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Original Article

Neurobehavioral effects of combined prenatal exposure to low-level mercury vapor and methylmercury

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ABSTRACT — We evaluated the effects of prenatal exposure to low-level mercury (Hg^0) or methylmercury (MeHg) as well as combined exposure (Hg^0 + MeHg exposure) on the neurobehavioral function of mice. The Hg^0 exposure group was exposed to Hg^0 at a mean concentration of 0.030 mg/m^3 for 6 hr/day during gestation period. The MeHg exposure was supplied with food containing 5 ppm of MeHg from gestational day 1 to postnatal day 10. The combined exposure group was exposed to both Hg^0 vapor and MeHg according to above described procedure. After delivery, when their offspring reached the age of 8 weeks, behavioral analysis was performed. Open field (OPF) tests of the offspring showed an increase and decrease in voluntary activity in male and female mice, respectively, in the MeHg exposure group. In addition, the rate of central entries was significantly higher in this group than in the control group. The results of OPF tests in the Hg^0 + MeHg exposure group were similar to those in the MeHg exposure group in both males and females. The results in the Hg^0 exposure group did not significantly differ from those in the control group in males or females. Passive avoidance response (PA) tests revealed no significant differences in avoidance latency in the retention trial between the Hg^0, MeHg, or Hg^0 + MeHg exposure group and the control group in males or females. Morris water maze tests showed a delay in the latency to reach the platform in the MeHg and Hg^0 + MeHg exposure groups compared with the control group in males but no significant differences between the Hg^0, MeHg, or Hg^0 + MeHg exposure group and the control group in females. The results of OPF tests revealed only slight effects of prenatal low-level Hg^0 exposure (0.03 mg/m^3), close to the no-observable-effect level (NOEL) stated by the WHO (0.025 mg/m^3), on the subsequent neurobehavioral function. However, prenatal exposure to 5 ppm of MeHg affected exploratory activity in the OPF test, and, in particular, male mice were highly sensitive to MeHg. The MeHg and Hg^0 + MeHg exposure groups showed similar neurobehavioral effects. Concerning the effects of prenatal mercury exposure under the conditions of this study, the effects of MeHg exposure may be more marked than those of Hg^0 exposure.

Key words: Mercury vapor, Methylmercury, Combined exposure, Mice, Neurobehavioral effects

INTRODUCTION

Health hazards due to methylmercury (MeHg) started with Minamata disease and MeHg poisoning in Iraq. At present, MeHg disasters around gold mines and health problems due to MeHg resulting from the consumption of a large amount of contaminated fish and shellfish have attracted attention in each country of the world. In addition, global concern about the effects of not only MeHg but also mercury vapor (Hg^0) generated from dental amal-

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gams has been increasing (Mutter *et al.,* 2004; Clarkson and Magos, 2006).

MeHg and Hg^0, unlike other heavy metals, are fat-soluble, and, therefore, readily pass the brain-blood and placental barriers, resulting in mercury accumulation in the brain and fetuses (Yoshida, 2002). Concerning prenatal mercury exposure, mercury toxicity more markedly affects the fetus than the mother (Clarkson, 2002).

Mercury exposure of pregnant females includes not only MeHg exposure associated with contaminated fish and shellfish consumption but also Hg^0 generated from dental amalgams. Experimental studies using pregnant animals have revealed an increase in the mercury concentration of fetal tissue depending on the number of dental amalgam fillings (Mackert and Berglund, 1997; Takahashi *et al.,* 2001). There have been many studies on the effects of MeHg or Hg^0 on fetuses as a highly sensitive group but few studies on the effects of combined exposure to MeHg and Hg^0. In addition, the effects of combined exposure to low-level MeHg and Hg^0 in the general environment, particularly via food, on fetal growth/development, especially neurobehavioral toxic effects, have not yet been clarified. We evaluated the effects of combined prenatal exposure to low-level Hg^0 and MeHg (Hg^0 + MeHg exposure) on fetal growth/development, particularly the effects on the neurobehavioral function. At the same time, sex difference in the offspring due to prenatal exposures to mercury was also examined.

MATERIALS AND METHODS

Animals and exposure procedure

Animals

Male and female mice of the C57BL/6 strain were purchased from Nippon CLEA Co. (Tokyo, Japan). The animal facility was maintained under a light/dark cycle of 12 hr, temperature of 24 ± 1°C, relative humidity of 55 ± 10%, and negative atmospheric pressure. The mice received mouse chow (CE-2, Japan Clea) and filtered tap water *ad libitum.* Males and females (one pair per cage) at 8 weeks of age were mated. Pregnancy was confirmed by the presence of a vaginal plug the following morning (defined as gestational day 0 = GD0).

Experimental methods

When reaching the age of 10 weeks, male and female mice were housed in pairs for mating, and pregnancy was confirmed by the presence of a vaginal plug the following morning (defined as GD0). After then, Hg^0 exposure, MeHg exposure, and combined exposure to Hg^0 and MeHg were carried out in the pregnant mice as follows:

Hg^0 exposure group

Pregnant mice were placed in an Hg^0 exposure chamber and exposed to Hg^0 at a mean concentration of 0.030 mg/m^3 (range, 0.017-0.038 mg/m^3) for 6 hr daily until GD18. The Hg^0 concentration in the exposure chamber was determined using a mercury gas monitor for a work environment, Mercury/EMP-1A (Nippon Instruments Corp., Tokyo, Japan). After exposure, they were delivered in an animal facility (room temperature, 22.5 ± 0.5°C; humidity, 55 ± 5%). After delivery, the number of siblings was adjusted to 3 males and 3 females. Ten days after delivery, 2 male and 2 female mice were sacrificed under ether anesthesia, and the brain, kidneys, and liver were resected and stored at -80°C. The mice were weaned 28 days after delivery, and behavioral analysis was performed at the age of 8 weeks. For behavioral analysis, 1-2 male and female mice delivered by each mother were used, and each experimental group consisted of 5-6 mice.

MeHg exposure group

Pregnant mice were immediately supplied with food containing 5 ppm of MeHg until 10 days after delivery and subsequently fed MeHg-free food. After delivery, the number of siblings was adjusted to 3 males and 3 females. Ten days after delivery, 2 male and 2 female mice were sacrificed under ether anesthesia, and the brain, kidneys, and liver were resected and stored at -80°C. The mice were weaned 28 days after delivery, and behavioral analysis was performed at the age of 8 weeks. For behavioral analysis, 1-2 male and female mice delivered by each mother were used, and each experimental group consisted of 5-6 mice.

Combined exposure group

Pregnant mice were exposed to both Hg^0 vapor and MeHg according to above described procedure. The mice were weaned 28 days after delivery, and behavioral analysis was performed at the age of 8 weeks. For behavioral analysis, 1-2 male and female mice delivered by each mother were used, and each experimental group consisted of 5-6 mice.

The mice were treated humanely and with regard to alleviation of suffering according to the National Institute for Environmental Studies' Guidelines for Animal Welfare and the guideline of St. Marianna University.

Behavioral analysis

Behavioral functions of the mice were evaluated employing three commonly used methods: the open field (OPF) test, passive avoidance test, and Morris water

maze. The rationale for choosing the former two tests is described elsewhere (Yoshida *et al.,* 2005). The third method, the Morris water maze, was developed for the evaluation of spatial memory, with which the animals were required to learn the spatial location of a hidden submerged platform in a water pool (Morris, 1984).

OPF test

The locomotory activity of mice was assessed using an OPF, for which the methodological details are given elsewhere (Yoshida *et al*., 2005). Briefly, each mouse was moved from its home cage to the center square (10 x 10 cm) of the OPF (50 x 50 cm), and covered with a black Plexiglas box (10 x 10 x 10 cm). After 20 sec, the box was gently removed, and the behavior of the mouse was video-recorded for the following 5 min. The video image was analyzed by Image OF, software for image analysis (O'Hara & Co., LTD., Tokyo, Japan). Two parameters of activity were calculated: the distance (in cm) moved and the positioning of the mouse. For the latter, the 25 squares making up the floor area (each 10 x 10 cm) were classified as either peripheral (the 16 squares adjacent to the wall) or central (the nine remaining squares in the center).

Passive avoidance response (PA) test

Passive avoidance learning was assessed employing a step-though procedure; the details are also given elsewhere (Yoshida *et al.,* 2005). The apparatus (PA-2010A, O'Hara & Co., Ltd.) consisted of a dark and an illuminated compartment, which were separated by a sliding door. On the first day (training trial), the mouse was placed in the illuminated compartment for 30 sec, and then the door was opened. When the mouse entered the dark compartment, it received an unavoidable, brief electric shock to the feet, and escaped immediately to the illuminated compartment. The door was closed after the mouse re-entered the illuminated compartment, and the mouse was removed. Twenty-four hours later (retention trial), the test was repeated again but without administering the electric shock. In both trials, the "latency" was defined as the interval between the opening of the door and the entry of the mouse into the dark compartment. The cut-off time of the retention session was 300 sec.

Morris water maze test

Spatial learning was assessed using a Morris water maze test. The water maze was a circular plastic pool of 100 cm in diameter and filled with water to a depth of 20 cm. The water was kept at room temperature (23 ± 1°C) and was made opaque by adding white paint to prevent the animal from seeing the submerged platform. In the "hidden platform" trials, a round 10-cm-diameter platform made of white Plexiglas was placed 1 cm below the water surface in the center of one of the four quadrants. A mouse was released into the water at one of four randomly selected positions near the wall and facing the wall. The latency, defined as the time from the release of the mouse to climbing on the platform, was recorded. When the mouse could not find the platform within 60 sec from the time of release, it was led to the platform and placed on it for 20 sec before being removed. In such cases, a latency of 60 sec was recorded. Each mouse underwent four trials on each of five consecutive days. The pool was fixed at the same position in the room, and the investigator always stood at the same position beside the pool during the experiment. Around the pool were also situated a video device, steel animal racks, and water supply pipes. All were visible from the inside of the pool, and served as distant visible cues for the mice. A visible platform trial, in which mice located the submerged platform by placing a marker on it, was performed after the hidden platform trial was completed.

Analysis of mercury concentrations in tissues

Mercury concentrations in the tissues were measured with a cold vapor atomic absorption spectrophotometer (RA-2A Mercury Analyzer; Nippon Instruments) after digestion with a concentrated acid mixture [HNO_3/$HClO_4$ 1:3 (v/v)] (Satoh *et al*., 1997). The detection limit of this method was 0.5 ng Hg with an intra-assay coefficient of variation (n = 10) of 4%.

Statistical analysis

Data were analyzed statistically with Student's t-test or Wilcoxon rank sum test (for behavioral tests) for comparison between the non-exposed control and exposed group with a preset probability.

RESULTS

Ten days after delivery, male and female mice in the control, Hg^0, MeHg, and Hg^0 + MeHg exposure groups were weighed. The body weight did not significantly differ between the control group and the Hg^0, MeHg, or Hg^0 + MeHg exposure group (data not shown).

Fig. 1 shows the total locomotory distances in the OPF test at the age of 8 weeks in the males and females in the control group and the Hg^0, MeHg, and Hg^0 + MeHg exposure groups. The total locomotory distance in the Hg^0 exposure group did not differ from that in the control group in both the males and females. The total locomoto-

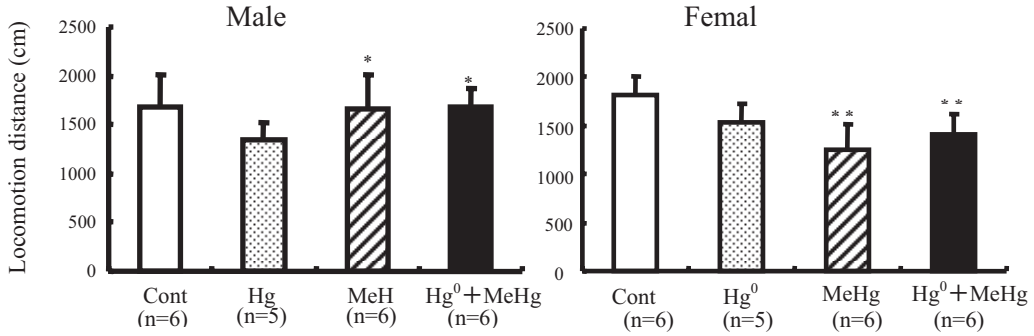

Fig. 1. Total locomotory activity of mice exposed in utero to Hg^0, MeHg, and Hg^0 + MeHg in the OPF task. Data shown are means ± S.D. for each group. The number of animals is shown in parentheses. *Significant difference from control animals at $p < 0.05$. ** Significant difference from control animals at $p < 0.01$.

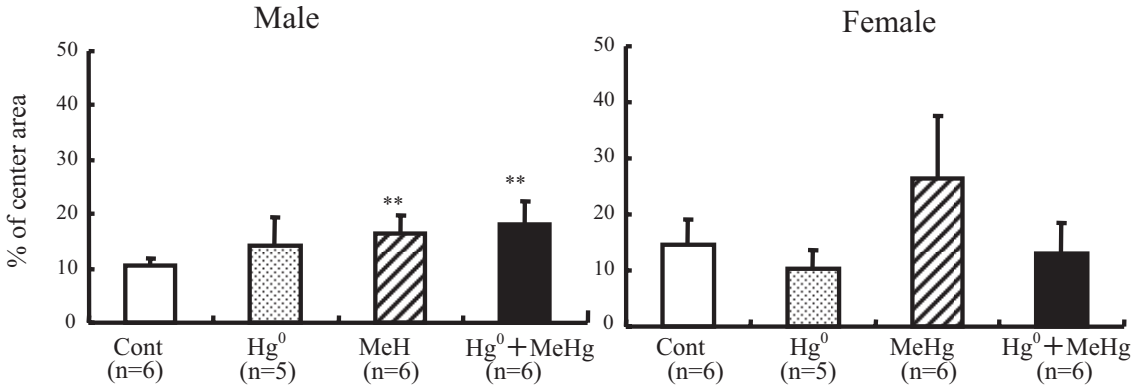

Fig. 2. The rate of central entries of mice exposed in utero to Hg^0, MeHg, and Hg^0 + MeHg in the OPF task. Data shown are means ± S.D. for each group. The number of animals is shown in parentheses. **Significant difference from control animals at $p < 0.01$.

ry distance in the MeHg exposure group was longer ($p < 0.05$) than that in the control group in males, but significantly shorter than that in the control group in females ($p < 0.01$). The total locomotory distance in the Hg^0 + MeHg exposure group as well as the MeHg exposure group was significantly longer than that in the control group in males ($p < 0.05$), but it was significantly shorter in females ($p < 0.01$).

Fig. 2 shows the rate of central entries in the OPF test. No significant difference was observed between the Hg^0 exposure and control groups in males or females. The rate of central entries in the MeHg exposure group was significantly higher than that in the control group ($p < 0.01$) in males, but did not significantly differ from that in the control group in females. The Hg^0 + MeHg exposure group as well as the MeHg exposure group showed a significantly higher rate than the control group in males ($p < 0.01$) but no significant difference from the control group in females.

Fig. 3 shows the results of training and retention trials in the PA test. The avoidance latency in the retention trial did not differ between the control and Hg^0, MeHg, or Hg^0 + MeHg group.

Changes in the avoidance latency in the Morris water maze test for 5 days are shown in Fig. 4. The avoidance latency in the Hg^0 exposure group did not significantly differ from that in the control group on all 5 days in males or females. The avoidance latency decreased with repeated training for 5 days in both males and females in the control group, but did not decrease in the females in the Hg^0 exposure group.

In the MeHg exposure group, the avoidance laten-

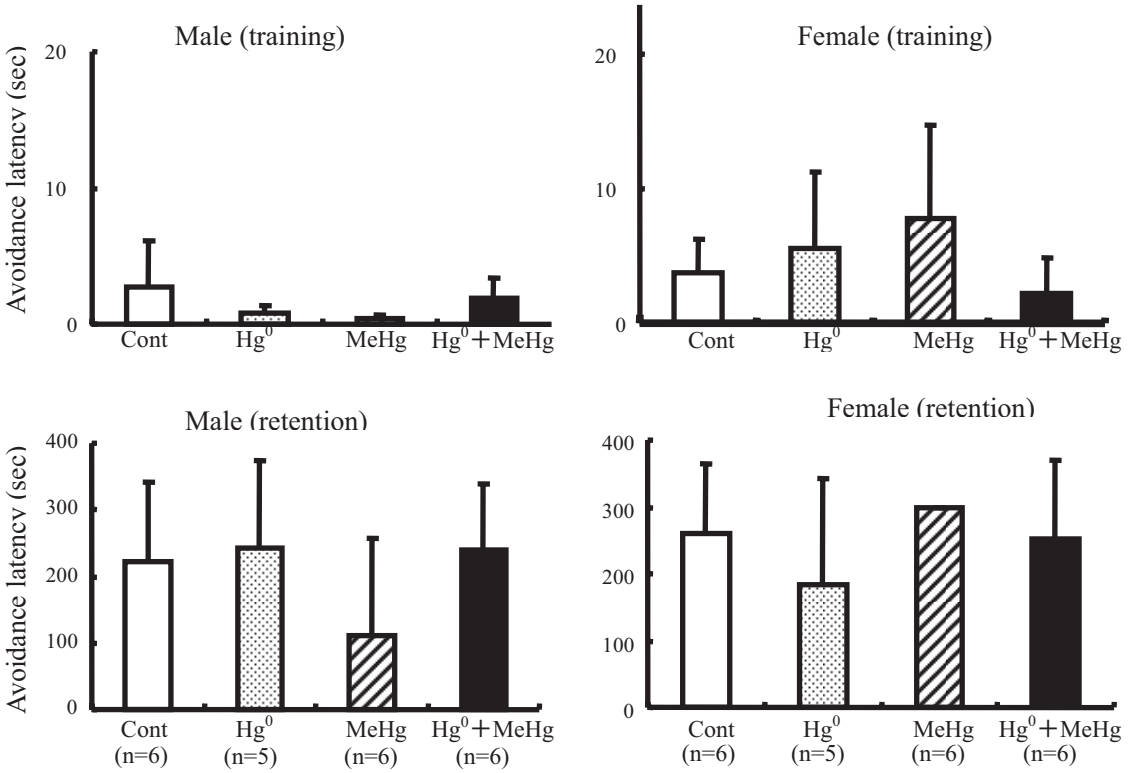

Fig. 3. Avoidance latency of mice exposed in utero to Hg^0, MeHg, and Hg^0 + MeHg in the passive avoidance test. Data shown are means ± S.D. for each group. The number of animals is shown in parentheses.

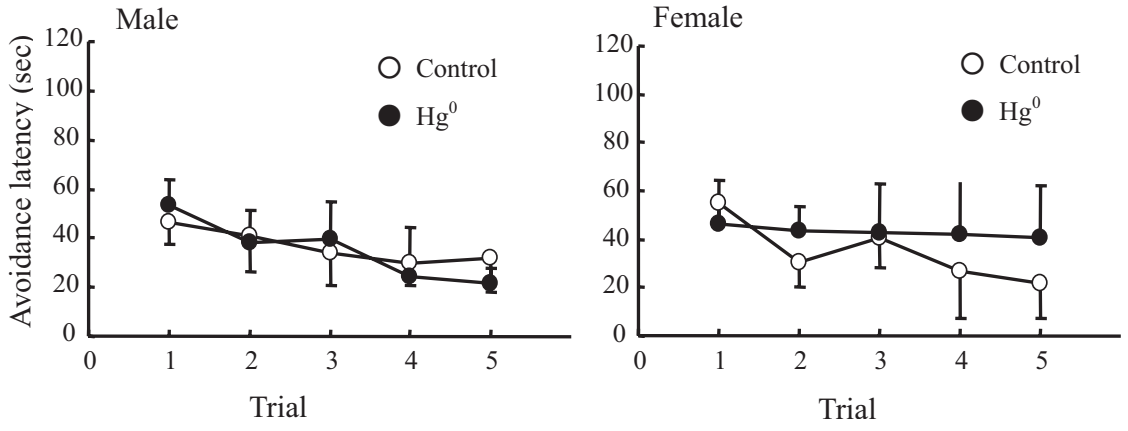

Fig. 4. Latency to reach the platform in the Morris water maze for mice exposed in utero to Hg^0. Data shown are means ± S.D. for exposed (●) and control (○) mice.

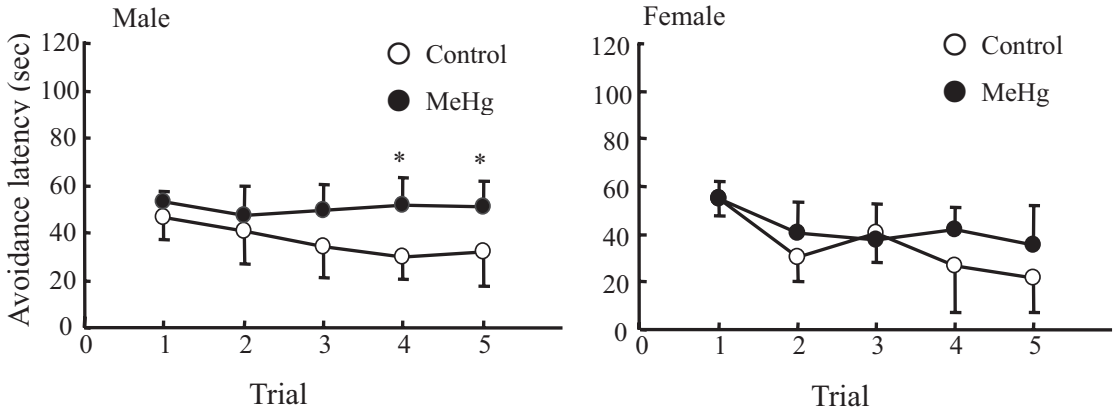

Fig. 5. Latency to reach the platform in the Morris water maze for mice exposed in utero to MeHg. Data shown are means ± S.D. for exposed (●) and control (○) mice. *Significant difference from control animals at $p < 0.05$.

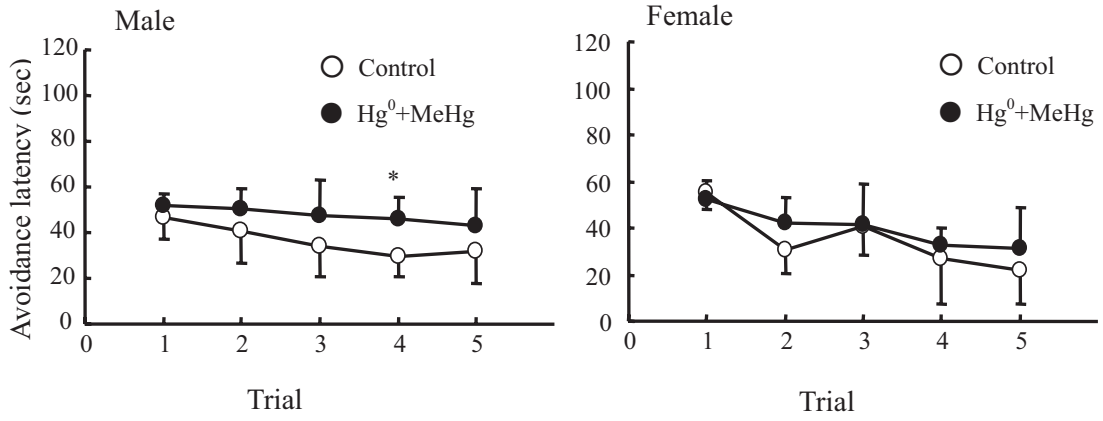

Fig. 6. Latency to reach the platform in the Morris water maze for mice co-exposed in utero to Hg^0 and MeHg. Data shown are means ± S.D. for exposed (●) and control (○) mice. *Significant difference from control animals at $p < 0.05$.

cy did not decrease even after 5-day training in males or females. In males, significant differences from the control group were observed on training days 4 and 5 ($p < 0.05$). In females, no significant difference from the control group was observed on any day (Fig. 5).

In the Hg^0 + MeHg exposure group as well as the MeHg exposure group, the avoidance latency did not decrease even after 5-day training in males or females. In males, a significant difference from the control group was observed on training day 4 ($p < 0.05$). In females, no significant difference from the control group was observed (Fig. 6).

The mercury concentrations in the brain, kidneys, and liver in male and female mice 10 days after delivery are shown in Table 1. In the Hg^0 exposure group, the mercury concentration in the brain was about twice that in the control group in both males and females ($p < 0.05$), but its concentration in the kidneys or liver in this group did not differ from that in the control group. In the MeHg exposure group, the mercury concentration in the brain was about 180 times that in the control group in both males and females ($p < 0.01$), and its concentration in the kidneys was about 20 times ($p < 0.01$) and that in the liver was about 60 times ($p < 0.01$) that in the control group. The Hg^0 + MeHg exposure group showed a brain mercury concentration of about 380 and 170 times that in the control group in males and females, respectively ($p < 0.01$) and about twice that in the MeHg exposure group in males ($p < 0.01$). The mercury concentration in the kidneys in the Hg^0 + MeHg exposure group was about 20

Table 1. Mercury concentration in the brain, liver, and kidneys of offspring at 10 days after birth

			Exposed		
		Control	Hg^0	MeHg	Hg^0 + MeHg
Male	Brain	1.7 ± 0.4	3.0 ± 0.3*	340 ± 91**	652 ± 33**
	Liver	5.3 ± 2.5	6.7 ± 0.6	308 ± 45**	425 ± 81**
	Kidneys	19.5 ± 0.9	23.7 ± 5.5	397 ± 50**	356 ± 76**
Female	Brain	2.1 ± 0.4	3.2 ± 0.6*	380 ± 89**	341 ± 69**
	Liver	6.0 ± 1.0	8.0 ± 1.7	444 ± 39**	588 ± 62**
	Kidneys	18.0 ± 1.6	18.2 ± 1.5	267 ± 28**	425 ± 99**

Mercury concentration is expressed as ng Hg/g tissue. Values are means ± S.D. *Significant difference from control animals at $p < 0.05$. ** Significant difference from control animals at $p < 0.01$.

times that in the control group ($p < 0.01$), and that in the liver in this group was about 80 times ($p < 0.01$) in both males and females.

DISCUSSION

As studies on neurobehavioral toxicity due to MeHg exposure during the prenatal period, Goulet *et al.* (2003) gave drinking water containing 0, 4, 6, and 8 ppm MeHg to C57BL/6 mice as the same species as that used in this study from the prenatal to breastfeeding period, and observed no changes in voluntary activity in the OPF test in male or female mice. The results of this study showed an increase in voluntary activity in males but its decrease in females, demonstrating the effects of MeHg exposure at a concentration of 6 or 8 ppm on short-term (work) memory in the female compared with the male group. Experiments on long-term memory also showed no effects of MeHg in males or females, but a higher susceptibility of female compared to male mice concerning the effects on long-term memory. In this study, PA tests were performed to evaluate learning memory of an adverse experience. The avoidance latency did not significantly differ between the MeHg exposure and control groups, showing no decrease in learning memory. In contrast, concerning the learning ability for space perception employing the Morris water maze test, males in the MeHg exposure group showed no decrease in the avoidance latency compared with the control group even after 5-day training, suggesting the effects of MeHg on the learning ability for space perception. However, acquisition of the spatial learning ability did not differ between the MeHg exposure and control groups. Although these effects of MeHg on memory differed from the results reported by Goulet *et al.* (2003), these findings also suggest the marked effects of prenatal MeHg exposure on the neurobehavioral function.

On the other hand, as experiments to evaluate the effects of Hg^0 exposure on behavior, Danielsson *et al.* (1993) evaluated rat behavior during the neonatal period after prenatal exposure to Hg^0 at a concentration of 1.8 mg/m^3 for 3 hr on GD11-14 and GD15-16. Voluntary activity in newborns after prenatal Hg^0 exposure decreased at the age of 3 weeks but increased at the age of 14 months. In spatial learning, delays in learning in a radial maze, simple learning, and habituation to a novel environment were observed. An experimental study (Newland *et al.,* 1996), in which pregnant squirrel monkeys were exposed to mercury at a concentration found in industrial settings (0.5 or 1.0 mg/m^3) for 4 or 7 hr/day on 5 days/week, showed a longer lever-press duration and more marked variability in the same individuals in the exposure group than in the control group under a reinforcement factor stimulation condition at the age of 0.8-4 years. In the present study, we used an exposure concentration that was even lower than the experimental condition employed in the study by Danielsson *et al.* (1993) and close to the recommended limit (0.025 mg/m^3) of occupational mercury vapor exposure of the WHO. Under a condition of this mercury vapor exposure, the brain mercury concentration was relatively low in both male and female mice. Therefore, it is considered that no behavioral effects of Hg^0 exposure alone were observed.

There have been only a few studies on the behavioral effects of combined prenatal exposure to Hg^0 and MeHg. Fredriksson *et al.* (1996) evaluated behavioral changes after combined prenatal exposure in male rats. Rats orally received MeHg (2 mg/kg/day) on GD6-9 and were

exposed to Hg^0 (1.8 mg/m^3) for 90 min daily on GD14-19, and behavioral tests were performed at the age of 16-20 weeks. Voluntary activity increased in the Hg^0 exposure group compared with the control group and further increased in the Hg^0 + MeHg exposure group. The avoidance latency to reach the platform in the Morris water maze test was delayed in the Hg^0 exposure group compared with the control group and further delayed in the Hg^0 + MeHg exposure group compared with the Hg^0 exposure group. Fredriksson *et al.* (1996) reported that the neurobehavioral effects of combined prenatal exposure, where Hg^0 exposure level was higher than the present study, are more marked than those of prenatal exposure to mercury alone. In this study, the neurobehavioral effects of Hg^0 + MeHg exposure were similar to those of MeHg exposure in the OPF, PA, and Morris water maze tests, showing no increase in neurobehavioral toxicity on combined exposure. From these facts, it is proved that prenatal exposure to Hg^0 at levels close to the recommended occupational exposure limit does not enhance the neurotoxicity of MeHg exposure.

The mercury concentration in the brain as the target organ for Hg^0 and MeHg was markedly higher in both the MeHg and combined exposure groups than in the Hg^0 exposure group. These results suggest that neurobehavioral toxicity in the Hg^0 + MeHg exposure group is due to the toxicity of MeHg rather than that of Hg^0.

Based on these results, even after exposure to Hg^0 at a concentration (0.03 mg/m^3) close to the prenatal NOEL recommended by the WHO (0.025 mg/m^3), its neurobehavioral effects were slight. However, prenatal exposure to 5 ppm of MeHg affected voluntary and exploratory activities in the OPF test and the avoidance latency in the Morris water maze test, and, in particular, male mice were highly sensitive to MeHg. Based on the analysis of the sex ratio of babies including stillbirth cases in Minamata city, Sakamoto *et al.* (2001) reported that male fetuses were more susceptible to the pollution than their female counterparts. It became clear in animal experiment that prenatal MeHg exposure had an influence on male fetuses than female fetuses.

In addition, the effects of the combined exposure to Hg^0 and MeHg were evaluated in this study. The Hg^0 + MeHg exposure group did not show more marked effects than the MeHg exposure group. Under the conditions of the present study, the effects of prenatal mercury exposure may be more marked on MeHg exposure compared to Hg^0 exposure. Therefore, we indicate that the combined exposure to MeHg at levels relevant to human exposure and Hg^0 at levels relevant to recommended limit of occupational Hg^0 exposure of the WHO may not cause additive and/or synergic effect of neurotoxicity.

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