**TABLE 1. Subject Characteristics ($n$ = 63) and Values, Including Maternal and Fetal Mercury Concentrations in Red Blood Cells and Hematocrit Values**

| category | mean (geomean) | SD | min | max |
|---|---|---|---|---|
| maternal age (yr) | 29.6 | 4.37 | 21 | 41 |
| maternal RBC-Hg level (ng/g) | 9.12 (8.41) | 3.63 | 3.76 | 19.1 |
| maternal Htc value | 31.5 | 3.18 | 23.9 | 38.4 |
| fetal RBC-Hg level (ng/g) | 14.7 (13.4) | 6.37 | 4.92 | 35.4 |
| fetal Htc value | 45.2 | 3.64 | 38.1 | 53.7 |
| fetus/mother RBC-Hg ratio | 1.6 | 0.27 | 1.08 | 2.19 |

involves sample digestion with $HNO_3$, $HClO_4$, and $H_2SO_4$ followed by reduction to $Hg^0$ by $SnCl_2$. The detection limit was 0.1 ng/g. Accuracy was ensured by using certified reference material (DORM-2, dogfish muscle prepared by the National Research Council of Canada) as the quality control material; the Hg concentration found averaged 4.53 $\mu$g/g, as compared to the assigned value of 4.64 ± 0.26 $\mu$g/g. The total analytical precision of this analysis was estimated to be 3.9%. Fatty acid composition analysis in plasma was performed by SRL Inc. (Tokyo, Japan). Lipid was extracted from the sample according to the method of Folch et al. (*30*), and tricosanoic acid (C23:0) was added as an internal standard and then hydrolyzed with 0.5 M HCl. Free fatty acids were extracted with chloroform, and methylated with 0.4 M potassium methoxide–methanol solution and 14 wt % boron trifluoride–methanol. Fatty acid methyl esters were separated by capillary gas chromatography (GC17A, Shimadzu Co., Japan) and identified by comparison with standards (Sigma Chemical Co., Poole, U.K.). Fatty acid compositions were expressed as concentration ($\mu$g/mL of plasma) and percentage by weight of total fatty acids.

**Statistics.** The differences in RBC-Hg concentrations between paired samples were determined by paired $t$-tests. The associations between RBC-Hg and plasma fatty acid concentrations were studied by Pearson and Spearman correlation analysis. Each fetal/mother ratio of fatty acid was analyzed by a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Significant differences were compared with the sum of the saturated and monounsaturated fatty acids assumed as a reference value. A $p$ value less than or equal to 0.05 was considered to demonstrate statistical significance.

## Results

Table 1 presents the subject characteristics and values, including maternal and fetal mercury concentrations in red blood cells and Htc values. The geometric mean of fetal RBC-Hg at 13.4 ng/g was 1.6 times higher ($p < 0.001$) than that of maternal RBC-Hg (8.41 ng/g). There was a considerable difference in Htc values between maternal and fetal blood. The mean fetal Htc value (45.2 ± 3.6) was about 1.4 times higher than the maternal level (31.5 ± 3.2). In all 63 cases fetal RBC-Hg levels were higher than maternal RBC-Hg levels. A strong correlation was observed in RBC-Hg between mothers and fetuses ($r$=0.92, $p < 0.001$; Figure 1), the average fetal/maternal RBC ratio was 1.6, and the individual ratios varied from 1.08 to 2.19 (Figure 2).

All of the fatty acid concentrations were lower in the fetuses' plasma than in their mothers' plasma (Table 2). However, the fetal/maternal ratio varied with each fatty acid. The ratio for the sum of saturated and monounsaturated fatty acid concentrations was 0.27. The ratios of $n$-3 and $n$-6 fatty acid concentrations were compared with the value for the sum of saturated and monounsaturated fatty acids

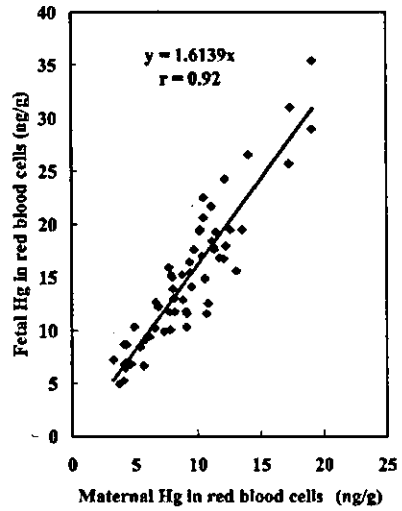


**FIGURE 1. Correlation between maternal and fetal mercury concentrations in red blood cells in 63 maternal–fetal pairs. In all 63 cases fetal RBC-Hg levels were higher than maternal RBC-Hg levels. A strong correlation was observed in RBC-Hg between mothers and fetuses ($r$ = 0.92, $p < 0.001$).**

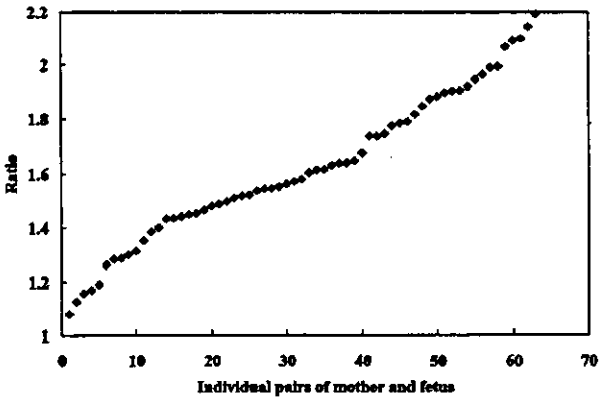


**FIGURE 2. Individual fetus/mother ratios of Hg concentrations in red blood cells in 63 maternal–fetal pairs. The average fetal/maternal RBC ratio was 1.6, and the individual ratios varied from 1.08 to 2.19.**

assumed as a reference value. The ratios for linoleic acid (LN, C18:2$n$-6)) and linolenic acid (LnN, C18:3$n$-3) were significantly ($p < 0.01$) lower than the value for the sum of saturated and monounsaturated fatty acids. On the other hand, those for arachidonic acid (AA, C20:4$n$-6), DHA, and dihomo-$\gamma$-linolenic acid (DGLA, C20:3$n$-6) were significantly ($p < 0.01$) higher than the reference value. Further, there were significant correlations in LN ($r$=0.31, $p < 0.05$), DGLA ($r$ = 0.34, $p < 0.01$), AA ($r$ = 0.39, $p < 0.01$), EPA ($r$ = 0.39, $p < 0.01$), and DHA ($r$ = 0.37, $p < 0.01$) concentrations between maternal and fetal plasma (Table 2).

Maternal RBC-Hg concentrations showed significant correlation coefficients with maternal plasma EPA ($r$=0.36, $p < 0.01$) and DHA ($r$=0.33, $p < 0.05$) concentrations (Table 3). Further, fetal RBC-Hg concentrations showed a significant positive correlation with fetal plasma EPA ($r$=0.32, $p < 0.05$) and DHA ($r$ = 0.35, $p < 0.01$) (Table 3 and Figure 3).

## Discussion

MeHg is one of the most risky substances to fetus brain, and most of the human exposure to MeHg is through maternal fish consumption. On the other hand, DHA, which is important for the fetus brain and its growth, is derived also from maternal fish consumption. If human exposure to MeHg were independent of nutrition from fish, we would aim at

**TABLE 2. Comparison of Maternal and Plasma Fatty Acid Concentrations and Correlation Coefficient between Maternal and Fetal Plasma Fatty Acid Concentrations in 63 Maternal–Fetal Pairs[a]**

| fatty acid | mother ($n = 63$) μg/mL | mother ($n = 63$) % | fetus ($n = 63$) μg/mL | fetus ($n = 63$) % | fetus/mother | correlation coeff |
|---|---|---|---|---|---|---|
| saturated and monounsaturated | 2361 (476) | 61.6 | 619 (156) | 65.0 | 0.273 (0.08) | |
| linoleic (C18:2*n*-6) | 1013 (177) | 26.4 | 118 (34) | 12.4 | 0.118 (0.03)[b] | 0.31[c] |
| linolenic (C18:3*n*-3) | 30 (9) | 0.8 | 1.1 (1.3) | 0.1 | 0.070 (0.04)[b] | 0.23 |
| dihomo-γ-linolenic (C20:3*n*-6) | 55 (17) | 1.4 | 33 (9.2) | 3.5 | 0.628 (0.20)[b] | 0.34[b] |
| arachidonic (C20:4*n*-6) | 187 (45) | 4.9 | 115 (29) | 12.0 | 0.637 (0.16)[b] | 0.39[b] |
| eicosapentaenoic (C20:5*n*-3) | 35 (19) | 0.9 | 7.1 (3.7) | 0.7 | 0.308 (0.13) | 0.39[b] |
| docosahexaenoic (C22:6*n*-3) | 149 (41) | 3.9 | 59 (16) | 6.2 | 0.417 (0.12)[b] | 0.37[b] |

[a] Saturated = palmitic (C16:0) and stearic (C18:0); monounsaturated = palmitoleic (C16:1*n*-7) and oleic (C18:1*n*-9). Values are mean (SD). Fetal/mother plasma fatty acid concentration ratio for *n*-3 and *n*-6 series against the sum of saturated and monounsaturated fatty acids as a reference value. Data were analyzed by a one-way ANOVA followed by Dunnett's multiple comparison test. The associations between maternal and fetal fatty acids were studied by correlation analysis. [b] $p < 0.01$. [c] $p < 0.05$.

**TABLE 3. Correlation Coefficient between Mercury Concentrations in Red Blood Cells and Fatty Acids in Both Mothers and Fetuses[a]**

| | | maternal mercury | fetal mercury |
|---|---|---|---|
| maternal ($n = 63$) | linoleic (C18:2*n*-6) | −0.09 | −0.10 |
| | linolenic (C18:3*n*-3) | 0.23 | 0.19 |
| | dihomo-γ-linolenic (C20:3*n*-6) | −0.20 | −0.01 |
| | arachidonic (C20:4*n*-6) | −0.05 | −0.14 |
| | eicosapentaenoic (C20:5*n*-3) | 0.24 | 0.17 |
| | docosahexaenoic (C22:6*n*-3) | 0.27[c] | 0.21 |
| fetal ($n = 63$) | linoleic (C18:2*n*-6) | −0.06 | −0.04 |
| | linolenic (C18:3*n*-3) | 0.06 | 0.10 |
| | dihomo-γ-linolenic (C20:3*n*-6) | −0.08 | −0.08 |
| | arachidonic (C20:4*n*-6) | −0.23 | −0.20 |
| | eicosapentaenoic (C20:5*n*-3) | 0.36[b] | 0.32[c] |
| | docosahexaenoic (C22:6*n*-3) | 0.33[b] | 0.35[b] |

[a] The associations between maternal and fetal fatty acids were studied by correlation analysis. [b] $p < 0.01$. [c] $p < 0.05$.

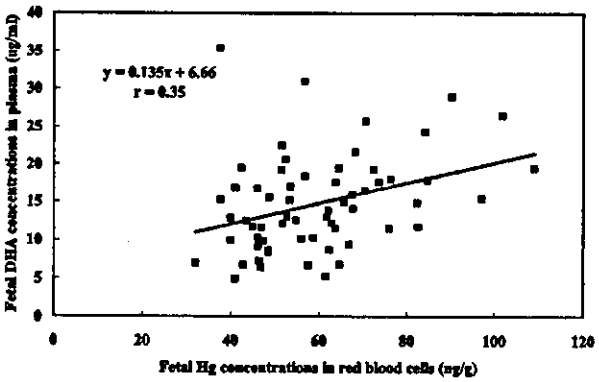


**FIGURE 3. Correlations between RBC-Hg concentrations and plasma DHA concentrations in 63 fetuses.**

zero exposure. However, the exposure is through fish, which is an important source of protein especially for Japanese and other Asian people. Fish also contain *n*-3 PUFA and other nutrients (*31*). Therefore, this study was designed mainly to determine the relationship between MeHg exposure and *n*-3 PUFA concentrations in fetus to consider the risks and benefits of maternal fish consumption during the gestation period.

The RBC-Hg level in umbilical cord blood was about 1.6 times higher than those in the mothers, and there was a significant correlation between them. This suggests that MeHg actively transfers to the fetus across the placenta via a neutral amino acid carrier, as demonstrated by previous studies (*9, 10*). This higher Hg accumulation in the fetuses than in mothers is widely acknowledged from human and animal studies (*1–3, 8–10*). However, the individual fetal/maternal RBC-Hg ratio varied from 1.08 to 2.19, indicating the individual differences in MeHg concentrations between maternal and fetal circulations at late gestation. This will be partly explained by the individual differences in MeHg transfer from mother to fetus through the placenta. The maternal MeHg level tends to be influenced by the latest meal. On the other hand, blood/organ ratios of MeHg concentration will be settled at parturition in fetal circulation. The results suggest that not the maternal side biomarker but the fetal side biomarker is much more advantageous to evaluate the subtle effects of MeHg exposure on the fetus during gestation.

DHA and AA are abundant in the brain (*22–25, 28*), and the DGLA concentration is higher than those of LN, EPA, and LnN (*22*). During rapid brain growth, large amounts of DHA and AA from maternal circulation must reach the fetus to meet its needs for development (*23, 25, 33*). The rapid quantitative accretion of both DHA and AA during the third trimester of pregnancy was noticed in human brain (*25, 28, 33*). Breast milk also contains these fatty acids (*22, 33*). The result of the high fetal/maternal ratio of DHA, AA, and DGLA (Table 2) also may indicate that the fatty acids which are important for the brain and its growth were selectively transferred from maternal circulation to fetal circulation, as was demonstrated in the previous study by Sakamoto and Kubota (*34*).

There were significant correlations in the EPA ($r = 0.39$, $p < 0.01$) and DHA ($r = 0.37$, $p < 0.01$) concentrations between maternal and fetal plasma (Table 2), indicating that EPA and DHA in fetal circulation which originated from fish consumption reflected the existence of these fatty acids in maternal circulation. Maternal RBC-Hg concentrations had significant correlation coefficients with both fetal plasma ($r = 0.36$, $p < 0.01$) and DHA ($r = 0.33$, $p < 0.01$) levels; further, fetal RBC-Hg concentrations had significant correlation coefficients with both fetal plasma EPA ($r = 0.32$, $p < 0.05$) and DHA ($r = 0.35$, $p < 0.01$) levels, indicating that both the MeHg and these *n*-3 PUFAs existing in fetal circulation showed a positive correlation (Table 3 and Figure 3). This is, to our knowledge, the first report to indicate significant correlation coefficients between the MeHg level and these fatty acids originating from fish consumption. These two results indicate that both MeHg and DHA, which act contrary to the normal growth and function of the developing brain, were taken into maternal circulation through maternal fish consumption and transfer to fetal circulation, and that they showed positive correlations. Therefore, if the ordinary fish consumed are low in MeHg but rich in DHA, children's health will especially benefit from fish consumption. However, if the fish MeHg concentration is high enough to ruin the effect

of DHA, fish consumption will retard children's development. Pregnant women in particular would do well to consume at least smaller fish, thereby reducing the risk from large fish but allowing them to continue to eat them to confer the benefits. The different outcomes of the two main cohort studies in the Faroe Islands (*11*) and Seychelles Islands (*13*) regarding the effect of fetal MeHg exposure on children's development may be partly explained by the difference in the amount of DHA. However, the average MeHg exposure level was slightly higher in the Seychelles Islands than in the Faroe Islands. The Seychelles study (*13*) concluded there was no adverse effect from MeHg exposure through fish consumption, whereas the Faroe Islands study (*11*) demonstrated a negative developmental effect due to MeHg exposure. In any event, DHA concentrations in the fetal biomarker should be measured as a confounding factor to examine the subtle effects of MeHg exposure from fish consumption.

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# A Cohort Study of Effects of Perinatal Exposures to Methylmercury and Environmentally Persistent Organic Pollutants on Neurobehavioral Development in Japanese Children: Study design and status report

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**Abstract:** Adverse effects of perinatal exposures to methylmercury (MeHg) and environmentally persistent organic pollutants (POPs) have been apparent from several birth cohort studies, but little is known about the hazardous effects in Japanese, whose fish consumption is high. The present study was designed to examine the effects of perinatal exposures to MeHg, polychlorinated biphenyls (PCB), dioxins, pesticides, and other chemicals in Japanese children. Six hundred eighty-seven pregnant women were participated in this study with their written informed consent. Maternal peripheral blood, cord blood, cord tissue, placenta, and breast milk samples were collected for chemical analysis. Maternal hair was also taken for MeHg analysis. Infants born at full term were assessed by a battery of neurobehavioral tests. The children will be continuously followed up to ages 6-7. The results of this cohort study will allow us to evaluate associations between the neurobehavioral development of children and perinatal exposures to MeHg and environmentally POPs in Japan.

**Key words:** epidemiology, methylmercury, pregnant women

## INTRODUCTION

The neurobehavioral effects of prenatal exposures to methylmercury (MeHg) and environmentally persistent organic pollutants (POPs) including polychlorinated biphenyls (PCBs), dioxins, and pesticides are of great concern worldwide (NAKAI, 2002). It was shown that prenatal MeHg exposure causes the delay of development of cognitive functions in Faroe Islands (GRANDJEAN, 1997), although studies conducted in the Seychelles showed the absence of toxic effects of prenatal exposures to MeHg (DAVIDSON, 1998). Several epidemiological studies have also shown the evidence of the adverse effects of perinatal PCB exposure on neurodevelopment.

In this report we present a protocol of our cohort study, the Tohoku Study of Child Development, of the effects of perinatal exposures to MeHg and POPs on neurobehavioral development among Japanese children (NAKAI, 2004). We hypothesize that the prenatal/postnatal exposures to the above chemicals delay or disturb the normal growth and neurobehavioral development of children.

## STUDY DESIGN

Healthy pregnant women were recruited with their informed consent at obstetrical wards of two hospitals in Tohoku, Japan. To establish an optimal study population, only infants born at term (36 to 42 weeks of gestation) without congenital anomalies or diseases are included. Pregnancy and delivery should have been completed without overt signs of serious illness or complications. The study protocol was approved by the Medical Ethics Committee of the Tohoku University Graduate School of Medicine.

The hair samples were collected from the mothers after delivery. Maternal peripheral blood samples were collected at 28 weeks of pregnancy. They were centrifuged within 4 hours for 20 minutes at 3000 rpm; plasma and whole blood were stored at –80 °C until analysis. A blood sample from the umbilical cord was collected into a bottle using heparin as the anticoagulant after the delivery. Placenta and cord tissues were also collected after the delivery. The mothers were finally asked to provide a sample of breast milk one month after the delivery.

Questionnaire. Several types of questionnaire were administered after the delivery. To assess the fish-intake and the general nutrition status of the mothers a food-intake frequent questionnaire (FFQ) for 122 individual foods and recipes, and some additional items regarding seafood was administered. This is a standardized FFQ that enables the assessment of the intake of not only major nutrients but also several essential nutrients including retinol and folic acid in the Japanese population. Other questionnaires were administered with the following items: educational background, occupation, income, smoking habit including passive smoking, alcohol consumption during pregnancy, hair treatments including bleaching, permanent wave and coloring, and dental amalgam treatment.

Neurodevelopment assessment. All testers who performed neurodevelopment assessments were not informed of exposure information including alcohol consumption/ smoking habit, FFQ data, and feeding method. The Brazelton Neonatal Behavioral Assessment Scale (NBAS) was administered when the infants were 3 days old. Cognitive functions of the infants at 7 months old were evaluated using the Bayley Scale of Infant Development, second edition (BSID), the Kyoto Scale of Psychological Development (KSPD), and the Fagan Test of Infant Intelligence (FTII). BSID and KSPD were also used for the assessment of neurobehavioral development when the children were 18 months old. The Japanese version of Kaufman Assessment Battery for Children (K-ABC) was employed to assess the development and intelligence of children when they are 42 months old. The growth and development of the children will be followed up until they are 6-7 years old.

Chemical determinations. Total mercury analysis was carried out by cold vapor atomic absorption spectrometry. Briefly, without washing the hair samples, each sample was acid digested with $HNO_3$, $HClO_4$ and $H_2SO_4$ at 200 °C for 30 minutes. The resultant ionic mercury was then reduced to mercury vapor by tin chloride to a flameless atomic absorption monitor (HG-201, Sanso Co., Ltd., Tokyo). Analytical accuracy was ensured by analyzing the Human Hair Reference Material NIES CRM No. 13 from the National Institute of Environmental Studies (Lot #650, Tsukuba). In fish-eating populations, total mercury in hair consists mostly of MeHg (more than 95 %).

Assessment of PCB exposure was performed by determining PCB levels in cord blood, placenta, breast milk, and maternal blood. All 209 PCB congeners were analyzed by high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) using the isotope dilution method.

A reporter gene assay of the toxic potency of dioxins and related chemicals was used for the assessment of dioxins. The CALUX (Chemically Activated LUciferase gene eXpression) assay was developed by Xenobiotic Detection Systems (XDS, NC, USA) using a patented recombinant mouse cell line that contains the luciferase reporter gene under the control of dioxin-responsive elements. This assay has several advantages including its high sensitivity, easy pretreatment, and rapid determination, in comparison with HRGC/HRMS.

Cadmium and lead were determined by graphite furnace atomic absorption spectrometry and inductively coupled plasma mass spectrometry, respectively, after samples were digested in a microwave oven with ultrapure nitric acid. Other major biochemical analyses of maternal and cord blood samples included those of plasma selenium and thyroid hormones. Selenium was determined fluorometrically. The assay of TSH, and total/free T4 and T3 were performed using a radioimmunoassay technique.

Potential confounders/covariates. The quality of the home environment was assessed using a questionnaire, the Evaluation of Environmental Stimulation (EES), which has been established in Japan modified after the Home Observation for Measurement of the Environment (HOME) score. The parental socioeconomic status (SES) was rated using the Hollingshead Four Factor Index of Social. Other major potential confounders included were as follows: intelligence quotient by the Raven standard progressive matrices, age at examination (days), gestational age (weeks), and alcohol consumption/smoking habits during pregnancy for the mothers, and the Apgar score, neonatal illness/jaundice, delivery type, parity, chronic diseases, and duration of breastfeeding (months) for the infants.

## RESULTS AND DISCUSSION

The present report describes the study design and protocol for the prospective cohort study of the effects of prenatal exposures to MeHg and other environmentally POPs on neurobehavioral development in Japanese children. To our knowledge, this is the first cohort study that examines these hazardous risks to children in Japan.

We recruited 687 healthy pregnant women between January 2000 and September 2003. Although the final number of babies registered in this study is not yet determined because the delivery of pregnant women registered in this study is ongoing, the percentage of babies fulfilling the criteria for inclusion with the mothers' consent to participate in the assessment using NBAS was 85 %. The percentage of babies participating in the next assessment at 7 months old was 86 % of those participating in the assessment using NBAS. This reduction was mainly due to family relocation to other places.

The results of this cohort study will allow us to evaluate associations between the neurobehavioral development of children and prenatal exposures to MeHg and environmentally POPs in Japan. A recent report from the cohort at Faroe Islands (MURATA ET AL., 2004) indicated that the adverse effects of prenatal exposure to MeHg were still observed in the children at age 14 years by neurophysiological tests, suggesting that some neurotoxic effects from prenatal exposures are irreversible. To clarify this issue, the subjects should be followed until their adolescent ages. The present report describes the study design for children aged 0 to 42 months. When any significant associations between child development and chemical exposures is observed in this study, the further follow-up is essential to know the persistency of adverse effects.

## Acknowledgments

We thank all parents and their children for their participation in this study. This study was supported by grants from the Ministry of Health, Labour and Welfare (Risk Analysis Research on Food and Pharmaceuticals, H15-006), and from the Japan Public Health Association, Japan.

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# Organochlorine Pesticide Residues in Human Breast Milk and Placenta in Tohoku, Japan

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## Introduction

Organochlorine pesticides are compounds widespread in the environment due to their persistence and highly lipophilic nature, and they accumulate in biological systems. Newborns are exposed to these organochlorine compounds across the placenta and through breastfeeding. Perinatal exposure to these compounds may induce several adverse effects such as lower birth weight [1], neurodevelopmental delay [2], and disturbance of thyroid hormone status [3]. DDT, especially, has been suggested to be a neuroendocrine disruptor as well as a functional teratogen in humans [4 5]. Other pesticides such as dieldrin and endosulfan were also recognized to have estrogenic hormonal activity in animal studies.

Recently, we have started a birth cohort study to examine the effects of exposure to persistent organochemical pollutants and heavy metals on neurodevelopment in Japanese children, The Tohoku Study of Child Development [6]. In this cohort study, biological samples, including maternal peripheral blood, cord blood, placenta, cord tissue, and breast milk have been collected from more than six hundred mother-infant pairs for chemical determinations. The growth of infants has been monitored using neurodevelopmental tests, including the Brazelton Neonatal Behavioral Assessment Scale, the Bayley Scale of Infant Development, the Kyoto Scale of Psychological Development, and others. Exposures to dioxin and related compounds, polychlorinated biphenyls, methylmercury, and several heavy metals were assessed. Additionally, since perinatal exposure to organochlorine pesticides may affect the neurodevelopment of children, we examined the effects of those pesticides in the cohort study.

In the present study, several organochlorine pesticides were analyzed in human breast milk and placenta from 20 mothers to identify the major pesticide compounds found in the cohort subjects. The relationship between pesticides in breast milk and the placenta was analyzed to examine the utilization of the placenta as the material for exposure assessment. Some information regarding the factors affecting the contamination of breast milk and the placenta with organochlorine pesticides

are also discussed.

**Methods and Materials**

This study was performed as part of our prospective cohort study [6]. Healthy pregnant women were recruited with their informed consent at obstetrical wards of two hospitals in Tohoku between January 2001 and September 2003. Twenty subjects were randomly selected from the registered subjects of the cohort study, and pairs of breast milk samples and placenta samples were used. The ages of mothers ranged from 21 to 39. The placenta was taken immediately after the delivery, and divided into 20-30 pieces that were randomly separated into 4 groups. Each bottle contained 50-100 g of tissue. The representative samples were finally prepared by homogenization. The mothers were asked to provide breast milk one month after the delivery. The breast milk sample was taken directly into a clean glass bottle. These samples were frozen at –80°C until analysis. Each mother completed a questionnaire to provide personal information such as the number of births, smoking, alcohol consumption during pregnancy, occupation, educational background, food intake, and place of residence. The study protocol was approved by the Medical Ethics Committee of the Tohoku University Graduate School of Medicine.

The pesticides examined were hexachlorobenzene (HCB), α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH, δ-HCH, cis-chlordane, trans-chlordane, oxy-chlordane, cis-nonachlor, trans-nonachlor, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, p,p'-DDD, o,p'-DDD, aldrin, endrin, dieldrin, α-endosulfun, β-endosulfun, heptachlor, heptachlorepoxide, and methoxychlor. Gas chromatographic determination of these organochlorine pesticides was performed with the collaboration of SRL, Inc. (Tokyo, Japan) for sample extraction and Toray Research Center (Tokyo, Japan) for gas chromatography. Briefly, after the samples were spiked with $^{13}C_6$-HCB, $^{13}C_6$-β-HCH, $^{13}C_{12}$-p,p'-DDT, $^{13}C_{12}$-endosulfun, and $^{13}C_{10}$-chlordane, they were extracted with ethanol/hexane. The organic extracts were finally purified with the use of a Florisil column, and the eluates were concentrated and spiked with $^{13}C_{12}$-pentaPCB(#118). A mass spectrometer (AutoSpec, Micromass) coupled to a Hewlett-Packard model HP6800 capillary gas chromatograph equipped with a capillary column (BPX-35, 0.25 mm ID x 25 m, film thickness 0.33 μm, SGE) was used for determination of pesticides. Residue levels were expressed as ng/g extracted fat.

**Results and Discussion**

HCB, β-HCH, oxy-chlordane, cis-nonachlor, trans-nonachlor, p,p'-DDT, p,p'-DDE, dieldrin, and heptachlorepoxide were found from all breast milk samples and placenta samples as shown in Table 1, whereas levels of α-HCH, γ-HCH, δ-HCH, cis-chlordane, trans-chlordane, o,p'-DDT, o,p'-DDE, p,p'-DDD, o,p'-DDD, aldrin, endrin, α-endosulfun, β-endosulfun, heptachlor, and methoxychlor were very low or below the detection limit (data not shown). Since using of these organochlorine compounds had been prohibited in the field in the 1970-1980s in Japan, these results reconfirmed their environmentally persistent nature. In Japan, the concentrations of PCBs, β-HCH, and DDTs in breast milk declined gradually from the peak levels observed at the mid-1970s and almost reached equilibrium states [7]. However, it remains to be elucidated whether the current low levels of organochlorine pesticides affect the neurodevelopment of children.

The concentration of organochlorine pesticides in breast milk mainly depends on their accumulation in the maternal fatty tissue and their subsequent mobilization. Indeed, numerous studies around the world have used human breast milk samples to determine maternal body burden and lactational transfer of pesticides to infants. Since there were excellent correlations of all major pesticides between breast milk samples and placenta samples (Table 1, and the two typical relationships in Fig. 1), placenta is also suggested to be the useful material to estimate the maternal body burden. In addition, the concentrations of some organochlorine pesticides such as HCB, oxy-chlordane, and trans-nonachlor, in the placenta samples had significant negative correlations with parity (Table 2). This finding clearly shows that the mothers eliminate these pesticides during pregnancy and by breastfeeding them into their children. Considering that the concentration of pesticides in breast milk samples had no significant correlation with parity, monitoring of the placental pesticide concentration may contribute to determining the prenatal exposure of infants to organochlorine pesticides. The placenta is a relatively large organ, and is usually discarded after delivery. Utilization of the placenta is possibly suggested for the purpose of assessment of exposure to chemicals.

**Table 1:** Organochlorine pesticide concentrations in the human milk samples and placenta samples, and the relationship between the 2 samples.

| Pesticide | Milk (ng/g-fat) | Placenta (ng/g-fat) | Correlation Coefficient Milk x Placenta |
|---|---|---|---|
| Hexachlorobenzene | 17.1±10.1 | 9.9±4.1 | 0.693** |
| β-HCH | 83.4±55.1 | 21.5±12.6 | 0.919** |
| oxy-Chlordane | 7.2±3.4 | 2.3±0.9 | 0.644** |
| cis-Nonachlor | 3.7±1.7 | 0.8±0.4 | 0.589** |
| trans-Nonachlor | 18.8±8.6 | 3.8±2.2 | 0.679** |
| p.p'-DDT | 6.2±3.5 | 1.4±0.6 | 0.746** |
| p.p'-DDE | 142.3±73.5 | 46.0±34.6 | 0.569** |
| Dieldrin | 5.0±3.6 | 1.7±1.1 | 0.808** |
| Heptachlorepoxide | 3.7±1.4 | 1.4±0.3 | 0.881** |

Spearman's correlation analysis, $^{**}p<0.01$, $^{*}p<0.05$

**Table 2:** Correlation coefficient values of organochlorine pesticides with fish intake, maternal age at delivery, and parity.

| Pesticide | Fish consumption | | Maternal age | | Parity | |
|---|---|---|---|---|---|---|
| | Milk | Placenta | Milk | Placenta | Milk | Placenta |
| Hexachlorobenzene | 0.023 | -0.127 | -0.421 | -0.223 | -0.429 | -0.625** |
| β-HCH | 0.064 | 0.034 | 0.244 | 0.375 | 0.025 | -0.064 |
| oxy-Chlordane | 0.609** | 0.515* | -0.208 | 0.033 | -0.428 | -0.521* |
| cis-Nonachlor | 0.486* | 0.356 | -0.085 | 0.234 | -0.093 | -0.354 |
| trans-Nonachlor | 0.701** | 0.475* | -0.133 | 0.155 | -0.282 | -0.471* |
| p.p'-DDT | 0.341 | 0.267 | -0.179 | 0.06 | -0.053 | 0.089 |
| p.p'-DDE | 0.054 | 0.412 | -0.165 | 0.169 | -0.174 | -0.131 |
| Dieldrin | 0.463* | 0.518* | -0.109 | 0.004 | 0.12 | 0.033 |
| Heptachlorepoxide | 0.566** | 0.711** | -0.185 | -0.169 | -0.054 | -0.235 |

Spearman's correlation analysis, ** $p<0.01$, * $p<0.05$

Some organochlorine pesticides have been thought to be introduced to humans partly through the consumption of fish and related products [8]. The concentrations of oxy-chlordane, nonachlors, dieldrin, and heptachlorepoxide in breast milk samples and placenta samples were indeed correlated with fish consumption; however, HCB, HCH, and DDE had no association. These results indicated that the contribution of fish consumption to the intake of pesticides was dependent on the kind of pesticide. More information regarding risk analysis of pesticide intake is needed for risk management. Maternal age at the time of delivery and parity have been shown to be important factors affecting the concentration of pesticides in breast milk samples [8]. Although parity was a potent factor in our data (Table 2), maternal age had no significant relationship with the concentrations of pesticides in breast milk samples and placenta samples. However, since parity correlated significantly with maternal age (data not shown), multiple regression analysis should be performed to control for the effects of covariates. These issues, and identification of the factors affecting the contamination levels of organochlorine pesticides in breast milk and placenta will be readdressed when we increase the sample size.

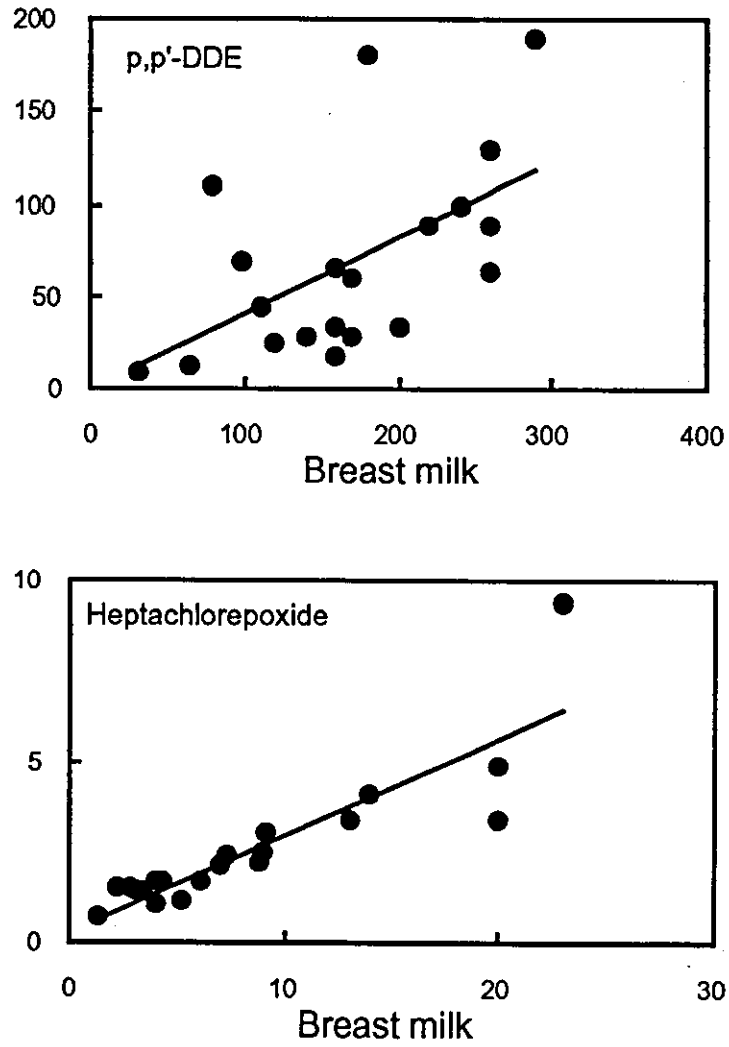


**Fig. 1.:** Relationship of p,p'-DDE (upper) and heptachlorepoxide (lower) between breast milk and placenta. ng/g-fat, N=20.

*Acknowledgment*

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*Invited Review*

# Behavioral Teratology of Mercury and Its Compounds

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SATOH, H. *Behavioral Teratology of Mercury and Its Compounds.* Tohoku J. Exp. Med., 2003, **201** (1), 1-9 —— Mercury and its compounds have a wide spectrum of toxicities depending upon the chemical forms and modes of exposure. Among the various chemical forms, mercury vapor and methylmercury are well known and established as neurotoxic agents. Since the disasters in Minamata and Iraq, in which fetuses were more susceptible than adults to methylmercury exposure, much attention has been focused on prenatal exposure to mercury and its consequence. Recently postnatal effects of in utero exposure to methylmercury through fish (and marine mammals) consumption by mothers have been concerned and several epidemiological studies have been conducted. Therefore, one of the most seriously concerned issues is the postnatal effects of in utero exposure to methylmercury. Because of these observations in humans, animal experiments have been conducted employing prenatal exposure to low levels of mercury. This paper reviews the animal (rodents) experiments concerning "behavioral teratology" of mercury for better understanding of effects of prenatal exposure to mercury and its compounds in addition to commentary on history and framework of behavioral teratology. ———— mercury; methylmercury; prenatal exposure; postnatal effects; behavioral teratology


Mercury and its compounds have a wide spectrum of toxicities depending upon the chemical forms and mode of exposure (Clarkson 2002). Among its various chemical forms, mercury vapor and alkylmercury compounds, especially methylmercury, are well known as neurotoxic agents. In human subjects repeated exposure to mercury vapor at low concentration caused mercurial erethism, which is characterized by behavioral and personality changes (Hunter 1969). Methylmercury exposure has been repeatedly shown to cause neurotoxicity;

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the typical signs and symptoms are described as Hunter-Russell syndrome (Hunter 1969).

Since the disasters in Minamata (Harada 1978) and Iraq (Bakir et al. 1973), in which fetuses were more susceptible than adults to methylmercury exposure, much attention has been focused on prenatal exposure to mercury and its consequence. Recently postnatal effects of in utero exposure to methylmercury through fish (and marine mammals) consumption by mothers have been concerned (Davidson et al. 1995; Myers et al. 1995b; Grandjean et al. 1997). Several epidemiological studies have been conducted (Kjellstrom et al. 1986; Kjellstrom et al. 1989; Myers et al. 1995a; Grandjean et al. 1997). The source of mercury is naturally occurring and there are populations who depend on fish as main protein source. Therefore, one of the most seriously concerned issues is the postnatal effects of in utero exposure to low levels of methylmercury.

Based on these observations in humans, animal experiments have been conducted employing prenatal exposure to methylmercury at low concentrations. In this paper, the animal (rodents) experiments concerning "behavioral teratology" of mercury are reviewed for better understanding of effects of prenatal exposure to mercury and its compounds.

*What is behavioral teratology ?*

"Behavioral teratology" is a field of science where postnatal effects of prenatal exposure to any foreign stimulant are investigated. It is considered that the concept of behavioral teratology was first established by Werboff in 1960s (Werboff and Gottleib 1963). He showed behavioral effects on the offspring of the maternal rats that had taken tranquillizers during pregnancy (Werboff 1966). He reviewed earlier and his studies concerning the behavior of the offspring born to mother animals given psychotropic drugs during pregnancy and claimed, "the behavior, functional adaptation of the offspring to its environment, is susceptible to teratogenic effects of drugs" (Werboff and Gottleib 1963). This behavioral teratology has later expanded the harmful agents by including environmental pollutants.

*The dawn of behavioral teratology in mercury toxicology*

Spyker and colleagues are the pioneers to study the postnatal effects of in utero methylmercury exposure. They revealed impaired swimming ability in offspring mice exposed to methylmercury in utero (Spyker et al. 1972). The control mice were able to swim easily but the treated mice showed "freezing; floating in a vertical position with only head above water" and swimming with legs askew. They also found changes in behaviors in the open field test. These results indicated the important conclusion that motor dysfunction and emotional change are detectabe postnatally. It was noteworthy that the offspring mice did not show any physical retardation or overt neurological signs and were considered to be normal until being examined by the above tests.

Spyker and colleagues (Weiss and Spyker 1974; Spyker 1975a, b) defined "behavioral teratology" as the overlapping area between behavioral toxicology and teratology, the biological study of malformations. This means that the cause of abnormality occurs during pregnancy and the effects become overt after birth over the lifetime of an individual. This was clearly shown by the "Six D's" in behavioral teratology (Spyker 1975a):

*Abnormal Development*
*Behavioral Deviation*
*Neurological Disorder*
*Immunological Deficiency*
*Generalized Debilitation*
*Premature Death*

In spite of prenatal exposure to an environmental stimulant offspring may be born as "normal" at birth. During lactational period, abnormal development may be observed; examples will be shown later. When the off-

spring become more mature, various kinds of behavioral examinations are possible and behavioral deviation and neurological disorder may be found. With aging, immunological deficiency and generalized debilitation may be observed and finally the individual may die earlier than expected indicates as premature death. Every item contains "D," thus they are called six D's.

As the framework of a behavioral teratology study, observations such as above were proposed by Spyker. Behavioral teratology tries to find effects of prenatal exposure not only at the early stage of life but also over the lifetime after development and aging.

*Current framework of behavioral teratology study: Eight D's*

However, six D's do not include very important item for the life, reproduction. Later Tanimura (Tanimura 1980) proposed another D, *Reproductive Debility*. As the issues concerning endocrine disrupting chemicals are being argued, reproductive debility must be seriously concerned. He also emphasized *Birth Defects* that are found at birth because of his expertise, anatomy. Thus, current framework of behavioral teratology study can be shown as Eight D's (Fig. 1), although all the D's in the framework are not examined by the actual studies.

*Subtle consequences: Important observation items in the investigation*

As emphasized by Spyker and colleagues, subtle consequences are the important findings of behavioral teratology (Spyker et al. 1972; Zbinden 1981). Subtle consequences may mean, 1. so slight as to be difficult to detect or 2. not immediately obvious. Therefore, birth defect that is obvious at once may not be considered by actual studies on behavioral teratology. Abnormal development, behavioral deviation and neurological disorder are items usually examined in behavioral teratology studies.

Immunological deficiency, generalized debilitation or premature death is not usually considered, probably because immunological deficiency may not be directly detected by the behavioral methods. As for generalized

**Eight D's in Behavioral Teratology**

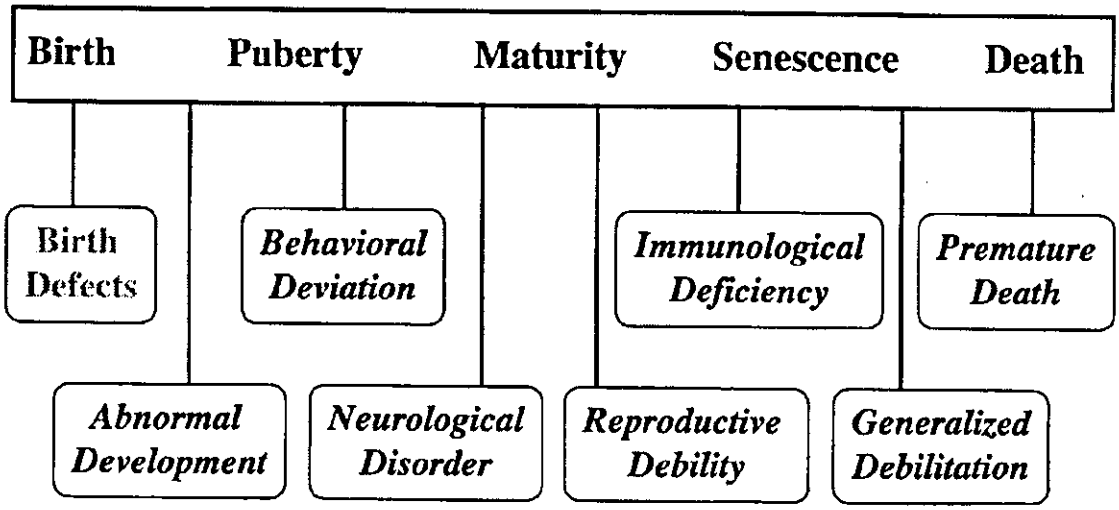


Fig. 1.

debilitation and premature death, previous experiments did not continue such long. Scientists are forced into publishing their studies quickly; they are unable to wait until experimental animals age enough to evaluate debilitation or to observe their death. Generalized debilitation and premature death are two important items, because an individual develops its abilities depending upon its age and accelerated aging may deteriorate the abilities. If deteriorating earlier than normal aging, the individual may not feel totally happy. Therefore, evaluating the possible effect by animal is important.

*Findings in behavioral teratology studies*

A considerable number of investigations on the effects of in utero methylmercury exposure using animal experimental models have been reported so far (see reviews by Shimai and Satoh 1985; Watanabe and Satoh 1996). The following were observed and reported; development of reflexive behavior during lactational period, swimming ability before and after weaning, passive and active avoidance learning, maze and water escape learning, operant learning, sensory function, spontaneous activity, open field test, susceptibility to convulsion and seizure, and ultrasonic vocalization. Most of the reports revealed retardation, impairment or change, although some failed.

The observed effects above are classified as follows according to the functional category (Table 1, Shimai and Satoh 1985; Watanabe and Satoh 1996). Sensory functions are difficult to examine in animal experiments (Evans et al. 1975; Rice and Gilbert 1995). Most of the experiments examining this functional category were done with primates on visual functions. Although Elsner (1991) trained rats to press a lever with predetermined forces and found impaired performance in methylmercury exposed rats. They had been given methylmercury during the period between 2 weeks before conception and lactation at the concentrations of 1.5 or 5.0 mg Hg/liter in drinking water. It is also interesting that they were examined at 300 days old.

Each function is important for an individual animal to survive. Further, most of these functions develop with age and deteriorate later in the lifetime. It is important that these functions develop under controlled conditions and in the right order of time. If a function does not develop at appropriate time, the individual may have difficult time to survive. Therefore, in behavioral teratology abnormality in ontogeny or ontogenesis must be considered as well as deficit of functions.

*Examples of investigations*

1. Development of righting reflex and walking activity

As mentioned above, behavioral teratology study has tendency to examine the offspring animal in early development stage. Satoh and colleagues (Satoh et al. 1985) examined the effects on development of righting reflex and walking activity by prenatal exposure. Preg-

TABLE 1. *Functional categories of behavioral teratology*

| Functions | Behavior and response |
|---|---|
| Motor development and functions | Reflexive behavior; Swimming ability |
| Cognitive functions | Maze, Avoidance, or Operant learning |
| Motivation and arousal behavior | Spontaneous or Open field activity; Susceptibility to convulsion and seizure |
| Social functions | Ultra sonic vocalization |
| Sensory functions | |

nant mice were injected with methylmercury at the dose of 6.0 mg Hg/kg on gestational day 9. They were allowed to litter and offspring mice were examined postnatal days 1, 3 and 8 for development of righting reflex and walking activity. The average righting reflex scores of methylmercury treated offspring were lower than the control on postnatal days 1 and 3. On postnatal day 8, however, no difference was found between the two groups. The methylmercury treated offspring showed similar ontogenic pattern (score increases with age of days) to the control, though the scores postnatal days on 1 and 3 were lower. Therefore methylmercury treatment makes the progress slightly being retarded. Similar findings were observed for the score of walking activity.

2. Avoidance learning

With young adult offspring, two-way avoidance can be conducted: An animal has to move from a compartment of a shuttle box to another compartment to avoid electric shock. The animal is warned by sound before electric shock is applied. The learning ability is evaluated how many trials are necessary to avoid electric shock (moving to the safe compartment before electric shock by hearing the sound). On gestational day 8 or 15, methylmercury (4.0 or 6.4 mg Hg/kg) was given to maternal rats by gastric intubations (Eccles and Annau 1982). When the offspring were 63 days old, acquisition was evaluated in a shuttle box. The offspring born to the treated mother needed more trials to learn avoidance. The difference was more distinct among the groups whose mother were given methylmercury on gestational day 15 than those given on gestational day 8. The control offspring needed 50 trials to acquire avoidance and the offspring born to the methylmercury treated mothers needed more than 200 trials. There is no difference between the two doses. Among the offspring given methylmercury on gestational day 8, the effect was not distinguished from the control.

The offspring were also examined later for reacquisition. After acquisition was established the same procedure is repeated without electric shock and thus offspring soon learn that electric shock does not come and they do not move and stay in the same compartment. This is the extinction training. Then again electric shock is applied and reacquisition is evaluated. Learning deficit was clearly shown among the offspring groups with prenatal methylmercury exposure in a dose-dependent manner. The control needed approximately 40 trials for reacquisition, while offspring treated with methylmercury on gestational day 15 with 4.0 or 6.4 mg Hg/kg of methylmercury needed 100 or 150 trials, respectively.

3. Maze learning test with methylmercury exposure originated from marlin or tuna meat

In these experiments described above mostly methylmercury compounds were given. In one experiment (Olson and Boush 1975), however, they gave marlin meat containing methylmercury to maternal rats and found deficit of the offspring in a maze test. Maternal rats were given one of the three diets from gestational day 0 throughout the experiment. Thus, first the maternal rats were exposed and later after weaning offspring rats were exposed. The diets were 1) rat chow for the control, 2) marlin meat+rat chow to adjust methylmercury concentration at 2 mg Hg/kg diet and 3) tuna meat+rat chow+methylmercury hydroxide to adjust methylmercury concentration at 2 mg Hg/kg diet. Offspring rats of 60 day old were examined in symmetrical mazes. It is interesting that offspring given marlin meat diet showed more errors than did the control, while offspring rats given tuna meat did not show more errors. Does methylmercury from different sources affect differently, or tuna meat possibly contained protective agent? No difference between selenium concentrations in both fish meats was reported.

4. Differential reinforcement of high rate; the lowest dose that produced prenatal methylmercury effects experimentally

The doses of methylmercury were various among experiments. They range roughly from the tenth of mg to ten mg Hg/kg body weights of maternal animals. Dosing regimens were single or repeated injections or gastric intubations. Only a few experiments employed dosing through diet (or drinking water).

The lowest dose that produced postnatal effects of prenatal methylmercury exposure in the animal experiment was, however, much smaller (Musch et al. 1978; Bornhausen et al. 1980). The offspring showed deficit in operant learning. In their experiment using the lowest dose (Bornhausen et al. 1980) maternal rats were given methylmercury chloride during gestational day 6-9 by gastric intubations. The doses were 0.004, 0.008 or 0.04 mg Hg/kg per day. At 4 month old, offspring rats were examined by an operant test called differential reinforcement of high rate (DRH). In this test schedule a rat is required to press a small lever at a predetermined number of times within a predetermined time interval to obtain a small pellet of food. Thus, DRH2/1 means two lever presses within one second. No difference was found among the four different dose groups (0, 0.004, 0.008 or 0.04 mg Hg/kg) for the success rate of the DRH2/1 test. But as for the DRH4/2 and 8/4, which require more lever presses, performance decreased in a dose-dependent manner. It is noteworthy that the total dose given to a mother rat is 0.16 mg Hg/kg at the highest dose group. This experiment has shown the lowest amount of methylmercury to produce behavioral changes ever.

5. Postnatal effects of mercury vapor exposure during gestation

Experiments investigating postnatal effects of in utero exposure to mercury vapor are scarce. One (Danielsson et al. 1993) of the few studies is as follows: Maternal rats were exposed to mercury vapor at 1.8 mg $Hg^0$ for 1 hour or 4 hours during gestational day 11-14 and 17-20. Spatial learning of offspring rats was tested in a radial arm maze. This test is to time the latency to obtain all the food pellets in distal ends of the radial arms. Reentry to the arm of which pellet was already taken was counted as an error. Offspring rats with in utero mercury vapor exposure needed longer time to get the pellets and made more errors. This result indicates neurobehavioral effects of prenatal exposure to mercury vapor in utero.

6. Interaction of methylmercury and mercury vapor co-exposure

It is likely that people in real life are exposed to various kinds of pollutants. As for mercury, people may be exposed to methylmercury by fish consumption and to mercury vapor with dental amalgam. Therefore, an experiment where animals were exposed to both mercury vapor and methylmercury was done (Fredriksson et al. 1996). The test procedure is similar to the previous one. Maternal rats were exposed to mercury vapor at 1.8 mg $Hg^0$ for 1.5 hour/day during gestational day 14-19. They were also given methylmercury chloride at a daily dose of 2 mg Hg/kg during gestational day 6-9. Co-exposure to mercury vapor and methylmercury caused longer time and more errors in the radial maze test comparing with single exposure to either methylmercury or mercury vapor.

7. Postnatal effects of maternal stress and methylmercury exposure during pregnancy

Whether postnatal development and behavior are affected by interaction with maternal stress was also examined (Colomina et al. 1997). Pregnant mice were exposed to methylmercury (1.6 mg Hg/kg/day) during gestational day 15-18. Stress was given as immobilization in a cylinder, 2 hours/day. However, no significant interaction on developmental landmarks or neurobehavioral development of offspring mice

TABLE 2. *Factors that may influence the effects of prenatal mercury exposure*

| |
|---|
| Species, strain and sex of experimental animals |
| Time of exposure during pregnancy |
| Heat exposure during pregnancy |
| Maternal behavior, Maternal care and Fostering |
| Nutrition, e.g. Selenium and Poly unsaturated fatty acids |
| Environment after birth, e.g. Enriched environment |
| Behavioral examinations employed |
| Age at examination |
| Miscellaneous |

during lactational period was observed.

*Factors that may influence the postnatal behavioral effects of prenatal mercury exposure*

Retardation of development of reflexes was partly counteracted by co-administration of selenium (Satoh et al. 1985). Moreover, offspring mice born to the dams fed with selenium deficiency diet and given methylmercury injections during gestation were more severely affected than the offspring of groups of selenium deficiency alone or methylmercury administration alone (Satoh et al. 1997). Maternal heat exposure before methylmercury administration during gestation did not enhance the postnatal effects of methylmercury, though heat exposure showed interactions (Yin et al. 1997). Since in the studies of behavioral teratology, the main goal is to elucidate postnatal effects of in utero exposure that does not cause overt maternal, fetal or neonatal toxicities, the doses become necessarily low. This also means the effect is more easily influenced by other factors and agents in the environment. Therefore, recognition of these factors and agents are important to evaluate experimental results. In Table 2 factors to be considered are listed.

Most behavioral evaluations were done during lactational periods or at several tens days of age. Few examined the effects at the elderly, though aging is an important factor to evaluate full spectrum of methylmercury toxicity.

## Conclusions

This review shows that behavioral teratology reveals subtle consequences of in utero exposure to mercury vapor and methylmercury; namely, behavioral teratology is sensitive to detect postnatal effects of prenatal exposure to mercury. However, some experiments, which were not described here, failed to detect the effects. In these experiments the doses used were similar to those above mentioned. Therefore, behavioral teratology is not always sensitive. For example, the behavioral effects may be masked by the age of examination.

Another problem of behavioral teratology is the underlying mechanism(s) of behavioral changes have not been fully investigated. What is the mechanism of behavioral changes? It may be alteration(s) in 1) developmental and ontogenetic, 2) physiological and psychological, 3) neurochemical and pharmacological function(s), or 4) histopathological changes. Further studies including investigation into mechanisms are necessary with the considerations on possible "interactions" such as selenium status and other environmental exposure and with the efforts expanding of investigation period over the lifetime of animals as indicated by the framework, namely, "Eight D's."

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