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Original Article

Neurobehavioral toxicity related to the exposure of weaning mice to low-level mercury vapor and methylmercury and influence of aging

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ABSTRACT — Female C57BL mice were exposed to a low level of mercury vapor (Hg^0, 0.096 mg/m^3) and was given the solution containing 5-ppm methylmercury (MeHg) during the growth period to examine the influence on the neurobehavioral function after birth. Exposure period was 4 weeks at 3 to 7 weeks of age. At 10 weeks of age, three behavioral tests were conducted; open field (OPF) test, passive avoidance response (PA) test, and eight-arm radial maze (RM) test. To evaluate the influence of aging, additional behavioral tests were performed at 79 weeks of age. With respect to the results of the three behavioral tests conducted at 10 to 14 weeks of age, there were no significant differences between the Hg^0/MeHg/Hg^0+MeHg and control groups. Furthermore, there were also no significant differences between each exposure and control group on behavioral tests performed at 79 to 83 weeks of age after the completion of mercury exposure. The concentration of mercury in the brain after the completion of exposure was the highest in the Hg^0+MeHg group, followed by the MeHg and Hg^0 groups. The values in the Hg^0+MeHg and MeHg groups were ≤ 3.0 μg/g. The value in the Hg^0 group was ≤ 1.0 μg/g. There were no differences in the brain concentration of mercury after 1 year between the Hg^0/MeHg and control groups. However, in the Hg^0+MeHg group, it was significantly higher than in the control group, suggesting that the disappearance of mercury in the brain is delayed in comparison with the exposure to Hg^0 or MeHg alone. These results showed that there was no influence of low-level Hg^0+MeHg exposure during the growth period on neurobehavioral manifestation. However, the disappearance of mercury in the brain was delayed in comparison with the exposure to Hg^0 or MeHg alone.

Key words: Mercury vapor, Methylmercury, Combined exposure, Behavioral toxicity, Growing mice

INTRODUCTION

Since the development of Minamata disease, health damage related to environmental pollution with mercury has been an important issue in the world. In particular, gold mining-related mercury pollution in the Amazon basin raises a similar issue not only in Brazil but also in gold-producing countries in Africa, Asia, and Eastern Europe (Veiga *et al*., 2006; Kristensen *et al*., 2013). Currently, gold mining at small-scale gold mines is conducted in more than 50 developing countries in Latin America, Southeast Asia, and Africa. According to the International Labor Organization (ILO), the number of miners working at small-scale gold mines was estimated to be 11,000,000 to 13,000,000, including 2,500,000 females and 250,000 children (ILO, 1999). As many children are working to support a living, the influence of mercury vapor exposure on health has been indicated (Bose-O' Reilly *et al*., 2008). If children are exposed to mercury during the growth period, they may be more sensitive to mercury than adults, and mercury poisoning may lead to sequelae, differing from that in adults (Counter and Buchanan, 2004).

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Yoshida *et al*. (2005, 2014) conducted an animal experiment, and reported that mercury vapor exposure during the fetal period or in infancy influenced subsequent neurobehaviors. Recently, around small-scale gold mines, it has been indicated that gold mining causes mercury pollution in water environments, leading to the fish and shellfish accumulation of methylmercury related to the methylation of metal mercury used for mining and residents' health damage associated with consumption. In China, soil around a mercury mine was polluted with mercury, and a high concentration of methylmercury was detected in grains harvested in the area, suggesting non-seafood-ingestion-dependent rice diet-related methylmercury exposure (Li *et al*., 2008; Qiu *et al*., 2008). However, the influence of combined exposure to a low concentration of mercury vapor (Hg^0) and methylmercury (MeHg) for a long period during the growth period (childhood) on neurobehaviors remains to be clarified. In this study, we examined the influence of combined low-level mercury exposure during the growth period on the neurobehavioral function.

MATERIALS AND METHODS

Animals

Sixty dams nursing 2-week-old C57BL/6J mice were purchased from SLC, Inc., Shizuoka, Japan, and acclimated in the animal room of the School of Pharmacy, Aichi Gakuin University, during which the mercury exposure were conducted. One week before the behavior tests, the mice were transferred to the conventional room in Department of Human Ecology, Graduate School of Medicine, The University of Tokyo, where all the behavior tests were conducted. Each room was maintained as 24 ± 1°C room temperature, 55 ± 10% humidity, and 12 hr automatic lighting cycle. All the mice were given solid food (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. At 3 weeks of age, only the female offspring were divided into 4 groups: control, Hg^0, MeHg, and Hg^0+MeHg group. Two to three female mice delivered by mothers were used (n = 21 per group). All the animal experiments were performed in accordance with the Regulation on Animal Experimentation Committee both at School of Pharmacy, Aichi Gakuin University, Nagoya, and at Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.

Exposure procedures

The female mice (n = 21/group), except control group, were exposed to any mercury form for 4 weeks from PND 21 to PND 49. For Hg^0 exposure, the mice were placed in a chamber for mercury vapor exposure, and exposed to Hg^0 for 8 hr daily at a mean concentration of 0.096 mg/m^3 (ranged from 0.055 to 0.143 mg/m^3). The mercury concentration in the chamber was measured every day using a mercury survey meter (Type; EMP-1A, Nippon Instruments Co., Tokyo, Japan). For MeHg exposure, methylmercury chloride (GL Sciences Inc., Tokyo, Japan) was diluted with distilled water to prepare 5-ppm solution. The solution containing 5-ppm MeHg was given ad libitum instead of tap water. For Hg^0+MeHg combined exposure, the mice were placed in a mercury vapor exposure chamber and 5-ppm MeHg solution was simultaneously given.

Behavioral experiments

Behavioral functions were evaluated with three commonly used methods: the open field test, passive avoidance test, and eight-arm radial maze test as described below. These behavioral tests were performed in this order either at 10 to 14 weeks of age (n = 7/group) or at 79 to 83 weeks of age (n = 7/group) after the completion of mercury exposure.

Open field (OPF) test

The locomotor activity of mice was assessed using an open field, for which the methodological details are given elsewhere (Yoshida *et al*., 2004). Briefly, each mouse was moved from its home cage to the center square (10 x 10 cm) of the open field (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 subsequent 2 min. The video images were analyzed with Image OF, image analysis software (O'hara Co. & Ltd., Tokyo, Japan). Two parameters of activity were calculated: the locomotor distance (cm/30 sec) moved by the mouse and the mouse position on the field at every 0.5 sec. For the latter, 25 squares (each 10 x 10 cm) were classified as either peripheral (the 16 squares adjacent to the wall) or central (the 9 remaining squares in the center).

Passive avoidance (PA) test

Passive avoidance learning, a learning task motivated by strong aversive stimuli, was assessed by a step-though procedure; the details of which are given elsewhere (Yoshida *et al*., 2004). The apparatus (Neuroscience; Inc., Tokyo, Japan) consisted of a dark and 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 foot, 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 (the retention trial), the test was repeated again, but without giving the electric shock. In both trials, "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 for the retention session was 300 sec.

Eight-arm radial maze (RM) test

Eight-arm radial maze apparatuses (O'hara Co. & Ltd.) was used and the detail components were described elsewhere (Yamasaki *et al*., 2008). The mice were restricted food intake to 2 to 3 g/day by feeding 25-mg spherical pellets special for behavioral experiments 10 days before training of an eight-arm radial maze test so that the body weight was maintained at 80% of the mean body weight for 3 days before food restriction. As acclimation training, the mice of a group (n = 7/group) were placed in the central platform and allowed to wander in the maze for 10 min without any food pellets at the initial training day, followed by another training to consume pellets scattered on arms as well as food wells at the next day. After these group training trials, actual maze trials (RM tests) were carried out a 5-min session for each mouse (one mouse per session) to explore a pellet placed on each food well at the distal end of each arm, and a total of 13 sessions were acquired for consecutive days, excluding Saturday, Sunday, and holidays. On the RM test at 11 weeks of age, a 'delay' program, which was loaded closure of all the doors for 2 min after the acquisition of 4 pellets, was used for the 1st to 7th sessions in the former half, and a standard program without any 'delay' was used for the 8th to 13th sessions in the latter half. On the RM test at 80 weeks of age, a standard program without any 'delay' was used for all the 1st to 13th sessions. Working memory errors and total food intake, that is, number of visits on arms where pellets had been consumed in a session, and number of pellets which were acquired in a session were recorded by Image RM software (O'hara Co. & Ltd.) for evaluation.

Analysis of mercury concentrations in tissue

Mice kidney, liver, lung, cerebrum and cerebellum were dissected at 7 weeks, 14 weeks, and 83 weeks of age at the timing of termination of mercury exposure or the behavior tests. Mercury concentrations in the tissues were measured with a cold atomic absorption spectrophotometer (RA-2A Mercury Analyzer; Nippon Instruments Co.) 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 the Student's *t*-test or Wilcoxon rank sum test for comparison between the non-exposed control and exposed group with a preset probability.

RESULTS

The locomotion activity of mice on OPF tests at 10

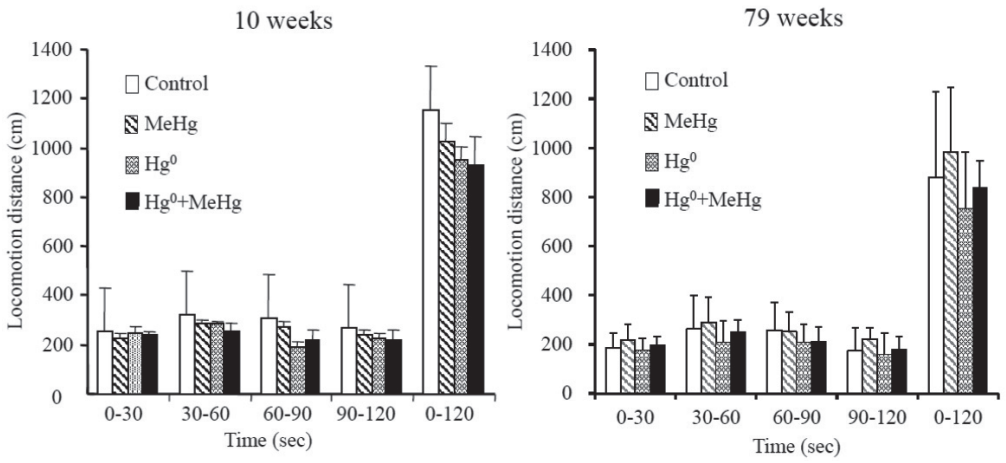

Fig. 1. Locomotion activity of mice at 10 and 79 weeks of age after exposure to MeHg, Hg^0 and Hg^0+MeHg during developing periods. Data shown are means ± standard deviation for seven animals from each group.

and 79 weeks of age is shown in Fig. 1. There were no significant differences in the locomotion distance at each point (0-30, 30-60, 60-90, and 90-120 sec) or total locomotion distance (0-120 sec) between the control and 3 exposure groups at 10 weeks of age. At 79 weeks of age, there were also no significant differences. The percent duration of stay at the center on the OPF test (%) is shown in Fig. 2. At 10 weeks of age, there were no differences between the control and 3 exposure groups. At 79 weeks of age, the percent duration of stay at the center in the Hg^0+MeHg group was 2 times higher than in the control group, but there was no significant difference. Furthermore, there were no differences between the control and other exposure groups.

The results of training and maintenance sessions on PA tests are shown in Fig. 3. There were no differences between the control and three exposure groups on training or maintenance sessions at 10 or 79 weeks of age.

The number of working memory errors per session on RM tests at 11 and 80 weeks of age is shown in Fig. 4. There were no differences in the number of working memory errors among the groups during 13 sessions at 11 or 80 weeks of age. Total food intake per session on RM tests at 11 and 80 weeks of age is shown in Fig. 5. There were no differences among the groups during 13 sessions at 11 or 80 weeks of age.

The tissue concentration of mercury after the completion of exposure is shown in Table 1. The cerebral concentration of mercury in the MeHg group was 80 times higher than in the control group. That in the Hg^0 group was 15 times higher, and that in the Hg^0+MeHg group was 90 times higher. The cerebellar concentrations of mercury in the MeHg, Hg^0, and Hg^0+MeHg groups were 140, 75, and 200 times higher than in the control group, respectively. In each exposure group, the lung, liver, and kidney concentrations of mercury were more than 10 times higher than in the control group. In the Hg^0+MeHg group, the concentrations of mercury in organs were significantly higher than in the Hg^0 group. The cerebellar concentration of mercury in the Hg^0+MeHg group was significantly higher than that in the MeHg group.

The tissue concentration of mercury at 14 weeks of age (8 weeks after the completion of exposure) is shown in Table 2. In the MeHg group, the cerebellar, lung, and kidney concentrations of mercury were 15, 18, and 22 times higher than in the control group, respectively. In the Hg^0 group, the cerebellar concentration of mercury was 8 times higher than in the control group. In the Hg^0+MeHg group, the cerebral, cerebellar, lung, and kidney concentrations of mercury were 20, 15, 18, and 20 times higher than in the control group, respectively. Furthermore, the cerebral and cerebellar concentrations of mercury in the Hg^0+MeHg group were higher than in the MeHg and Hg^0 groups. The tissue concentration of mercury at 81 weeks of age is shown in Table 3. There were no differences in any organ between the MeHg/Hg^0 and control groups. However, the cerebral and cerebellar concentrations of mercury in the Hg^0+MeHg group were significantly higher than in the control, MeHg, or Hg^0 group.

DISCUSSION

Methylmercury or mercury vapor exposure in the embryonic and lactation stages influences the neu-

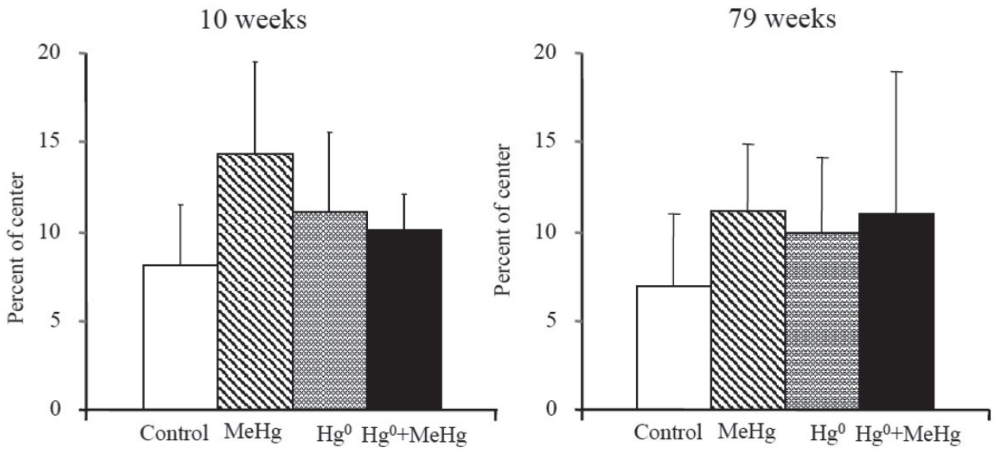

Fig. 2. The percent of central entry of mice at 10 and 79 weeks of age after exposure to MeHg, Hg^0 and Hg^0+MeHg during developing periods. Data shown are means ± standard deviation for seven animals from each group.

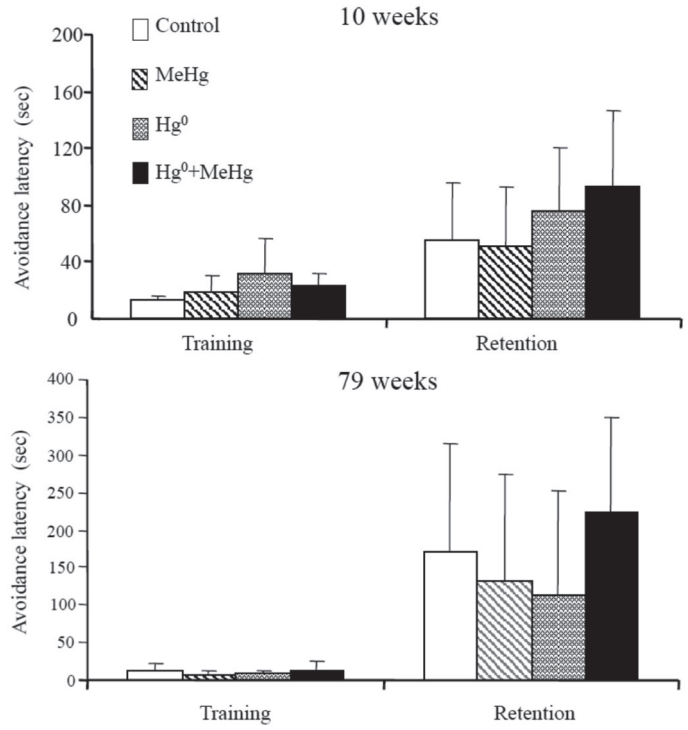

Fig. 3. Avoidance latency of mice at 10 and 79 weeks of age after exposure to MeHg, Hg^0 and Hg^0+MeHg during developing periods. Data shown are means ± standard deviation for seven animals from each group.

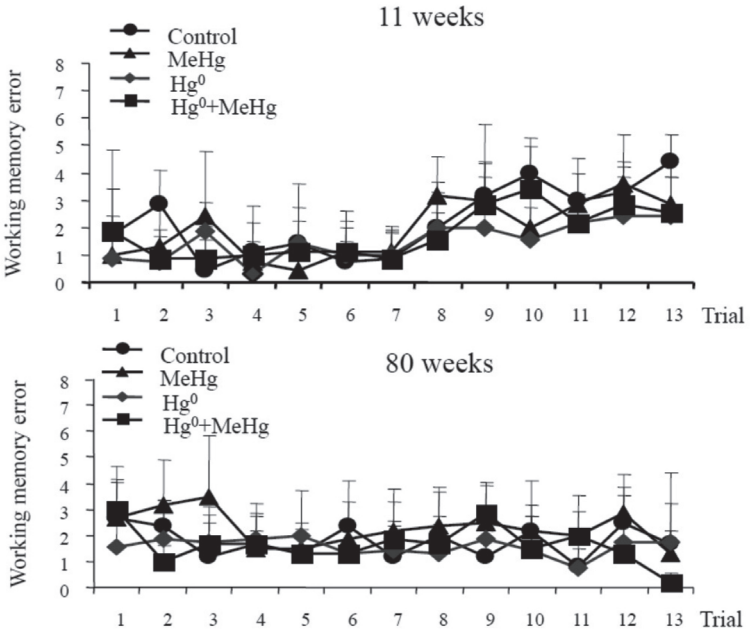

Fig. 4. Number of working memory error of mice at 11 and 80 weeks of age after exposure to MeHg, Hg^0 and Hg^0+MeHg during developing periods. Data shown are means ± standard deviation for seven animals from each group.

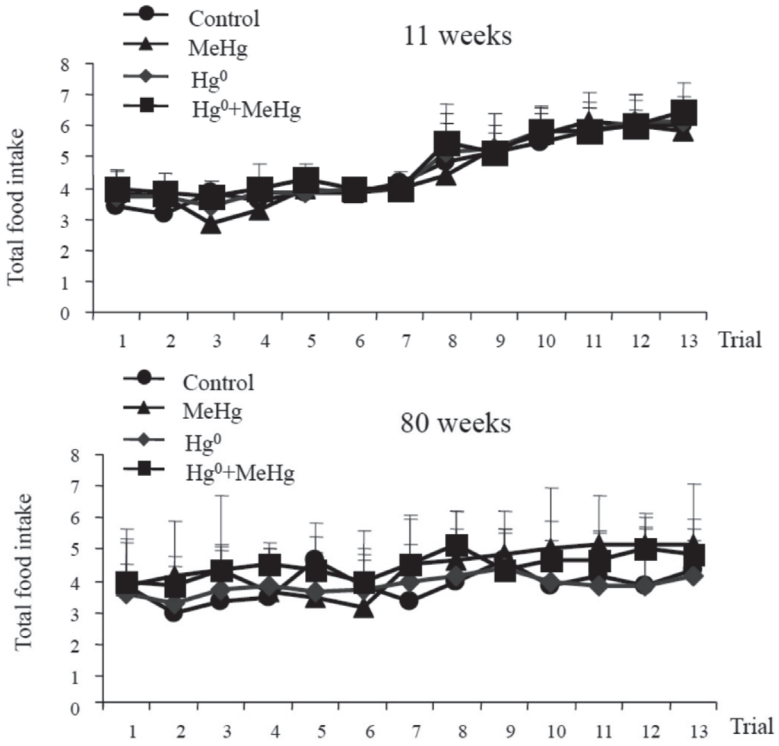

Fig. 5. Number of total food intake of mice at 11 and 80 weeks of age after exposure to MeHg, Hg^0 and Hg^0+MeHg during developing periods. Data shown are means ± standard deviation for seven animals from each group.

robehavioral function. Few studies have reported neurobehavioral toxicity related to combined exposure. Fredriksson *et al*. (1992) investigated the influence of combined exposure in the embryonic stage on male rats' behaviors. On Days 6 to 9 of pregnancy, 2 mg/m^3/day of MeHg was orally administered to pregnant rats. On Days 14 to 19 of pregnancy, they were exposed to 1.8 mg/m^3 of Hg^0 for 90 min per day. Behavioral tests were conducted using rats 16 to 20 weeks after birth. They indicated that, in the Hg^0 exposure group, spontaneous motility was enhanced in comparison with the control group, and that, in the Hg^0+MeHg exposure group, it was further enhanced in comparison with the Hg^0 exposure group. They also reported that, in the Hg^0 exposure group, the platform avoidance response time was markedly delayed in comparison with the control group on Morris' water maze test, and that, in the Hg^0+MeHg exposure group, it was further delayed in comparison with the Hg^0 exposure group. Furthermore, Yoshida *et al*. (2011) examined the influence of combined exposure (Hg^0+MeHg exposure) in the embryonic stage on the neurobehavioral function using mice. According to them, in the Hg^0 exposure group, mice were exposed to Hg^0 at a mean concentration of 0.030 mg/m^3 for 6 hr per day every day between Days 0 and 18 of pregnancy. In the MeHg exposure group, food containing 5-ppm MeHg was given from Day 0 of pregnancy until 10 days after delivery. A behavioral test at 10 weeks of age showed that spontaneous motility was enhanced in male mice in the MeHg and combined exposure groups, whereas it was reduced in female mice. On Morris' water maze test, the platform avoidance response time was delayed in male mice in the MeHg and combined exposure groups in comparison with the control group. However, they indicated that there was no difference between the Hg^0 exposure and control groups on a behavioral test, and that there was no marked influence of Hg^0 exposure at a no-observed-effect level (NOEL), 0.025 mg/m^3, recommended by the WHO, in the embryonic stage. In this study, we investigated the influence of combined (0.10 mg/m^3 Hg^0 + 5-ppm MeHg) exposure on the neurobehavioral function during the growth period (3 to 7 weeks after birth). Behavioral tests, including OPF, PA, and RM tasks, at 10 weeks of age did not show any significant difference between the control and 3 exposure

Table 1. Mercury concentration in the organs of mice at 24 hr after the cessation of exposure to MeHg, Hg^0 and Hg^0+MeHg during developing periods.

	Cerebrum	Cerebellum	Lung	Liver	Kidney
Control	26 ± 7**	8 ± 6**	6 ± 5**	26 ± 14**	8 ± 5**
MeHg	2080 ± 85##	1150 ± 186##,*	3470 ± 559##	9070 ± 1680##	27000 ± 5540##
Hg^0	352 ± 32##,**	599 ± 102##,**	1070 ± 151##,**	194 ± 90##,**	1340 ± 452##,**
Hg^0+MeHg	2320 ± 210##	1610 ± 256##	4270 ± 648##	10100 ± 2410##	27700 ± 2950##

Mercury concentration is expressed as ng Hg/g tissue. Data shown are the mean ± standard deviation for four exposed and control animals, respectively. ## Significant difference from control animals at $p < 0.01$. * Significant difference from the Hg^0+MeHg group at $p < 0.05$. ** Significant difference from the Hg^0+MeHg group at $p < 0.01$.

Table 2. Mercury concentration in the organs of mice at 14 weeks of age after exposure to MeHg, Hg^0 and Hg^0+MeHg during developing periods.

	Cerebrum	Cerebellum	Lung	Liver	Kidney
Control	8 ± 5**	21 ± 4**	14 ± 7**	44 ± 13	15 ± 9**
MeHg	23 ± 24**	148 ± 22##,**	111 ± 19##,*	26 ± 22	332 ± 28##
Hg^0	15 ± 9**	158 ± 82#,*	27 ± 14**	16 ± 8*	30 ± 28**
Hg^0+MeHg	167 ± 30##	325 ± 64##	251 ± 88##	29 ± 5	297 ± 22##

Mercury concentration is expressed as ng Hg/g tissue. Data shown are the mean ± standard deviation for four exposed and control animals, respectively. # Significant difference from control animals at $p < 0.05$. ## Significant difference from control animals at $p < 0.01$. * Significant difference from the Hg^0+MeHg group at $p < 0.05$. ** Significant difference from the Hg^0+MeHg group at $p < 0.01$.

Table 3. Mercury concentration in the organs of mice at 83 weeks of age after exposure to MeHg, Hg^0 and Hg^0+MeHg during developing periods.

	Cerebrum	Cerebellum	Lung	Liver	Kidney
Control	15 ± 4*	30 ± 16**	32 ± 4	15 ± 4	34 ± 2
MeHg	21 ± 6*	31 ± 7**	27 ± 4**	23 ± 5	41 ± 8
Hg^0	14 ± 6**	32 ± 6**	30 ± 3**	16 ± 2*	34 ± 4*
Hg^0+MeHg	39 ± 17#	64 ± 7##	48 ± 7	29 ± 11	44 ± 5

Mercury concentration is expressed as ng Hg/g tissue. Data shown are the mean ± standard deviation for four exposed and control animals, respectively. # Significant difference from control animals at $p < 0.05$. ## Significant difference from control animals at $p < 0.01$. * Significant difference from the Hg^0+MeHg group at $p < 0.05$. ** Significant difference from the Hg^0+MeHg group at $p < 0.01$.

groups. This suggests that the influence of combined mercury exposure during the growth period is less marked than in the embryonic stage.

Recently, Yoshida *et al*. (2013) conducted an experiment using mice, and exposed them to Hg^0 at 0.057 mg/m^3, which is approximate to an NOEL (0.025 mg/m^3), for 24 hr every day during the lactation period. Three behavioral tests, OPF, PA, and Morris' water maze (MM) tasks, at 3 months of age did not show any abnormalities in the exposure group. However, the behavioral tests at 15 months of age showed the marked influence of exposure on the percent duration of staying at the central segment of the field (central locomotion) on the OPF task; they indicated the tardive behavioral effects of low-level Hg^0 exposure during the lactation period. Furthermore, Yoshida *et al*. (2008) examined the behavioral effects of 5-ppm MeHg exposure in the embryonic stage using heavy metal toxicity-sensitive metallothionein-null mice. Behavioral tests at 12 weeks of age did not show any behavioral effects of MeHg, excluding an OPF task involving female MT-null mice. However, in 52-week-old wild-type and MT-null mice, behavioral effects were observed on OPF, PA, and MM tasks. In particular, the effects were more marked in the MT-null mice. They also suggested that low-level MeHg exposure in the embryonic stage results in tardive behavioral effects. Neither 10- nor 79-week-old mice showed any behavioral effect of low-level Hg^0, MeHg, or combined mercury exposure during the growth period. This suggests that the neurobehavioral toxicity of Hg^0, MeHg, or combined mercury exposure during the growth period (childhood) is weaker than in the embryonic stage or during the lactation period.

With respect to combined exposure-related brain mercury, Fredriksson *et al*. (1992) reported that the brain concentration of mercury in male mice exposed to combined mercury in the embryonic stage was higher than in mice exposed to Hg^0 or MeHg alone. In an experiment conducted by Yoshida *et al*. (2011), the brain concentration of mercury in male mice exposed to combined mercury in the embryonic stage was higher than in mice exposed to Hg^0 or MeHg alone, whereas there was no difference between female mice exposed to combined mercury and MeHg. Thus, there was a gender difference in brain uptake on combined mercury exposure. The brain concentration of mercury immediately after combined mercury exposure during the growth period (childhood) was similar to that after MeHg exposure. The brain concentration of mercury in mice exposed to combined mercury after 79 weeks of age was higher than in controls and mice exposed to Hg^0 or MeHg alone, but there were no differences in its concentration between the control and Hg^0- or MeHg-exposed groups. It was shown that, after combined mercury exposure, its disappearance in the brain was delayed in comparison with the exposure to Hg^0 or MeHg alone.

With respect to the relationship between Hg^0 exposure-related brain mercury and behavioral abnormalities, Kishi *et al*. (1978) conducted an experiment using adult rats, and reported that behavioral abnormalities appeared when the brain concentration of mercury exceeded 10 μg/g, and that the brain concentration of mercury was 5 μg/g when behavioral effects disappeared after the discontinuation of exposure. Yoshida *et al*. (2004, 2006) performed an Hg^0 exposure test using adult mice, and indicated that behavioral abnormalities were detected at brain mercury concentration of ≥ 1 μg/g. On the other hand, Burbacher *et al*. (1990) reviewed studies regarding MeHg exposure using rats and mice, and reported that behavioral abnormalities were observed at brain mercury concentration of 4 to 9 μg/g, and that whether or not such abnormalities appear at ≤ 3 μg/g was unclear. Furthermore, Sakamoto *et al*. (2004) exposed lactating rats to MeHg, and indicated that there were no behavioral abnormalities in newborn rats with brain mercury concentration of ≤ 3 μg/g. The brain concentration of mercury after MeHg or Hg^0+MeHg exposure at 3 to 7 weeks of age ranged from 2 to 3 μg/g, being a threshold at which there may be behavioral effects. In this study, there were no neurobehavioral effects in the Hg^0, MeHg, or Hg^0+MeHg groups. This was possibly because the brain concentrations of mercury were at the threshold.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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