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Chronic Effects of Methylmercury in Rats. I. Biochemical Aspects

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Yasutake, A., Nakano, A., Miyamoto, K. and Eto, K. *Chronic Effects of Methylmercury in Rats. I. Biochemical Aspects.* Tohoku J. Exp. Med., 1997, **182** (3), 185-196 —— To examine chronic effects of methylmercury (MeHg), male Wistar rats were fed on MeHg-contaminated diet, 0, 1 and 5 ppm Hg, under a restricted feeding schedule of 16 g/rat/day for 6 days a week. Rats were killed at 6-month intervals for examination of Hg accumulation, tissue levels of glutathione, metallothionein and lipid peroxide, as well as anti-oxidative enzyme activities. The survival of the 5 ppm Hg group, 50% of which died by the end of 32nd month of the exposure, was somewhat shorter than control and 1 ppm Hg groups, 50% of which survived for 34 months. Although the rats showed no neurological signs or decreased body weight gain even in 5 ppm Hg-exposed group until the end of the 2nd year, crossing of hind limb was evident after 2.5 years in all three groups. Accordingly, the neurological sign observed here possibly due to aging rather than MeHg toxicity. Tissue Hg levels showed a dose-dependent accumulation except for the kidney, where the highest Hg accumulation was observed among tissues examined. Renal Hg levels in the 1 ppm group showed about 40% of those in the 5 ppm group. Significant effects by MeHg were evident only in the kidney, where glutathione and metallothionein levels increased in both MeHg-exposed groups. However, lipid peroxide levels elevated only in 1 ppm group. Among the anti-oxidative enzymes examined, the renal glutathione peroxidase was found to be the most labile enzyme against MeHg exposure. Renal dysfunction suggested by increased plasma creatinine levels was also significant in 5 ppm Hg rats at 2 years. Furthermore, anemia which would be caused by reduced erythropoietin production in the kidney was also evident in this group. The present study suggested that the kidney was the most susceptible organ against MeHg toxicity under the present exposure schedule and that the renal dysfunction might at least partly account for the shortened survival in 5 ppm Hg rats. —— methylmercury; rats; Hg accumulation; oxidative stress

Toxic effects of methylmercury (MeHg) have well been studied using experimental animals with a wide variety of dosing schedules. Health effects of MeHg chronic exposure may be important in humans, because all the fish we eat are contaminated by a trace amount of MeHg. People who eat fish are thus exposed to MeHg, though the levels may be low. However, experimental information on

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chronic MeHg exposure throughout a lifetime is limited. Mitsumori et al. (1983) found that when rats were given MeHg contaminated diet at 10 ppm level, the animals showed typical neurological signs and a significant increase in mortality before the end of the 2nd year. Considering that the rat life-span is around 3 years, their experimental condition seems to be too severe to discuss effects of chronic exposure in humans. In the present study, rats were given MeHg-contaminated diet with levels of 5 and 1 ppm Hg, while expecting a minimum effect on their life-span.

In general, rats become obese with a high fat accumulation by ad libitum feeding for a long period, with consumption of 20 g of diet or more in a day. Such an animal model may be too different from the human case in order to be able to extrapolate the experimental result. It may be necessary to suppress a body weight gain in a long-term experiment. Accordingly, the amount of diet was restricted to 16 g a day in each rat to avoid age-dependent obesity.

Materials and Methods

Chemicals and diet

MeHg chloride was purchased from Tokyo Kasei Co. (Tokyo) and used in the experiment without further purification, because contamination by inorganic Hg was less than 0.02%. Glutathione (GSH), $HgCl_2$, $CdCl_2$ and sodium perborate were purchased from Wako Pure Chemical Ind. Ltd. (Osaka), and 2-thiobarbituric acid was product of Merck KGaA (Darmstadt, Germany). γ-Ray-sterilized CE-7 laboratory chow (18% protein; CLEA Japan, Osaka) was used as a basic diet. Total and inorganic Hg content of the diet were 8.6 ± 0.6 and 2.8 ± 0.3 ng/g, respectively. MeHg-contaminated chows were prepared by the manufacturer by supplementing appropriate amounts of MeHg. Each lot of chow was consumed within 2 months after production.

Animals

Wistar strain male rats of 9 weeks age (CLEA Japan) were housed inside a barrier system in TPX cages (2 or 3 rats/cage). Animals were divided into 3 groups, and each group was fed on one of three γ-ray-sterilized chow with different MeHg supplementation, 0, 1 or 5 ppm Hg. The amount of chow was restricted to 16 g/rat/day for 6 days a week. Chlorine-sterilized water was given ad libitum. For young adult control, 9-weeks old rats which had been fed on CE-7 chow under the restricted feeding schedule for 2 weeks were used.

Preparation of tissue samples

Rats were sacrificed at 6-month intervals. Blood was removed from the heart under ether anesthesia, and animals were then perfused from the heart with ice-cold saline. Aliquots of blood samples were immediately centrifuged (12,000 rpm $\times$ 3 min) to separate erythrocytes and plasma at 4°C. Aliquots of eryth-

rocytes and plasma thus obtained were mixed with ice-cold 5% perchloric acid containing 1 mM EDTA for GSH assay. A hemisphere of the brain and aliquots of liver and kidney were stored at −80°C for determinations of lipid peroxide, metallothionein (MT), total and inorganic Hg levels, and activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GSH-Px). Another hemispheric brain and other tissue aliquots were immediately homogenized in ice-cold 5% perchloric acid (1 mM EDTA) for GSH assay.

Analysis

Total Hg level in each tissue sample was determined by the oxygen combustion-gold amalgamation method (Jacobs et al. 1960). Samples for inorganic Hg were prepared by removing MeHg using benzene-petroleum ether extraction from the tissue homogenate as reported before (Yasutake and Hirayama 1990). The GSH level in each sample was determined by the enzymatic recycling method according to Tietze (1969). Lipid peroxide levels in plasma and organs were estimated by 2-thiobarbituric acid-reactive substance (TBARS) levels according to Yagi (1976) and Ohkawa et al. (1979), respectively. Tissue MT levels were determined by the Cd-Hg substitution method according to Naganuma et al. (1987) using non-radioactive $HgCl_2$. MT levels were shown as amount of Hg bound to thionein molecule. SOD activity was evaluated by an inhibitory effect of nitroblue tetrazolium reduction rate according to Misra and Fridovich (1972). Catalase and GSH-Px activities were examined by rates of sodium perborate decomposition (Thomson et al. 1978) and NADPH oxidation (Lawrence and Burk 1976), respectively. Plasma creatinine levels and blood cell numbers were determined using a TBA-20FR biochemical analyzer (Toshiba, Tokyo) and a Coulter JT automated hematology analyzer (Coulter Electronics Inc., Hialeah, FL, USA), respectively.

Results

Through dietary restriction to 16 g a day for each individual (25 to 30% reduction of normal diet comsunption), the body weight of the rats in three dietary groups, 0, 1 and 5 ppm Hg, increased gradually up to nearly 400 g by the end of the 2nd year. No difference was observed in body weight among the groups during this period (Fig. 1). However, some rats in 5 ppm group began to fail to consume all the diet given at 2.5 years, and showed a slight reduction of body weight thereafter. Fifty percent of them died by the end of 32nd month of the experiment (Fig. 2). On the other hand, 50% of the rats survived for 34 months in 0 and 1 ppm groups. Although neurological sign, such as crossing or weakness of hind limb, was not shown even in the 5 ppm group by the end of the 2nd year, the rats of all three groups showed a sign of hind limb-crossing after 2.5 years. Accordingly, the neurological sign observed here possibly due to aging rather than MeHg toxicity. On sacrifice, subperitoneal fat accumulation was as

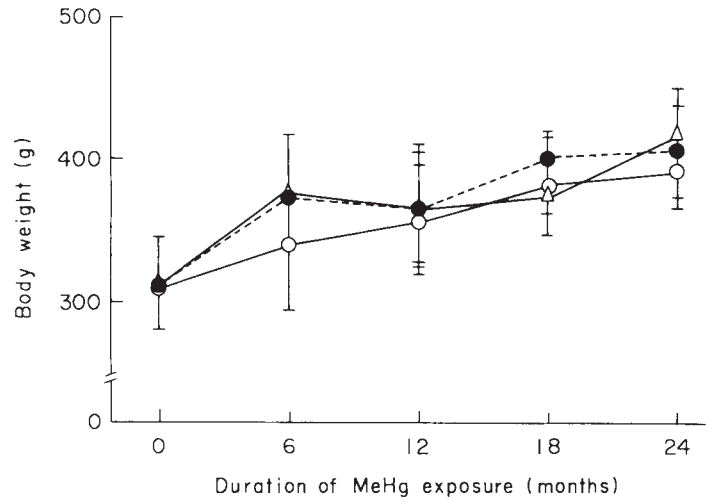

Fig. 1. Body weight of rats with dietary restriction.
Each rat was given 16 g per day of laboratory chow 6 days a week.
○—○, Control; ●---●, 1 ppm; △—△, 5 ppm.

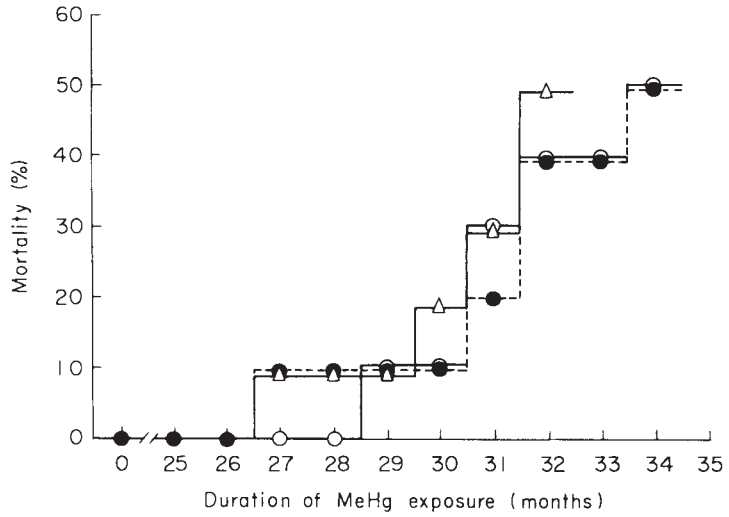

Fig. 2. Survival rates of control and MeHg-exposed rats.
○—○, Control; ●---●, 1 ppm; △—△, 5 ppm.

low as a young adult (9 weeks of age), suggesting that the dietary regimen employed in the present study makes a good aged rat model without obesity.

Hg accumulated in various rat tissues by feeding of the MeHg-contaminated diet quickly within the first 6 months, and the accumulation rates generally became lower thereafter (Figs. 3 and 4). Tissue Hg levels in 5 ppm group except for the kidney showed about 5-fold of 1 ppm group indicating that Hg accumulated in a dose-dependent manner. In the brain, total Hg level reached around 3 μg/g tissue in 5 ppm group after 2 years. The inorganic portions were at most 5% of total Hg both in the 5 and 1 ppm groups. The liver showed a somewhat higher Hg accumulation than the brain, and inorganic Hg accounted for less than 30% of total Hg. Since serum ALT and AST levels remained unchanged by MeHg exposure (data not shown), the hepatic Hg thus accumulated would be below the

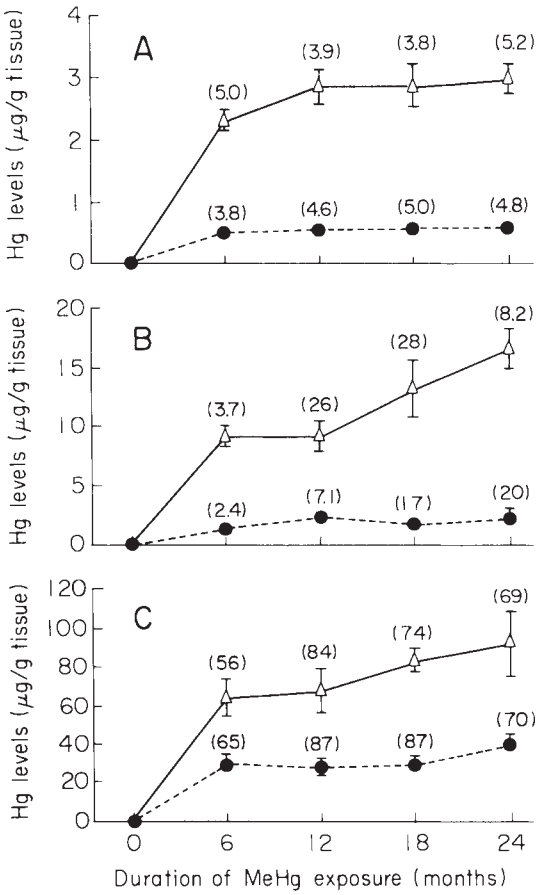

Fig. 3. Hg levels in brain (A), liver (B) and kidney (C) of rats fed on MeHg-contaminated diet.
Tissue Hg levels were determined by oxygen combustion-gold amalgamation method (Jacobs et al. 1960) and are shown as μg/g tissue. Numbers in parentheses represent percent of inorganic Hg determined by the benzene-extraction method (Yasutake and Hirayama 1990). Values represent mean $\pm$ S.D. obtained from 5 to 7 rats.
●---●, 1 ppm; △—△, 5 ppm.

toxic level. The kidney was the organ with high Hg accumulation; the levels reached nearly 100 μg/g by the end of the 2nd year in the 5 ppm group. Different from other organs, the renal Hg levels in the 1 ppm group showed about 40% of that in the 5 ppm group. Inorganic Hg was shown to be the major portion (56 to 87% of total Hg) throughout the experiment in both exposure groups, despite a continuous MeHg supply via the diet. Erythrocytes also showed a high Hg accumulation, its level was comparable to the renal level (Fig. 4). On the other hand, the plasma Hg level was extremely low, only 0.5% of the erythrocyte Hg level.

Oxidative stress was suggested to participate in a toxic mechanism of MeHg (Yonaha et al. 1983; Andersen and Andersen 1993). To know the extent of the stress, lipid peroxide, endogenous anti-oxidants and anti-oxidative enzyme activities would provide useful information. Effects of MeHg exposure or aging on lipid peroxide, GSH and MT levels and three kinds of anti-oxidative enzyme

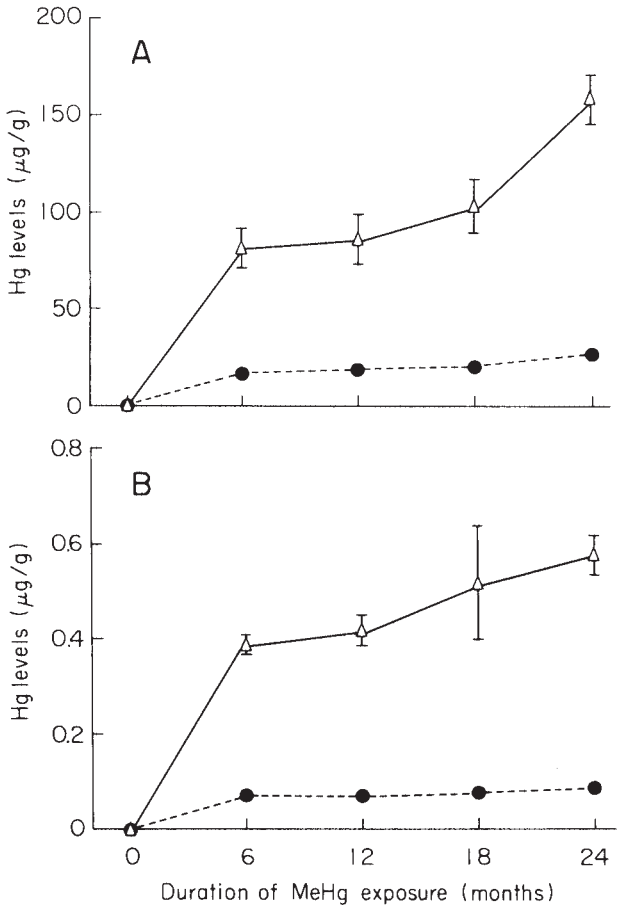

Fig. 4. Total Hg levels in erythrocytes (A) and plasma (B) of MeHg-treated rats. Blood samples freshly obtained from rats were centrifuged (12,000 rpm × 3 min) to separate plasma and erythrocytes. Total Hg levels were determined as in tissue levels. Values represent mean ± S.D. obtained from 5 to 7 rats. ●···●, 1 ppm; △——△, 5 ppm.

activities were prominent only in the kidney. Since MeHg or aging-dependent alteration of these indices were not significant in brain and liver (data not shown), the results were limited those in the kidney. Lipid peroxide levels estimated from the TBARS levels in the kidney markedly increased by MeHg exposure in the 1 ppm group, though that in the 5 ppm group showed low values (Fig. 5A). Furthermore, the renal levels tended to increase slightly with age. GSH is the major low molecular-weight cytosolic SH compound, and not only functions as a reductant but also has a close relationship to a fate of MeHg (Hirayama et al. 1987) due to high affinity for this heavy metal (Simpson 1961). The kidney level significantly increased by MeHg exposure at both dose levels to a similar extent (Fig. 5B). Since an activity of the renal γ-glutamylcysteine synthetase, a rate determining enzyme in GSH biosynthesis, was suggested to be enhanced in MeHg-treated animals in an acute (Yasutake and Hirayama 1994) and sub-acute phase (Woods et al. 1992), chronic MeHg exposure would affect similarly on this enzyme in the present study. MT is one of the stress-proteins induced by heavy metals or various kinds of stress, and functions to chelate various metals and to

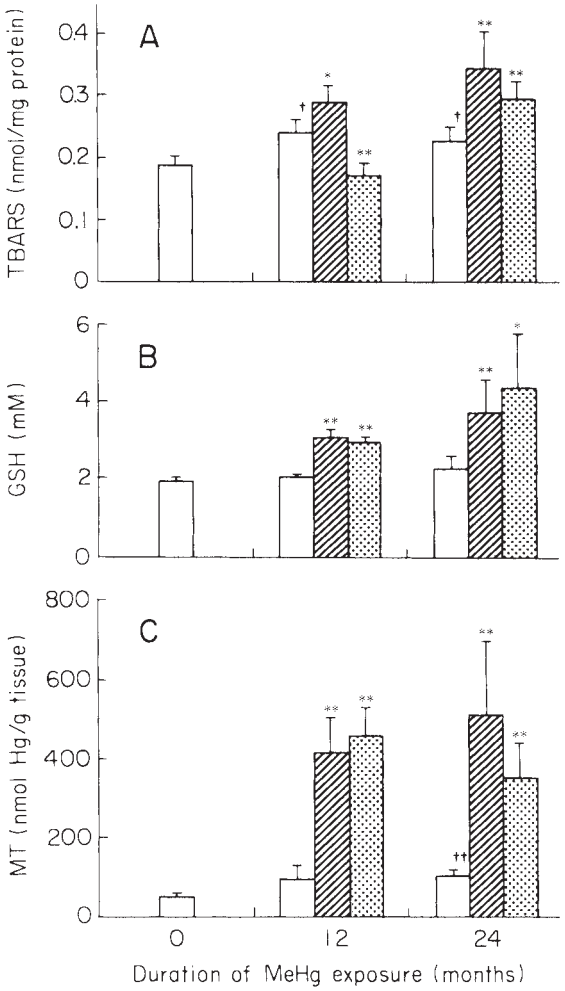

Fig. 5. Lipid peroxide (A), glutathione (B) and metallothionein levels (C) in the kidney of control (□), 1 ppm (▨) and 5 ppm groups of rats (⁘).
Lipid peroxide levels were estimated from 2-thiobarbituric acid-reactive substance (TBARS) levels according to Ohkawa et al. (1976). Total glutathione levels were determined by enzymatic recycling method according to Tietze (1969). Metallothionein levels were determined by Cd-Hg saturation method according to Naganuma et al. (1987), and were shown as nmol Hg bound to the thionein molecule in g wet tissue. Young control values were obtained from 9-weeks rats which were fed on the same diet as control group for 2 weeks. Values represent mean±S.D. obtained from 5 to 7 rats.
Significant difference from control rats of each experimental time by $^{*}p<0.05$ and $^{**}p<0.01$, and from young adult was shown by $\dagger p<0.05$ and $\dagger\dagger p<0.01$.

scavenge free radicals. In the rat kidney MT levels increased slightly with aging and greatly (5 to 8 times) with MeHg exposure (Fig. 5C). Although induction rates were similar in the two dose groups after 1-year exposure, the rate in 5 ppm rats became somewhat lower than that in 1 ppm rats after 2 years.

SOD catalyzes to decompose superoxide to form H_2O_2 and O_2 with the aid of protons. Interestingly, the renal activity was stimulated in the 1 ppm group at 1 year, whereas the activity in the 5 ppm group was similar to the control activity (Fig. 6A). When MeHg exposure was prolonged to 2 years, the Hg-induced stimulation became smaller, and the 5 ppm group showed a rather reduced value compar-

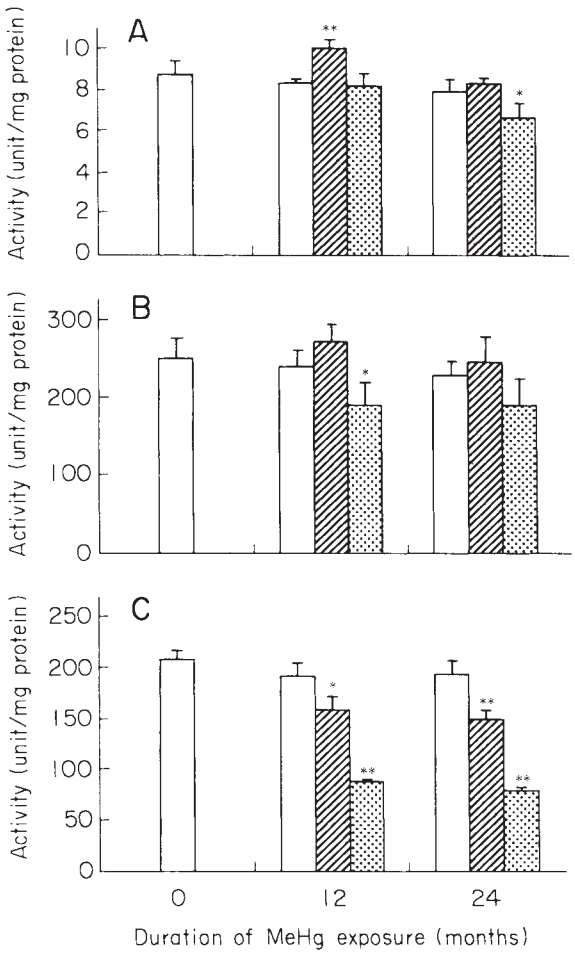

Fig. 6. Anti-oxidative enzyme activities in kidney of MeHg-exposed rats.
Superoxide dismutase activity (A) was evaluated from inhibition rate of nitroblue tetrazolium reduction according to Misra and Fridovich (1972). Catalase (B) and glutathione peroxidase (C) activities were determined by sodium perborate decomposition rate (Thomson et al. 1978) and NADPH oxidation rate (Lawrence and Burk 1976), respectively. Values represent mean ± S.D. obtained from 5 to 7 rats.
Significant difference from control rats at each experimental time was shown by $^{*}p<0.05$ and $^{**}p<0.01$. □, Control; ▨, 1 ppm; ⁘, 5 ppm.

ed with the control. A similar effect of MeHg was observed in the renal catalase activities (Fig. 6B). Different from the above two enzymes, GSH-Px activities in the kidney were sensitively reduced by MeHg exposure in a dose-dependent manner. The activities in the 5 ppm group were less than 50% of the control levels from 1 to 2 years exposure (Fig. 6C). These results suggested that the kidney in 5 ppm group rats seemed to be under more stressed condition compared to 1 ppm group.

Toxic effects of MeHg exposure on the kidney were demonstrated also by hematological examination. Increase in plasma creatinine levels in 5 ppm group became significant after 2-year exposure (Fig. 7A). It should be noted that reduction of erythrocyte number became significant after 6 months of MeHg exposure (Fig. 7B).

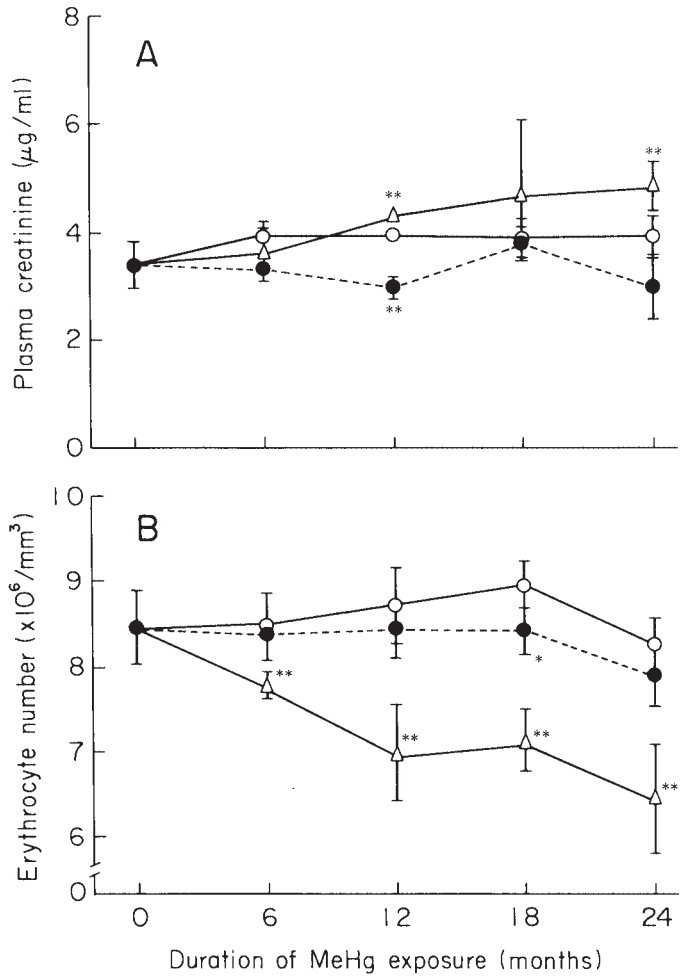

Fig. 7. Plasma creatinine levels (A) and numbers of erythrocytes (B) in control and MeHg-exposed rats. Values represent mean±S.D. obtained from 5 to 7 rats.
Significant difference from control rats at each experimental time was shown by $*p<0.05$ and $**p<0.01$.
○—○, Control; ●---●, 1 ppm; △—△, 5 ppm.

Thus, long duration of MeHg exposure from 5 ppm Hg-contaminated diet, though its level was too low to cause neurological signs, caused adverse effects on the rat kidney.

Discussion

The present study with rats demonstrated that dietary MeHg at a level of up to 5 ppm Hg throughout a lifetime caused no MeHg-derived neurological effects, like crossing of hind limb, a typical sign of MeHg intoxication in rat. Mitsumori et al. (1983) have reported that rats manifested neurological symptoms after 22-weeks exposure on 10 ppm MeHgCl (8 ppm Hg)-contaminated diet with brain Hg levels about 5 μg/g. The brain Hg levels in the present model rats, a plateau level of around 3 μg/g, would be below a critical level if it lasted for more than 1 year. The critical brain Hg level to manifest toxic signs would lie between 3 and 5 μg/g in rat. Similarly, no evidence which suggested hepatic damage was

obtained, though up to 20 μg/g of Hg accumulated in the liver of 5 ppm Hg-exposed rats.

On the other hand, in the kidney, various alterations could be detected after MeHg exposure at both dosing groups. The effects of MeHg exposure, however, was somewhat different between two dose groups. Low level (1 ppm Hg) exposure would give sufficient stress to the kidney to cause elevation of the TBARS levels and induction of MT. This stress also stimulated the activities of SOD, catalase and possibly γ-glutamylcysteine synthetase, a rate-determining enzyme for GSH synthesis. The activation of these enzymes, as well as induction of MT, would affect in a favorable way to protect the organ against an oxidative challenge originated from the heavy metal exposure. Nevertheless, GSH-Px activity, the susceptibility of which might be extremely high against the Hg challenge, was significantly reduced even by 1 ppm Hg exposure. However, it might be that other anti-oxidative reactions, such as elevations in catalase activity and cytosolic thiol (GSH and MT) levels, would compensate for the reduced potency of GSH-Px activity to scavenge reactive oxygen species. Thus, the Hg accumulated through chronic MeHg exposure by 1 ppm Hg-diet might not be hazardous for rat kidney. The fact that a survival rate of 1 ppm group was same as that of control group would support this speculation.

In the kidney of the 5 ppm Hg group rats, an extremely high level of Hg (up to 100 μg/g) accumulated there, and drastic changes had occurred. Although induction of MT and GSH were common in both exposed groups, though the former in 5 ppm group became rather lower after 2 years. Since SOD and catalase activities were elevated only in 1 ppm group, Hg accumulation of a moderate level (less than 50 μg/g) would result in the enhancement of these enzyme activities in the kidney, but higher accumulation would inhibit them. On the other hand, GSH-Px activity might be affected by Hg in a similar manner in accordance with the Hg accumulation. In the preliminary experiment, even if kidney homogenate from control rat was treated with MeHg or Hg^{2+} of comparable level with that from 5 ppm Hg-exposed rat, GSH-Px activity was not affected at all. This might indicated that reduction of the renal GSH-Px activity observed in the present model rat would not be direct action of Hg on the enzyme. Since GSH-Px is a Se-containing enzyme, reduction or impaired utility of Se in the kidney might account for the reduced enzyme activity. This was supported by the fact that when 5 ppm rats were given 2 ppm Se-containing water throughout the initial 6 months, the renal GSH-Px activity returned to the level of control rats which were also given Se-containing water (data not shown).

Lipid peroxidation, estimated by TBARS value, was used to evaluate a degree of oxidative stress in tissues. It was quite strange that TBARS levels in the kidney were enhanced only in 1 ppm Hg group, though oxidative damage expected to be enhanced in 5 ppm group. Since TBARS levels also depend on the content of unsaturated fatty acid in the lipid membrane, composition of fatty

acids in the kidney membrane might be altered by high-dose MeHg exposure.

Hematological examination also suggested hazardous effects of MeHg exposure on the kidney. Increase in plasma creatinine levels observed in the late stage of high dose group would indicate renal dysfunction. Decreased erythrocyte number (anemia) was significant as early as 6 months in 5 ppm group. Mitsumori et al. (1983) also documented similar effect by 10 and 2 ppm MeHgCl-contaminated diet. Since leukocytes number was not affected at all (data not shown), effect of the heavy metal on the hematopoietic function would be little. Considering the various Hg-induced effects observed in the kidney as mentioned above, the production of erythropoietin in this organ might also be inhibited by Hg toxicity to cause the anemia.

MeHg is well known to be changed to inorganic form in vivo by the action of intestinal flora (Rowland et al. 1984) and reactive oxygen species (Suda et al. 1991; Suda and Hirayama 1992). Since kidney is a terminal tissue for inorganic Hg excretion, this Hg species transformed from MeHg in other tissues, such as liver and spleen, would accumulate here via the circulation. Although MeHg itself could induce renal dysfunction, nephrotoxic action of Hg^{2+} was much more potent than its organic form (Yasutake et al. 1990). Since, despite a continuous exposure to MeHg, the renal Hg was detected mostly as inorganic form, it was difficult to say which form of Hg participated in various effects observed in the kidney.

In conclusion, a chronic MeHg exposure by 5 ppm Hg-diet might be insufficient to accumulate Hg in the brain to cause neurotoxic action of MeHg even if it lasted throughout a lifetime. On the other hand, long lasting of high Hg accumulation in the kidney would be hazardous enough to cause various adverse effects, such as reduction of anti-oxidative enzyme activities, dysfunction, histological abnormalities in the tissue (Eto et al. 1997) and a slight reduction of a life-span.

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