dependent effect was not observed. Considering the slightly lower effect of 10% BP than of 5% BP on the urinary Hg excretion, it may be that overload of BP as high as 10% in the diet for 3 weeks might be less effective with regard to MeHg elimination from the kid-

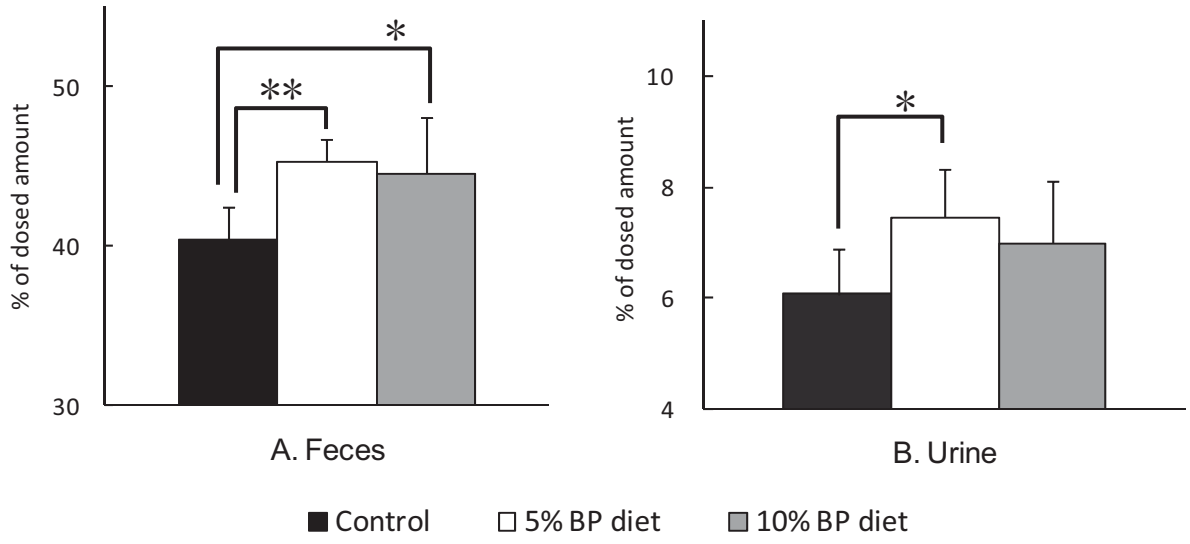

Fig. 1. The amounts of mercury excretion in feces (A) and urine (B) for 21 days after MeHg administration. Values represent the mean ± S.D. obtained from 6 mice. Significant differences are shown by * ($p < 0.05$) and ** ($p < 0.01$).

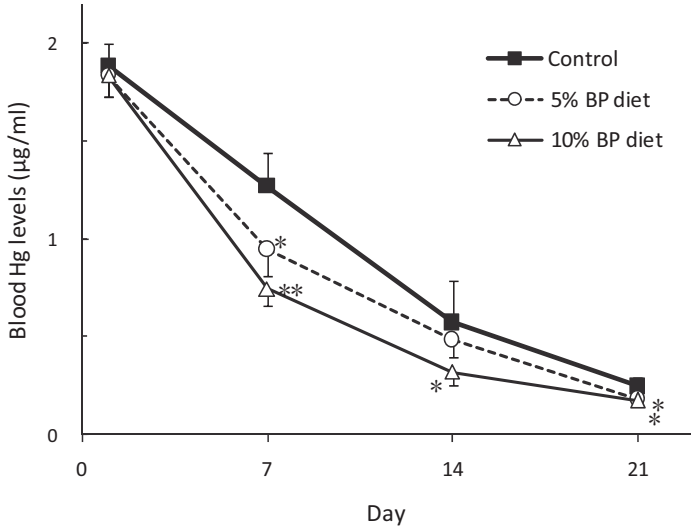

Fig. 2. The change in blood Hg levels for 21 days after MeHg administration. Values represent the mean ± S.D. obtained from 6 mice. Significant differences from the control group are shown by * ($p < 0.05$) and ** ($p < 0.01$).

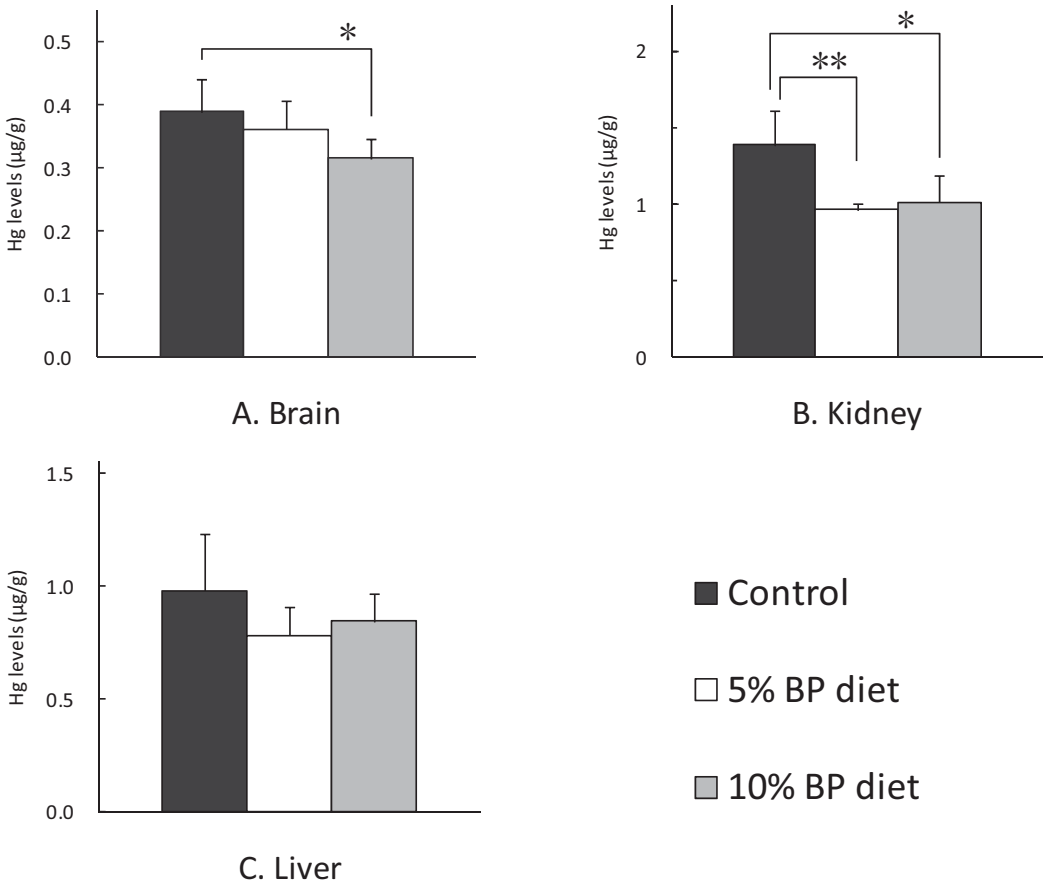

Fig. 3. The mercury levels of the brain (A), kidney (B), and liver (C) on the 21st day after MeHg administration. Values represent the mean ± S.D. obtained from 6 mice. Significant differences are shown by * ($p < 0.05$) and ** ($p < 0.01$).

ney, which is the terminal organ for urinary Hg excretion. Since a dose-dependent reduction in blood Hg levels was observed for up to 2 weeks (Fig. 2), dose-dependency in the renal Hg levels might have been observed at an earlier phase.

On the other hand, alterations in the hepatic Hg levels were not significant, though levels tended to be lower in the BP groups (Fig. 3C). We attributed the lowered tissue Hg accumulation in both BP groups to the enhanced excretion of absorbed Hg. The slightly lower hepatic Hg levels of the BP groups compared to those of the control group might be a result of MeHg excretion via the enterohepatic circulation. The BP-induced increase in fecal Hg excretion should indicate enhanced bile secretion of the hepatic Hg. If the Hg excretion via the enterohepatic circulation was accelerated by the BP intake, the hepatic Hg levels of the BP groups would also decrease, as observed in the kidney and brain Hg levels. On the other hand, absorbed MeHg would be topically accumulated in the liver to be excreted via enterohepatic circulation, and BP would stimulate the accumulation, leading to a temporal elevation of the hepatic Hg levels. Thus, BP-induced stimulation of bile secretion and hepatic accumulation of MeHg would make the alteration of hepatic Hg levels unclear at 3 weeks after administration. Different from the liver and kidney, a dose-dependent effect of BP on brain Hg levels was evident. Since the brain is the major target organ of MeHg toxicity, the BP-induced alterations of tissue Hg may be considered to be a beneficial effect.

Tissue MeHg accumulation and its elimination are reported to be closely related to GSH status (Hirayama *et al.*, 1987; Yasutake *et al.*, 1989). To examine the involvement of GSH in BP-induced alterations of tissue Hg levels, we measured the GSH levels of the blood, liver, and kidney in the 10% BP group and the control group. Contrary to expectations, no BP-induced difference was found in the GSH levels of any of the tissues examined (data not shown). These results suggest that the intake of BP may affect the urinary and fecal excretory systems individually without involving a modification of GSH metabolism.

As one factor in increased fecal Hg excretion, BP-derived components may be involved in this excretion via the enterohepatic circulation. It has previously been reported that BP-derived components such as compound lipids, phospholipids, and dietary fibers inhibit the reabsorption of bile acid in the intestinal tract and accelerate bile secretion in the enterohepatic circulation (Sano, 1982). In this study, continuous BP intake would have caused a sustained increase in bile secretion from the liver, leading to increased glutathione-MeHg (GS-MeHg) secretion with bile acid.

Regarding the increase in the levels of Hg excreted in urine, the BP-derived metal-binding factor might be involved in urinary excretion. Nagano *et al.* (1983) have reported an increase in urinary Cd excretion in response to the co-administration of *Chlorella*-derived metal-binding protein in rats. When absorbed MeHg is converted to inorganic Hg in animal cells, a *Chlorella*-derived metal-binding factor may interact with inorganic Hg and accelerate the urinary Hg excretion from the kidney. Selective quantification of the urinary inorganic Hg and analysis of its chemical form may be necessary to support this hypothesis. Alternatively, the enzymes such as γ-glutamyl transpeptidase (γ-GTP) and dipeptidase in the renal tubule might be influenced by BP intake. GS-MeHg complex secreted to the tubular lumen is rapidly converted to Cys-MeHg by these enzymes, but the Cys-MeHg formed is easily re-absorbed in the renal tubule (Yasutake *et al.*, 1989; Tanaka-Kagawa *et al.*, 1993). If these enzymatic activities were influenced by BP intake, alterations in urinary MeHg excretion may have occurred. In a human experiment regarding *Chlorella* supplementation (6 g/day) for 6 weeks, the γ-GTP values in serum were significantly lower than those before *Chlorella* intake (Toyomasu *et al.*, 2010). If BP had a similar effect on the renal γ-GTP activity in the present study, this effect may account for the increased MeHg elimination observed in the BP groups. We plan to research the effect of BP intake on renal γ-GTP activity.

The results obtained in this study have demonstrated that continuous BP intake for 3 weeks is effective in reducing MeHg accumulations in tissues, including the brain, through enhanced excretions in feces and urine. These results suggest that *Chlorella* may be a food material that can detoxify MeHg. Further study must be carried out to clarify the mechanisms of the enhanced urinary and fecal Hg excretions.

REFERENCES

European Commission (2004): Methyl mercury in fish and fishery products.

FDA (2001): Fish and fisheries products hazards and controls guidance. Third edition.

Fott, B. and Nováková, M. (1969): A monograph of the genus *Chlorella*. The fresh water species. In Studies in phycology (Fott, B., ed.), pp.10-74, Academia, Prague.

Fujiwara, Y., Sinpo, K., Imae, Y., Nonomura, M. and Hirakawa, K. (1998): Effect of Chlorella vulgaris strain CK-5 on the frequency of bowel movement in humans. Jpn. J. Nutr., **56**, 253-263.

Hirayama, K., Yasutake, A. and Inoue, M. (1987): Effect of sex hormones on the fate of methylmercury and on glutathione metabolism in mice. Biochem. Pharmacol., **36**, 1919-1924.

Krienitz, L., Hegewald, E.H., Hepperle, D., Huss, V.A.R., Rohr, T.

and Wolf, M. (2004): Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae). Phycologia, **43**, 529-542.

Ministry of Environment of Japanese Government (2004): The manual of mercury analysis. 10-27.

Morita, K., Ogata, M. and Hasegawa, T. (2001): Chlorophyll derived from *Chlorella* inhibits dioxin absorption from the gastrointestinal tract and accelerates dioxin excretion in rats. Environ. Health Perspect, **109**, 289-294.

Nagano, T., Suketa, Y. and Okada, S. (1983): Comparative absorption and excretion of *Chlorella ellipsoidea* cadmium-binding protein and inorganic cadmium in rats. Nippon Eiseigaku Zasshi, **38**, 741-747.

Sano, T. (1982): The effect of *Chlorella* on alimentary hyperlipemia in rats. Kurume Igaku Zasshi, **45**, 1130-1152.

Tanaka-Kagawa, T., Naganuma, A. and Imura, N. (1993): Tubular secretion and reabsorption of mercury compounds in mouse kidney. J. Pharmacol. Exp. Ther., **264**, 776-782.

Tietze, F. (1969): Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem., **27**, 502-522.

Toyomasu, K., Ueno, S., Nakashima, Y., Konishi, F. and Maruyama, I. (2010): *Chlorella* supplementation reduced risk factors of metabolic syndrome in white-collar workers. Oyo Yakuri, **78**, 87-92.

Uchikawa, T., Ueno, T., Hasegawa, T., Maruyama, I., Kumamoto, S. and Ando, Y. (2009): *Parachlorella beyerinckii* accelerates lead excretion in mice. Toxicol. Ind. Health, **25**, 551-556.

Uchikawa, T., Yasutake, A., Kumamoto, Y., Maruyama, I., Kumamoto, S. and Ando, Y. (2010): The influence of *Parachlorella beyerinckii* CK-5 on the absorption and excretion of methylmercury (MeHg) in mice. J. Toxicol. Sci., **35**, 101-105.

Yasutake, A., Hirayama, K. and Inoue, M. (1989): Mechanism of urinary excretion of methylmercury in mice. Arch. Toxicol., **63**, 479-483.