Neurobehavioral effects of postnatal exposure to low-level mercury vapor and/or methylmercury in mice

Minoru Yoshida¹, Jin-Yong Lee², Masahiko Satoh² and Chiho Watanabe³

¹Faculty of Health and Medical Care, Hachinohe Gakuin University, 13-98 Mihono, Hachinohe 031-8588, Japan
²Laboratory of Pharmaceutical Health Sciences, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan
³Department of Human Ecology, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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ABSTRACT — This study examined the effects on neurobehavioral function of exposure to low-level mercury vapor (Hg⁰), methylmercury (MeHg) in female mice and the combination of Hg⁰ and MeHg during postnatal development. Postnatal mice were exposed to Hg⁰ at a mean concentration of 0.188 mg/m³ Hg⁰ and supplied with food containing 3.85 μg/g of MeHg from day 2 to day 28 after delivery. The combined exposure group was exposed to both Hg⁰ and MeHg, using the same procedure. When their offspring reached the age of 11 weeks, behavioral analyses were performed. The behavioral effects in mice were evaluated based on locomotive activity and rate of center entries in the open field (OPF), learning activity in the passive avoidance response (PA) and spatial learning ability in the radial maze (RM). Total locomotive activity in the OPF significantly decreased in the Hg⁰, MeHg and combined exposure groups compared with the control group. The proportion of entries to central area in the OPF was significantly higher in the combined exposure group than in the control group, while those in the Hg⁰ or MeHg exposure group did not differ from the control group. Other behavioral tests did not reveal significant differences among the groups. Behavioral anomalies were more distinctive after combined exposure compared to Hg⁰ or MeHg exposure alone. The brain Hg concentration of offspring, immediately after exposure, was highest in the combined exposure group, exceeding 2 μg/g, followed by the MeHg and Hg⁰ exposure groups. Thus, the enhancement of neurobehavioral effects in the combined exposure group was associated with higher brain mercury concentration.

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

INTRODUCTION

A couple of mercury compounds are considered serious environmental pollutants, with toxic effects on all organisms. Since the occurrence of Minamata disease, the health problems due to methylmercury (MeHg) resulting from the consumption of a large amount of contaminated fish and shellfish have attracted attention around the world. Mercury vapor (Hg⁰) also exerts neurotoxic effects, and gold miners’ exposure to high levels of Hg⁰ in mercury amalgamation and burning amalgam has become a great concern in developing countries (Hilton et al., 2003; Veiga et al., 2006). The International Labor Organization (ILO) estimates that the number of miners working in small-scale gold mines is 11,000,000 to 13,000,000, including 2,500,000 females and 250,000 children (ILO, 1999). The exposure of children to Hg⁰ has increased due to the use of mercury in amalgam to extract gold from ore (Bose-O’Reilly et al., 2008). Further, working people at the artisanal and small-scale gold mining were exposed to Hg⁰ exceeding the Permissible Exposure Limit (PEL) of 100 μg/m³ (Black et al., 2017).

Recently, it has been indicated that gold mining causes mercury pollution in water environments around small-scale gold mines, leading to accumulation of MeHg in fish and shellfish by the methylation of metal mercury used for mining and associated health problems in local residents. In China, soil around a mercury mine was polluted with mercury, and a high concentration of MeHg was detected in grains harvested in the area, suggesting non-seafood-
ingestion-dependent rice diet-related MeHg exposure (Li et al., 2008; Qiu et al., 2008). Therefore, gold miners and residents living near gold mines are exposed to low levels both of Hg\textsuperscript{0} and MeHg over long periods, which can have serious effects on their health. In particular, neurobehavioral toxicities due to Hg\textsuperscript{0} and MeHg exposure during childhood are of concern. We previously investigated the effects of prenatal or postnatal low-level Hg\textsuperscript{0} exposure on the neurobehavioral function of mice and reported that Hg\textsuperscript{0} exposure during growth and/or development results in neurobehavioral changes (Yoshida et al., 2004, 2014). In addition, maternal MeHg exposure after parturition is known to cause exposure of the suckling offspring via breast milk (Yoshida et al., 1994). However, little is known about the later consequences with respect to neurobehavioral toxicity when the suckling offspring are exposed to a low-level combination of Hg\textsuperscript{0} and MeHg during the lactation period.

The purpose of the study was to assess any neurobehavioral changes in offspring mice exposed to low-level Hg\textsuperscript{0}, MeHg or the combination of Hg\textsuperscript{0} and MeHg during the developmental period. Exposure levels close to the threshold limit value (TLV), as recommended by the WHO, and approximately 7-fold higher than the TLV were chosen in the present study.

MATERIALS AND METHODS

Animals and exposure procedure

Animals

Forty-eight female C57BL/6J mice at one day postpartum were purchased from SLC, Inc. (Shizuoka, Japan), and were divided into 4 groups: control, Hg\textsuperscript{0}, MeHg, and Hg\textsuperscript{0}+MeHg. The animal facility was maintained under a light/dark cycle of 12/12-hr, temperature of 24 ± 1°C, relative humidity of 55 ± 10% in the animal room of the School of Pharmacy, Aichi Gakuin University. During this period, solid food (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were given ad libitum.

Experimental methods

Hg\textsuperscript{0} exposure group

The mice were exposed to Hg\textsuperscript{0} from 2 days to 28 days after delivery. For Hg\textsuperscript{0} exposure, the mice were placed in a mercury vapor exposure chamber, and exposed for 24 hr every day at a mean concentration of 0.188 (range: 0.125 to 0.252) mg/m\textsuperscript{3}. To exchange the animal bedding, drinking water and food, however, Hg\textsuperscript{0} exposure was discontinued every 3 or 4 days. The concentration of mercury in the exposure chamber was measured every day using a mercury survey meter (Type; EMP-1A, Nippon Instruments Co., Tokyo, Japan).

MeHg exposure group

For MeHg exposure, methylmercury chloride (purity 98%, GL Science Inc., Tokyo, Japan) was mixed with the natural MF food (Oriental Yeast Co.) and food containing 3.85 μg/g of MeHg was prepared. Offspring mice were immediately supplied with the food containing MeHg from 2 days to 28 days after delivery.

Combined exposure group

Twelve offspring mice were exposed to both Hg\textsuperscript{0} and MeHg as described above. The mice were placed in a mercury vapor exposure chamber, and food containing 3.85 μg/g MeHg was simultaneously given.

Both dams and neonates were exposed. For all exposure groups, 3 to 5 mice were sacrificed 24 hr after the completion of exposure to analyze the level of mercury in organs. The remaining mice were acclimated with food and tap water until the start of behavioral tests. Animals received humane care throughout the experiment according to the guidelines established by the Aichi Gakuin University for animal welfare.

Behavioral analysis

Behavioral functions were evaluated with three commonly-used methods: the open field test, passive avoidance test, and radial maze test, as described below.

Open field (OPF) test

The locomotor activity of mice was assessed using an open field, for which the methodological details were previously described (Yoshida et al., 2004). Briefly, each mouse was moved from its home cage to the center square (10 x 10 cm) of an 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 next 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 distance (in cm/2 min) moved by the mouse and the positioning of the mouse. 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 response (PA) test

Passive avoidance learning, a learning task motivated by strong aversive stimulus, was assessed by a step-through procedure (Yoshida et al., 2004). The apparatus
(PA-2010A; O’Hara & Co., Ltd.) comprised dark and illuminated compartments 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.

Radial maze (RM) test
Spatial learning was assessed using an 8-arm radial maze test (Yoshida et al., 2016). Briefly, in 12-week-old female C57BL/6J mice (n = 7/group x 4 groups), starting from 10 days before the experiment, food intake was restricted to 2 to 3 g/day using a 25-mg spherical pellet diet (Ohara Ika Sangyo, Tokyo, Japan). The amount of food pellets was adjusted such that the body weight was maintained at 80-85% of the free-fed body weight (mean body weight for 3 days before the food restriction). As acclimation training (2 days preceding the behavioral experiment), mice were allowed to freely wander in the apparatus for 10 min, with pellets scattered on each arm. The behavioral test was conducted for 5 minutes/session/animal/day (n = 7/group), and a total of 13 sessions was performed every day. After completion of the behavioral tests, the mice were sacrificed, and tissue concentrations of mercury were measured as described below.

Analysis of mercury concentrations in tissue
Mercury concentrations in the tissues were measured with a cold atomic absorption spectrophotometer (RA-2A Mercury Analyzer; Nippon Instruments, Tokyo, Japan) after digestion with a concentrated acid mixture [HNO₃/HClO₄ 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 behavioral tests) for comparison between the non-exposed control and exposed groups with a preset probability.

RESULTS
Locomotor activity in the open field task is shown in Fig. 1. There were significant differences in the total locomotion distances (cm) for a 2-min observation between the control and MeHg, Hg⁰, and Hg⁰+MeHg exposure groups. The percent duration of stay at the center (%) on the open field task is shown in Fig. 2. No significant difference was observed between the control group and the MeHg or Hg⁰ exposure groups. However, the Hg⁰+MeHg exposure group showed a significantly higher percentage than the control group.

Fig. 1. Locomotion activity of 11-week old mice exposed to MeHg, Hg⁰ and Hg⁰+MeHg during developmental period. Data shown are means ± standard deviation for seven to eight animals from each group. **Significant difference from control animals at p < 0.01. *Significant difference from control animals at p < 0.05.

Fig. 2. The percent of central entry of 11-week old mice exposed to MeHg, Hg⁰ and Hg⁰+MeHg during developmental period. Data shown are means ± standard deviation for seven to eight animals from each group. * Significant difference from control animals at p < 0.05.
The results of training and retention trials in the passive avoidance test are shown Fig. 3. There were no differences in avoidance latency between the control and three exposure groups on training or retention trials.

The numbers of working memory errors per session on the eight-arm radial maze task at 12 weeks of age are shown in Fig. 4. There were no differences in the number of working memory errors among the groups during the 8 sessions. The number of total arm entries (i.e., when an animal placed all their paws in an arm) is shown in Fig. 5. No significant differences were noted in the number of arm choices between the control and three exposure groups.

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 about 210 times (p < 0.01) higher than in the control group. That in the Hg⁰ group was about 10 times (p < 0.01) higher, and that in the Hg⁰+MeHg group was about 280 times (p < 0.01) higher. The cerebellar concentrations of mercury in the MeHg, Hg⁰, and Hg⁰+MeHg groups were about 480, 330, and 580 times (p < 0.01) higher than in the control group, respectively. Furthermore, the cerebral and cerebellar concentrations of mercury in the Hg⁰+MeHg group were higher than in the MeHg and Hg⁰ groups. In the liver and kidney, the mercury concentrations were markedly higher in the three exposure groups than in the control group.

**DISCUSSION**

Several heavy metal pollutants have toxic effects on specific tissues. Each mercury compound adversely affects the brain; cadmium has renal toxicity; arsenic...
induces skin cancer (Counter and Buchanan, 2004; Lee et al., 2015; Cohen et al., 2013). However, there have been few studies on neurobehavioral toxicity due to combined exposure to mercury vapor (Hg0) and methylmercury (MeHg) during the developmental period. Fredriksson et al. (1996) investigated the behavioral effects of combined prenatal Hg0 and MeHg exposure using male rats. Rats orally received 2 mg/kg/day of MeHg on Days 6 to 9 of pregnancy, and were exposed to 1.8 mg/m3 of Hg0 for 90 min per day. Behavioral tests were conducted using rats 16 to 20 weeks after delivery. They showed that spontaneous motility increased in the Hg0 exposure group compared with the control group and further increased in the combined exposure group. Moreover, the avoidance latency to reach the platform in the Morris water maze test was markedly greater in the Hg0 exposure group compared with the control group and either the Hg0 or MeHg exposure group; however, the combined exposure group exhibited a higher percentage than the control group. In contrast, the learning activity in the passive avoidance response and eight-arm radial maze was not significantly different between the control group and three exposure groups. The results of the OPF task indicated that the combined exposure increased neurobehavioral effects compared to Hg0 or MeHg exposure.

Regarding the mercury concentration in the brain of animals with combined Hg0+MeHg exposure during the prenatal period, Fredriksson et al. (1996) reported that combined exposure resulted in a slightly higher brain concentration in rats than the concentrations obtained after exposure to Hg0 or MeHg alone. Yoshida et al. (2011) also found that the mercury concentrations in the brains of mice prenatally exposed to the combination Hg0 and MeHg were higher than those with only Hg0 or MeHg exposure. Furthermore, the brain mercury concentration in the mice with combined exposure to a low concentration of Hg0 and MeHg for a long period during the growth period (childhood) was found to be significantly higher than either Hg0 or MeHg exposure immediately after exposure or one year later (Yoshida et al., 2016). This experiment revealed that combined exposure increased the mercury concentration in the brain more than Hg0 or MeHg exposure, exceeding 2 μg/g.

Regarding the mercury concentration in the brain and

**Table 1.** Mercury concentration in the organs of offspring mice at 24 hr after the cessation of MeHg, Hg0 and Hg0+MeHg exposure during developmental period.

<table>
<thead>
<tr>
<th></th>
<th>Cerebrum</th>
<th>Cerebellum</th>
<th>Liver</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 5)</td>
<td>7.6 ± 2.3</td>
<td>2.3 ± 6</td>
<td>21.0 ± 5.1</td>
<td>35.1 ± 11.7</td>
</tr>
<tr>
<td>MeHg (n = 5)</td>
<td>1,630 ± 361**,*</td>
<td>1,110 ± 107**,*</td>
<td>1,340 ± 1660**</td>
<td>4,680 ± 1,010**,*</td>
</tr>
<tr>
<td>Hg0 (n = 3)</td>
<td>758 ± 63**,*</td>
<td>763 ± 180**,*</td>
<td>679 ± 359**</td>
<td>8,510 ± 1,680**,*</td>
</tr>
<tr>
<td>Hg0+MeHg (n = 5)</td>
<td>2,130 ± 188**</td>
<td>1,340 ± 328**</td>
<td>5,720 ± 1,160**</td>
<td>22,800 ± 5,730**</td>
</tr>
</tbody>
</table>

Mercury concentration is expressed as ng Hg/g tissue. Data shown are the mean ± standard deviation. The number of animals is shown in parentheses.

* Significant difference from control animals at p < 0.01.
** Significant difference from the Hg0+MeHg group at p < 0.05.
## Significant difference from the Hg0+MeHg group at p < 0.01.
behavioral abnormalities, Kishi et al. (1978) observed in an experiment using adult rats that behavioral abnormalities appeared when the brain concentration of mercury exceeded 10 μg/g. Yoshida et al. (2004, 2006) performed a Hg⁰ exposure test using adult mice, and indicated that behavioral abnormalities were detected at brain mercury concentrations of ≥ 1 μg/g. In contrast, Burbacher et al. (1990) reviewed studies regarding MeHg exposure using rats and mice, and reported that behavioral abnormalities were observed at brain mercury concentrations of 4 to 9 μg/g, and that whether or not such abnormalities appear at ≤ 3 μg/g was unclear. The central nervous system is known to be susceptible to mercury, and mercury affects neonates more strongly than adults (Counter and Buchanan, 2004). Fredricksson et al. (1992) reported behavioral effects at lower brain mercury concentrations of less than 0.1 μg/g after exposure of neonatal rats to Hg⁰. Yoshida et al. (2011) also demonstrated that neurobehavioral abnormality was not observed when the brain mercury concentration in neonatal mice exposed to Hg⁰ was less than 0.5 μg/g. Sakamoto et al. (2004) indicated that there were no behavioral abnormalities in neonatal rats with brain mercury concentrations of ≤ 3 μg/g. In this study, behavioral effects were found in total locomotive activity in the OPF test at brain mercury concentrations of more than 0.7 μg/g after Hg⁰ exposure, and of more than 1.0 μg/g after both MeHg and combined exposure. Regarding mercury concentration in the brain after combined exposure, our previous study demonstrated that mercury concentration in the brain of the mice exposed to combined mercury during the growth period is significantly higher than either Hg⁰ or MeHg exposure immediately after exposure (Yoshida et al., 2016). Therefore, it is suggested the tendency of mercury accumulation in the brain of the mice exposed to mercury compounds may be complementary even in different period of exposure. In this study, the brain mercury concentration in mice with combined exposure exceeded 2 μg/g, which was the highest value in the three exposure groups. Moreover, abnormal rates of central entries in the OPF task were observed, while there were no abnormalities in other behavioral performance. From these results, we provide that postnatal combined (0.188 mg/m³ Hg⁰ + 3.85 μg/g MeHg) exposure causes an enhanced behavioral effect compared with Hg⁰ or MeHg exposure alone, which is due to a higher brain mercury concentration following combined exposure than single exposure.

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

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