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The Tohoku Study of Child Development: A Cohort Study of Effects of Perinatal Exposures to Methylmercury and Environmentally Persistent Organic Pollutants on Neurobehavioral Development in Japanese Children

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NAKAI, K., SUZUKI, K., OKA, T., MURATA, K., SAKAMOTO, M., OKAMURA, K., HOSOKAWA, T., SAKAI, T., NAKAMURA, T., SAITO, Y., KUROKAWA, N., KAMEO, S. and SATOH, H. *The Tohoku Study of Child Development: A Cohort Study of Effects of Perinatal Exposures to Methylmercury and Environmentally Persistent Organic Pollutants on Neurobehavioral Development in Japanese Children*. Tohoku J. Exp. Med., 2004, **202** (3), 227-237 —— Several birth cohort studies have shown adverse effects of perinatal exposures to methylmercury (MeHg) and environmentally persistent organic pollutants (POPs). These chemicals are ingested mainly through fish consumption, but little is known about the hazardous effects in Japanese, whose fish consumption is high. The present study, the Tohoku Study of Child Development, was designed to examine the effects of perinatal exposures to MeHg, polychlorinated biphenyls (PCB), dioxins, pesticides, and other chemicals in Japanese children. Six

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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 neurobehavioral tests: the Brazelton Neonatal Behavioral Assessment Scale at three days old, the Kyoto Scale of Psychological Development and the Bayley Scales of Infant Development at 7 and 18 months old, and the Fagan Test of Infant Intelligence at 7 months old. The children will be continuously followed up to ages 6-7 years. Maternal food intake frequency, maternal IQ, socioeconomic status, and home environment were assessed as covariates. 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. ——— cohort; development; dioxin; methylmercury; polychlorinated biphenyls

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 and Satoh 2002). It was shown that prenatal MeHg exposure causes the delay of development of cognitive functions in Faroe Islands (Grandjean et al. 1997), Madeira Islands (Murata et al. 1999), and New Zealand (Kjellstorm et al. 1986), although studies conducted in the Seychelles showed the absence of toxic effects of prenatal exposures to MeHg (Davidson et al. 1998). Several epidemiological studies have also shown the evidence of the adverse effects of perinatal PCB exposure on neurodevelopment. Cohort studies in North Carolina (Rogan et al. 1986), Michigan (Jacobson et al. 1985, 1990), NewYork (Darvill et al. 2000; Stewart et al. 2000), The Netherlands (Patandin et al. 1999; Vreugdenhil et al. 2002), Germany (Winneke et al. 1998; Walkowiak et al. 2001), and Faroe Islands (Grandjean et al. 2001) demonstrated negative associations between perinatal PCB exposure and cognitive functions in children.

MeHg and POPs constitute a group of persistent environmental chemicals. Due to their hydrophobic nature and resistance towards metabolism, they are found in every level of the food chain. Consequently, these chemicals accumulate in humans mostly through the consumption of food, particularly that of fish and shellfish origins. Indeed, the consumption of fish and shellfish is the major route of dioxin exposure (>80% of all food sources) in Japan (Ministry of Health, Labour and Welfare 2002). From the nutritional perspective, fish is usually recommended for pregnant women because it is rich in some nutrients such as n-3 polyunsaturated fatty acids (PUFA) essential for the perinatal growth of the brain. Therefore, from the perspective of risk assessment, the above health hazard issues are particularly of importance in fish-eating populations.

In this report we present a protocol of our cohort study, the Tohoku Study of Child Development, on the effects of perinatal exposures to MeHg and POPs on neurobehavioral development among Japanese children. We hypothesize that the prenatal/postnatal exposures to the above chemicals delay or disturb the normal growth and neurobehavioral development of children. Exposure assessment includes measurements of multiple chemicals that may potentially affect the child development. Health risk of children was mainly evaluated by neurobehavioral tests. In studies designed to examine neurobehavioral development, multiple confounding factors including food intake habit, home environment,

TABLE 1. *Inclusion criteria for the Tohoku study*

Mother
1. Absence of thyroid dysfunction, mental and psychological diseases, hepatitis, immune deficiency, malignant tumor, diabetes mellitus requiring antidiabetic agents, and any other severe diseases that may affect the normal growth of fetus
2. No severe preeclampsia and severe gestational diabetes mellitus
3. No in vitro fertilization
4. Japanese as the mother tongue
5. Written consent
Infant
1. Absence of congenital anomalies or severe diseases
2. Singleton birth at term from 36 to 42 weeks of gestation
3. Body weight of more than 2400 g, and when the term was 36 weeks of gestation, body weight of more than 2500 g

socioeconomic status, and others must be considered. These issues that must be considered in a study design are reported.

Study design

Recruitment of cohort. Healthy pregnant women were recruited with their informed consent at obstetrical wards of two hospitals in Sendai. 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 inclusion criteria are shown in Table 1. The study protocol was approved by the Medical Ethics Committee of the Tohoku University Graduate School of Medicine.

Sample collections. The hair samples were collected from the mothers after delivery. Most epidemiological studies on MeHg exposure have used mercury concentration in hair to estimate the body burden (WHO 1990). Since hair growth rate is independent of gender or racial differences (Cernichiari et al. 1995), by assuming a constant rate of hair growth equal to 1.1 cm per month (Cox et al. 1989), it is possible to generate a profile of MeHg exposure based on the mercury concentrations in serial segments of scalp hair. The hair samples were cut next to the scalp, in the nape area, with stainless steel scissors. The samples were placed in a plastic bag and kept in a desiccator until analysis.

Since most commercially available plastic and glass materials are possibly contaminated with a significant amount of chemicals such as POPs, all glassware used for sample collection and storage was treated by heating at 400°C in a chemically clean chamber to exclude the possible contamination with PCBs and dioxins. All other materials were confirmed to be clean before use.

Blood samples were collected from mothers at 28 weeks of pregnancy. For blood collection, a vacuum system heparin tube confirmed to be without contamination was used to collect peripheral blood (30 ml), and centrifuged within 4 hours for 20 minutes at 3000 rpm; plasma and whole blood were stored at −80°C until analysis.

A blood sample (more than 50 ml) 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. Since the placenta is a large organ, which is a heterogeneous mixture of placental cells and decidual tissues containing maternal and fetal blood, representative samples of placenta were obtained as follows: the placenta was divided into 20-30 pieces that were randomly separated

into four groups. Each bottle contained 50-100 g tissue. The representative samples were finally prepared by homogenization (Iyengar and Rapp 2001). The entire cord was stored in a clean glass tube without any preparation.

The mothers were finally asked to provide a sample of breast milk (more than 50 ml) one month after the delivery. A clean glass bottle was used for the shipping of breast milk.

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 (Date et al. 1996) 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. The testers had been trained in the training center at Nagasaki University School of Medicine, Japan.

Cognitive functions of the infants at 7 months old were evaluated using the Bayley Scale of Infant Development (BSID), second edition, the Kyoto Scale of Psychological Development (KSPD), and the Fagan Test of Infant Intelligence (FTII). BSID, an established psychodevelopmental test tool, consists of three major scales: the Mental Scale, the Psychomotor Scale, and the Behavior Rating Scale; only the first two scales are used. The mental scale assesses the infant's level of cognitive function (memory, learning, and problem solving), language development (expressive/receptive language, and vocalization), and personal/social development. The motor scale assesses fine and gross motor functions. Since there is no Japanese version of the standardized protocol of BSID, we translated the original manual into Japanese. To examine its reliability, the evaluation of testers were examined on the basis of the Gold Standard developed at the University of Rochester School of Medicine (Davidson et al. 1995). In addition, raw scores were used in the analysis because of the lack of Japanese age norms. KSPD is a Japanese standard developmental test (Maehara et al. 2002); therefore, the developmental performance of the infants is expressed as the developmental age (DA) for each behavior area and for all areas. The developmental quotient (DQ) is obtained by dividing the estimated DA by the chronological age and then multiplying the quotient by 100. FTII is a noninvasive test of information processing that may be applied to infants up to one year of age (Fagan and Detterman 1992).

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, but the battery of neurobehavioral tests is as yet undetermined.

Chemical determinations. Total mercury analysis was carried out by cold vapor atomic absorption spectrometry (Akagi and Nishimura 1991) with minor modifications. Briefly, without washing the hair samples, each sample, weighing approximately 20 mg, was acid digested with 0.5

ml of HNO_3, 0.5 ml of $HClO_4$ and 2 ml of H_2SO_4 at 200°C for 30 minutes. The resultant ionic mercury was then reduced to mercury vapor by adding 0.5 ml of 10% 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. Indeed, a few samples were analyzed to know the exact MeHg concentration by the method of Akagi and Nishimura (1991). MeHg in hair first extracted with hydrochloric acid and then with benzene. The organic layer was subjected to electron-capture detection gas chromatography (ECD-GC) at the National Institute for Minamata Diseases. The concentration of MeHg was confirmed to be more than 95% of the total mercury content. Total mercury analysis was also applied to other samples similarly.

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. The analytical method was as follows: after biological samples were spiked with the ^{13}C-labeled standard mixture of PCBs, lipids in a sample were extracted and weighed. The extract redissolved by an organic solvent was purified in a multi-layer silica gel column. The purified solution was concentrated and analyzed for PCB after the addition of ^{13}C-labeled syringe spike. Four nonplanar PCB congeners (International Union for Pure and Applied Chemistry (IUPAC) nos. 118, 138, 153, and 180) are the predominant congeners found in human tissues and typically account for approximately 50-60% of total PCB (data not shown). Some earlier epidemiological studies attempted to assess PCB exposure using the sum of the above four major PCB congeners. For comparison with those earlier studies, the sum is also calculated in the present study.

A reporter gene assay of the toxic potency of dioxins and related chemicals was used for the assessment of dioxins. The Chemically Activated LUciferase gene eXpression (CALUX) assay was developed by Xenobiotic Detection Systems (XDS, Durham NC, USA) using a patented recombinant mouse cell line that contains the luciferase reporter gene under the control of dioxin-responsive elements (Denison et al. 1998). This analytical process consisted of the first extraction process as in PCB analysis and then column purification using sulfuric acid-impregnated silica gel and activated carbon column. The last purified extracts were given to the cells to produce luciferase, and the amount of light generated by the luciferase was directly related to dioxin toxic equivalent (TEQ) value. This assay has several advantages including its high sensitivity, easy pretreatment, and rapid determination, in comparison with HRGC/HRMS. This assay also requires only a smaller sample volume, which is another important advantage for epidemiological studies.

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. The standard reference material for analysis was NIST 1577b (bovine liver). Other major biochemical analyses of maternal and cord blood samples included those of plasma selenium and thyroid hormones including TSH, and total/free T4 and T3. Selenium was determined fluorometrically (Watkinson 1966). The assay of thyroid hormones 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) (Anme et al. 1998), which has been established in Japan modified after the Home Observation for Measurement of the Environment (HOME) score (Caldwell and Bradley 2001).

HOME is a validated instrument for the assessment of the home environment, but there is no Japanese version that matches the Japanese cultural context. The EES is a questionnaire that directly evaluates the interaction between the child and the caregiver. It was shown that the results of EES highly correlated with those of HOME (Anme et al. 1998).

The parental socioeconomic status (SES) was rated using the Hollingshead Four Factor Index of Social Status (Hollingshead 1975) with several modifications to make the category and prestige of occupation match the Japanese economical context.

Maternal intelligence quotient was measured using the Raven standard progressive matrices. Only the Raven colored progressive matrices have already been introduced in Japan only for people older than 40 years old. We therefore used the original Raven standard version and analyzed results using the raw data.

Other major potential confounders included were as follows: age at examination (days), gestational age (weeks), and alcohol consumption/smoking habits during pregnancy for the mothers, and the Apgar score, neonatal illness/jaundice, spontaneous delivery, parity, chronic diseases, and duration of breastfeeding (months) for the infants.

TABLE 2. *Variables measured at the Tohoku study*

Measurement	Description
Exposure assessment	
PCBs	Cord blood, placenta, breast milk, and maternal blood
Dioxins	Cord blood, placenta, and breast milk, expressed by CALUX-TEQ
Pesticides	Breast milk and placenta, but the exact assay method has not been decided.
MeHg	Maternal hair at delivery, maternal blood, cord blood, and placenta
Heavy metals	Other heavy metals including Pb and Cd, in cord blood, maternal blood, and placenta
Other biochemical measurements	
Selenium	Cord blood and maternal blood
TSH, T4/T3	Cord blood and maternal blood
Neurodevelopment assessments	
NBAS	Infants at 3 days old
BSID	Infants at 7 and 18 months old
KSPD	Infants at 7 and 18 months old
FTII	Infants at 7 months old
K-ABC	Children at 42 months old
Confounders/covariates	
EES	A questionnaire regarding the home environment
SES	Hollingshead four factor index with modifications for application in Japan
Maternal IQ	Raven standard progressive matrices
FFQ	An interview method, with 122 single foods and recipes, and some additional seafood items
Questionnaires	Alcohol consumption/smoking during pregnancy, educational background, hair cosmetic treatments, dental amalgam, and duration of breastfeeding (months),
Other factors	**Mother**: age at delivery, spontaneous delivery/cesarean section, and chronic diseases **Infant**: Apgar score, body weight, body height, head circumference at birth, gestational age (weeks), neonatal illness/jaundice, parity, and age at examination (days)

Results and Discussion

The present report describes the study design and protocol for the prospective cohort study on the effects of perinatal exposures to MeHg and other environmentally POPs on neurobehavioral development in Japanese children. All variables measured are summarized in Table 2. To our knowledge, this is the first cohort study that examines these hazardous risks to children in Japan.

Recruitment. We recruited 687 healthy pregnant women between January 2001 and September 2003 at the obstetrical wards of two hospitals in Sendai, but 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 from Sendai to other places. Sample size is essential for the statistical power, and this is especially important to test whether exposures to low levels of chemicals have the hazardous effects. In addition to the theoretical approach to decide the appropriate sample size, recent epidemiological studies that assessed neurobehavioral consequences of perinatal exposure to PCBs are useful in considering this issue. The Dutch cohort study was started with 418 healthy infants and 395 children were examined at 42 months of age (94% of the original cohort) (Patandin et al. 1999). The German cohort study consisted of 171 mother-infant pairs; 126 mothers provided milk samples and 91 mothers remained in the final examination of children at 42 months of age (approximately 70% of the mothers participating in the postnatal follow-up cohort) (Winneke et al. 1998). In the Faroe cohort study, PCBs could be analyzed in cord tissues from 435 of 1022 children who underwent neurodevelopment examination at 7 years old (Grandjean et al. 2001). These cohort studies showed a negative correlation between prenatal/postnatal PCB exposure and neurobehavioral development in children. Considering that the exposure level of Japanese women was similar to that of European women, and that the potential risk is almost identical, our sample size is probably sufficient.

Neurodevelopment assessment. There are six sets of cohort studies on health hazardous effects of perinatal PCB exposure in children, and all these studies approached this issue by the method of neurodevelopment assessment. Four sets of studies employed BSID to measure the development of infants, and three of them found a significant correlation between the outcomes of BSID and PCB exposure (Schantz et al. 2003). Based on these findings, BSID is expected to be a useful tool for evaluating the risks and the results can be easily compared among the studies. This was the reason why we employed BSID as one of the major components in our tests. On the other hand, BSID is a developmental test based on the developmental milestone concept, and there are no standardized data in Japan. Thus, BSID does not provide us information on MPI and PDI, the two standard indexes of the relative status of development in a population. We therefore used KSPD, the most commonly used neurodevelopmental test in Japan, to calculate DQ. Both BSID and KSPD were originally developed based on the work of Gessell (Ikuzawa et al. 1985; Black and Matula 1999). We also applied FTII and K-ABC to assess children at the ages of 7 and 42 months, respectively. The present study was the first trial to use FTII in Japan. FTII is a novelty preference task designed to predict the later development and intelligence of children (Fagan and Detterman 1992). These two intelligence tests were shown to be sensitive in detecting the adverse effects of low levels of perinatal PCB exposure (Jacobson et al. 1985; Patandin et al. 1999; Darvill et al. 2000; Walkowiak et al. 2001).

Chemical determinations. In a review (Schantz et al. 2003) of epidemiological studies on the possible adverse effects of perinatal expo-

sure to PCBs, it was concluded that a more complete information regarding the neurotoxicity of individual congeners or congener groups may be helpful for risk assessment. There are 209 PCB congeners, and a large number of these congeners were indeed found to be present in human tissues. Since their relative potency to produce nerve system effects is entirely unknown, a congener-specific analytical technique is essential for risk assessment. Despite the fact that several recent studies have used sophisticated congener-specific analytical techniques, there have been no attempts to analyze individual PCB congeners probably present in cord blood, mainly due to the lack of assay sensitivity. The delay of cognitive development may be more related to prenatal PCB exposure, as measured by the sum of concentrations of three or four major PCB congeners in either cord or maternal blood, but not with the postnatal PCB exposure, as measured by the sum of concentrations of PCBs in breast milk samples (Schantz et al. 2003). These findings suggest the importance of PCB congener-specific analysis in cord blood. In the present study, the detailed assessment of individual PCB congeners in cord blood and other samples was designed using a very sensitive HRGC/HRMS.

Only the Dutch cohort study (Patandin et al. 1999) examined the adverse effects of dioxin exposure on neurobehavioral development in children, in which the perinatal exposure, as measured by GC/MS in breast milk samples collected at 2 weeks postpartum, showed no noticeable correlation with cognitive functions measured later. However, the interpretation of these findings is complicated by the results that total PCB in breast milk samples showed no correlation with cognition functions, even though the same study showed negative correlation when total PCB in cord blood was used for analysis. These findings suggest that the characterization of prenatal exposure is more important to clarify the adverse effects of dioxins; the effects of prenatal dioxins exposure should be examined by analyzing levels of dioxins in cord blood. Because dioxins could not be measured by HRGC/HRMS in small volume of cord blood and maternal blood samples, the CALUX assay, a reporter gene assay to determine the all dioxin-like substances, is useful for this purpose. Previously, we already confirmed that data obtained by CALUX assay showed an extremely good correlation with TEQs obtained by HRGC/HRMS in environmental materials (Nakamura et al. 2002).

However, in practice, several problems in exposure assessment remain. First, the metabolites of PCBs are likely included in the adverse effects of PCB exposure. The main hypotheses are that PCB effects on neurodevelopment include the disruption of thyroid hormone homeostasis (Porterfield and Source 2000), and that candidate PCB congeners that may disturb the homeostasis may include several minor congeners and their OH-metabolites (Cheek et al. 1999; Chauhan et al. 2000). The measurement of all possible metabolites of PCBs is not realistic. Second, although there are limited available data describing the neurotoxicity of pesticides in humans, these chemicals may indeed affect the neurodevelopment of children (Schettler 2001). In the present study, however, the assay methods for pesticides including organochlorine and organophosphorus chemicals are not yet determined because the number of chemicals is too large. Third, the measurement of all chemicals including PCBs, dioxins, heavy metals and pesticides from cord blood is difficult because of the shortage of sample volume and the insufficient detection limit. Other biological samples such as placenta are promising for identifying the surrogate marker for exposure assessment. A recent report suggested a good correlation of total PCB in placenta with that in cord blood, maternal blood, and breast milk samples (Wang et al. 2004). Further studies are necessary in order to examine the importance and usefulness of placenta and cord tissues in the assessment of prenatal exposure effects.

Confounders. Despite the major source of MeHg and POPs is via fish intake, fish consumption itself is though to have several beneficial

aspects. Selenium is considered to play an essential role in protection against MeHg toxicity (Watanabe 2002). Fish is usually rich in selenium, and almost 70% of the daily total selenium is through the fish intake in Japan (Miyazaki et al. 2002). However, the bioavailability of fish-derived selenium is still controversial. Fish is also rich in PUFA which may be essential for the normal development of an infant brain (Horwood and Fergusson 1998). However, the beneficial effects of increased amount of PUFA in cord blood on the later developmental period are also still controversial (Bakker et al. 2003). In the present study, these confounding factors including selenium and PUFA were considered from nutritional perspectives in the risk assessment of eating fish.

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. 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 are observed in this study, the further follow-up is essential to know the persistency of adverse effects.

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